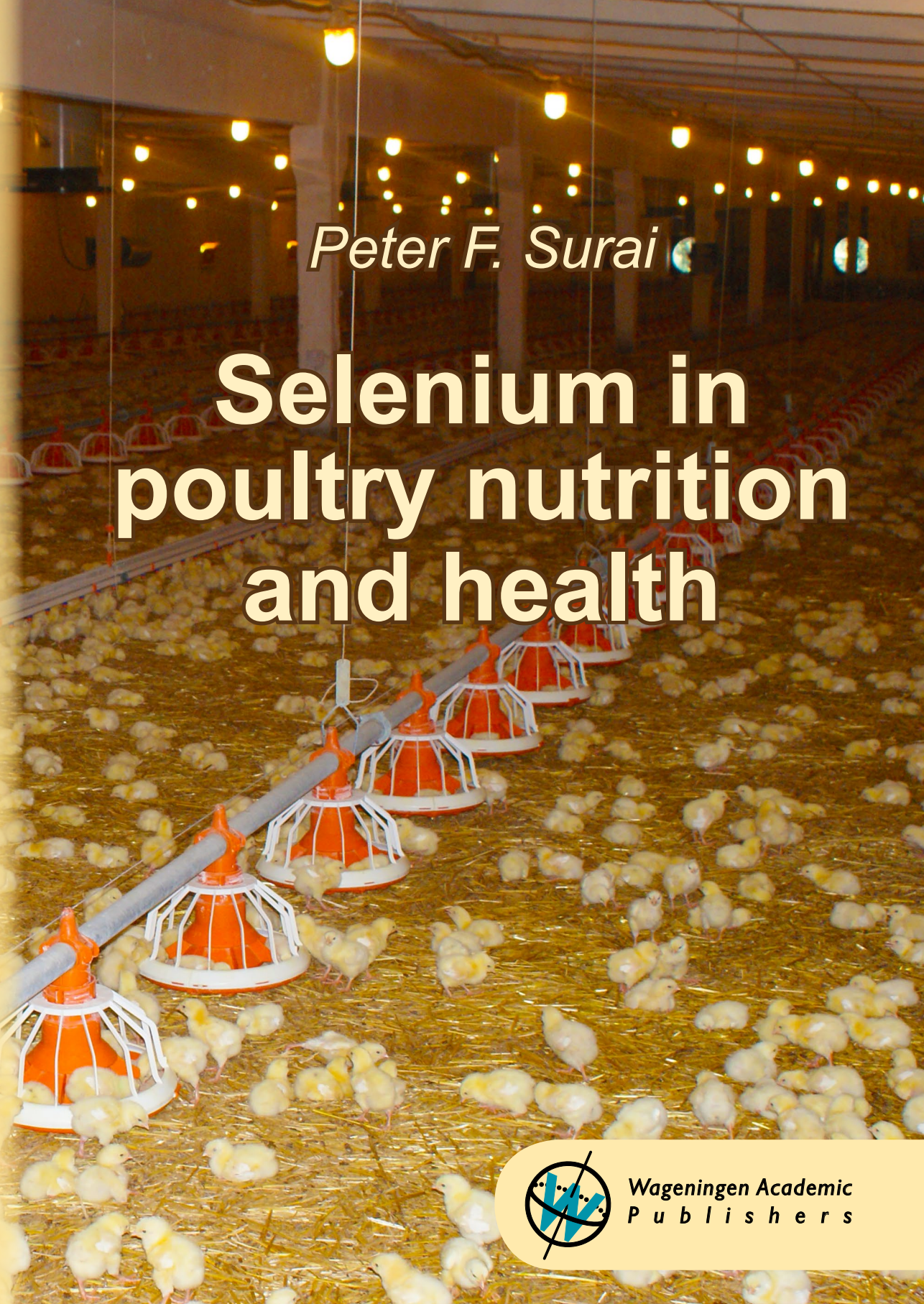

Peter F. Surai

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Dedication

To my wife Helen, my daughter Katie, my son Anton,
my grandsons Oscar, Arthur and Henry
and my granddaughter Aiste
who gave me inspiration for writing this book.

About the author

Dr. Peter Surai started his studies at Kharkov University, Ukraine, where he obtained his PhD and DSc in biochemistry studying effects of antioxidants on poultry. Later he became Professor of Human Physiology. In 1994 he moved to Scotland to continue his antioxidant related research in poultry and in 2000 he was promoted to a full Professor of Nutritional Biochemistry at the Scottish Agricultural College. Recently he was awarded Honorary Professorships in 5 universities in various countries, including UK, Hungary, Bulgaria and Ukraine. In 2010 he was elected to the Russian Academy of Sciences as a foreign member. He has more than 750 research publications, including 150 papers in peer-reviewed journals and 13 books. In 1999 he received the prestigious John Logie Baird Award for Innovation for the development of 'super-eggs' and, in 2000, The World's Poultry Science Association Award for Research in recognition of an outstanding contribution to the development of the poultry industry. In 2017 he became a member of the team at the Moscow State Academy of Veterinary Medicine and Biotechnology named after K.I. Skryabin to conduct a research under a mega-grant of the Government of Russian Federation (Contract No. 14.W03.31.0013). For the last 15 years he has been lecturing all over the world visiting more than 70 countries.

Table of contents

Preface

Among many minerals selenium has a special place being the most controversial trace element. Indeed a narrow gap between essentiality and toxicity and environmental issues on the one hand and global selenium deficiency on the other hand, fuel research in this field. There were several breakthroughs in selenium research. The first one was the discovery of Se essentiality in early 1960s. The second one was the discovery in 1973 that glutathione peroxidase is a selenoprotein. The third one came almost 30 years later with characterisation of main selenoproteins in human and animal body and further understanding the role of selenium in nutrition and health. Indeed, this third breakthrough is really a selenium revolution creating many hypotheses, stimulating new research and providing practical applications in medicine and agriculture. New insight in the role of free radicals as signalling molecules, understanding the role of nutrients in gene expression and maternal programming, tremendous progress in human and animal genome work created new demands for further research related to biological roles of selenium.

Several comprehensive monographs and reviews have been recently published addressing various Se-related issues. However, most of them were dealing with Se roles in human health. Animal food-producing industry is developing very quickly and a great body of information was accumulated indicating importance of Se in maintenance of animal health, productive and reproductive performance. Our previous comprehensive book 'Selenium in Nutrition and Health' was published in 2006 and a lot of important Se-related information has been accumulated for the last 10 years. Therefore, the goal of this volume is to provide up to date information about the roles of Se in poultry nutrition and health. In Chapter 1 a special emphasis is given to the role of selenium as an essential part of the integrated antioxidant system of the body with regulatory functions providing necessary connections between different antioxidants. In fact selenium is called 'the chief executive of the antioxidant defence'. Chapter 2 is addressing molecular mechanisms of Se action describing major functions of the selenoproteins. Indeed, the family of selenoproteins includes 25 members and functions of many of them are still not well understood. Selenium in feed is described in Chapter 3. The main idea of this chapter is to describe an organic Se concept. Indeed, in grains and some other important food ingredients selenomethionine is the main Se form. The idea was put forward that during evolution the digestive system of human and animals was adapted to natural form of selenium consisting of SeMet and other organic selenocompounds. Therefore, this form of Se is more efficiently assimilated in the body than inorganic forms of selenium. In fact SeMet is considered to be the storage form of selenium in the body. Accumulation of the Se reserves in the body as a result of organic selenium consumption is considered as an adaptive mechanism providing additional antioxidant defences in stress conditions. The three generations of Se supplements for poultry are characterised. Chapter 4 is devoted to Se-deficiency diseases in poultry with a specific emphasis to new data on the effect of Se deficiency on the expression of various selenoproteins in different chicken tissues. Indeed, oxidative stress is considered to be a driving force in the development of such Se-deficiency diseases as encephalomalacia, exudative diathesis, nutritional muscular

dystrophy, nutritional pancreatic atrophy, impaired immunocompetence and decreased productive and reproductive performance of chickens. The data presented in Chapter 5 indicate importance of Se in growth, development and reproduction of poultry. The main idea of the chapter is to show benefits of various forms of organic Se on antioxidant defences in the body leading to improvement of productive and reproductive performance of poultry and poultry product quality. Indeed, organic selenium is proven to be the most effective form of Se supplementation for poultry and farm animals. Chapter 6 is devoted to the link between animal industry and human health and describing some features of new technologies for production of Se-enriched eggs and meat. In fact, production of a range of Se-enriched products is considered as an important solution for global Se deficiency. Se-enriched eggs are already on supermarket shelves in many countries worldwide with millions of such eggs sold daily. Chapter 7 is devoted to the role of selenium in immunity. It is difficult to overestimate immunomodulating properties of selenium and increased resistance to various diseases of poultry/animals is a result of optimal Se status. The possibility of virus mutation in the body of animals deficient in selenium is of great importance for understanding mechanisms of spreading such diseases as chicken influenza, etc. The last chapter is devoted to the antioxidant-prooxidant balance in the digestive tract. It seems likely that this balance has been overlooked by scientists. However, the specific roles of selenoproteins in such a balance need further investigation. Indeed, chicken health starts from its gut. I understand that my views on the role of selenium in poultry nutrition and health are sometimes different from those of other scientists and therefore I would appreciate very much receiving any comments from readers which will help me in my future research. I would like to thank my colleagues with whom I have had the pleasure to collaborate and share my ideas related to natural antioxidants and selenium in particular, who helped me at various stages of this research by providing reprints of their recent publications. I am also indebted to the World's Poultry Science Association for the Research Award and a grant of the Government of Russian Federation (Contract No. 14.W03.31.0013) supporting my research.

Peter F. Surai

Abbreviations

5-LO	5-lipoxigenase
9-oxoODE	9-oxo-octadecadienoic acid
AA	ascorbic acid
Ab	antibody
AEC	abdominal exudate cells
AFB_1	aflatoxin B_1
ALS	amyotrophic lateral sclerosis
AO	anti-oxidant
APR	acute phase response
AvBD	avian beta-defensin
BD	basal diet
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
C/EBP	CCAAT-enhancer-binding protein
CAT	catalase
CDS	coding sequence
CNS	central nervous system
ConA	concanavalin A
CoQ	coenzyme Q
COX-2	cyclooxygenase-2
CVB3	Coxsackie virus B3
DAA	dehydroascorbic acid
DC	dendritic cells
DDT	dichlorodiphenyltrichloroethane
DHA	docosahexaenoic acid
DHT	dihydrotestosterone
Dio	iodothyronine deiodinase
DON	deoxynivalenol
DTH	delayed-type hypersensitivity
EC-SOD	extracellular superoxide dismutase
ED	exudative diathesis
EFX	enrofloxacin
ER	endoplasmic reticulum
ERO1	endoplamic reticulum oxidoreductin 1
FAK	focal adhesion kinase
FB_1	fumonisin B_1
FCR	feed conversion ratio
FcγR	phagocytic Fcγ receptors
FDA	US Food and Drug Administration
FO	fish oil
FT3	free triiodothyronine
FT4	free thyroxine
GI-GSH-Px	gastrointestinal glutathione peroxidase
GIT	gastrointestinal tract

GR	glutathione reductase
GSH	reduced glutathione
GSH-Px	glutathione peroxidase
GSSG	oxidised glutathione
GST	glutathione S-transferase
H/L ratio	heterophil to lymphocyte ratio
H_2O_2	hydrogen peroxide
HETE	15-hydroxyeicosatetraenoic acid
HI	hemaglutination inhibition
HMSeBA	selenomethionine hydroxyanalogue, 2-hydroxy-4-methylselenobutanoic acid
HO-1	haeme oxygenase-1
HPETE	15-hydroperoxyeicosatetraenoic acid
HS	heat stress
Hsf1	heat shock factor 1
HSP	heat shock proteins
IBD	Infectious bursal disease
ID	iodothyronine deiodinase
IELs	intraepithelial lymphocytes
IFN	interferon
Ig	immunoglobulin
IL-1	interleukin 1
IL-2R	interleukin 2 receptor
IL-6	interleukin 6
iNOS	inducible nitric oxide synthase
IκB	inhibitor of kappa B
Keap1	Kelch-like-ECH-associated protein 1
LA	linoleic acid
LAK	lymphokine-activated killer
LDH	lactate dehydrogenase
LOOH	lipid hydroperoxide
LOX	lipoxygenase
LP	lipid peroxidation
LPS	lipopolysaccharide
LTA	lymphocyte transformation assay
LXA_4	lipoxin A_4
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein-1
MD	Marek's disease
MDA	malondialdehyde
Met	methionine
MHC	major histocompatibility complex
MIF	macrophage inflammatory protein 2
MLTC	mixed lymphocyte/tumour cell cultures
Msr	methionine sulfoxide reductase
NE	nutritional encephalomalacia

NecE	necrotic enteritis
NF-κB	nuclear factor-kappa B
NK cells	natural killer cells
NKT	natural killer T cells
NMD	nutritional muscular dystrophy
NO	nitric oxide
NPA	nutritional pancreatic atrophy
NRC	National Research Council
Nrf2	NF-E2-related factor 2
OCP	organochlorine pesticides
$ONOO^-$	peroxynitrite
OTA	ochratoxin A
PAMP	pathogen-associated molecular patterns
PCB	polychlorinated biphenyls
PCV2	porcine circovirus type 2
PFC	plaque-forming cell
PGE2	prostaglandin E2
pGSH-Px	plasma glutathione peroxidase
PHA	phytohemagglutin
PH-GSH-Px	phospholipid glutathione peroxidase
PI3K	phosphatidylinositol 3-kinase
PLA2	phospholipase A2
PMN	polymorphonuclear leukocytes
POP	persistent organic pollutants
PPAR	peroxisome proliferator-activated receptor
PPRE	peroxisome proliferator response element
PRR	pattern recognition receptors
Prx	peroxiredoxin
PTGE	prostaglandin E
PUFA	polyunsaturated fatty acid
PWM	pokeweed mitogen
RDA	recommended daily allowance
RNS	reactive nitrogen species
RXR	retinoid-X receptor
SBP2	SECIS-binding protein
SECIS	selenocysteine insertion sequence
SeCys	selenocysteine
SelN	selenoprotein N
SelP	selenoprotein P
SelP-L	long-form selenoprotein P
SelR	selenoprotein R
SeMet	selenomethionine
SeS	selenoprotein S
SeW	selenoprotein W
SM	silymarin
SO	Selisseo, OH-SeMet

SOD	superoxide dismutase
SP	selenoproteins
SPS	selenophosphate synthetase-2
SRBC	sheep red blood cells
SS	sodium selenite
SSC	spermatogonial stem cells
SY	selenium enriched yeast
T	testosterone
T3	triiodothyronine
T4	thyroxine
T-AOC	total antioxidant capacity
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
TCR	T-cell receptor
Th cells	T helper cells
TLR	Toll-like receptors
TNF-α	tumour necrosis factor alpha
Toc	tocopherol
Trx	thioredoxin
TrxR	thioredoxin reductase
TSH	thyroid-stimulating hormone
vMDV	virulent Marek's disease virus
VSMCs	vascular smooth muscle cells
ZEA	zearalenone

Chapter 1
Antioxidant systems in animal body

Self-preservation is the first law of nature

1.1 Introduction

For the majority of organisms on Earth, life without oxygen is impossible. Animals, plants and many microorganisms rely on oxygen for efficient production of energy. However, the high oxygen concentration in the atmosphere is potentially toxic for living organisms. It is interesting that oxygen toxicity was first described in laboratory animals in 1878 (see Knight, 1998). For the last three decades free radical research has generated valuable information for further understanding not only the detrimental, but also the beneficial role of free radicals in cell signalling and other physiological processes. The benefit or harm of free radicals ultimately depend on the level of their production and efficiency of antioxidant defence.

1.2 Free radicals and reactive oxygen and nitrogen species

Free radicals are atoms or molecules containing one or more unpaired electrons. Free radicals are highly unstable and reactive and are capable of damaging biologically relevant molecules, such as DNA, proteins, lipids or carbohydrates. The animal body is under constant attack from free radicals, formed as a natural consequence of the body's normal metabolic activity and as part of the immune system's strategy for destroying invading microorganisms. The internal and external sources of free radicals are shown in Table 1.1. Collective terms reactive oxygen species (ROS) and reactive nitrogen species (RNS) have been introduced (Halliwell and Gutteridge, 1999) including not only the oxygen or nitrogen radicals, but also some non-radical reactive derivatives of oxygen and nitrogen (Table 1.2).

Superoxide ($O_2\bullet^-$) is the main free radical produced in biological systems during normal respiration in mitochondria and by autoxidation reactions with half-life at 37 °C in the range of 1×10^{-6} seconds. Superoxide can inactivate some enzymes due to formation of unstable complexes with transition metals of enzyme prosthetic groups, followed by oxidative self-destruction of the active site (Chaudiere and Ferrari-Iliou, 1999). Depending on the conditions, superoxide can act as oxidizing or as reducing agent. It is necessary to mention that superoxide, by itself, is not extremely dangerous and does not rapidly cross the lipid membrane bilayer (Kruidenier and Verspaget, 2002). However, superoxide is a precursor of other, more powerful ROS. For example, it reacts with nitric oxide with the formation of peroxynitrite ($ONOO^-$), a strong oxidant, which leads to the formation of reactive intermediates due to spontaneous

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Table 1.1. Internal and external sources of free radicals (adapted from Surai, 2006).

Internally generated	Factors promoting ROS formation
Mitochondria (ETC)	Cigarette smoke
Phagocytes (NADPH-oxidase)	Radiation
Xanthine oxidase	UV light
Reactions with Fe^{2+} or Cu^{+}	Pollution
Arachidonate pathways	Certain drugs
Peroxisomes	Chemical reagents
Inflammation	Industrial solvents
Biomolecule oxidation (adrenaline, dopamine, tetrahydrofolates, etc.)	High level of ammonia
	Mycotoxins

Table 1.2. Reactive oxygen and nitrogen species (adapted from Surai, 2006).

Radicals	Non-radicals
Alkoxyl, RO•	Hydrogen peroxide, H_2O_2
Hydroperoxyl, HOO•	Hypochlorous acid, HOCl
Hydroxyl, •OH	Ozone, O_3
Peroxyl, ROO•	Singlet oxygen, $^1O_2$
Superoxide, O_2•	Peroxynitrite, $ONOO^-$
Nitric oxide, NO•	Nitroxyl anion, NO^-
Nitrogen dioxide, NO_2•	Nitrous acid, HNO_2

decomposition (Kontos, 2001; Mruk *et al.*, 2002). In fact $ONOO^-$ was shown to damage a wide variety of biomolecules, including proteins (via nitration of tyrosine or tryptophan residues or oxidation of methionine or selenocysteine residues), DNA and lipids (Groves, 1999). Superoxide can also participate in the production of more powerful radicals by donation of an electron, thereby reducing Fe^{3+} and Cu^{2+} to Fe^{2+} and Cu^{+}, as follows:

$$O_2^- + Fe^{3+}/Cu^{2+} \longrightarrow Fe^{2+}/Cu^{+} + O_2$$

Further reactions of Fe^{2+} and Cu^{+} with H_2O_2 are the source of the hydroxyl radical (•OH) in the Fenton reaction:

$$H_2O_2 + Fe^{2+}/Cu^{+} \longrightarrow {}^{\bullet}OH + OH^- + Fe^{3+}/Cu^{2+}$$

The sum of the reaction of superoxide radicals with transition metals and of transition metals with hydrogen peroxide is known as the Haber-Weiss reaction. It is necessary to underline that the superoxide radical is a 'double-edged sword'. It is beneficial when

produced by activated polymorphonuclear leukocytes and other phagocytes as an essential component of their bactericidal activities, but in excess it may result in tissue damage associated with inflammation.

Hydroxyl radicals are the most reactive species with an estimated half-life of only about 10^{-9} seconds. It can damage any biological molecule it touches, however, its diffusion capability is restricted to only about two molecular diameters before reacting (Yu, 1994). Therefore, in most cases, the damaging effect of a hydroxyl radical is restricted to the site of its formation. In general, hydroxyl radicals can be generated in the human/animal body as a result of radiation exposure from natural sources (radon gas, cosmic radiation) and from man-made sources (electromagnetic radiation and radionuclide contamination). In fact, in many cases, hydroxyl radicals are a trigger of the chain reaction in lipid peroxidation.

Therefore, ROS/RNS (Table 1.2) are constantly produced *in vivo* in the course of the physiological metabolism in tissues. It is generally accepted that the electron-transport chain in the mitochondria is responsible for the major part of superoxide production in the body (Halliwell and Gutteridge, 1999). Mitochondrial electron transport systems consume more than 85% of all oxygen used by the cell and, because the efficiency of electron transport is not 100%, about 1-3% of the electrons escape from the chain and the univalent reduction of molecular oxygen results in superoxide anion formation (Chow *et al.*, 1999; Halliwell, 1994; Singal *et al.*, 1998). About 10^{12} O_2 molecules are processed daily by each rat cell and if leakage of partially reduced oxygen molecules is about 2%, this will yield about 2×10^{10} molecules of ROS per cell per day (Chance *et al.*, 1979). An interesting calculation has been made by Halliwell (1994), showing that in the human body about 1.72 kg/year of superoxide radicals is produced. In stress condition this would be substantially increased. Clearly, these calculations show that free radical production in the body is substantial and many thousands of biological molecules can be easily damaged if they are not protected. The activation of macrophages in stress conditions is another important source of free radical generation. Immune cells produce ROS/RNS and use them as an important weapon to destroy pathogens (Kettle and Winterbourn, 1997; Schwarz, 1996).

The most important effect of free radicals on the cellular metabolism is due to their participation in lipid peroxidation reactions (Surai, 2006). The first step of this process is called the initiation phase, during which carbon-centred free radicals are produced from a precursor molecule, for example polyunsaturated fatty acid (PUFA):

$$LH \xrightarrow{\text{Initiator}} L\bullet$$

The initiator in this reaction could be the hydroxyl radical, radiation or some other events or compounds. In presence of oxygen, these radicals (L•) react with oxygen producing peroxyl radicals starting the next stage of lipid peroxidation called the propagation phase:

$$L\bullet + O_2 \longrightarrow LOO\bullet$$

At this stage a relatively unreactive carbon-centred radical (L•) is converted to a highly reactive peroxyl radical. The resulting peroxyl radical can attack any available peroxidazable material producing hydroperoxide (LOOH) and new carbon-centred radical (L•):

$$LOO\bullet + LH \longrightarrow LOOH + L\bullet$$

Therefore, lipid peroxidation is a chain reaction and a potentially large number of cycles of peroxidation could cause substantial damage to cells. In membranes the peroxidazable material is represented by PUFAs. It is generally accepted that PUFA susceptibility to peroxidation is proportional to the amount of double bounds in the molecules. In fact, docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (20:4n-6) are among the major substrates of peroxidation in the membrane. It is necessary to underline that the same PUFAs are responsible for maintenance of physiologically important membrane properties, including fluidity and permeability. Therefore, as a result of lipid peroxidation within the biological membranes, their structure and functions are compromised. Proteins and DNA are also important targets for ROS.

It has been shown that the DNA in each cell of a rat is hit by about 100,000 free radicals a day and each cell sustains as many as 10,000 potentially mutagenic (if not repaired) lesions per day arising from endogenous sources of DNA damage (Ames, 2003; Ames and Gold, 1997; Diplock, 1994; Helbock *et al.*, 1998). Therefore, some oxidative lesions escape repair and the steady state level of oxidative lesions increases with age; an old rat has accumulated about 66,000 oxidative DNA lesions per cell (Ames, 2003). Oxidation, methylation, deamination and depurination are four endogenous processes leading to significant DNA damage with oxidation to be most significant one and approximately 20 types of oxidatively altered DNA molecules have been identified. The chemistry of attacks by ROS on DNA is very complex and lesions in chromatin include damage to bases, sugar lesions, single strand breaks, basic lesions and DNA-nucleoprotein cross-links (Diplock, 1994).

The complex structure of proteins and the variety of oxidisable functional groups of amino acids makes them susceptible to oxidative damage. In fact, the accumulation of oxidised proteins has been implicated in the aging process and in other age-related pathologies. A range of oxidised proteins and amino acids has been characterised in biological systems (Dean *et al.*, 1997; Kehrer, 2000). In general, the accumulation of oxidised proteins depends on the balance between antioxidants, prooxidants and removal/repair mechanisms. Oxidation of proteins leads to the formation of reversible disulfide bridges. More severe protein oxidation causes a formation of chemically modified derivatives, e.g. shiff's base (Tirosh and Reznick, 2000). Nitric oxide, hydroxyl radicals, alkoxyl and peroxyl radicals, as well as carbon-centred radicals, hydrogen peroxide, aldehydes or other products of lipid peroxidation can attack protein molecules. Usually oxidative modification of proteins occurs by two different mechanisms: a site-specific formation of ROS via redox-active transition metals and non-metal-dependent ROS-induced oxidation of amino acids (Tirosh and Reznick, 2000). The modification of a protein occurs by either a direct oxidation of a specific

amino acid in the protein molecule or by cleavage of the protein backbone. In both cases biological activity of the modified proteins would be compromised. The degree of protein damage depends on many different factors (Grune *et al.*, 1997):

- the nature and relative location of the oxidant or free radical source;
- nature and structure of protein;
- the proximity of ROS to protein target;
- the nature and concentrations of available antioxidants.

Free radicals are implicated in the initiation or progression phase of various diseases, including cardiovascular disease, some forms of cancer, cataracts, age-related macular degeneration, rheumatoid arthritis and a variety of neuro-degenerative diseases (Hogg, 1998; McCord, 2000). In poultry production, overproduction of free radicals and oxidative stress are considered to be related to various type of stresses, including, nutritional, technological, environmental and internal stresses (Figure 1.1; Surai and Fisinin, 2016a,b,c,d). In general, it is widely believed that most human and animal/poultry diseases at different stages of their development are associated with free radical production and metabolism (Surai, 2002, 2006). Normally, there is a delicate balance between the amount of free radicals generated in the body and the antioxidants to protect against them. For the majority of organisms on Earth, life without oxygen is impossible, animals, plants and many micro-organisms rely on oxygen for the efficient production of energy. However, they pay a high price for the pleasure of living in an oxygenated atmosphere since high oxygen concentration in the atmosphere is potentially toxic for living organisms.

1.3 Three levels of antioxidant defence

During evolution, living organisms have developed specific antioxidant protective mechanisms to deal with ROS and RNS (Halliwell and Gutteridge, 1999). Therefore, it is only the presence of natural antioxidants in living organisms which enable them to survive in an oxygen-rich environment (Halliwell, 1994, 2012). These mechanisms are described by the general term 'antioxidant system'. It is diverse and responsible for the protection of cells from the actions of free radicals. This system includes:

- natural fat-soluble antioxidants (vitamin E, carotenoids, ubiquinones, etc.);
- water-soluble antioxidants (ascorbic acid, uric acid, carnitine, taurine, etc.);
- antioxidant enzymes: glutathione peroxidase (GSH-Px), catalase (CAT) and superoxide dismutase (SOD);
- thiol redox system, consisting of the glutathione system (glutathione/glutathione reductase/glutaredoxin/glutathione peroxidase) and the thioredoxin system (thioredoxin/thioredoxin peroxidase/thioredoxin reductase) (Figure 1.2; for details see Chapter 2).

The antioxidant capacity of a compound is determined by multiple factors in addition to the reactivity towards free radicals, including chemical reactivity towards free radicals; fate of antioxidant-derived radicals; interaction with other antioxidants; concentration, distribution, mobility, and metabolism at the micro-environment

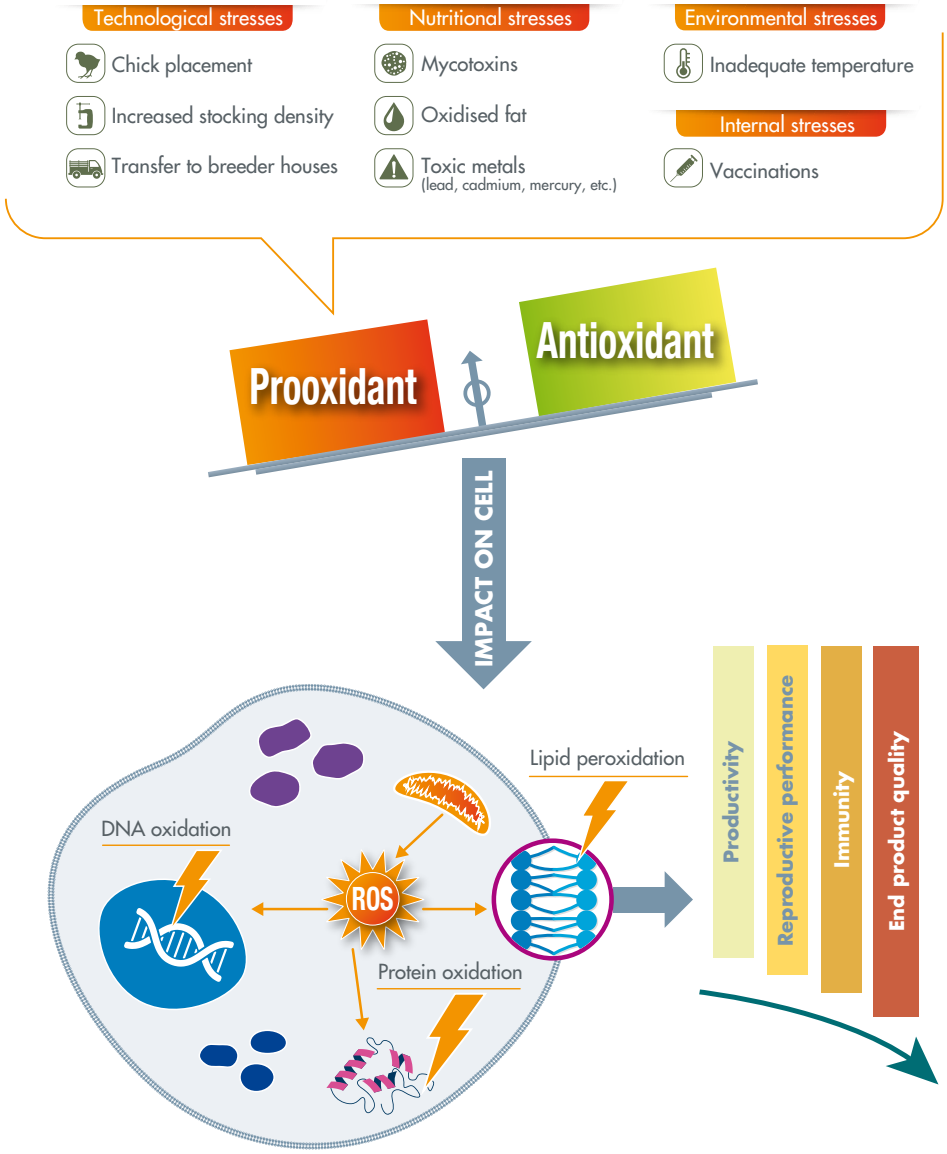

Figure 1.1 External stresses impact cellular functions and animal productivity.

(Niki, 2014, 2016). The protective antioxidant compounds are located in organelles, subcellular compartments, or the extracellular space enabling maximum cellular protection to occur. Thus, the antioxidant system of the living cell includes three major levels of defence (Niki, 1996; Surai, 1999, 2002, 2006).

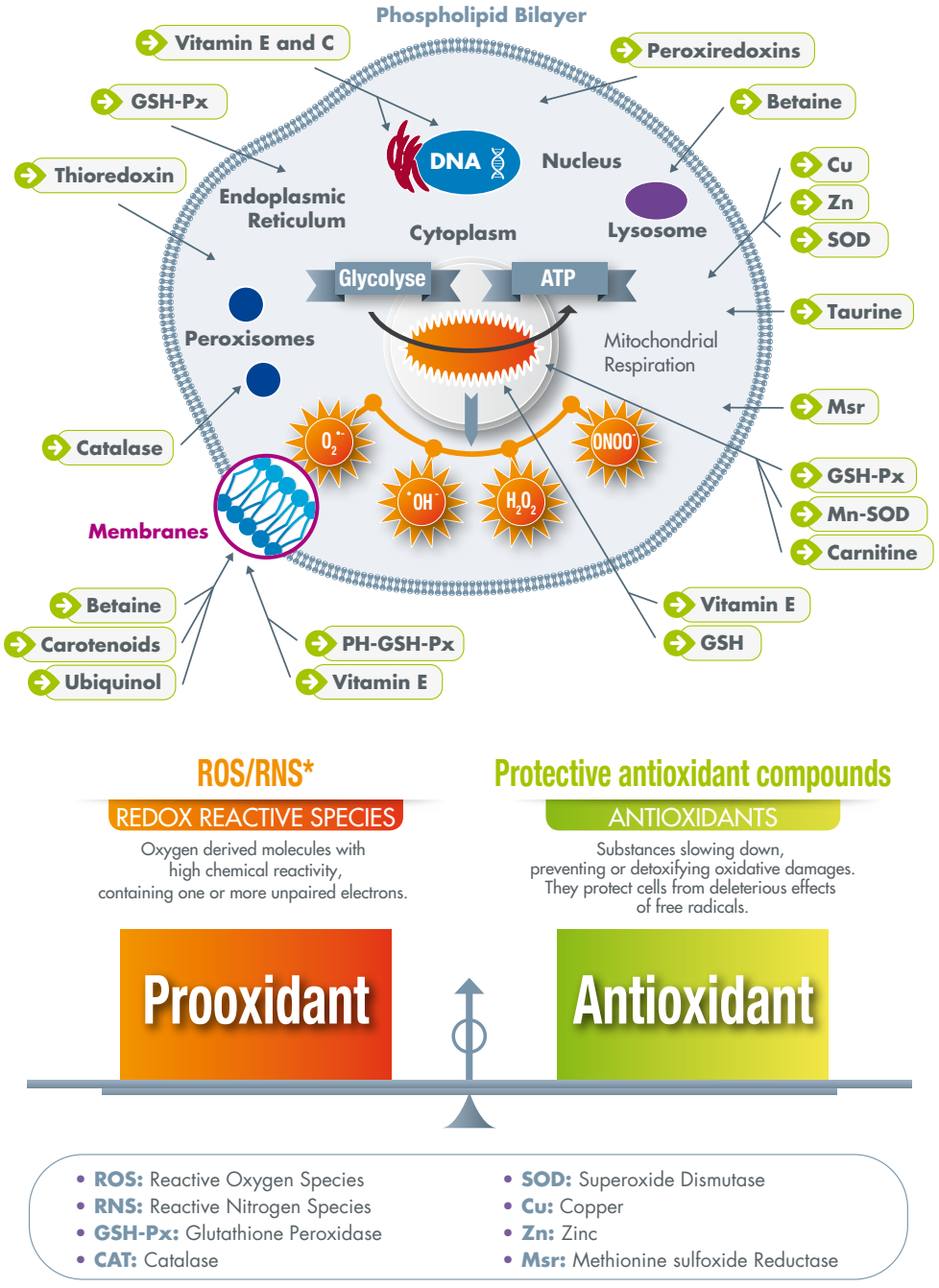

Figure 1.2 The right balance to maintain cellular functions.

The first level of defence is responsible for prevention of free radical formation by removing precursors of free radicals or by inactivating catalysts and consists of three antioxidant enzymes, namely, SOD, GSH-Px and CAT plus metal-binding proteins (Figure 1.3).

Since the superoxide radical is the main free radical produced in physiological conditions in the cell (Halliwell, 1994), superoxide dismutase (EC 1.15.1.1) is considered to be the main element of the first level of antioxidant defence in the cell (Surai, 1999). This enzyme dismutates the superoxide radical in the following reaction:

$$2O_2^{\bullet} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

1.4 Superoxide dismutase in biological systems

Superoxide dismutase was discovered by McCord and Fridovich in 1969 as an enzymatic activity in preparations of carbonic anhydrase or myoglobin that inhibited the aerobic reduction of cytochrome C by xanthine oxidase. Therefore, haemocuprein, which was discovered much earlier, became copper-zinc superoxide dismutase (Bannister, 1988). This discovery opened a new era in free radical research. At present, three distinct isoforms of SOD have been identified in mammals, and their genomic structure, cDNA, and proteins have been described (Zelko *et al.*, 2002). A fourth

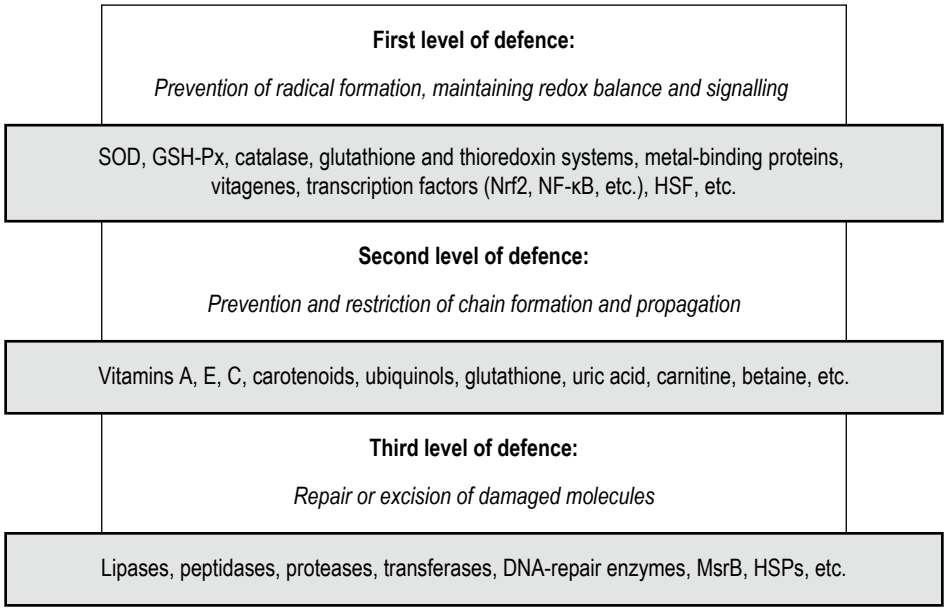

Figure 1.3. Three lines of antioxidant defence in animal cells (adapted from Surai, 1999).

form of the enzyme Fe-SOD was isolated from various bacteria but not found in animals. Furthermore, a novel type of nickel-containing SOD was purified to apparent homogeneity from the cytosolic fractions of *Streptomyces* spp. (Youn *et al.*, 1996). The biosynthesis of SODs, in most biological systems, is well controlled. In fact, exposure to increased pO_2, increased intracellular fluxes of O_2^-, metal ions perturbation, and exposure to several environmental oxidants have been shown to influence the rate of SOD synthesis in both prokaryotic and eukaryotic organisms (Hassan, 1988). A range of transcriptional factors, including nuclear factor-kappa B (NF-κB), AP-1, AP-2, and Sp1, as well as CCAAT-enhancer-binding protein (C/EBP), have been shown to regulate the constitutive or inducible expression levels of all three SODs (Miao and St. Clair, 2009). Furthermore, it seems likely that in addition to transcriptional control, epigenetic regulation and posttranscriptional modifications are responsible for the regulation of SOD functional activity (Miao and St. Clair, 2009). Comparative characteristics of SOD1, SOD2 and SOD3 are shown in Table 1.3.

SOD1, or Cu,Zn-SOD, was the first enzyme of this family to be characterised and is a copper and zinc-containing homodimer that is found almost exclusively in intracellular cytoplasmic spaces. It exists as a 32 kDa homodimer and is present in the cytoplasm and nucleus of every cell type examined (Zelko *et al.*, 2002). The chromosomal localization and characteristics of the SOD1 gene have been identified in rodents, bovines and humans; the human SOD1 gene is localised on chromosome

Table 1.3. Biochemical properties of mammalian superoxide dismutase (adapted from Huang *et al.,* 2012; Miao and St. Clair, 2009).

Enzymes	Cu,Zn-SOD	Mn-SOD	EC-SOD
Gene designation (human/mouse)	SOD1/Sod1	SOD2/Sod2	SOD3/Sod3
Chromosome location (human/mouse)	HAS21/MMU16	HAS6/MMU17	HAS4/MMU5
Disease caused by enzyme defects	amyotrophic lateral sclerosis (ALS)	none	none
Metal co-factor(s)	Cu^{2+} – catalytically active; Zn^{2+} – maintains enzyme stability	Mn^{2+} – catalytically active	Cu^{2+} – catalytically active; Zn^{2+} – maintains enzyme stability
Active form	dimer	tetramer	tetramer
Molecular mass, kDa	88	32	135
Subcellular locations	cytosol, intermembrane space of mitochondria, nucleus	mitochondria matrix	extracellular matrix and circulation
Tissue distribution (from high to low)	liver, kidney, brain, heart	heart, brain, skeletal muscle	blood vessels, lung, kidney, uterus
Post-translational modification	nitration, phosphorylation, glutathiolation, glycation	acetylation, nitration, phosphorylation	glycosylation
Inducibility	not inducible	inducible	induced by antioxidants and regulated through NRF

21q22. Furthermore, the SOD1 gene consists of five exons interrupted by four introns, which is significantly similar in different species in terms of the size of exons, particularly the coding regions (Miao and St. Clair, 2009).

The second member of the family (SOD2) has manganese (Mn) as a cofactor and therefore is called Mn-SOD. SOD2 is shown to have an unique genetic organisation and little similarity with SOD1 and SOD3 (Miao and St. Clair, 2009). The primary structure of SOD2 genes is shown to be highly conserved and it shares more than 90% sequence homology in the coding region in mouse, rat, bovine and human. The human sod2 is located on chromosome 6q25.3 (Miao and St Clair, 2009). It was shown to be a 96 kDa homotetramer and located exclusively in the mitochondrial matrix, a prime site of superoxide radical production (Halliwell and Gutteridge, 1999). Therefore, the expression of Mn-SOD is considered to be essential for the survival of all aerobic organisms from bacteria to humans and it participates in the development of cellular resistance to oxygen radical-mediated toxicity (Fridovich, 1995). Indeed, Mn-SOD is shown to play a critical role in the defence against oxidant-induced injury and apoptosis of various cells.

In fact, Mn-SOD is an inducible enzyme and its activity is affected by cytokines and oxidative stress. Mn-SOD has been shown to play a major role in promoting cellular differentiation and in protecting against hyperoxia-induced pulmonary toxicity (Fridovich, 1995) being a crucial determinant of redox status of the cell. Furthermore, Mn-SOD influences the activity of transcription factors (such as HIF-1α, AP-1, NF-κB and p53) and affects DNA stability (Miriyala *et al.,* 2012). A critical role of Mn-SOD under physiological and pathological conditions has recently been reviewed in detail and the following findings confirm the critical role of Mn-SOD in the survival of aerobic life (Holley *et al.,* 2012; Indo *et al.*, 2015; Miriyala *et al.,* 2011):

- *Escherichia coli* and yeasts lacking the Mn-SOD gene are highly sensitive to oxidative stress.
- Mn-SOD gene knockout mice can only survive a few days after birth, with pathological symptoms of many/various diseases due to mitochondrial disorders, suggesting a critical role of the enzyme.
- Cells transfected with Mn-SOD cDNAs have increased resistance to various free-radical-generating toxicants (paraquat, tumour necrosis factor, doxorubicin, mitomycin C, irradiation, ischemic reperfusion, smoking, etc.).
- Human Mn-SOD gene transgenic mice show reduced severity of free-radical-induced pulmonary damage and adriamycin-induced myocardial damage.

In 1982, a third SOD isozyme was discovered by Marklund and co-workers and called extracellular superoxide dismutase (EC-SOD), due to its exclusive extracellular location. EC-SOD is a glycoprotein with a molecular weight of 135 kDa with high affinity for heparin. However, there are some species-specific variations in molecular weight. The human EC-SOD gene has been mapped to chromosome 4q21 and consists of three exons and two introns (Nozik-Grayck *et al.,* 2005). EC-SOD is the only antioxidant enzyme that scavenges superoxide specifically in the extracellular space. EC-SOD is present in various organisms as a tetramer or, less commonly, as a dimer and contains one copper and one zinc atom per subunit, which are required for enzymatic

activity (Fattman *et al.*, 2003). The expression pattern of EC-SOD is highly restricted to the specific cell type and tissues where its activity can exceed that of Cu,Zn-SOD or Mn-SOD. As a copper-containing enzyme, the activity of EC-SOD is regulated by copper availability (Nozik-Grayck *et al.,* 2005). EC-SOD is comparatively resistant to high temperatures, extreme pH, and high urea concentrations, it can be inhibited by a various agents including azide and cyanide and inactivated by diethyldithiocarbamate and hydrogen peroxide. Oxidative stress and post-translational modification of EC-SOD have been shown to cause loss of EC-SOD activity. The full-length mouse EC-SOD cDNA is shown to be 82% identical to that of rat and 60% identical to human EC-SOD (Miao and St. Clair, 2009).

Understanding the molecular mechanisms of regulation of SOD gene expression and the factors involved in tissue- and cell-specific expression of SOD genes are of great importance for a developing novel strategies for preventing negative consequences of various stresses in poultry production.

1.5 Superoxide dismutase in avian biology

Chicken SOD was described and purified in the early 1970s. Indeed, in chicken liver two types of SOD were identified, one of which was localised in the mitochondria, while the other was found in the cytosol (Weisiger and Fridovich, 1973). The cytosol SOD was inhibited by cyanide, whereas the mitochondrial enzyme was not. Later this feature was used to distinguish between two forms of enzymes during assays. The cytosol SOD was purified to homogeneity with an apparent molecular weight in presence of mercaptoethanol of 30,600 Da and to contain copper and zinc, being similar to the other Cu,Zn-SOD that have been isolated from diverse eukaryotes. In fact, purified cytosol SOD from chicken liver contained 0.30% copper and 0.25% zinc. This calculates to 0.9 atom of copper and 0.8 atom of zinc per subunit. It was also shown that this chicken liver Cu,Zn-SOD had a tendency to polymerise (Weisiger and Fridovich, 1973). In contrast, the mitochondrial SOD in chicken liver was found to be a manganoprotein which had a molecular weight of 80,000 Da. It is composed of four subunits of equal size, which are not covalently joined. It contains 2.3 atoms of Mn per molecule and is strikingly similar to the SOD previously isolated from bacteria. This supports the theory that mitochondria have evolved from aerobic prokaryotes. In fact, Mn-SOD was first isolated from chicken liver (Weisiger and Fridovich, 1973). MnSOD was found primarily in the mitochondrial matrix space, whereas Cu,Zn-SOD, previously isolated from the cytosol, was found in the intermembrane space (Weisiger and Fridovich, 1973a).

Cu,Zn-SOD was purified from chicken liver with a subunit Mr of 16,900 (Dameron and Harris, 1987). Low dietary copper was associated with a decrease in SOD activity and when the 10 day old deficient chicks were injected with 0.5 mol of $CuSO_4$ intraperitoneally, SOD activity in aorta was restored to control levels in about 8 h. Indeed, dietary copper regulates SOD activity in the tissues of young developing animals. The authors also suggested that copper deficiency suppresses Cu,Zn-SOD

activity without inhibiting synthesis or accumulation of the Cu,Zn-SOD protein in this tissue (Dameron and Harris, 1987). Interestingly, molecular properties (amino acid composition, molecular mass and subunit composition) of the chicken enzyme was shown to be similar to those of a bovine erythrocyte Cu,Zn-SOD (Michalski and Prowse, 1991). Purified chicken liver Cu,Zn-SOD was confirmed to contain two subunits having Cu and Zn elements with a molecular weight of 16,000±500 for each (Oztürk-Urek and Tarhan, 2001). The optimum pH of purified Cu,Zn-SOD was determined to be 8.9. The enzyme was found to have fair thermal stability up to 45 °C at pH 7.4 over an 1 h incubation period. The SOD enzyme was not inhibited by dithiothreitol and β-mercaptoethanol, but inhibited by CN^- and H_2O_2 (Oztürk-Urek and Tarhan, 2001). SOD purified from chicken heart has a molecular weight 31.0±1.0 kDa and is composed of two equally sized subunits each having 1.1±0.03 and 0.97±0.02 atoms of Cu and Zn elements, respectively (Demirel and Tarhan, 2004). The Mn-SOD cDNA in chicken heart was shown to be 1,108 bp in length. The molecular weight of the mature peptide was 22 kDa. A comparison of the deduced amino acid sequence with that of human, rat, *Caenorhabditis elegans* and *Drosophila melanogaster* showed that the amino acid homology rates were 82.4, 84.7, 62.4 and 59.3%, respectively (Bu *et al.,* 2001). SOD activity in avian tissues depends on many different factors, including genetics, nutrition and various stress factors. For example, SOD activity in the Jungle Fowl feather melanocytes was shown to be 2- and 4-fold higher than that in Barred Plymouth Rock and White Leghorn tissue, respectively (Bowers *et al.,* 1994).

The hydrogen peroxide formed by SOD action can be detoxified by GSH-Px or CAT which reduce it to water as follows:

$$H_2O_2 + 2GSH \xrightarrow{\text{GSH-Px}} GSSG + 2H_2O$$

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

Protective roles of SOD in animal/poultry physiology are shown in Figure 1.4.

1.6 Other antioxidant mechanisms

Catalase (EC 1.11.1.6) is a tetrameric enzyme consisting of four identical subunits of 60 kDa containing a single ferriprotoporphyrin group per subunit. It plays an important role in the acquisition of tolerance to oxidative stress in the adaptive response of cells (Mates *et al.*, 1999). In mammalian cells, NADPH is bound to catalase protecting it from inactivation by H_2O_2 (Chaudiere and Ferrari-Illiou, 1999). Since GSH-Px has a much higher affinity for H_2O_2 than CAT (Jones *et al.,* 1981) and wider distribution in the cell (catalase is located mainly in peroxisomes), the H_2O_2 removal from the cell is very much dependent on GSH-Px. Details of GSH-Px action are shown in Chapter 2. Recently, it has been shown that thioredoxin peroxidases are also capable of directly reducing hydrogen peroxide (Nordberg and Arner, 2001). It is interesting

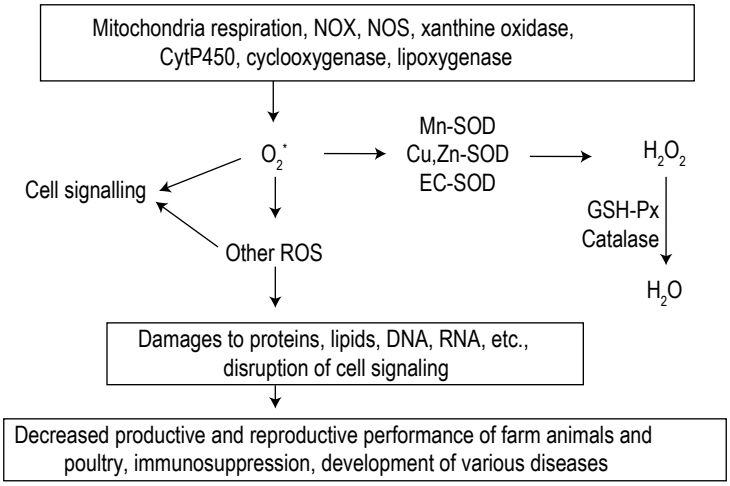

Figure 1.4. Protective roles of SOD in animal/poultry physiology.

that the levels of antioxidant enzymes are regulated by gene expression as well as by post-translational modifications (Fujii and Taniguchi, 1999).

Hydrogen peroxide can also be detoxified by peroxiredoxins (Prx):

$$\text{Prx (reduced)} + H_2O_2 \longrightarrow \text{Prx (oxidised)} + 2H_2O$$

The oxidised form of Prx is inactive, requiring the donation of electrons from reduced thioredoxin (Trx) to restore its catalytic activity:

$$\text{Prx (oxidised)} + \text{Trx (reduced)} \longrightarrow \text{Prx (reduced)} + \text{Trx (oxidised)}$$

Furthermore, Se-dependent enzyme TrxR reduces oxidised Trx to its reduced active form:

$$\text{Trx (oxidised)} \xrightarrow{\text{TR}} \text{Trx (reduced)}$$

Mammalian cells express 6 Prx isoforms, including Prx3 and Prx5 in the mitochondria. Prx functions by undergoing oxidation by H_2O_2 at an active site cysteine and followed by subsequent reduction by thioredoxin, thioredoxin reductase, and NADPH. There are eight GSH-Pxs, which are oxidised by H_2O_2 and reduced by glutathione (GSH) (Sena and Chandel, 2012). More details on the role of the thioredoxin system in antioxidant defences are shown in Chapter 2.

Transition metal ions also accelerate the decomposition of lipid hydroperoxides into cytotoxic products, such as aldehydes, alkoxyl radicals and peroxyl radicals:

$$LOOH + Fe^{2+} \longrightarrow LO\bullet + Fe^{3+} + OH^-$$

$$LOOH + Fe^{3+} \longrightarrow LOO\bullet + Fe^{2+} + H^+$$

Therefore, metal-binding proteins (transferrin, lactoferrin, haptoglobin, hemopexin, metallothionein, ceruloplasmin, ferritin, albumin, myoglobin, etc.) also belong to the first level of defence. It is necessary to take into account that iron and copper are powerful promoters of free radical reactions and therefore their availability in 'catalytic' forms is carefully regulated *in vivo* (Halliwell, 1999). Therefore, organisms have evolved to keep transition metal ions safely sequestered in storage or transport proteins. This way the metal-binding proteins prevent formation of hydroxyl radicals by preventing them from participation in radical reactions. For example, transferrin binds iron (about 0.1% of the total body reserves), transports it in the plasma pool and attaches it to the transferrin receptor. The important point is that iron associated with transferrin will not catalyse a free radical reaction. Ferritin is considered to be involved in iron storage (about 30% of total body reserves) within the cytosol in various tissues, including liver and spleen. A major part of the iron in the body (55-60%) is associated with haemoglobin within red cells and about 10% with myoglobin in muscles (Galey, 1997). A range of other iron-containing proteins (mainly enzymes) can be found in the body including NADH dehydrogenase, cytochrome P450, ribonucleotide reductase, proline hydroxylase, tyrosine hydroxylase, peroxidases, catalase, cyclooxygenase, aconitase, succinate dehydrogenase, etc. (Galey, 1997). Despite an importance of iron in various biochemical reactions, iron can be extremely dangerous when not carefully handled by proteins. In fact, in many stress conditions a release of free iron from its normal sites and its participation in Fenton chemistry mediates damages to cells. For example, a superoxide radical can release iron from ferritin and H_2O_2 degrades the haeme of haemoglobin to liberate iron ions (Halliwell, 1987).

Ceruloplasmin, being a copper-binding protein, is another major protein mediating free radical metabolism. Under physiological conditions it binds six or seven copper ions per molecule preventing their participation in free radical generation. About 5% of human plasma copper is bound to albumin or to amino acids and the rest is bound to ceruloplasmin. Furthermore, ceruloplasmin possesses antioxidant properties itself being able to scavenge superoxide radicals (Yu, 1994). Thus, it is quite clear that metal sequestration is an important part of extracellular antioxidant defence.

Unfortunately, this first level of antioxidant defence in the cell is not sufficient to completely prevent free radical formation. Some radicals escape the antioxidant safety screen and initiate lipid peroxidation, causing damage to DNA and proteins. Therefore, a second level of defence consists of chain-breaking antioxidants – vitamin E, ubiquinol, carotenoids, vitamin A, ascorbic acid, uric acid and some other antioxidants. The glutathione and thioredoxin systems also play a substantial role in the second level of antioxidant defence (for details see Chapter 2). Chain-breaking antioxidants inhibit peroxidation by keeping the chain length of the propagation

reaction as short as possible. As such, they prevent the propagation step of lipid peroxidation by scavenging peroxyl radical intermediates in the chain reaction:

$$LOO\bullet + Toc \longrightarrow Toc\bullet + LOOH$$

where LOO• = lipid peroxyl radical; Toc = tocopherol; Toc• = tocopheroxyl radical; LOOH = lipid hydroperoxide.

Vitamin E, the most effective natural free radical scavenger identified to date, is the main chain breaking antioxidant in the cell. However, hydroperoxides, produced in the reaction of vitamin E with the peroxyl radical, are toxic and if not removed, impair membrane structure and functions (Gutteridge and Halliwell, 1990). In fact, lipid hydroperoxides are not stable and in the presence of transition metal ions can decompose, thereby producing new free radicals and cytotoxic aldehydes (Diplock, 1994). Therefore, hydroperoxides have to be removed from the cell in the same way as H_2O_2. However, catalase is not able to detoxify these compounds and only Se-dependent GSH-Px can deal with them, converting hydroperoxides into non-reactive products (Brigelius-Flohe, 1999) as follows:

$$ROOH + 2GSH \xrightarrow{\text{GSH-Px}} ROH \text{ (non-toxic)} + H_2O + GSSG$$

Thus, vitamin E performs only half the job in preventing lipid peroxidation by scavenging free radicals and forming hydroperoxides. The second part of this important process of antioxidant defence is taken care of by Se-GSH-Px. It is necessary to underline, that vitamin E and selenium work in tandem; and even very high doses of dietary vitamin E cannot replace Se that is needed (in the form of GSH-Px and thioredoxin reductase) to complete the second part of antioxidant defence as mentioned above. Thus, Se as an integral part of the GSH-Px and thioredoxin reductase belongs to the first and second levels of antioxidant defence.

Coenzyme Q, known also as ubiquinone, was discovered in 1957. The name ubiquinone is related to its 'ubiquitous' presence in all cells and the name coenzyme Q reflects the chemical structure of the compound containing one quinone group and 10 isoprenyl units. Coenzyme Q_{10} (CoQ_{10}) exists both in an oxidised and a reduced form, ubiquinone and ubiquinol, respectively (Overvad *et al.*, 1999). Importantly, ubiquinone is considered to be an important fat-soluble antioxidant and electron carrier synthesised in mitochondria (Stefely and Pagliarini, 2017). In general, dietary supplementation of CoQ does not affect the endogenous synthesis of CoQ in tissues. However, oxidative stress (physical exercise, thyroid hormone treatment, cold adaptation, vitamin A deficiency, etc.) is associated with increased CoQ synthesis reflecting cellular adaptation (Ernster and Dallner, 1995). Therefore, CoQ synthesis is considered to be an adaptive mechanism in response to stress conditions when other antioxidants are depleted. For example, in vitamin E and Se deficient rats CoQ concentration is elevated and the CoQ-dependent reductase system activated (Navarro *et al.*, 1998).

Antioxidant properties of CoQ are directly related to the protection in the gastrointestinal tract. For example, in rats treated *per os* with sodium nitrite, increases TBARS in small intestinal mucosa and liver were observed. Pre-treatment of nitrite-poisoned rats with CoQ_{10} mitigated lipid peroxidation and increased total antioxidant status in animal blood (Grudzinski and Frankiewicz-Jozko, 2001). It is directly involved in the protection of biological molecules (lipids, proteins and DNA) from oxidative damage by quenching free radicals, regenerating other antioxidants (vitamins E and C) and regulating mitochondrial integrity (Varela-López *et al.,* 2016). It was suggested that Se inadequacy could compromise the cells ability to synthesise/obtain the optimal concentration of CoQ_{10}, as optimal function of Se depends on the levels of CoQ_{10} (Alehagen and Aaseth, 2015). It seems likely that additional synthesis of CoQ in stress conditions could be considered as an adaptive mechanism to deal with overproduction of free radicals.

Carotenoids comprise a family of more than 750 compounds responsible for a variety of bright colours in fall leaves, flowers (narcissus, marigold), fruits (pineapple, citrus fruits, paprika), vegetables (carrots, tomatoes), insects (ladybird), bird plumage (flamingo, cock of the rock, ibis, canary) and marine animals (crustaceans, salmon) (Maoka, 2009; Pfander, 1992). These pigments provide different colours from light yellow to dark red and when complexed with proteins they can produce green and blue colours (Ong and Tee, 1992). Carotenoids – important elements of the antioxidant system – possess antioxidant activities and participate directly or indirectly (for example, by recycling vitamin E or regulating expression of various genes) in antioxidant defences. Recently, an important role of canthaxanthin with a special emphasis to carotenoid antioxidant activities in breeder nutrition has been described (Surai, 2012a,b). Biological functions of these natural pigments in relation to animals or humans are not well defined, but their antioxidant properties seem to be of major importance. Therefore, antioxidant interactions, including their recycling, provide an effective and reliable system of defence from free radicals and toxic products of their metabolism.

Vitamin C is a hydrophilic antioxidant functioning in an aqueous environment and possessing high free-radical-scavenging activity (Yu, 1994). It directly reacts with O_2^- and OH• and various lipid hydroperoxides and is taking part in vitamin E recycling (Halliwell, 1996; Yu, 1994). Ascorbic acid is protective against a number of ROS (Carr and Frei, 1999; Halliwell, 1999, 1996).

The major advantages of ascorbate as an antioxidant have been described as follows (Carr and Frei, 1999):

- Both ascorbate and ascorbyl radicals have low reduction potential and can react with most other biologically relevant radicals and oxidants.
- Ascorbyl radicals have a low reactivity as a result of resonance stabilisation of unpaired electrons and readily dismutate to ascorbate and dehydroascorbic acid (DAA).
- Ascorbyl radicals and DAA can be converted into active ascorbate forms by enzyme-dependent or independent pathways. In particular, ascorbyl radicals can

be reduced by NADH-dependent semi-dehydroascorbate reductase or thioredoxin reductase. At the same time, DAA can be reduced to ascorbic acid by GSH, lipoic acid or glutaredoxin.
- Recent data from the epigenetics field indicate that ascorbate could play an important role in the demethylation of DNA and histone. In fact, by regulating the epigenome, ascorbate can be involved in embryonic development, postnatal development and in health maintenance in general (Camarena and Wang, 2016).

Glutathione (GSH) is the most abundant non-protein thiol in avian and mammalian cells, and considered to be an active antioxidant in biological systems, providing cells with their reducing milieu (Meister, 1992). Indeed, GSH is shown to be one of the most important non-enzymatic antioxidants in animals/poultry participating in redox balance maintenance and signalling, regulation of transcription factors and gene expression and many other important pathways/processes, including epigenetic mechanisms (García-Giménez *et al.,* 2017). Cellular GSH plays a key role in many biological processes (Sen and Packer, 2000):
- the synthesis of DNA and proteins;
- cell growth and proliferation;
- regulation of programmed cell death;
- immune regulation;
- the transport of amino acids;
- xenobiotic metabolism;
- redox-sensitive signal transduction.

Furthermore, GSH thiol group can react directly with (Lenzi *et al.*, 2000; Meister and Anderson, 1983):
- H_2O_2;
- superoxide anion;
- hydroxyl radicals;
- alkoxyl radicals;
- hydroperoxides.

Therefore, the crucial role for GSH is as a free radical scavenger, particularly effective against the hydroxyl radical (Bains and Shaw, 1997), as there are no enzymatic defences against this type of radical. Usually, decreased GSH concentration in tissues is associated with increased lipid peroxidation (Thompson *et al.*, 1992). Furthermore, in stress conditions, GSH prevents the loss of protein thiols and vitamin E (Palamanda and Kehrer, 1993) and plays an important role as key modulator of cell signalling (Elliott and Koliwad, 1997). Animals and human are able to synthesise glutathione.

Taurine, a sulphur containing amino acid (2-aminoethane sulfonic acid) is synthesised from methionine and cysteine in the presence of vitamin B6 and found in almost all tissues in mammals. It is the most abundant intracellular amino acid in humans that is not incorporated into proteins. Antioxidant properties of taurine were shown in various model systems *in vivo* and *in vitro* (Cozzi *et al.*, 1995; Haber *et al.*, 2003; Sethupathy *et al.*, 2002; Shimada *et al.,* 2015). It seems likely that taurine does not

function as a classical free radical scavenger, but rather as an indirect antioxidant, able to lower the production of ROS or enhance the other antioxidant defence mechanisms (Shimada *et al.,* 2015). However, the antioxidant effect of taurine is not restricted to PUFAs. For example, Ogasawara *et al.* (1993, 1994) showed an inhibiting effect of taurine against the modification of proteins, as well as an antioxidative effect through the reaction of taurine with aldehydes *in vivo*. It seems likely that in many cases in biological systems taurine can interact with other antioxidants, becoming an important part of an integrated antioxidant system. For example, taurine supplementation of rats restored kidney GSH content and GSH-Px activity and reduced malondialdehyde (MDA) production levels in the kidney tissue following cisplatin treatment (Saad and Al-Rikabi, 2002). In streptozotocin-diabetic rats, dietary taurine supplementation ameliorates MDA levels, GSSG/GSH, and NAD^+/NADH (Obrosova and Stevens, 1999). Taurine was shown to have a protective effect against glutathione antioxidant system deterioration. In fact, it protects tissues against GSH pool depletion and prevents a decrease of glutathione reductase and glutathione peroxidase activities in acute severe hypoxia (Mankovska *et al.*, 1998). Recently, taurine was shown to increase expression of antioxidant enzymes, including HO-1, through increased nuclear factor-erythroid-2-(NF-E2-)related factor 2 (Nrf2) activity and decreased NF-κB-mediated generation of proinflammatory cytokines (Lee *et al.,* 2017). Similarly, taurine could reverse arsenic-inhibited Nrf2 and thioredoxins in rats (Bai *et al.,* 2016).

Uric acid is traditionally considered to be a metabolically inert end-product of purine metabolism in man, without any physiological value. However, this ubiquitous compound has proven to be a selective antioxidant (Becker, 1993; Maples and Mason, 1988) which can:

- react with hydroxyl radicals and hypochlorous acid, itself being converted to innocuous products;
- serve as an oxidisable co-substrate for the enzyme cyclooxygenase;
- protect against reperfusion damage induced by activated granulocytes;
- prevents oxidative inactivation of endothelial enzymes in stress conditions;
- chelate transition metal ions and scavenging of ROS.

Carnitine has been shown to be a new type of antioxidant, regulating free radical production in mitochondria (Surai, 2015,a,b). The antioxidant action of carnitine is related to:

- its direct scavenging of free radicals;
- chelating catalytic metals-promoters of ROS, such as Fe and Cu;
- maintaining mitochondrial integrity in stress conditions and preventing ROS formation;
- inhibiting ROS-generating enzymes, such as XO and HADPH-oxidases;
- affecting redox signalling via activation of Nrf2 and PPARα and inhibition of NF-κB with additional synthesis of antioxidant enzymes (SOD, GSH-Px, glutathione reductase (GR), glutathione S-transferase (GST), CAT, etc.) and small antioxidant molecules (GSH);
- regulating vitagenes and synthesis of heat shock proteins (HSPs), sirtuins, thioredoxins and other antioxidant molecules.

Betaine can also be included into the antioxidant family. Indeed, evidence accumulates showing that betaine also possess antioxidant properties (Alirezaei *et al.,* 2012, 2015; Tsai *et al.,* 2015).

Polyphenolic compounds, including flavonoids, have received tremendous attention as natural antioxidants. However, their direct involvement in antioxidant defences as free-radical scavengers has been questioned (Surai, 2014). In fact, polyphenolic concentrations in target tissues (except gut) are too low to show direct antioxidant activities. However, there are other mechanisms where polyphenolics are involvemed in antioxidant defences. For example, silymarin (SM), an extract from the plant *Silybum marianum* (milk thistle), containing various flavonolignans (with silybin being the major one) has received tremendous attention for the last decade as herbal remedy for treatment of various liver diseases. Possible antioxidant mechanisms of SM include (Surai, 2015c):

- Direct scavenging free radicals and chelating free Fe and Cu are mainly effective in the gut.
- Preventing free radical formation by inhibiting specific ROS-producing enzymes or improving an integrity of mitochondria in stress conditions are of great importance.
- Maintaining an optimal redox balance in the cell by activating a range of antioxidant enzymes and non-enzymatic antioxidants, mainly via hermetic mechanisms of Nrf2 activation is probably the main driving force of anti-oxidant (AO) action of SM.
- Decreasing inflammatory responses by inhibiting NF-κB pathways is an emerging mechanism of SM protective effects in the liver toxicity and various liver diseases.
- Activating vitagenes, responsible for synthesis of protective molecules, including HSP, thioredoxin and sirtuins and providing additional protection in stress conditions deserves more attention.
- Affecting microenvironment of the gut, including SM-bacteria interactions, awaits future investigations.

Some specific enzymes that hydrolyse oxidised bases, preventing their incorporation into DNA, can also be considered as a part of the second level of antioxidant defence (Slupphaug *et al.*, 2003). The role of ubiquinones and uric acid in the farm animal and poultry development has not yet been studied.

However, even the second level of antioxidant defence in the cell is not able to prevent all damaging effects of ROS and RNS on lipids, proteins and DNA. In this case, the third level of defence is based on systems that eliminate damaged molecules or repair them. This level of antioxidant defence includes lipolytic (lipases), proteolytic (peptidases or proteases) and other enzymes (DNA repair enzymes, ligases, nucleases, polymerases, proteinases, phospholipases and various transferases).

Since maintaining the integrity of the genome is of the vital importance, living organisms have evolved a range of systems that can recognise, signal the presence of, and repair various forms of DNA damage. In fact, DNA repair is one of the

fundamental processes of life (130 human DNA repair genes have been identified; Wood *et al.*, 2001) and if the systems are compromised devastating consequences can be expected. In order to deal with the deleterious effects of such lesions, leading to genomic instability, cells have evolved a number of DNA repair mechanisms. They include the direct reversal of the lesion, mismatch repair, base excision repair, nucleotide excision repair, nucleotide incision repair, transcription-coupled repair, global genome repair, homologous recombination and non-homologous end-joining (Gros *et al.*, 2002; Slupphaug *et al.*, 2003). These repair pathways are universally present in living cells and extremely well conserved.

DNA repair systems include a number of enzymatic processes ranging from base recognition and excision to ligation of DNA strands. In particular, the DNA glycosylases recognise damaged purines and pyrimidines and hydrolyse the bond linking the abnormal base to the sugar-phosphate backbone (Halliwell and Gutteridge, 1999); the 5'-apurinic endonucleases process strand breaks, sites of base loss, and the products of DNA glycosylase/apurinic lyase action. DNA polymerase fills in the one-nucleotide gap with the correct base. DNA ligases complete the repair process by sealing the 3' end of the newly synthesised stretch of DNA to the original portion of the DNA chain (Cardozo-Pelaez *et al.*, 2000; Croteau and Bohr, 1997; Wallace *et al.*, 1997).

It is believed that most damaged or inappropriate bases in the DNA are removed by excision repair, while a minority are repaired by direct damage reversal (Krokan *et al.*, 2000). The importance of the DNA repair systems is confirmed by the fact that defects in these systems result in cell death and hypersensitivity to endogenous or environmental mutagens (Jackson, 1999). Therefore, removing mutagenic lesions in DNA is a vital task for repair systems. In general, the DNA repair mechanisms in bacteria are well-defined, whereas in higher eukaryotes the genes and proteins responsible for repair await further investigation (Croteau and Bohr, 1997). It seems likely that DNA repair is integrated with cell cycle regulation, transcription and replication and use some common factors (Slupphaug *et al.*, 2003). However, the enzymes of the third level of antioxidant defence are not able to achieve the complete repair or removal of damaged DNA molecules, and this could lead to cell cycle arrest and cell death. In fact, programmed cell death (apoptosis) is involved in maintenance of the genetic integrity by removing genetically altered cells.

In spite of the important role of protein oxidation in pathogenesis of various diseases, mechanisms for the control of protein oxidation and their repair have not been well studied and this has been a topic of great interest for the last few years. The oxidative damage to proteins is associated with alteration of transport proteins and ion disbalance, disruption to the receptors and impairment of signal transduction, enzyme inactivation, etc. It is believed that conversion of –SH groups into disulphides and other oxidised species (e.g. oxyradicals) is one of the earliest events during the radical-mediated oxidation of proteins. Thioredoxin plus thioredoxin reductase counters these changes by reducing protein disulphides to thiols and regulating redox-sensitive transcription factors (Dean *et al.*, 1997). It is worth mentioning that

the main cellular mechanisms that effectively control protein homeostasis in the cell include (Goloubinoff *et al.*, 2016):

- molecular chaperones, acting as aggregate unfolding and refolding enzymes;
- chaperone-gated proteases, acting as aggregate unfolding and degrading enzymes;
- aggresomes, acting as aggregate compacting machineries; and
- autophagosomes, acting as aggregate degrading organelles.

Among molecular chaperones, HSPs play a very important role. HSPs are highly conserved families of proteins discovered in 1962 (Ritossa, 1962). Later, it was realised that most HSPs have strong cytoprotective effects and are molecular chaperones for other cellular proteins. Taking the current knowledge of the mode of action of HSPs into account, the name 'stress proteins' would be more appropriate for them, but for historical reasons they are still called HSPs. In the case of oxidative stress, the HSP network participates in the detection of intracellular changes, protection against protein misfolding and prevention of downstream activation of events related to inflammation and apoptosis (Kalmar and Greensmith, 2009). Some HSPs are constitutively expressed, whereas others are strictly stress-inducible. Under physiologic conditions, HSPs play an important role as molecular chaperones by promoting the correct protein folding and participating in the transportation of proteins across intracellular membranes and repair of denatured proteins. Therefore, HSPs participate in the regulation of essential cell functions, such as protein translocation, refolding, assembly and the recognition, prevention of protein aggregation, renaturation of misfolded proteins, degradation of unstable proteins, etc. (Zilaee *et al.*, 2014). It should be mentioned that the events of cell stress and cell death are linked and HSPs induced in response to stress appear to function at key regulatory points in the control of apoptosis (Garrido *et al.*, 2001). A key feature of HSPs are their ability to provide cytoprotection. Indeed, in a recently developed model, it was proposed that the HSP-based chaperone machinery played a major role in determining the selection of proteins that have undergone oxidative or other toxic damage for ubiquitination and proteasomal degradation (Pratt *et al.*, 2010).

It is interesting that reversible oxidation of cysteine can be an important cellular redox sensor in some proteins (Finkel, 2000). Methionine residues in proteins are also very susceptible to oxidation by methionine sulfoxide formation, which has been detected in native proteins (Gao *et al.*, 1998). This could affect the activity of various proteins. In fact, almost all forms of ROS oxidise methionine residues of proteins to a mixture of the R- and S-isomers of methionine sulfoxide (Stadtman *et al.*, 2002). Methionine sulfoxide reductase (Msr) can reduce either the free or the protein-bound methionine sulfoxide back to methionine. Therefore, Msr is considered a repair mechanism for products of the reaction of oxidants with methionine residues (Levine *et al.*, 1996). These authors hypothesised that methionine residues function as a 'last chance' antioxidant defence system for proteins. It has been shown that in bacterial glutamine synthetase surface-exposed methionine residues surrounding the entrance to the active site are preferentially oxidised and other residues (e.g. cysteine) within the critical regions of the protein are protected without loss of catalytic activity of the protein (Levine *et al.*, 1996). Indeed, due to Msr activity the methionine-methionine

sulfoxide pair can function catalytically. MsrA is present in most living organisms and encoded by a single gene; the mammalian enzyme has been detected in all tissues studied. In particular, it was found in the cytosol and mitochondria of rat liver cells (Vougier *et al.*, 2003). Msr is considered to have at least three important functions in the cellular metabolism, including antioxidant defence, repair enzyme, and as regulator of certain enzyme functions, and possibly participates in signal transduction (Bar-Noy and Moskovitz, 2002; Stadtman *et al.*, 2002). In particular, mice that lack the MsrA gene (Moskovitz *et al.*, 2001):

- exhibit enhanced sensitivity to oxidative stress;
- have a shorter lifespan;
- develop an atypical walking pattern;
- accumulate higher tissue levels of oxidised protein under oxidative stress;
- are less able to up-regulate expression of thioredoxin reductase under oxidative stress.

MsrA is known for a long time, and its repairing function well characterised, however, recently, a new methionine sulfoxide reductase has been characterised (Grimaud *et al.*, 2001). It was named MsrB and its presence was shown in genomes of eubacteria, archaebacteria, and eukaryotes. Thus, in mammals, two methionine sulfoxide reductases, MsrA and MsrB, are expressed with different substrate specificity (Grimaud *et al.*, 2001). They catalyse the thioredoxin-dependent reduction of the *S*-isomer and *R*-isomer of methionine sulfoxide to methionine, respectively.

Recently, the major mammalian MsrB has been identified as a selenoprotein (Kryukov *et al.*, 2002; Moskovitz *et al.*, 2002). In fact, selenoprotein R is a zinc-containing stereo-specific Msr (Kryukov *et al.*, 2002). Furthermore, it was shown that there is a loss of MsrB activity in $MsrA^{-/-}$ mice in parallel with losses in the levels of MsrB mRNA and MsrB protein (Moskovitz and Stadtman, 2003). Therefore, the authors suggested that MsrA might have a role in MsrB transcription. Moreover, Se deficiency in mouse was associated with a substantial decrease in the levels of MsrB-catalytic activity, MsrB protein, and MsrB mRNA in liver and kidney tissues (Moskovitz and Stadtman, 2003). It has been reported that human and mouse genomes possess three MsrB genes responsible for synthesis of the following protein products: MsrB1, MsrB2 and MsrB3 (Kim and Gladyshev, 2003; Lee, 2016). In particular, MsrB1 (Selenoprotein R) was present in the cytosol and nucleus and exhibited the highest methionine-R-sulfoxide reductase activity due to presence of selenocysteine (Sec) in its active site. The other mammalian MsrBs are not selenoproteins and contain cysteine in place of Sec and are less catalytically efficient (Kim and Gladyshev, 2003; Lee, 2016).

The reduced glutathione itself can also participate in maintenance of protein -SH groups. At the same time the thioredoxin system has alkyl hydroperoxide reductase activity. Protein disulphide isomerase is also involved in re-pairing of -SH groups in proteins (Dean *et al.*, 1997). Furthermore, the cells can generally remove oxidised proteins by proteolysis. In fact, damaged proteins are degraded by the proteasome, multicatalytic proteinase (an intracellular, non-lysosomal threonine type protease, EC

3.4.99.46), which is responsible for degradation of the majority of cytosolic proteins (Rock *et al.*, 1994).

It is well recognised now that the proteasome is the major enzymatic system in charge of cellular 'cleansing' and plays a key role in the degradation of damaged proteins, thereby controlling the level of altered proteins in eukaryotic cells (Friguet *et al.*, 2000). It is suggested that protein unfolding is associated with enhanced susceptibility to degradation by proteinases (Dean *et al.*, 1997), however, heavily oxidised proteins are characterised by an increased resistance to proteolytic attack by most proteinases. The proteasome complex recognises hydrophobic amino acid residues, aromatic residues, and bulky aliphatic residues that are modified during oxidative stress and catalyse the selective removal of oxidatively modified cell proteins (Grune *et al.*, 1997). By minimising protein aggregation and cross-linking and by removing potentially toxic protein fragments, the proteasome forms an active part of the cellular defence system against oxidative stress. The selective degradation of oxidatively damaged proteins enables cells to restore vital proteins, including enzymes during physiological metabolism and during moderate stress conditions (Grune *et al.*, 1997). Oxidised proteins may also be recognised as 'foreign' by the immune system with corresponding antibody formation (Halliwell and Gutteridge, 1999). Clearly, further work is needed to clarify the molecular mechanisms of the third level of antioxidant defence.

All these antioxidants are operating in the body in association with each other forming an integrated antioxidant system. The co-operative interactions between antioxidants in the cell are vital for maximum protection from the deleterious effects of free radicals and toxic products of their metabolism. For example, it is well established that vitamin E is the major antioxidant in biological membranes, a 'headquarter' of antioxidant network. It is usually present in low molar ratios (one molecule per 2,000-3,000 phospholipids), however, vitamin E deficiency is difficult to induce in adult animals. This is probably due to the fact that oxidised vitamin E can be converted back into the active reduced form in reaction with other antioxidants: ascorbic acid, glutathione, ubiquinols or carotenoids (Figure 1.5).

Figure 1.5 shows a connection of the antioxidant defence to the general body metabolism (the pentose phosphate cycle is the major producer of reducing equivalents in the form of NADPH) and the involvement of other nutrients in this process. For example, dietary proteins are a source of essential amino acids for glutathione synthesis; riboflavin is an essential part of glutathione reductase; niacin is a part of NADPH; and Se is an integral part of thioredoxin reductase. At the same time thiamine is required for transketolase in the pentose phosphate pathway.

A recent major finding is the possibility of direct or indirect vitamin E recycling (Surai, 2002, 2006, 2014; Surai and Fisinin, 2014). The rate of regeneration, or recycling, of vitamin E radicals that were formed during their antioxidant action may affect both its efficiency in antioxidant action and its lifetime in biological systems. A greater recycling activity is associated with an increased efficiency of inhibition of

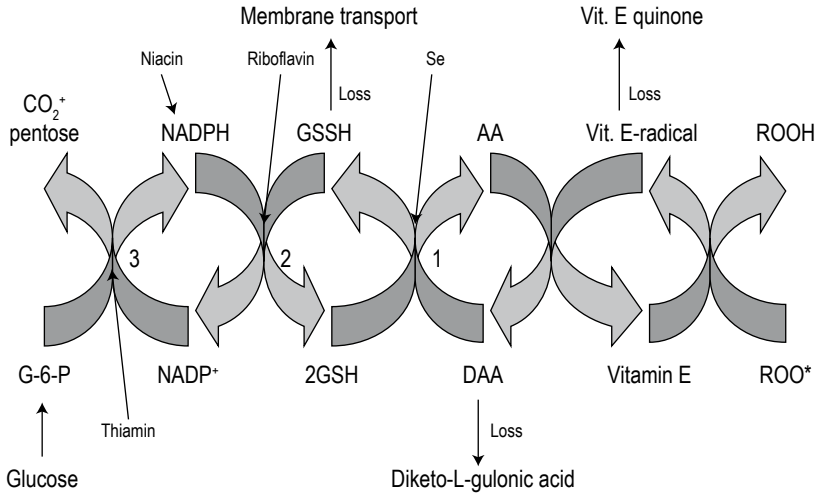

Figure 1.5. Redox cycle of vitamin E (adapted from Surai, 1999; Winkler *et al.*, 1994).As a result of the antioxidant action of vitamin E, an tocopheroxyl radical is formed. This radical can be reduced back to an active form of α-tocopherol by coupling it to ascorbic acid oxidation. Ascorbic acid can be regenerated back from its oxidised form with glutathione, which can receive a reducing potential from NADPH, synthesised in the pentose phosphate cycle of the carbohydrate metabolism. Enzymes involved in vitamin E recycling are: (1) thioredoxin reductase; (2) glutathione reductase; (3) glucose-6-phosphate dehydrogenase. Due to the incomplete regeneration (efficiency of recycling is usually less than 100%) in biological systems, antioxidants have to be obtained from the diet (vitamin E and carotenoids) or synthesised in the tissues (ascorbic acid and glutathione).

lipid peroxidation (Packer, 1995). It seems likely that vitamin E efficacy is very often more dependent on its recycling efficiency than on its concentration *per se*.

Thus, antioxidant protection in the cell depends not only on vitamin E concentration and location, but also relies on effective recycling. Indeed, if recycling is effective, then even low vitamin E concentrations are able to maintain high antioxidant protection under physiological conditions. For example, this was demonstrated using a chicken brain as a model system. Data (Surai, 2002) indicated that the brain was characterised by extremely high concentrations of long chain PUFAs predisposing this tissue to lipid peroxidation. Furthermore, the brain contained much lower levels of vitamin E than the other body tissues. However, in fresh chicken brain, the levels of products of lipid peroxidation were very low, which could be a reflection of an effective vitamin E recycling by ascorbic acid, which is present in this tissue in comparatively high concentration. Antioxidant recycling is the most important element in understanding mechanisms involved in antioxidant protection against oxidative stress. The rate of regeneration, or recycling, of the vitamin E radicals may affect both its antioxidant efficiency and its lifetime in biological systems.

Living cells permanently balance the process of formation and inactivation of ROS and as a result ROS levels remain low but still above zero. Adverse environmental conditions

initiate attempts of organisms to resist the aggressive environment (Skulachev, 1998). Cells can usually tolerate mild oxidative stress by additional synthesis of various antioxidants (glutathione, antioxidant enzymes, etc.) in an attempt to restore antioxidant/oxidant balance. At the same time, energy expenditures increased and respiration activated leading to the increased yield of ROS (Skulachev, 1998). However, these adaptive mechanisms have limited ability to deal with overproduction of free radicals. Once the free radical production exceeds the ability of the antioxidant system to neutralise them, lipid peroxidation develops and causes damage to unsaturated lipids in cell membranes, amino acids in proteins and nucleotides in DNA, and, as a result, membrane and cell integrity is disrupted. Membrane damage is associated with a decreased efficiency of absorption of different nutrients and leads to an imbalance of vitamins, amino acids, inorganic elements and other nutrients in the organism. All these events result in a decreased productive and reproductive performance of animals. Immunity incompetence and unfavourable changes in the cardio-vascular system, brain and neurones, and muscle system due to increased lipid peroxidation make the situation even worse. The antioxidant defence includes several options (Surai, 2015,a,b,c,d, 2016; Surai and Fisinin, 2014, 2015):

- decrease localised oxygen concentration;
- decrease activity of prooxidant enzymes (carnitine, silymarin);
- improve efficiency of electron chain in the mitochondria and decrease electron leakage leading to superoxide production (carnitine);
- induction of various transcription factors (e.g. Nrf2, NF-κB and others) and antioxidant response element (ARE)-related synthesis of AO enzymes (SOD, GSH-Px, CAT, GR, GST, etc.);
- binding metal ions (metal-binding proteins) and metal chelating;
- decomposition of peroxides by converting them to non-radical, nontoxic products (Se-GSH-Px);
- chain breaking by scavenging intermediate radicals, such as peroxyl and alkoxyl radicals (vitamins E, C, GSH, uric acid, carnitine, ubiquinol, bilirubin, etc.);
- repair and removal of damaged molecules (methionine sulfoxide reductase, DNA-repair enzymes, chaperons, etc.);
- redox signalling and vitagene activation with synthesis and increased expression of protective molecules (GSH, thioredoxins, SOD, HSPs, sirtuins, etc.);
- antioxidant recycling mechanisms, including vitamin E recycling;
- apoptosis activation and removal of terminally damaged cells, and restriction of mutagenesis.

As shown above, all antioxidants in the body are working as a 'team' responsible for antioxidant defence and we call this team the antioxidant system. In this team one member helps the other working efficiently. In general, vitamin E and coenzyme Q are considered to be the 'headquarter' of the antioxidant defences, while Se is a 'chief executive', as from the 25 known selenoproteins, more than half participate in antioxidant defences. Furthermore, a central role in antioxidant system regulation belongs to vitagene expression and additional synthesis of protective molecules during stress conditions ('ministry of defence') in order to improve the adaptive ability to stress (Figure 1.6; Surai, 2017).

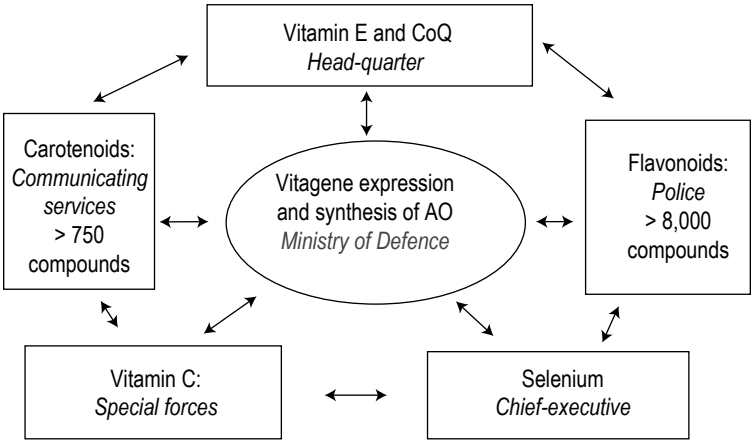

Figure 1.6. Antioxidant system.

Therefore, if relationships in this team are effective, which happens only in case of a balanced diet and sufficient provision of dietary antioxidant nutrients, then even low doses of antioxidants, such as vitamin E, can be effective. On the other hand, when this team is subjected to high stress conditions, free radical production is increased dramatically. During these times, without external help it is difficult to prevent damage to major organs and systems. This 'external help' is dietary supplementation with increased concentrations of natural antioxidants. For a nutritionist or feed formulator it is a great challenge to understand when the internal antioxidant team in the body requires help, how much to provide of this help, and what the economic return will be. Again, it is necessary to remember the essentiality of keeping the right balance between free radical production and antioxidant defence (Figure 1.7). Indeed, ROS and RNS have another more attractive face by participating in the regulation of a variety of physiological functions.

In physiological conditions the right and left parts of the so-called 'balances' are in equilibrium i.e. free radical generation is neutralised by the antioxidant system. Exogenous factors are among the most important elements that increase the efficiency of the antioxidant system of an organism. Natural and synthetic antioxidants in feed, as well as optimal levels of Mn, Cu, Zn and Se help maintain efficient levels of endogenous antioxidants in the tissues. Optimal diet composition allows antioxidants in feed to be efficiently absorbed and metabolised. Optimal temperature, humidity and other environmental conditions are also required for the effective protection against free radical production. The prevention of different diseases by antibiotics and other drugs is an integral part of efficient antioxidant defence as well.

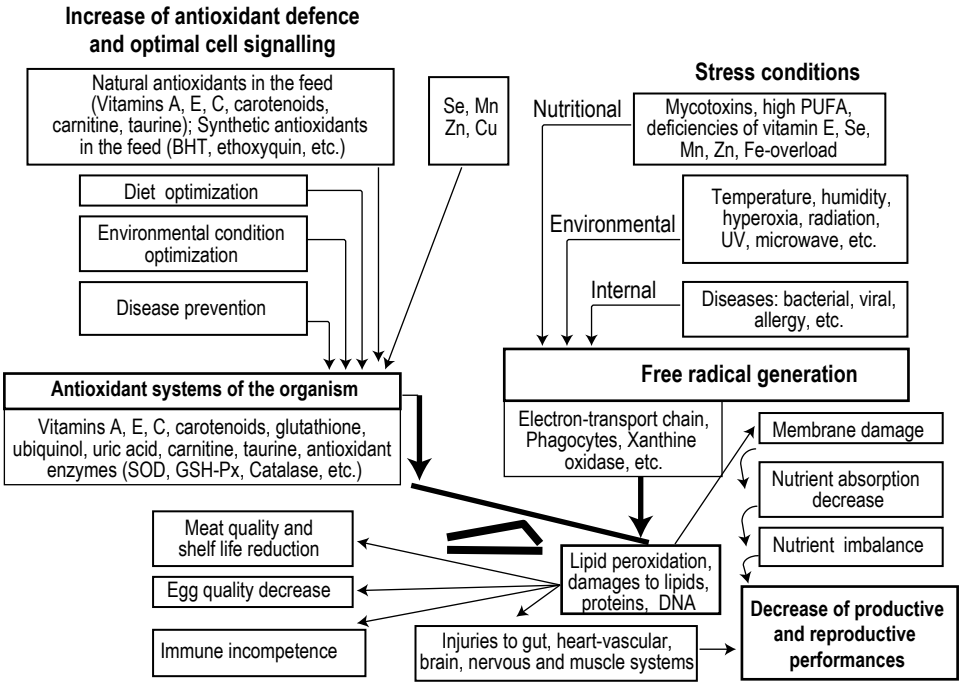

Figure 1.7. Anti-oxidant vs prooxidant balance in the organism (adapted from Surai, 1999).

Different stress conditions are associated with overproduction of free radicals and cause oxidative stress (Surai and Fisinin, 2016a,b,c,d), i.e. a disturbance in the prooxidant vs antioxidant balance leading to potential tissue damage (Jaeschke, 1995). Stress conditions can be generally divided into four main categories: nutritional, environmental, technological and internal stress (Surai and Fisinin, 2016a,b,d; Table 1.4).

The most important category are nutritional stress conditions, including high levels of PUFAs; deficiencies of vitamin E, Se, Zn or Mn; Fe overload; hypervitaminosis A; presence of different mycotoxins and other toxic compounds; low water quality; and usage of coccidiostats or other drugs via feed or water. A second stress category includes environmental conditions: inadequate temperature, humidity or lightning, inadequate ventilation and increased dust, etc. A third category includes technological stress factors, such as chick placement, increased stocking density, weighing, grading, group formation, catching, transferring to breeder houses, prolonged egg storage, egg transportation, inadequate egg storage conditions, incorrect incubation regimes, etc. The fourth group includes internal stress factors, such as vaccination, microbial or viral challenges, gut disbacteriosis, pipping and hatching (Surai and Fisinin, 2016b).

Table 1.4. Stresses in poultry production (adapted from Surai and Fisinin, 2016a,b).

Technological stress	• Chick placement
	• Increased stocking density
	• Weighing, grading, group formation, catching, transferring to breeder houses
	• Prolonged egg storage, egg transportation, inadequate egg storage conditions, incorrect incubation regimes
Environmental stress	• Inadequate temperature
	• Inadequate ventilation and increased dust
	• Inadequate lightning
Nutritional stress	• Mycotoxins
	• Oxidised fat
	• Toxic metals (lead, cadmium, mercury, etc.)
	• Imbalance of minerals (Se, Zn, Cu, etc.) and other nutrients
	• Low water quality
	• Usage of coccidiostats and other drugs via feed or water
Internal stress	• Vaccinations
	• Microbial or virus challenges
	• Gut disbacteriosis
	• Pipping and hatching

1.7 Oxidative stress and transcription factors

It is important to mention that ROS are no longer viewed as just toxic by-products of mitochondrial respiration, but are now appreciated for their role in regulating a myriad of cellular signalling pathways (Reczek and Chandel, 2015). It has been suggested that the signalling ROS are produced in a subtly regulated manner, while many deleterious ROS are produced and react randomly (Niki, 2014). Therefore, it is unlikely that nutritional antioxidants detrimentally affect physiologically important signalling functions, since the antioxidants do not scavenge signalling ROS/RNS nor do they inhibit the formation of signalling molecules (Niki, 2012, 2016). Recent evidence suggests that several selenoproteins could participate in cell signalling. In particular, selenoprotein W and six other small thioredoxin-like mammalian selenoproteins (SelH, SelM, SelN, SelT, SelV and Sep15) may serve to transduce hydrogen peroxide signals into regulatory disulfide bonds in specific target proteins (Hawkes and Alkan, 2010). Similarly, GSH-Px and TrxR are also involved in cellular redox balance regulation (Labunskyy *et al.,* 2014).

Oxidation-reduction (redox) based regulation of gene expression is a fundamental regulatory mechanism in cell biology acting at the cell-signalling level. In fact, redox signalling is the overlap of signal transduction with redox biology. Redox signalling is essential in physiological homeostasis and alterations in redox signalling are observed in stress conditions and aging; sustained deviation from redox homeostasis results in disease (Forman, 2016), and decreased productive and reproductive performance

of poultry. Since ROS are damaging to many biological molecules, the antioxidant systems are responsible for the prevention of this damage. However, a basal level of oxidative stress is essential for cell adaptation and survival. Therefore, a moderate level of oxidative stress can create adaptive responses and improve the adaptive ability to stressful challenges/conditions (Yan, 2014). Indeed, in animals, redox-signalling pathways use ROS as signalling molecules to activate genes responsible for regulation of various functions, including growth, differentiation, proliferation and apoptosis. Furthermore, the antioxidant defence systems are also under regulation by various transcription factors (Kweider *et al.,* 2014; Ma and He, 2012; Majzunova *et al.,* 2013; Song and Zou, 2014). In fact, the redox balance is controlled by a battery of transcriptional factors, including Nrf2, NF-κB, PPARs, PGC-1a, p53, FoxO, MAPK, AP-1, etc. (Lushchak, 2011; Wang and Hai, 2016). They regulate redox status by modulating ROS-generating enzymes and antioxidant enzymes in a cooperative and interactive way. In recent years great attention has been paid to basic leucine zipper transcription factor, Nrf2, NF-κB and peroxisome proliferator-activated receptors (PPARs).

1.7.1 Transcription factor Nrf2

It is known that Nrf2 is the redox-sensitive master regulator of oxidative stress signalling and stress response, and critical for cell survival under stressful conditions (Itoh *et al.,* 2010). It has been shown that the Nrf2 antioxidant response pathway is an important player in the cellular antioxidant defence. Indeed, it is responsible for activation of a variety of genes involved in early defence reactions of higher organisms (Ma, 2013; Van der Wijst *et al.,* 2014). High expression of Nrf2 in organs that face environmental stress, including lungs and the small intestine (Itoh *et al.,* 2015), is a confirmation of its importance in stress adaptation processes. Clearly, Nrf2 has a significant role in adaptive responses to oxidative stress, being involved in the induction of the expression of various antioxidant molecules to combat oxidative and electrophilic stress (Howden, 2013; Keum and Choi, 2014; Tang *et al.,* 2014; Vriend and Reiter, 2015).

It is suggestive that under normal physiological conditions, Nrf2 is kept in the cytoplasm as an inactive complex with the negative regulator, Kelch-like-ECH-associated protein 1 (Keap1), which is anchored to the actin cytoskeleton. In fact, Keap1 sequesters Nrf2 in the cytoplasm and forwards it to a Cul3-based E3 ligase which is followed by rapid ubiquitin-proteasome degradation leading to a short (about 20 min) half-life of Nrf-2 under physiological conditions (for review see Choi *et al.,* 2014). It seems likely that, Keap-1 is an important cellular redox sensor and upon exposure to oxidative or electrophilic stress, critical cysteine thiols of Keap1 are modified/oxidised and Keap1 loses its ability to ubiquitinate Nrf2 resulting in preventing its degradation. There are also other ways of Nrf2 activation. For example, phosphorylation of Nrf2 at specific serine and/or tyrosine residues also causes an Nfr2-Keap1 dissociation resulting in Nrf2 release and translocation to nucleus, where it combines with a small musculoaponeurotic fibrosarcoma protein called Maf to form a heterodimer (Bhakkiyalakshmi *et al.,* 2015). Indeed, by binding to ARE in the

upstream promoter region of genes encoding various antioxidant molecules, Nrf2 regulates the expression of antioxidant proteins, thiol molecules and other protective molecules. This includes enzymes of the first line of the antioxidant defence, namely SOD, GSH-Px and catalase, detoxification enzymes (HO-1, NQO1, and GST), GSH-related proteins (γ-GCS), NADPH-producing enzymes and others stress-response proteins contributing to the prevention of oxidative and inflammatory damage (Lee *et al.,* 2013; Zhou *et al.,* 2014). In fact, hundreds of cytoprotective genes are regulated by Nrf2 (Itoh *et al.,* 2015) and gene products (proteins) are involved in the maintenance and responsiveness of the cellular antioxidant systems. Indeed, an orchestrated change in gene expression via Nrf2 and ARE is a key mechanism of the protective effect against oxidative stress (Lee *et al.,* 2003). It is suggestive that Nrf2 is controlled through a complex transcriptional/epigenetic and post-translational network that provides regulatory mechanisms ensuring Nrf2 activity increases in response to redox disturbances, inflammation, growth factor stimulation and nutrient/energy fluxes orchestrating adaptive responses to diverse forms of stress (Hayes and Dinkova-Kostova, 2014). As mentioned above, there is a range of Nrf2 activating mechanisms, including stabilisation of Nrf2 via Keap1 cysteine thiol modification and phosphorylation of Nrf2 by upstream kinases (Surh, 2008; Surh *et al.,* 2008). It is proven that effects of Nrf2 on the adaptive ability of cells is quite broad and goes beyond activation of synthesis of antioxidant molecules. Indeed, Nrf2 also contributes to homeostasis by up-regulating the repair and degradation of damaged macromolecules, and by modulating intermediary metabolism conducting directs metabolic reprogramming during stress (Zhou *et al.,* 2014).

Recently molecular mechanisms of regulating roles of transcription factors in cellular adaptation to stress have been extensively studied. In particular, it has been suggested that low intensity oxidative stress is predominantly sensed by the Keap1/Nrf2 system (Lushchak, 2011) followed by downstream up-regulation of the protective AO genes. It is interesting to note that intermediate oxidative stress also increases the activity of antioxidant enzymes, but mainly via NF-κB and AP-1 pathways (Lushchak, 2011). Furthermore, at both, low and intermediate intensity oxidative stresses, MAP-kinases and other kinases seem to be involved in signal sensing and cellular response, leading to enhanced antioxidant potential (Zhou *et al.,* 2014). Emerging evidence clearly indicates that Nrf2 can interact with other transcription factors, including heat shock factor (Hsf1; Dayalan Naidu *et al.,* 2015) to provide additional options for AO system regulation. As mentioned above, the Nrf2 stress pathway intimately communicates with mitochondria to maintain cellular homeostasis during oxidative stress (Itoh *et al.,* 2015).

1.7.2 Transcription factor nuclear factor-kappa B

NF-κB is an inducible transcription factor that regulates many cellular processes including immunity and inflammation. NF-κB consists of a group of five related proteins that are capable of binding to DNA. This transcription factor is activated by a wide range of stimuli including oxidative stress. It has been shown that NF-κB regulates the transcription of many different genes, including pro-inflammatory

cytokines and leukocyte adhesion molecules, acute phase proteins and anti-microbial peptides (Buelna-Chontal and Zazueta, 2013; Pedruzzi *et al.,* 2012; Tkach *et al.,* 2014). There are some similarities in regulation of Nrf2 and NF-κB. For example, in physiological conditions, NF-κB is found in cytoplasm in an inactive state associated with the inhibitory IκB (inhibitor of kappa B) proteins preventing its binding to target sites. It has been proven that activation of NF-κB is an effective mechanism of host defence against infection and stress (Pal *et al.,* 2014). As a result of action of cytokines and other stressors, IκB proteins are rapidly phosphorylated by IκB kinase on specific serine residues, followed by ubiquitination, and degradation by the 26S proteasome. The following release of NF-κB and its translocation to the nucleus is responsible for the transcription of target genes, for cell survival, and involved with inflammation, immunity, apoptosis, cell proliferation and differentiation (Hayden and Ghosh, 2014). NF-κB transcription factors, such as p65, can combine to form hetero- and homo-dimers of different composition, providing a tool for effective regulation of different sets of gene targets (Grilli and Memo, 1997). There is a range of additional stimuli implicated into the NF-κB activation including, cell-surface receptors, inhibitory κB kinases, IB proteins, and factors that are involved in the posttranslational modification of the Rel proteins, etc. (Buelna-Chontal and Zazueta, 2013; Hayden and Ghosh, 2014; Pal *et al.,* 2014; Pedruzzi *et al.,* 2012; Tkach *et al.,* 2014). Accumulating evidence indicates that there is a complex interplay/crosstalk between Nrf2 and NF-κB pathways. For example, several Nrf2 activators can inhibit NF-κB pathway. NF-κB may also directly antagonise the transcriptional activity of Nrf2 (for review see Tkach *et al.,* 2014). In recent years, several compounds, including LC, have been shown to have inhibitory activities against multiple components of NF-κB activation pathway.

1.7.3 Transcription factors peroxisome proliferator-activated receptors

PPARs are a group of three nuclear receptor isoforms, PPARγ, PPARα, and PPARδ/β, identified in the 1990s in rodents and named after their property of peroxisome proliferation (Kota *et al.,* 2005). PPARs are ligand-regulated transcription factors that control gene expression by binding to specific response elements (PPREs) within promoters and they affect various important cellular events including proliferation, differentiation, and apoptosis (Berger and Moller, 2002). PPARs are shown to form a heterodimer with retinoid-X receptor (RXR) and bind a peroxisome proliferator response element (PPRE) on target genes (Nakamura *et al.,* 2014). It is proven that PPARs control expression of various genes that are crucial for lipid and carbohydrate metabolism being 'master' transcriptional regulators of nutrient metabolism and energy homeostasis that modulate the expression of unique constellations of genes (Berger *et al.,* 2005). In particular, PPARγ is considered to be the master transcription factor for adipogenesis, while PPARα mainly distributes in tissues with a high efficiency of mitochondrial fatty acid oxidation, which highly expresses in the liver, whereas PPARβ/δ expression is found to be highly expressed in the small intestine and keratinocyte (Neels and Grimaldi, 2014). It seems likely that expression levels of PPARs are subject to regulation by diets and nutrient status in a tissue-dependent manner, and the activities of PPARα and PPARγ can be regulated by phosphorylation (Juge-Aubry *et al.,* 1999). It is also known that the three PPAR members share a high

degree of homology, however, they differ in tissue distribution, ligand specificity, and physiological roles (Berger and Moller, 2002). In fact, all three PPARs play essential roles in lipid and fatty acid metabolism by directly binding to and modulating genes involved in fat metabolism (Fan and Evans, 2015). Recently, a considerable number of papers have reviewed PPARs importance in the regulation of various physiological and biochemical processes within the body (Giordano Attianese and Desvergne, 2015; Musso *et al.,* 2010; Ndisang, 2014; Zhang *et al.,* 2015) and also the evolutionary pattern and regulation characteristics of PPARs have been analysed (Zhou *et al.,* 2015). In particular, PPARα is activated by adiponectin and could inhibit the NF-κB pathway, while PPAR (gamma) enhances insulin action, free fatty acid oxidation, adiponectin secretion, and inhibits secretion of proinflammatory cytokines (Musso *et al.,* 2010). It seems likely that PPAR signalling is a part of the body's antioxidant system playing an important role in various stress conditions. In fact, the antioxidant effect of PPARα has been shown and PPAR-responsive elements (PPREs) have been identified in the promoter regions of several antioxidant genes, including catalase and Cu^{2+}/Zn^{2+}-SOD. Therefore, PPARα can bind to PPREs to promote the expression of antioxidants to inhibit oxidative stress (Khoo *et al.,* 2013; Kim and Yang, 2013; Li *et al.,* 2012; Liu *et al.,* 2012) having a regulatory effect over the production of oxidative, proinflammatory and profibrotic mediators (Diep *et al.,* 2002). Furthermore, induction of PPARα by PPARα-agonist WY14643 treatment ameliorated the oxidative stress and severity of liver injury and restored expression of genes altered by ethanol treatment (Kong *et al.,* 2011).

A synergistic relationship between PPAR-signalling and the HO-system exists related to the regulation of various physiological functions. For example, PPARs suppress inflammation/oxidative stress and attenuate excessive immune responses, while agonists of PPARγ and PPARα have been shown to upregulate the HO-system. At the same time, the HO-system can enhance PPARα, and potentiates the expression and activity of PPARγ. Similar to PPARs, the HO-system has been shown to suppress inflammation/oxidative stress and modulate immune response (Ndisang *et al.,* 2014).

1.8 Vitagene concept development

Since at the molecular level most stresses are associated with overproduction of free radicals and oxidative stress, the development of effective antioxidant solutions to decrease negative consequences of commercially-relevant stress is an important task for poultry scientists. One of such approaches is based on the possibility of modulation of vitagenes, a family of genes responsible for animal/poultry adaptation to stress. The term 'vitagene' was introduced in 1998 by Rattan who wrote 'our survival and the physical quality of life depends upon an efficient functioning of various maintenance and repair processes. This complex network of the so-called longevity assurance processes is composed of several genes, which may be called vitagenes' (Table 1.5).

Later, the vitagene concept has been further developed in medical sciences by professor Calabrese and colleagues in 2004-2016. In accordance with Calabrese *et*

Table 1.5. Major components of the 'vitagene' network (adapted from Calabrese *et al.*, 2007; Rattan, 1998; Surai, 2015d).

Molecular level	**Cellular level**
AO defence	Cell proliferation
DNA-repair systems	Cell differentiation
Transfer of genetic information	Stability of cell membrane
Stress protein synthesis	Stability of intracellular milieu
Proteosomal function	Macromolecular turnover
Tissue and organ level	**Physiological and redox control level**
Neutralisation and removing toxic chemicals	Stress response
Tissue regeneration and wound healing	Hormonal response
Tumour suppression	Immune response
Cell death and cell replacement	Thermoregulation
	Neuronal response

al. (2007, 2009, 2014) the term vitagenes refers to a group of genes that are strictly involved in preserving cellular homeostasis during stress conditions. The vitagene family includes HSPs, including haeme oxygenase-1 (HSP32, HO-1) and HSP70, the thioredoxin system and sirtuins. The list of potential candidates to vitagene family was later further extended. In particular, SOD, a major inducible enzyme of the first level of antioxidant defence, has been included into the vitagene family (Surai, 2016; Surai and Fisinin, 2016e). The products of the mentioned genes actively operate in detecting and controlling diverse forms of stress and cell injuries.

The cooperative mechanisms of the vitagene network were in detail considered in recently published reviews. It was proven that the vitagene network in cell and whole organism plays an essential regulatory role of adaptation to various stresses. Indeed, cellular stress response is mediated via the regulation of pro-survival pathways and vitagene activation followed by synthesis of a range of protective antioxidant molecules is the central event in such an adaptation. The vitagene concept helped to develop effective strategies to fight oxidative stress in various human diseases, including neurodegenerative disorders, neuroprotection, aging and longevity, dermatology, osteoporosis and Alzheimer pathology, and other free radical-related diseases (for a review see Surai and Fisinin, 2016e). Indeed, HSPs, including HO-1 and HSP70, are responsible for protein homeostasis under stress conditions in poultry production (Surai, 2015d), while the thioredoxin system is the major player in maintaining redox status of cells involved in protein and DNA synthesis and repair, as well as in regulation of expression of many important genes (Surai and Fisinin, 2016e). Furthermore, sirtuins regulate the biological functions of various molecules post-translationally by removing acetyl groups from protein substrates, ranging

from histones to transcription factors, and orchestrate cellular stress response by maintenance of genome integrity and protein stability. Finally, SODs belong to the first level of antioxidant defence preventing lipid and protein oxidation at the very early stages (Surai, 2016). All the aforementioned vitagenes operate in concert building a reliable system of stress detection and adequate response and are considered to be key elements in stress adaptation.

Recently, the vitagene concept has been successfully transferred from medical to poultry science (Fisinin and Surai, 2011a,b; Surai, 2015a,b,c,d; Surai and Fisinin, 2012a,b, 2015). The new concept of fighting stress is based on the idea that supplying birds with various antioxidants through drinking water could help them to effectively deal with stress conditions. Indeed, a decreased feed consumption at time of stress is observed and existing feeding systems do not allow to include anything into the feed loaded into the feed storage bins. Therefore, water-soluble additive supplementation via the drinking system is shown to be a valuable option. In fact, modern commercial poultry houses are equipped with water medication systems, which can be effectively used for the aforementioned supplementations. It has been proven that inclusion of vitagene-regulating compounds (carnitine, betaine, vitamin E, etc.) as well as some minerals, vitamins, electrolytes and organic acids in water, could be effective in fighting various stresses (Fisinin and Surai, 2011a,b; Surai and Fisinin, 2012a,b). This helps at chick placement, when the antioxidant system is crucial for the digestive and immune system development (Fisinin and Surai, 2012a; Surai and Fisinin, 2015). In particular, it was proven in an university-conducted trial that inclusion of anti-stress composition (PerforMax) into the drinking water improved chicken growth and feed conversion ratio (FCR; Fotina *et al.,* 2011, 2014). Using the same anti-stress composition under commercial conditions improved FCR during a 39 day broiler growth trial. The improvement in FCR due to the anti-stress composition during the first three days post-hatch, as well as before and after vaccination was highly significant (Velichko and Surai, 2014; Velichko *et al.,* 2013). The importance and efficacy of the anti-stress composition for rearing birds and adult egg type parent stock (Hy-Line) at one of the biggest egg producing farms in Russia (Borovskaya poultry farm, Tumen region) have been recently reviewed (Shatskich *et al.,* 2015).

In particular, it was shown that adding the anti-stress composition to drinking water at specific periods of the increased stress could improve breeder's performance. Egg peak production increased by 2%, and the peak plateau lasted about 50 days longer than that in the control birds. It is interesting to note that hen housed egg production in the control group (260.8 eggs) was higher than the target for the line (253.4 eggs) and in the experimental group this was increased by 6 eggs. Furthermore, improved egg production was associated with increased weight of the oviduct in the experimental layers. It is also important to mention that FCR (feed per 10 eggs) was improved when using the anti-stress composition and better than the target for the line. Notably, shell strength at age 26, 36 and 56 weeks was improved in the experimental group by 2.8, 5.6 and 5.6%, respectively. The most interesting finding was related to a significant increase in the carotenoid level in the egg yolk of experimental birds. Since carotenoids were not supplied with the anti-stress composition, this increase could be

due to improved absorption of nutrients resulting from anti-stress composition usage. This can also explain the improved FCR in the experimental birds. The vitamin A level in egg yolk from the experimental group was also increased, probably reflecting its transfer from the anti-stress composition. In particular, anti-stress composition usage was associated with improved fertility at 16, 40, 48 and 56 weeks by 2.5, 2.7, 2.8 and 3.7%, respectively. In the same experimental group the hatch of viable chicks improved at 26, 32, 40, 48 and 56 weeks by 3.6, 2.1, 3.4, 4.9 and 4.3%, respectively (Shatskih *et al.,* 2015). In addition, it was shown that the anti-stress composition had an immune-modulating effect in broilers (Fotina *et al.,* 2011) and growing ducklings (Surai *et al.,* 2012). Improvement of the antioxidant system through the supply of an antioxidant composition via drinking water could also help dealing with various mycotoxins in feed, including deoxynivalenol (Fisinin and Surai, 2012b,c), ochratoxin (Fisinin and Surai, 2012d,e) and T-2 toxin (Fisinin and Surai, 2012f,g). Furthermore, such a technology could help fight heat stress (Surai and Fotina, 2013) and immunosuppression (Fisinin and Surai, 2013a,b). However, further work is required to understand the molecular mechanisms of the interactions of vitagenes with various signalling systems and transcription factors in the cell to build an adequate adaptive response to minimise detrimental consequences of commercially-relevant stress in poultry production.

1.9 Conclusions

Antioxidant-prooxidant balance in the cell is an important determinant of various physiological functions. Indeed, oxidative stress occurs when this balance is disturbed due to overproduction of free radicals or compromised antioxidant defences. Free radical overproduction and oxidative stress are considered as a pathobiochemical mechanism involved in the initiation or progression phase of various diseases. In animal production free radial generation and lipid peroxidation are responsible for the development of various diseases, as well as for the decrease of animal productivity and product quality. Dietary antioxidants may be especially important in protecting against the development of the oxidative stress.

In general, ingestion of excessive amounts of antioxidants is presumed to shift the oxidant-antioxidant balance toward the antioxidant side. This is supposed to be beneficial; however, this may also adversely affect key physiological processes that are dependent on free radicals, including prostaglandin production, cell division and differentiation. Recent evidence suggests that cellular oxidation can induce changes in gene expression during normal development. Conversely, antioxidants, such as ascorbate, glutathione, α-tocopherol or carotenoids are inhibitory to differentiation in many types of cells.

Free radicals are now considered to take part in signal transduction in the cell and at least two well-defined transcription factors, NF-κB and AP-1, are regulated by the intracellular redox state. The regulation of gene expression by oxidants, antioxidants, and redox state has emerged as a novel subdiscipline in molecular biology that has

promising implications for the feed industry and animal production. Thus the redox state of the cell, which reflects antioxidant/prooxidant balance, can be considered as an important element of gene regulation. Therefore, the effect of antioxidants on animal health is much deeper than one could expect several years ago. The mechanisms by which natural antioxidants act at the molecular and cellular level include roles in gene expression and regulation, apoptosis and signal transduction, and antioxidants are involved in fundamental metabolic and homeostatic processes. However, there are still many gaps in our knowledge of the basic molecular mechanisms of oxidative damage and antioxidant defence.

Indeed, a range of selenoproteins (see Chapter 2) is involved in the regulation of key elements of antioxidant defence. By maintaining optimal activities of antioxidant enzymes, such as GSH-Px, TrxR and MsrB, as well as directly participating in regulation of DNA-repair enzymes Se belongs to all three major levels of antioxidant defence. Furthermore, Se also interacts with other antioxidants, such vitamin E, ascorbic acid and glutathione. That is why I would call selenium the 'chief executive of antioxidant defences'. In the next chapters information will be presented proving that Se is a key to effective antioxidant defence and to maintaining animal and human health. Indeed, 'diet cures more than the lancet'.

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Chapter 2
Molecular mechanisms of selenium action: selenoproteins

Diet cures more than the lancet

2.1 Introduction

It is generally accepted that in biological systems Se participates in various physiological functions as an integral part of a range of selenoproteins. In fact, it is well known that sulphur and selenium occur in proteins as constituents of the amino acids cysteine, methionine, selenocysteine, and selenomethionine. The redox activity of those amino acids under physiological conditions allows a wide variety of posttranslational protein modifications, metal free redox pathways, and unusual chalcogen redox states. For example, unlike any other amino acid, cysteine and SeCys can participate in several distinct redox pathways, including exchange and radical reactions, as well as atom-, electron-, and hydride-transfer reactions. Furthermore, the position of selenium in the periodic table between the metals and the non-metals makes selenoproteins effective catalysts for many biological redox transformations (Surai, 2006).

2.2 The selenoprotein family

In avian species, 25 different SeCys-containing selenoproteins have been identified (Li and Sunde, 2016; Mariotti *et al.*, 2012). Recently, a family of chicken selenoproteins has been updated to include 24-25 genes responsible for selenoprotein synthesis (Lei, 2017; Zhao *et al.*, 2017). Of these, glutathione peroxidases (GSH-Px), thioredoxin reductases (TrxR) as well as selenoprotein P are known to directly detoxify oxidants. Furthermore, selenoprotein R (MsrB1) reduces free and protein-bound oxidised Met. In general, selenoproteins are involved in regulation of many different physiological and biochemical processes, including:

- glutathione-dependent hydroperoxide removal;
- reduction of thioredoxins;
- selenophosphate synthesis;
- activation and inactivation of thyroid hormones;
- repair of oxidised methionine residues; and
- endoplasmic reticulum associated protein folding and degradation.

This explains the important role of selenium in animal health, including roles in antioxidant defence, immune system regulation and other functions.

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2.3 Selenocysteine: the functional selenium

As mentioned above, the main effects of Se in animals, including chickens, are caused by specific SeCys-containing selenoproteins. In fact, SeCys is considered to be the 21st amino acid, and understanding selenoprotein synthesis has substantially helped with understanding the genetic code (Figure 2.1). Indeed, in 1970th as a great surprise to many scientists, it was found that in mammalian and avian species UGA served as both a terminal codon and a SeCys codon (Hatfield *et al.*, 1970). Selenocysteine (Sec) tRNA was described in the same year (Mäenpää *et al.*, 1970). The dual role of the UGA codon confounds the identification of novel selenoprotein genes. The presence of SeCys in the catalytic site of selenium-dependent antioxidant enzymes enhances the kinetic properties and broadens the catalytic activity of enzymes against biological oxidants, when compared to sulphur-containing enzymes. Therefore, SeCys is unique among proteinogenic amino acids in that it is the only one containing an essential dietary micronutrient (selenium) that requires complex tRNA-dependent synthetic machinery for its synthesis, delivery to the ribosome, and insertion into the nascent selenoprotein. In fact, selenium availability and stress are the major factors that determine selenoprotein expression in various chicken tissues. Synthesis of selenoproteins is associated with insertion of SeCys into the primary protein structure during translation, and SeCys incorporation at UGA codons requires cis-acting mRNA secondary structures and several specialised trans-acting factors. The latter include a selenocysteine-specific tRNA, an elongation factor specific for this tRNA and a SECIS-binding protein, SBP2, which recruits the elongation factor to the selenoprotein mRNA (for review see Hatfield *et al.*, 2016; Surai, 2006).

Expression of individual eukaryotic selenoproteins is characterised by high tissue specificity, depends on Se availability, can be regulated by hormones, and if compromised contributes to various pathological conditions (Hatfield *et al.*, 2016; Surai, 2006). Selenoprotein synthesis includes:

- charging of serine onto a specialised tRNA, $tRNA^{Sec}$;
- selenocysteine synthase catalyses the seryl- to selenocysteyl-conversion on the $tRNA^{Sec}$;
- SeCys cotranslationally incorporated into selenoprotein active centres.

All selenoprotein genes have two characteristic features: (1) an UGA codon that designates Sec; and (2) a Sec insertion sequence element (Gladyshev *et al.*, 2016). Most selenoprotein mRNAs (but not selenoprotein P) contain a single UGA codon encoding a single SeCys residue per polypeptide chain and a single specific RNA secondary structure, termed a selenocysteine insertion sequence (SECIS) element, that directs incorporation of SeCys. Therefore, the SECIS element is an RNA hairpin in the 3'UTR of selenoprotein mRNAs required for decoding UGA selenocysteine codons. In general, there is a strong hierarchy in selenoprotein expression in various tissues with the brain being more conservative in terms of Se manipulation than other tissues (Pillai *et al.*, 2014). Furthermore, within the selenoprotein family also a hierarchy exists in selenoprotein expression under various conditions, including Se deficiency. For example, the GI-GSH-Px and PH-GSH-Px are most preferentially

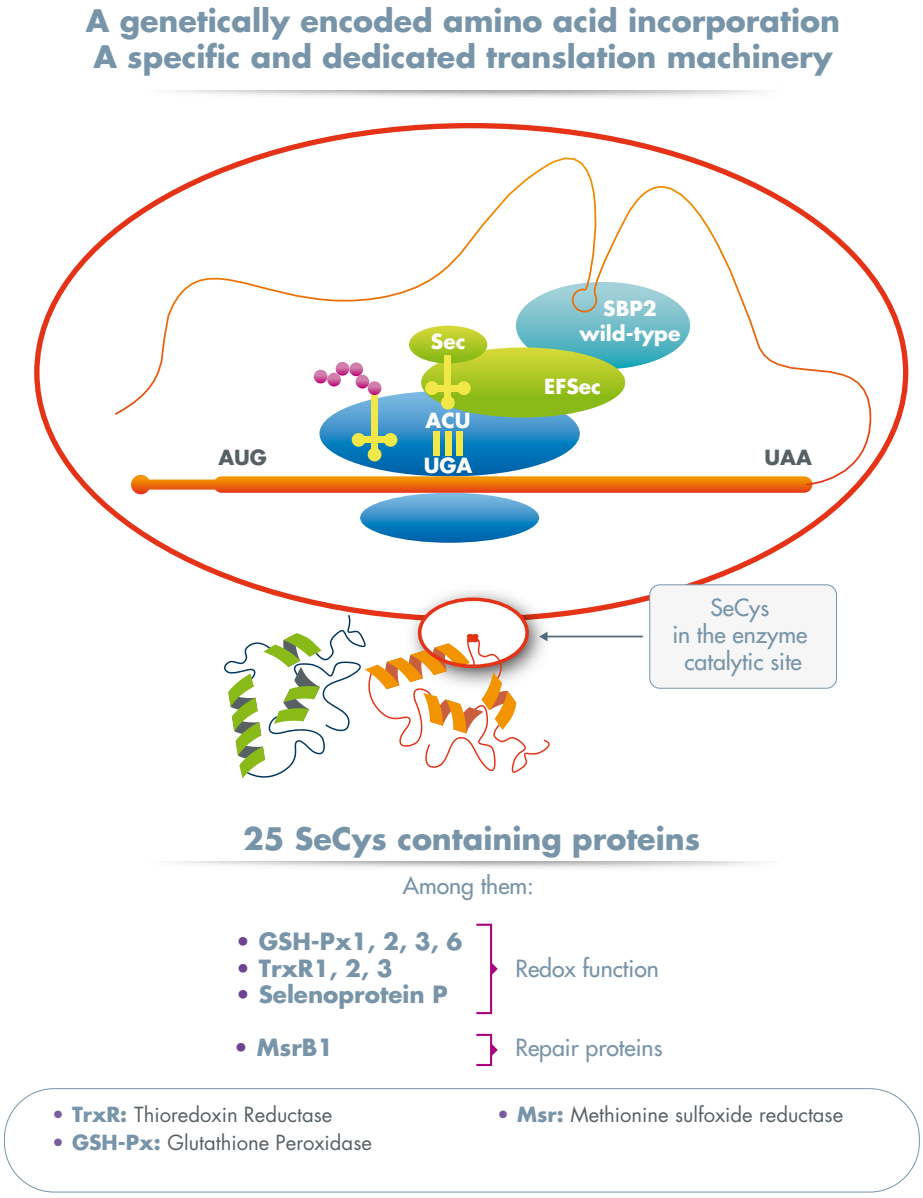

Figure 2.1 Seleniumcysteine – the 21st amino acid.

supplied with Se compared to other members of the GSH-Px family. This kind of regulation has important implications for the development of signs of Se deficiency. In fact, selenoproteins that are less affected by Se-deficiency are considered to be more physiologically important than others (Flohe and Brigelius-Flohe, 2016).

All known up-to-date specific selenoproteins can be divided into two distinct groups depending on the location and functional properties of SeCys (Gladyshev, 2016):

- Most of selenoproteins, including GSH-Px, belongs to the first group (sometimes called GSH-Px group) which includes proteins in which SeCys is located in the N-terminal region or the middle of the protein portion of a functional domain.
- The selenoproteins belonging to the second group, contain a redox-active SeCys located in the C-terminal sequences and include TrxRs, selenoproteins K, S, O and I.

Table 2.1 summarises known eukaryotic/avian selenoproteins and in this chapter a brief overview of selenoproteins that have been functionally characterised will be provided. Readers are referred to individual chapters in the recent book by Hatfield *et al.* (2016) for additional information on various selenoproteins. Glutathione peroxidase (GSH-Px) and thioredoxin reductase (TrxR) are the most abundant antioxidant Se-containing proteins in mammals (Gladyshev, 2016). Major characteristics of GSH-Px and TrxR are shown in Table 2.2 and 2.3 (for a review see Surai *et al.*, 2018a,b).

2.4 Glutathione peroxidases

Glutathione peroxidase (GSH-Px) is the first described selenoprotein and is considered to be the most important element of the antioxidant defence system of the cell and of the body in general (Figure 2.2). The PubMed search conducted on November 14th 20016 on the 'glutathione peroxidase' gave 31,191 publications, including more than 1,600 references from 2016. Therefore, the interest in this subject is really tremendous. It is important to note that old biological textbooks, written 20 or more years ago, include only one GSH-Px, but the GSH-Px family includes at least 8 members and 5 of them are Se-dependent forms of the enzyme. They differ in molecular weight, substrate specificity, cell distribution and perform a variety of functions. There are 4 different glutathione peroxidases in avian species, namely GSH-Px1, GSH-Px2, GSH-Px3 and GSH-Px4. General characteristics of various GSH-Px proteins are shown in Table 2.2.

2.4.1 Cytosolic glutathione peroxidase (cGSH-Px or GSH-Px1).

Glutathione peroxidase (glutathione H_2O_2 oxidoreductase E.C. 1.11.1.9) was discovered by Mills in 1957, who showed that this enzyme had a protective effect in erythrocytes against H_2O_2- or ascorbate-induced haemolysis. Sixteen years later it became clear that GSH-Px was a selenoenzyme (Flohe *et al.*, 1973; Rotruck *et al.*, 1973). In fact, Rotruck *et al.* (1973) were the first to show that in rat red cells Se was tightly bound to the enzyme and demonstrated the uptake of ^{75}Se by GSH-Px. In the same year, Flohe *et al.* (1973) reported that bovine GSH-Px contained one Se atom per subunit. It is interesting that 1973 was a year of Se discoveries, since in that year

Table 2.1. Characteristics of human selenoproteins (adapted from Kryukov, 2003; Mariotti *et al*., 2012; Pappas *et al.*, 2008).

Protein	Protein length	SeCys position	Cellular distribution/tissues/species	Functions
Cytosolic GSH-Px (GSH-Px1)	201	47	cytosol	AO protection
GI-GSH-Px (GSH-Px2)	190	40	gastrointestinal tract	AO protection
pGSH-Px (GSH-Px3)	226	73	extracellular space and plasma	maintenance of cellular redox status
PH-GSH-Px (GSH-Px4)	197	73	cell membrane, many other tissues	detoxification of lipid hydroperoxides
Cytosolic TrxR1 (TrxR1)	499	498	cytosol, liver, kidney, heart	part of the Trx system, AO defence, redox regulation, cell signalling
Mitochondrial TrxR2 (TrxR2)	523	522	mitochondria, liver kidney	part of the Trx system, AO defence, redox regulation, cell signalling
TR3 (testicular) (TGR, TrxR3)	656	655	Testes	part of the Trx system, AO defence, redox regulation, cell signalling
Iodothyronine deiodinase 1 (Dio1)	249	126	many tissues like liver, kidney, thyroid	conversion of T4 to T3 and T4 to reverse T3
Iodothyronine deiodinase 2 (Dio2)	265	133	liver, kidney, thyroid, brown adipose tissue	conversion of T4 to T3
Iodothyronine deiodinase 3 (Dio3)	278	144	placenta, brain, skin, (not in pituitary, thyroid, adult liver)	conversion T4 to reverse T3
Selenoprotein H (SepH, SelH)	122	44	widely distributed	upregulation of genes involved in GSH synthesis
Selenoprotein I (Sell,SepI)	397	387	widely distributed	lipid metabolism
Selenoprotein K (Selke, SepK)	94	92	cardiomyocytes	possible AO protection in cardiomyocytes
Selenoprotein M (SelM, SepM)	145	48	brain and other tissues	distantly related to Sel15.
Selenoprotein N (SelN, SepN)	556	428	endoplasmatic reticulum	it is linked with rigid spine syndrome
Selenoprotein O (SelO, SepO)	669	667	widely distributed	unknown
Selenoprotein P (SepP, SepP1)	381	59 [a]	plasma, other tissues	involved in Se transport, AO defence
Selenoprotein Pb			plasma, other tissues	unknown
Methionine-R-sulfoxide reductase 1a (MsrB1, SelR, SelX)	116	95	cytosol, nucleus	reduction of oxidised methionine residues in damaged proteins
Selenoprotein S (SelS, SepS)	189	188	endoplasmatic reticulum	cellular redox balance, possible influence of inflammatory response
Selenophosphate synthetase 2a (SPS, SPS2, SPS2a)	448	60	testes, many other tissues	synthesis of selenophosphate
Selenoprotein T (SelT, SepT)	182	36	ubiquitous	role in regulation of Ca^{2+} homeostasis and neuroendocrine secretion
Selenoprotein U (SelU, SepU1)			fish and chicken, but not higher eukaryotes	unknown
Selenoprotein W (SelW, SepW1)	87	13	muscle, heart and other tissues	antioxidant protection
15-kDa Selenoprotein (Sel15, Sep15)	162	93	endoplasmatic reticulum	antioxidant protection

[a] All positions of SeCys in SeP: 59, 300, 318, 330, 345, 352 367, 369, 376, 378.

[b] AO = anti-oxidant; GSH = glutathione; TrxR = thioredoxin reductase.

Table 2.2. Se-dependent glutathione peroxidase characteristics (adapted from Surai, 2006).[1]

Glutathione peroxidase	Nomenclature	Localisation	Subunit size (kDa)	Substrates	Electron donors	Other characteristics
Cytosolic GSH-Px	GSH-Px1	intracellular, cytosolic, partly mitochondria	21.9	H_2O_2, t-BHP	GSH	Erythrocytes, kidney and liver
Gastrointestinal GSH-Px	GSH-Px2	intracellular, cytosolic	21.9	H_2O_2, t-BHP	GSH	mucosal epithelial cells in GIT
Extracellular (plasma) GSH-Px	GSH-Px3	plasma	25.5	H_2O_2, t-BHP, phospholipid hydroperoxides	GSH, thioredoxin, gluta-redoxin	expressed in kidney
Phospholipid hydroperoxide GSH-Px	GSH-Px4	intracellular, partly cytosolic, mitochondrial, membrane-bound	22.1	H_2O_2, phospholipid hydroperoxides	GSH, DTT, 2-ME, L-cys	renal epithelial cells and testes

[1] t-BHP = tret-butylhydroperoxide; DTT = 1,4-ditiothreitol; 2-ME = 2-mercaptoethanol; L-cys = L-cysteine.; GIT = gastrointestinal tract; GSH = glutathione.

Table 2.3. Total glutathione peroxidase (GSH-Px) activity in the liver of various animals, U/mg protein (adapted from Tappel *et al.*, 1982).

Animal	GSH-Px activity	Animal	GSH-Px activity
Chicken	33	White mouse	468
Cattle	70	Ground squirrel	49
Sheep	64	Cat	67
Rat	245	Dog	20
Mouse	476	Rainbow trout	0.9
Guinea pig	12	Blue gill sunfish	3.4
Hamster	920	Carp	143
Rabbit	496	Fence lizard	22
Gerbil	683	American toad	2
Wild house mice	446	Western newt	1.5

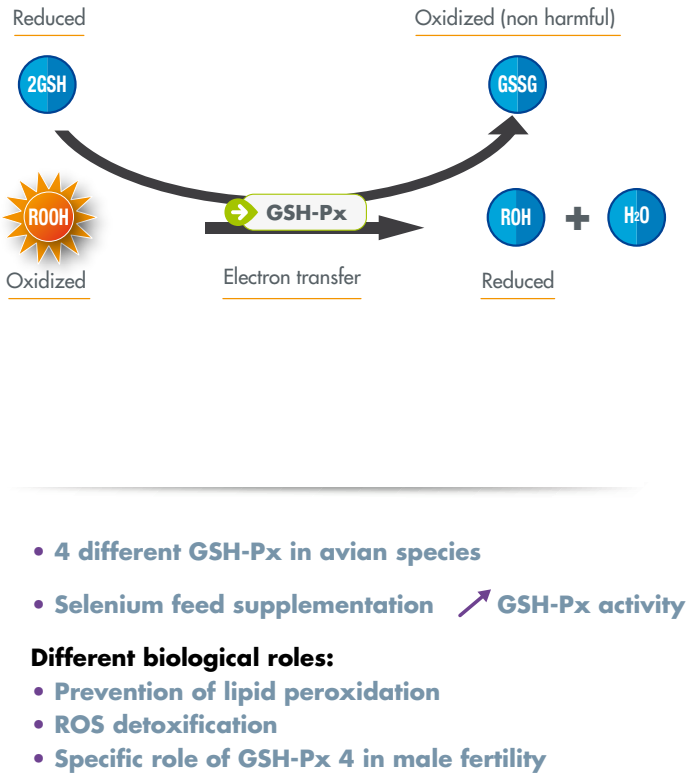

Figure 2.2 Glutathione peroxidase – an important enzyme of the antioxidant system.

selenoproteins also were described in micro-organisms. GSH-Px is responsible for detoxification of hydroperoxides and hydrogen peroxide in the following reactions:

$$ROOH + 2GSH \xrightarrow{\text{GSH-Px}} ROH + GSSG + H_2O$$

$$H_2O_2 + 2GSH \xrightarrow{\text{GSH-Px}} GSSG + 2H_2O$$

These reactions employ a ping-pong mechanism. In particular, SeCys in the active centre of the enzyme is oxidised with selenenic acid formation, which is reduced back in a reaction with 2 molecules of GSH. The Se atom in the enzyme catalytic site undergoes a redox cycle, while reduction of H_2O_2 and organic peroxides take place (Mugesh and Singh, 2000). The selenolate, is oxidised to selenenic acid, which reacts with GSH to form a selenosulfide adduct with the following reaction with GSH to regenerate the active form of the enzyme.

GSH-Px detoxifies peroxynitrite in the following reaction (Sies *et al.*, 1998):

$$ONOO^- + 2GSH \xrightarrow{\text{GSH-Px}} ONO^- + GSSG + H_2O$$

Glutathione reductase (GR) is responsible for a reduction of oxidised glutathione back to a reduced form:

$$GSSG + NADPH + H^+ \xrightarrow{\text{GR}} 2GSH + NADP^+$$

GSH-Px is characterised by high specificity for GSH as a donor of a reducing equivalent (substrate) and catalyses the reduction of a variety of hydroperoxides. However, its activity is related only to free peroxides and it is not able to reduce esterified fatty acid hydroperoxides. Therefore, in biological system hydroperoxides in membranes have to be released by other enzymatic systems (e.g. phospholipases) or another member of GSH-Px (PH-GSH-Px) can deal with them.

GSH-Px activity is dependent on the Se status of tissues. In fact, dietary Se supplementation has been shown to be effective in increasing GSH-Px in a variety of animal species including rat, mouse, chicken, quail, sheep, cattle, horse, pig, deer, salmon, etc. (Flohe and Brigelius-Flohe, 2016). On the other hand, there is a range of nutritional means of decreasing GSH-Px activities in various tissues including vitamin E excess, deficiencies of iron, zinc, riboflavin, vitamin B_6 or copper, as well as consumption of silver, tri-o-cresyl phosphate or doxorubicin (Surai, 2006). In fact, depending on concentration and duration of exposure various chemical elements and compounds can either decrease or increase GSH-Px activity in tissues. When Se is available, increased GSH-Px activity could be a compensatory mechanism to deal with stress conditions. An oxygen-responsive element was identified in the 5'-flanking region of the cGSH-Px gene (Flohe and Brigelius-Flohe, 2016). The earliest response in the specific activity of selenium-dependent GSH-Px occurred in chicken plasma at 8 hours and in liver at 24 hours after selenium administration (Bunk and Combs,

1980). It is important to mention that GSH-Px was found in the chicken egg proteome (Mann and Mann, 2008).

GSH-Px activity was shown to have species- and tissue-specificity. For example, GSH-Px in the mouse leg muscle was shown to be almost 10-fold higher than that in the chicken muscle (Omaye and Tappel, 1974). A comprehensive study of GSH-Px in various tissues of different animals was conducted by Tappel *et al.* (1982) and data on GSH-Px activity in various animal species are shown in Table 2.3).

It was shown that Guinea pig is the only species in which the selenoenzyme was virtually absent in most tissues. Also of interest is the fact that the selenium-dependent and selenium-independent enzymes were present in about equal amounts in rat liver. However, in the hamster liver non-Se-GSH-Px was practically absent, while in the guinea pig liver non-Se-GSH-Px activity was 5-fold higher than that of Se-GSH-Px. It is especially interesting to note the relative order of activities in the liver of various species: hamster > rabbit ~ mouse > rat. Among the various tissues, liver has the highest total GSH-Px activity. The total GSH-Px activities found in the study for chicken liver, heart and lung were 33, 27 and 10 nmol NADPH oxidised/min/mg protein, respectively. It is interesting to note that the GSH-Px activities of the stomach and intestine were similar in magnitude to those of the heart and lung. Concerning phylogenetic relationships, those animals of lower development, trout, sunfish, newt, toad and lizard, were shown to have low total GSH-Px activities in their tissues. In contrast, the rodent limb of the phylogenetic tree was characterised by the highest concentrations of GSH-Px in liver and kidney. Between these extremes are the tissues of animals of higher phylogenetic order including cattle, sheep, cats and dogs (Tappel *et al.*, 1982). Twenty years later, Chiaradia *et al.* (2002) determined lymphocyte GSH-Px activity, plasmatic GSH levels and the effect of H_2O_2 on the responsiveness of lymphocytes to proliferative stimuli. Among the three species considered, sheep presented the lowest plasmatic GSH and the highest lymphocyte GSH-Px activity. On the contrary, dogs showed an inverted pattern (high GSH – low GSH-Px). Horses displayed intermediate values for both parameters analysed. In particular, high rate of peroxide production in tissues, such as liver, kidney or lung, was associated with high GSH-Px activity. GSH-Px activity in chicken, duck and turkey muscles [*m. pectoralis* (breast), *m. gastrocnemius* and *m. peroneus longus* (thigh)], ostrich (steak and fillet) and lamb muscles [*m. longissimus dorsi* (LD), *m. psoas major* (PM)] were measured (Daun and Akesson, 2004). It was shown that the activity of GSH-Px varied more than 5-fold among the muscles from different species. The highest activity, found in duck muscles, was significantly higher than that in all other muscles. Moreover, lamb PM had a significantly higher GSH-Px activity than chicken and turkey breast and ostrich fillet. Another interesting comparison can be made between oxidative and glycolytic muscles for each species. In the oxidative muscles of chicken, duck, lamb and turkey, GSH-Px activities were significantly higher than those of the glycolytic muscles (Daun and Akesson, 2004). It is interesting to note that GSH-Px was shown to be 2.5-fold higher in duck embryo liver in comparison to chicken (Jin *et al.*, 2001) or 15-fold higher in the postnatal duck muscle in comparison to chicken muscle (Hoac *et al.*, 2006), while GSH-Px activity in chicken meat was almost 2-fold lower

than that in camel or cattle meat (Gheisari and Motamedi, 2010). It is important to mention that GSH concentration in the duck liver (5.66 mmol/g) was shown to be more than 2-fold higher than that in the chicken (1.91mmol/g) or goose (2.42 mmol/g) liver (Mezes *et al.*, 1998). In the liver of emperor penguins, GSH-Px activities were 2-3 times higher than those in other avian species (Zenteno-Savin *et al.*, 2010). GSH-Px activities in the liver of rat, chicken, lizard and frog were as follows 36, 6; 23.2; 14.3 and 9.2 μmol NADPH/min/g, respectively (Venditti *et al.*, 1999). In turkey, GSH-Px3 expression was high in all tissues except kidney, while GSH-Px1 expression was high in kidney (Sunde *et al.*, 2015). Whole blood GSH-Px in chicken reduced by age (Chadio *et al.*, 2015). The endogenous rhythm of GSH-Px in five regions (hippocampus, hypothalamus, striatum, cortex and cerebellum) of chick brain was studied. In particular it was shown that GSH-Px exhibited a marked 24 h rhythm with peak activity in each brain region which had acrophases about 8 hours after lights off and about 4 hours after the serum melatonin peak was detected (Pablos *et al.,* 1998). Furthermore, the exposure of chicks to constant light for 6 days eliminated the melatonin rhythm as well as the peaks in glutathione peroxidase.

This enzyme has a wide cell distribution and is present in mitochondria and the cytosol. In fact, in rat liver 75% of the enzyme was found in the cytosol and 25% in mitochondria (Flohe and Brigelius-Flohe, 2016). It is interesting that GSH-Px was also detected in peroxisomes (Singh *et al.*, 1994). The ranking order of tissue-specific stability of cGSH-Px is as follows (Flohe and Brigelius-Flohe, 2016): brain >> thymus > thyroid > heart > liver, kidney, and lung.

GSH-Px activity is dependent of Se dietary supply. For example, compared with Se-adequate rats, rats fed the basal amino acid diet retained only 1, 6, 4 and 9% of the Se-adequate GSH-Px activity in liver, heart, kidney and lung, respectively (Lei *et al.*, 1995). Therefore, GSH-Px activity ultimately depends on Se provision in the diet. In an experiment by Kim and Combs (1993), chicks produced from hens marginally deficient in Se and vitamin E were used. The hepatic activity of Se-GSH-Px was significantly greater in Se-adequate chicks than in Se-deficient ones, which was about 20% of the control level. When an experiment of the same design was conducted using chicks produced from hens that had been depleted of Se and vitamin E for a longer period of time (9 months), the hepatic activity of Se-GSH-Px of chicks in that treatment group was about one-fifth of the activity observed for the same dietary treatment in the previous experiment. In fact, in this experiment, Se- and vitamin E-deficient chicks showed only 8% of the hepatic Se-GSH-Px activities of Se- and vitamin E-fed controls. At 6 weeks of age, the hepatic activities of Se-GSH-Px in Se- and vitamin E-deficient chicks was 12% of that of Se- and VE-fed controls (Kim and Combs, 1993). There are substantial differences among different forms of GSH-Px with regard to response to Se deficiency (Flohe and Brigelius-Flohe, 2016). The selenoproteins retained in tissues for longer periods during progressive Se deficiency are considered to have higher physiological significance in comparison to those whose activities rapidly decline. In this respect, the main GSH-Px forms rank as follows (Flohe and Brigelius-Flohe, 2016): GI-GSH-Px > PH-GSH-Px > Plasma GSH-Px = Cytosolic GSH-Px. In fact, recently it has been suggested that GSH-Px, Txnrd1, SELP,

and SPS2 play a more important role than the other selenoproteins in poultry (Luan *et al.*, 2016).

To evaluate the role of GSH-Px in the body metabolism two approaches have been used, namely over-expression of GSH-Px in various types of cells and animals, and GSH-Px 'knockout' models. Mice deficient in cellular GSH-Px (GSH-Px knockout mice) were created in various laboratories by gene-targeting technology (Ho *et al.*, 1997, 2002). It is interesting that alteration of GSH-Px1 expression showed no impact on the expression of other selenoproteins and antioxidant enzymes in unstressed mice (Ho, 2002; Lei, 2001). To the surprise of many researchers and to the disappointment of those who considered GSH-Px as a 'wonder enzyme', the first experiments with GSH-Px knockout models showed that the contribution of GSH-Px1 to the cellular antioxidant mechanism under normal animal development and physiological conditions is very limited. Indeed, mice deficient in GSH-Px were apparently healthy. Their tissues exhibited neither a retarded rate in consuming extracellular hydrogen peroxide, nor an increased content of protein carbonyl groups and lipid peroxidation compared with those of wild-type mice (Ho *et al.*, 1997). Therefore, these mice developed normally and showed no marked pathologic changes under normal physiologic conditions (Ho *et al.*, 1997). Remarkably enough, a deficiency in these genes had no effects on animal survival under hyperoxia. It was shown that kidney GSH-Px1 mRNA levels and liver, kidney, lung, and testis total GSH-Px activities were affected by the GSH-Px1 knockout and dietary Se concentrations (Cheng *et al.*, 1998). In contrast, kidney extracellular or plasma GSH-Px3 mRNA levels and PH-GSH-Px4 activities in the four tissues were affected by only dietary Se concentrations. Therefore, GSH-Px1 is expressed independently of GSH-Px3 or GSH-Px4 and represents approximately 60% of the total hepatic Se in Se-adequate mice (Cheng *et al.*, 1997).

In general, studies of GSH-Px1 knockout mice lead De Haan *et al.* (2003) to conclude that GSH-Px1 functions as the primary protection against acute oxidative stress, particularly in neuropathological situations, such as stroke and cold-induced head trauma, where high levels of ROS occur during reperfusion or in response to injury. The GSH-Px knockout model is not perfect, since it is an unusual situation when only one selenoprotein is affected. For example, in the case of Se-deficiency a whole range of selenoproteins would be affected and therefore physiological changes in the body would be quite different from those observed in this model system. However, clearly, this model has helped understanding the crucial role of classical GSH-Px as an important antioxidant devise effective in various stress conditions. A review of results based on overexpression of GSH-Px (Surai, 2006) indicated that overexpression of GSH-Px is associated with an increased protection against oxidative stress, created as a result of various environmental or nutritional manipulations. Indeed, GSH-Px is well-regulated by enzymes and its increased activity can be considered as an additional protective mechanism in stress conditions. Most of research related to GSH-Px activity in poultry was exclusively related to GSH-Px1 and only in a few studies GSH-Px4 was determined, while GSH-Px2 and GSH-Px3 still await investigation in avian species.

In newly hatched chickens, the highest GSH-Px activity was found in liver and kidney, with intermediate activity in the heart, lung and yolk sac membrane (YSM) and comparatively low activity shown in muscles and brain (Surai *et al.*, 1999). In all the tissues, Se-dependent GSH-Px was the main enzymic form, comprising from 65% (lung) up to 90% (heart) of the total enzyme activity. It is interesting to note that the highest Se concentration was found in the YSM, intermediate Se concentration in liver and kidney, while lower Se concentration was a characteristic of other tissues studied (Surai *et al.*, 1999). The specific activity of GSH-Px in embryonic liver increased continuously during the second half of the *in vivo* developmental period, so that the activity at hatching was 3.0 times greater than that at day 10 (Surai, 1999). The most rapid increase occurred between days 11 and 15 with a much more gradual increase thereafter. The expression of GSH-Px in the YSM also increased (by 1.8 times) between days 11 and 15 but decreased thereafter. By contrast, the specific activity of GSH-Px in the brain was very low and relatively constant through the second half of embryonic development. Thus, by the time of hatching, the specific activity of the enzyme in the liver was 6.1 times greater than that in the brain. GSH-Px activities in the embryonic kidney, lung, heart and skeletal muscle were measured between day 15 of embryonic development and 1 day after hatching. During this period, the enzyme specific activity in the kidney increased gradually by 1.6 fold; the amount in the heart remained approximately constant; the specific activity in skeletal muscle decreased gradually by 30%; the specific activity in lung decreased by 27% between days 15 and 19, then increased by the same amount by day 1 after hatching (Surai, 1999). It was shown that GSH-Px activity in the prenatal normoxic lung demonstrated a sharp increase between day 16 and day 18 and remained constant until hatch (Starrs *et al.*, 2001). It is interesting to note that GSH-Px activity demonstrated great differences between normoxic and hypoxic conditions. In fact, under hypoxic conditions, GSH-Px activity was constant from day 14 to pip, and then increased significantly from pip to hatch. GSH-Px activity in hypoxic chicken embryos was elevated above normoxic values for day 14, day 16 and hatch (Starrrs *et al.*, 2001). According to GSH-Px activity tissues of 35 day old chickens can be placed in the following descending order: liver >> kidney > plasma = erythrocytes >> femoral muscle >> pectoral muscle. Dietary organic Se supplementation (0.3 mg/kg) was associated with increased GSH-Px activity in plasma, erythrocytes, liver, leg muscles, breast muscles and kidney by 44, 33, 30, 22, 11 and 10%, respectively (Arai *et al.*, 1994). In the chicken liver Se-dependent GSH-Px comprises about a half (48%) of total activity of the enzyme (Engberg *et al.*, 1996).

GSH-Px has been found to be expressed in chicken seminal plasma and spermatozoa (Surai *et al.*, 1998a,b). There are species-specific differences in activity and distribution of GSH-Px in avian semen. For example, in seminal plasma total GSH-Px activity was the highest in turkey and lowest in duck and goose (Surai *et al.*, 1998a). In spermatozoa, on the other hand, the highest GSH-Px activities were found for goose and duck and much lower GSH-Px activity was recorded for guinea fowl, turkey or chicken. In seminal plasma, the activity of GSH-Px was two times greater in the White Koluda ganders than in chickens (Partyka *et al.*, 2012). A process of freezing and thawing fowl semen was associated with increased GSH-Px activity in the seminal plasma (Partyka *et al.*, 2012a). It has also been shown that despite a high proportion of

PUFAs and a low level of vitamin E, duck spermatozoa have the same susceptibility to lipid peroxidation as chicken spermatozoa (Surai *et al.*, 2000b). It has been suggested that an increased activity of Se-GSH-Px in duck semen compensates for the relatively low concentrations of other antioxidants. If selenium is limiting in the diet (which is the case in many countries in the world), then dietary supplementation of this trace element should have a beneficial effect on the antioxidant defence in various tissues including sperm. This was confirmed in our studies. Inclusion of Se in the diet of male chickens significantly increased Se-GSH-Px activity in the liver, testes, spermatozoa and seminal plasma (Surai *et al.*, 1998c). As a result, a significant decrease in the sperm's and tissue susceptibility to lipid peroxidation was observed. This protective effect was more expressed in stored semen as compared to fresh. In this respect, it is extremely important that an inducible form of the enzyme (Se-GSH-Px) represents more than 75% of the total enzymatic activity in chicken spermatozoa and more than 60% in the testes and liver of cockerels. Increased GSH-Px activity in the UVJ compared to other regions of the lower oviduct (vagina, uterus) could be related to a necessity of AO defence during sperm storage in sperm-storage glands (Breque *et al.*, 2006).

2.4.2 Phospholipid glutathione peroxidase (GSH-Px4, PH-GSH-Px)

In 1985 Ursini and co-workers reported that another form of GSH-Px, which used a phosphatidyl choline hydroperoxide as a substrate (PH-GSH-Px, GSH-Px4), was Se-dependent. They showed that the enzyme was a monomer of 23 kDa. It contained 1 g of Se/22,000 g protein. Se was found here in the selenol form. The kinetic data were compatible with a tert-uni ping-pong mechanism, as in the case of the 'classical' glutathione peroxidase. The second-order rate constants (K_1) for the reaction of the enzyme with the hydroperoxide substrates indicated that, while H_2O_2 is reduced faster by the cGSH-Px, linoleic acid hydroperoxide is reduced faster by PH-GSH-Px. The authors suggested that this enzyme was active at the interface of the membrane and the aqueous phase of the cell. PH-GSH-Px is distinguished from classical GSH-Px as it is active in monomeric form and has a different amino acid composition (Sunde, 1993). In particular, arginine residues surrounding the reaction centre, which are responsible for a productive binding of GSH in GSH-Px1, are missing in PH-GSH-Px (Mauri *et al.*, 2003).

There are three different forms of PH-GSH-Px. It is synthesised as a long form (L-form; 23 kDa) and a short form (S-form, 20 kDa) from mRNA that is transcribed from two initiation sites in exon Ia of PH-GSH-Px genomic DNA (Imai and Nakagawa, 2003). S-form PH-GSH-Px is the nonmitochondrial PH-GSHPx and L-form PH-GSH-Px is the mitochondrial PH-GSH-Px. Recently, a third form of PH-GSH-Px, a 34 kDa selenoprotein, was detected in rat sperm nuclei and called sperm nuclei GSH-Px (snGSH-Px). However, in chicken there is no snGSH-Px (Bertelsmann *et al.*, 2007).

The PH-GSH-Px is unique in its capability of reducing ester lipid hydroperoxides even if they are incorporated in biomembranes or lipoproteins. For other members

of GSH-Px family, preliminary release of peroxides from the membrane by such enzymes as phospolipase C is an essential part of detoxification.

It is well known that PH-GSH-Px is widely expressed in normal tissues, and especially high in the testis (Imai *et al.*, 1995), where it has an important role in spermatogenesis and sperm function and is under gonadotropin control. In this organ, the relevant PH-GSH-Px activity is strongly linked to mitochondria of cells that undergo differentiation to spermatozoa. In testis mitochondria, PH-GSH-Px is electrostatically bound to the inner surfaces of the organelle (Roveri *et al.*, 1994). There was no difference between the soluble and the mitochondrial enzyme in terms of chromatographic properties, the electrophoretic mobility, the reactivity to antibodies and the fragmentation patterns and substrate specificity. However, two-dimensional electrophoresis followed by immunostaining with monoclonal antibodies, showed the presence of multiple isoforms with a different pattern between the soluble and the mitochondrial enzyme (Roveri *et al.*, 1994). It is interesting that PH-GSH-Px is localised in the midpiece of spermatozoa in various species, including *Drosophila melanogaster*, frog, fish, cock, mouse, rat, pig, bull, and human (Nayernia *et al.*, 2004). It is also important to mention that PH-GSH-Px mRNA expression in male reproductive organs is under oestrogen control (Nam *et al.*, 2003).

The most extraordinary discovery about PH-GSH-Px is related to its polymerisation and conversion from active enzyme to the structural protein. Indeed, PH-GSH-Px protein was identified as the major constituent of the keratin-like material that embeds the helix of mitochondria in midpiece of spermatozoa (Ursini *et al.*, 1999).

In 1991 chick liver cytosolic glutathione peroxidase activity was separated into three peaks by gel permeation chromatography (Miyazaki, 1991). The relative molecular weights and enzyme activities indicated that the first peak was Se-GSH-Px1 and the second non-Se-GSH-Px. The third peak was the monomeric GSH-Px, later called GSH-Px4. The proportions of the GSH-Px1, non-Se-GSH-Px and GSH-Px4 activities to total liver glutathione peroxidase were approximately 30, 42 and 28%, respectively (Miyazaki and Motoi, 1992). In the chick samples examined, the total glutathione peroxidase activity ranged from 118 nmol/min/mg in the kidney to 15.3 nmol/min/mg in plasma. In all tissues except plasma GSH-Px activity was separated into three peaks, while in plasma only one peak of GSH-Px1 was detected. In terms of percentage of total GSH-Px activity, Se-GSH-Px activity was high in plasma and erythrocytes, intermediate in testis, brain, kidney and liver, and low in duodenum. For example, in erythrocytes, about 80% of total activity was Se-GSH-Px. In terms of non-Se-GSH-Px, enzyme activity was high in duodenum, intermediate in kidney, brain, liver and testis, and low in erythrocytes, while about 70% of total glutathione peroxidase activity was non-Se-GSH-Px in the duodenum. All organs examined contained GSH-Px4 in different proportions. The proportions of GSH-Px4 to total activity ranged from 14% in testis to 28% in liver, while in erythrocytes the contribution of GSH-Px4 to total activity was very low comprising only about 4%. In terms of specific activity, GSH-Px4 activity was high in liver, duodenum and kidney, intermediate in testis and low in brain. The high GSH-Px4 activity in bird livers suggests that this enzyme is a

major enzymatic system for reducing membrane lipid hydroperoxides in birds. The livers of all the species examined by the authors had three kinds of GSH-Px, but there was variation between species in their relative proportions. Se-GSH-Px was the main glutathione peroxidase activity in rat liver while non-Se-GSH-Px was predominant in bovine liver. In avian livers, GSH-Px4 activity ranged from 10% of the total glutathione peroxidase activity in Japanese quail to 28% in chicks. The contribution of GSH-Px4 to total GSH-Px activity was very low in the livers of mammals. In terms of specific activity toward cumene hydroperoxide, GSH-Px4 activity of mammalian livers was below 6% of the activity of chick liver (Miyazaki and Motoi, 1992). Later, the same authors purified GSH-Px4 to homogeneity from a broiler chick liver cytosolic fraction using 5 different column chromatographic methods (Miyazaki and Motoi, 1996). The molecular weight of the purified enzyme, determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, was 19,500, which was confirmed by gel filtration chromatography. Therefore, it was suggested that the enzyme protein is a single polypeptide. The isoelectric point of the enzyme was determined as 7.0 and the optimum pH for the enzyme reaction as 7.0. The purified enzyme catalysed the reduction of hydrogen peroxide, cumene hydroperoxide, tert-butyl hydroperoxide and linoleic acid hydroperoxide. GSH-Px4 can deal with lipid peroxides inside membranes, since it reduced phosphatidylcholine hydroperoxide in the absence of phospholipase A2. By using an antiserum against the purified enzyme, it was shown that it reacted with the 19.5 kDa polypeptide in the liver cytosol of duck and quail, suggesting the presence of this enzyme in these avian species (Miyazaki and Motoi, 1996). GSH-Px4 has been shown to exist as both an 197 amino acid mitochondrial targeting protein and as an 170 amino acid non-mitochondrial protein (Kong *et al.*, 2003). The cDNA encoding the non-mitochondrial chicken GSH-Px (cGSH-Px4) was isolated from a chicken embryonic fibroblast cell line cDNA library. The nucleotide sequence of cGSH-Px4 was shown to be 802 bp in length with an open reading frame that encoded 170 amino acids, but lacked the N-terminal domain encoding the mitochondrial leader sequence. Chicken non-mitochondrial GSH-Px4 was highly expressed in brain and stromal tissues. The authors also showed that ovarian stromal tissue cGSH-Px4 expression is regulated according to the reproductive status of the bird and its hormone status, suggesting that GSH-Px may play an important role in avian reproduction (Kong *et al.*, 2003). GSH-Px4 in avian species is shown to be very sensitive to Se status. In fact, the liver had the highest GSH-Px activity in Se-adequate poults, and GSH-Px4 activity in Se-deficient liver decreased to 5% of Se-adequate levels (Sunde and Hadley, 2010). It is interesting that liver GSH-Px4 mRNA levels could be down-regulated by excess of Se in chicken diet (Zoidis *et al.*, 2010).

2.4.3 Plasma glutathione peroxidase (pGSH-Px, GSH-Px3)

GSH-Px from human plasma was purified to homogeneity by Takahashi and co-workers in 1987. This enzyme is a glycoprotein and synthesised in the kidney. It is an extracellular enzyme found in blood plasma (representing 20% of the Se found in plasma), chamber water of the eye or amniotic fluid. In the same year, Maddipati and Marnett (1987) showed that the human plasma glutathione peroxidase is a tetramer of identical subunits of 21.5 kDa molecular mass as determined by sodium dodecyl

sulphate-polyacrylamide gel electrophoresis. The plasma peroxidase is a selenoprotein containing one selenium per subunit (Maddipati and Marnett, 1987). In general, the protein has a molecular weight of approximately 92,000 Da with each subunit having a molecular weight of 23,000 Da containing four Se atoms per molecule (Cohen and Avissar, 1993). Unlike several other glutathione peroxidases this enzyme exhibits saturation kinetics with respect to glutathione (Km for glutathione = 4.3 mM). The peroxidase exhibits high affinity for hydroperoxides with Km values ranging from 2.3 microM for 13-hydroperoxy-9,11-octadecadienoic acid to 13.3 microM for hydrogen peroxide at saturating glutathione concentration (Maddipati and Marnett, 1987). The main source of chicken GSH-Px3 in the plasma is the kidney. The chicken GSH-Px3 gene is shown to encode a very homologous signal peptide sequence (Thu *et al.*, 2016).

Biological role of this enzyme remains speculative, since in blood plasma there are low concentrations of extracellular GSH or reduced thioredoxin. pGSH-Px is considered to have intermediate specificity to peroxides. It can reduce lipid hydroperoxides in low density lipoproteins, however, it is not active against peroxidised cholesterol esters (for review see Flohe and Brigelius-Flohe, 2016). Indeed, pGSH-Px is considered to be a redox buffer involved in a regulation of inflammatory reactions.

A comparison between pGSH-Px and cGSH-Px showed (Esworthy *et al.*, 1993) that:
- The rates of reactivity of human p-GSHPx and human GSH-Px-1 with LinOOH and H_2O_2 are in the same range.
- pGSH-Px is more reactive with LinOOH and GSH-Px1 is more reactive with H_2O_2.
- pGSH-Px has a low level of reducing activity toward cholesterol-7-alpha-OOH and no detectable activity with the 5-alpha-OOH isomer in contrast to PH-GSH-Px that readily reduces both isomers. However, it catalyses the reduction of phosphatidylcholine hydroperoxides to the corresponding hydroxy derivatives (Yamamoto *et al.*, 1993). In fact, the reductions of aromatic and small hydrophobic hydroperoxides (cumene hydroperoxide, t-amyl hydroperoxide, t-butyl hydroperoxide, paramenthane hydroperoxide) are better catalysed by pGSH-Px than the more 'physiological' substrates (linoleic acid hydroperoxide, hydrogen peroxide, peroxidised plasma lipids, and oxidised cholesterol) (Howard and Hawkes, 1998).
- pGSH-Px possesses catalytic activity towards phospholipid hydroperoxides in the absence of detergents, is enhanced at low concentrations by deoxycholate, and strongly inhibited by Triton X-100 and incorporation into liposomes.
- pGSH-Px exhibits a smaller GSH rate constant than GSH-Px-1.

2.4.4 Gastrointestinal glutathione peroxidase (GI-GSH-Px, GSH-Px2)

GI-GSH-Px was first described in 1993 (Chu *et al.*, 1993). The enzymatic properties of this enzyme are practically the same as those of cytosolic GSH-Px. The physical properties are also similar; and activity of both enzymes depends on Se supply (Chu *et al.*, 1993). The authors showed similar substrate specificities for GSHPx-1 and GI-GSH-Px; they both catalyse the reduction of H_2O_2, tert-butyl hydroperoxide, cumene hydroperoxide, and linoleic acid hydroperoxide with glutathione, but not of

phosphatidylcholine hydroperoxide. Furthermore, GI-GSH-Px mRNA was readily detected in human liver and colon, and occasionally in human breast samples, but not in other human tissues including kidney, heart, lung, placenta, or uterus. On the other hand, in rodent tissues, GI-GSH-Px mRNA was only detected in the gastrointestinal tract, and not in other tissues including liver (Chu *et al.*, 1993). In fact, GSHPx-GI appeared to be the major glutathione-dependent peroxidase activity in rodent GI tract.

GI-GSH-Px activity was present in both the villus and crypt regions of rat mucosal epithelium and its activity nearly equalled that of classical GSH-Px throughout the small intestine and colorectal segments (Esworthy *et al.*, 1998). GI-GSH-Px could be considered to be a barrier against hydroperoxide resorption (Flohe and Brigelius-Flohe, 2016). Furthermore, in the gastrointestinal tract there are at least three more selenoproteins including plasma GSH-Px, selenoprotein P and thioredoxin reductase (Mork *et al.*, 1998). The data on GI-GSH-Px clearly indicate that this enzyme should be considered as a major antioxidant defence in the intestine.

2.5 Glutathione peroxidase activity effectors

2.5.1 Selenium deficiency

Chicks fed a Se-deficient purified diet based on crystalline amino acids showed decreased Se-dependent GSH-Px activities in plasma and pancreas (Combs *et al.*, 1984). Furthermore, when fed low Se diets all chicks exhibited depressed growth and markedly reduced blood GSH-Px activities (Halpin and Baker, 1984). In fact, the activity of GSH-Px in plasma and liver varied inversely with the incidence of Exudative Diathesis and was increased by feed restriction (Zhou and Combs, 1984). Similarly, the pectoralis major muscle of chickens with muscular dystrophy disease by 7 days posthatch showed elevated GSH-Px activity (Murphy and Kehrer, 1986). Selenium- and vitamin E-depleted chicks reared on a low Se, amino acid-based diet containing 100 IU vitamin E per kilogram were found to have exceedingly low pancreatic activities of GSH-Px activity at 8 d of age (Whitacre *et al.*, 1987). Indeed, Se deficiency decreased GSH-Px in liver (Liu *et al.*, 2014b, 2015c; Walser *et al.*, 1988), brain (Xu *et al.*, 2013), pancreas (Zhao *et al.*, 2014a), muscles (Yao *et al.*, 2013a; 2014), thyroids (Lin *et al.*, 2014), duodenal mucosa (Liu *et al.*, 2016a), spleen (Peng *et al.*, 2012; Zhang *et al.*, 2012b) and other immune organs (thymus and bursa of Fabricius; Zhang *et al.*, 2012b). In fact, chickens fed diets deficient in vitamin E and selenium displayed the lowest GSH level and GSH-Px activity (Avanzo *et al.*, 2001). Se deficiency in turkey was associated with a decrease in GSH-Px4 mRNA levels in the liver (Sunde and Hadley, 2010). Enzyme activities in Se-deficient chicks for plasma GSH-Px, liver and gizzard GSH-Px1, and liver and gizzard GSH-Px4 decreased dramatically to 3, 2, 5, 10 and 5%, respectively, of Se-adequate levels (Li and Sunde, 2016). Similarly, Se deficiency in turkey, chicken, rat, mouse and lamb was associated with decreased GSH-Px1 in all species to <4% of selenium-adequate levels. At the same time, plasma GSH-Px3 activity decreased to <3% in all species except for mice.

Furthermore, liver GSH-Px4 activity was shown to fall to <10% in avians, but only to <50% of selenium-adequate levels in rodents (Sunde *et al.*, 2016). Se deficiency in chickens decreased mRNA expression of GSH-Px, GSH-Px production and activities in duodenum, jejunum and rectum (Yu *et al.*, 2015a). Compared with the +Se chicks, the Se-deficiency chicks had lower muscle mRNA levels of GSH-Px1, GSH-Px3, GSH-Px4, SepP1, SelO, SelK, SelU, SelH, SelM, SepW1, and Sep15 and decreased production of 6 selenoproteins (long-form selenoprotein P (SelP-L), GSH-Px1, GSH-Px4, Sep15, SelW, and SelN) (Huang *et al.*, 2015b).

2.5.2 Selenium supplementation

The addition of Se to various diets significantly elevated GSH-Px activity in chicken plasma (Combs and Regenstein, 1980; Maurice and Jensen, 1979; Rao *et al.*, 2013), blood (Placha *et al.*, 2014; Yoon *et al.*, 2007), liver (McGowan and Donaldson, 1987; Placha *et al.*, 2014; Wang, 2009), seminal plasma, spermatozoa, testes (Shi *et al.*, 2014; Surai *et al.*, 1998a) and other vital organs (Mercurio and Combs, 1987), including kidney, femoral muscle and heart (Salman *et al.*, 2009) as well as egg yolk (Wang *et al.*, 2010). Correlation analysis has shown that tissue Se concentration (pooled data) was correlated to Se added to feed ($r=0.529$, $P<0.01$, log values) and to glutathione peroxidase activity ($r=0.332$, $P<0.05$), with the latter not being correlated with Se added to feed (Zoidis *et al.*, 2014).

Evidence is actively accumulated to show that organic Se is as effective as sodium selenite and in some cases even more effective in upregulation of GSH-Px activity. Indeed, SeMet increased GSH-Px activity in plasma and breast muscles (Jiang *et al.*, 2009). The inclusion of organic Se (0.3 mg/kg) in the cockerel diets doubled seminal plasma GSH-Px activity (Ebeid *et al.*, 2009). Similarly, maternal SeMet increased GSH-Px in breast muscles of 1 day old chicks (Wang *et al.*, 2011a). Maternal SeMet supplementation increases GSH-Px activity in serum and breast muscles of 56 day old progeny chicks (Zhang *et al.*, 2014a). Organic Se in the form of Se-yeast at 0.3 mg/kg was shown to increase GSH-Px in egg in comparison to sodium selenite (SS) supplemented breeders (Rajashree *et al.*, 2014). Similarly, Se-yeast dietary supplementation increased GSH-Px activity in the liver and breast muscle of 42 day old chickens in comparison to SS-supplemented birds (Chen *et al.*, 2014). SeMet and Se-yeast were more effective than SS in increasing GSH-Px in chicken plasma (Jing *et al.*, 2015). Chickens fed organic selenium had elevated GSH-Px activity in both blood and liver in a thermoneutral environment and after heat distress (Mahmoud and Edens, 2003). Indeed, Se supplementation of heat stressed chickens increased GSH-Px activity in the liver (Liao *et al.*, 2012). Se-yeast supplementation in broiler diets resulted in greater tissue Se concentrations than SS supplementation, and pGSH-Px3 and tissue Se concentrations remained greater in birds previously fed a diet with Se-yeast than in those fed SS after a low-Se diet (Payne and Southern, 2005). *In vitro* studies confirmed that an optimal Se status is important for maximum GSH-Px activity. Compared to the control, Se significantly increased GSH-Px activity in chicken hepatocytes with maximal effects being observed at 2 μmol/l of SeMet and at

1.5 µmol/l of $Na2SeO_3$, respectively. Significant decreases in GSH-Px4 mRNA levels were observed in all the hepatocytes treated with Se (vs control) (Wu *et al.*, 2010).

However, when the diet is adequate in Se, usually there is no response to extra Se supplementation. For example, no effect of source of Se (SeMet vs Se-yeast) or dosage (0.1; 0.3 or 0.5 mg/kg) was observed on serum GSH-Px activities in chickens (Delezie *et al.*, 2014). Similarly, plasma GSH-Px activity was not affected by source of Se or concentration (Payne and Southern, 2005a).

2.5.3 Selenium excess

It has been shown that oxidative stress imposed by Se excess in the chicken diet is associated with decreased GSH-Px activity. For example, in acute Se toxicosis GSH-Px activity of the red blood cells was significantly elevated at the first sampling (3 h after treatment) and decreased to the control level thereafter (Mézes and Sályi, 1994). In fact, GSH-Px activity declined in the blood plasma and in the red blood cell hemolysate (Balogh *et al.*, 2004), spleen (Peng *et al.*, 2012) and liver (Zoidis *et al.*, 2010) as a result of Se excess in the diet. Dietary Se (3 mg/kg) depressed growth performance of chicken and down-regulated 9 and 3 selenoprotein genes in thymus and spleen, respectively, and only Sepp1 was up-regulated in the bursa of Fabricius (Tang *et al.*, 2017).

2.5.4 Heat stress

It seems likely that GSH-Px activation is an important adaptive mechanism to deal with oxidative stress imposed by heat stress. Indeed, heat stress increased GSH-Px in the chicken liver (Ramnath *et al.*, 2008; Tan *et al.*, 2010; Yang *et al.*, 2010), serum (Yang *et al.*, 2010), erythrocytes (Aengwanich and Suttajit, 2013) and thigh muscle (Huang *et al.*, 2015a), while it was not affected in the breast muscle. Se and vitamin E supplementation increased GSH-Px activity in skeletal muscles of heat stressed chickens, while there was no effect of such a supplementation in unstressed control chickens (Ghazi Harsini *et al.*, 2012). However, when stress is too high to be adapted to, GSH-Px activity decreases. In fact, heat stress decreased GSH-Px activity in serum (Liu *et al.*, 2014a; Sahin *et al.*, 2016), muscles (Sahin *et al.*, 2016), spleen (Xu *et al.*, 2014) and bursa of Fabricius (Xu and Tian, 2015).

2.5.5 Cold stress

Data on the effect of cold stress on GSH-Px activity are not consistent. For example, when broilers were exposed to a cool environment for 3 weeks, plasma GSH-Px activity was decreased compared to normal-temperature rearing chicks (Pan *et al.*, 2005; Ramnath and Rekha, 2009). In contrast, acute cold stress was associated with increased GSH-Px activity in spleen, thymus and bursa of Fabricius (Zhao *et al.*, 2014b). In fact, acute cold stress initially increased (1-3 hours) and then decreased (24 hours) GSH-Px activity in chicken heart tissue (Zhao *et al.*, 2013). In contrast,

in chronic cold stress, GSH-Px activity initially decreased (5 days) and later (10-20 days) recovered.

2.5.6 Other stress conditions

Various stress conditions could affect GSH-Px activity in chicken blood and tissues. For example, GSH-Px activity in the liver and muscle was significantly higher in layers exposed to feed withdrawal and low light intensity than in control birds (Naziroglu *et al.*, 2000). Similarly, the plasma GSH-Px activity of feed-restricted birds was markedly higher than that in *ad libitum* fed on day 35 and 42 (Pan *et al.*, 2005). In pullets, refeeding resulted in a significant increase of GSH-Px in the whole blood hemolysate (Milinković-Tur *et al.*, 2007). Chicken transportation increased GSH-Px in muscles (Wang *et al.*, 2015), while it did not affect erythrocyte GSH-Px activity (Perai *et al.*, 2015). Increased humidity is shown to increase GSH-Px activity in chicken pectoral muscles (Wei *et al.*, 2014). However, a simultaneous increase in humidity and ammonia concentrations did not change GSH-Px activity in chicken muscles. Exposure of broilers to blue light increased GSH-Px in their breast and leg muscles (Ke *et al.*, 2011). Outdoor access with scattered feeding was associated with an increase in GSH-Px activity in chicken serum (Jiang *et al.*, 2011). In great contrast, in pullets, fasting resulted in a significant decrease of whole blood hemolysate GSH-Px activity (Milinković-Tur *et al.*, 2007) and high nutrient density diet decreased GSH-Px in the chicken heart (Peng *et al.*, 2013). However, there were no differences in GSH-Px between groups of broilers with low and high feed efficiency (Ojano-Dirain *et al.*, 2005).

2.5.7 Dietary fat

In the early 1980s it was suggested that the type of dietary fat can affect plasma GSH-Px activity in chicks without altering the intestinal absorption of selenium (Mutanen and Mykkänen, 1984). In particular, incorporation of peroxidised corn oil into diets increased plasma GSH-Px activity when those diets contained supplemental selenium (Combs and Regenstein, 1980). Furthermore, olive oil was shown to increase liver GSH-Px (Tufarelli *et al.*, 2016) and yellow grease increased plasma GSH-Px in laying hens (Laika and Jahanian, 2015). However, there is a range of publications indicating that dietary fat does not affect GSH-Px in chickens. For example, dietary supplementation of oxidised oil (6%) with or without vitamin E (200 mg/kg) did not affect GSH-Px activity in chicken plasma (Açıkgöz *et al.*, 2011). Similarly, oxidised oil in the chicken diet did not change GSH-Px activity in the chicken liver (Engberg *et al.*, 1996). Furthermore, dietary fat sources (tallow or soybean oil, 5%) or supplementary α-tocopheryl acetate (220 mg/kg) did not affect serum GSH-Px (Khajali and Fahimi, 2010).

2.5.8 Diseases

Eimeria tenella challenge was associated with substantial (more than 2.5-fold) increase in GSH-Px activity in chicken plasma (Bun *et al.*, 2011). Lung mitochondrial GSH-Px activity was elevated in broilers with PHS compared to controls (Iqbal *et al.*, 2002). Similarly, GSH-Px was upregulated in the brain of cold-induced pulmonary hypertensive chickens (Hassanpour *et al.*, 2015). However, in most cases disease challenge was associated with a decreased GSH-Px activity. In particular, a decreased blood GSH-Px activity was shown in necrotic enteritis (Zhou *et al.*, 2016), infection bronchitis (Lin *et al.*, 2015) and *Ascaridia* infection (Gabrashanska *et al.*, 2007). Similarly, Newcastle disease infection decreased GSH-Px activity in chicken brain and liver (Subbaiah *et al.*, 2011).

2.5.9 Heavy metals

In some cases when heavy metals are in comparatively low concentrations in the diet, they can increase GSH-Px activity in chickens. For example, MeHg increased GSH-Px in chicken cerebellum (Carvalho *et al.*, 2008), arsenic increased GSH-Px activity in chicken erythrocytes (Aggarwal *et al.*, 2009), and Cr supplementation increased plasma GSH-Px (Rao *et al.*, 2012). However, most common responses to heavy metals in the diet are associated with a significant decrease GSH-Px in various chicken tissues. For example, Cd decreased GSH-Px in chicken kidney (Liu *et al.*, 2015a), liver (Li *et al.*, 2013), ovary and serum (Yang *et al.*, 2012a), as well as in testicular tissue (Li *et al.*, 2010) and in cultured granulosa cells from chicken ovarian follicles (Jia *et al.*, 2011). High vanadium in the chicken diet decreased GSH-Px in the liver and kidney (Liu *et al.*, 2012). Similarly dietary vanadium (45-60 mg/kg) decreased GSH-Px in chicken intestine, including duodenum, jejunum, ileum and caecal tonsil (Deng *et al.*, 2012). Arsenic decreased GSH-Px in the gastrointestinal tract (Guo *et al.*, 2015) and in chicken brain (Zhao *et al.*, 2017). Dietary $NiCl_2$ decreased GSH-Px in the chicken intestinal mucosa (Wu *et al.*, 2013b), caecal tonsil (Wu *et al.*, 2014b) and kidney (Guo *et al.*, 2014a). Dietary chromium decreased GSH-Px in brain (Cheng *et al.*, 2016) and kidney (Liu *et al.*, 2015b).

2.5.10 Other metals

It has been shown that dietary Mn decreased GSH-Px activity in the serum and immune organs (spleen, thymus, and bursa of Fabricius) of chickens (Liu *et al.*, 2013b). It also significantly lowered the activity of GSH-Px in the testis of cockerels (Liu *et al.*, 2013a) and in chicken splenic lymphocytes (Zhu *et al.*, 2016). Similarly, high Cu diet (250 mg/g) induced an oxidative stress characterised by increased concentrations of MDA and decreased activity of GSH-Px in the liver (Yigit *et al.*, 2012). On the other hand, dietary Zn supplementation (20-60 mg/kg) doubled GSH-Px activity in chicken plasma (Bun *et al.*, 2011a) and higher Zn doses (90 or 120 mg/kg) in the form of Zn-Gly led to an improvement of activity of GSH-Px in livers at 21 and 42 days (Ma *et al.*, 2011).

2.5.11 Mycotoxins

Among the studied mycotoxins, aflatoxin B_1 (AFB_1) showed the most consistent negative effect on GSH-Px activity in various chicken tissues, including liver (Liu *et al.*, 2016b; Yang *et al.*, 2012b; Yarru *et al.*, 2009), serum (Fan *et al.*, 2015) and spleen (Chen *et al.*, 2016; Liu *et al.*, 2016b; Wang *et al.*, 2013). Furthermore, AFB_1 downregulated the expression of GSH-Px mRNA in the chicken livers (Ma *et al.*, 2015). Depending on the experimental conditions, including doses used, T-2 toxin was shown to decrease hepatic activity of GSH-Px (by 36.8%; Dvorska *et al.*, 2007) or increase hepatic enzymatic GSH-Px activity 3-fold (Leal *et al.*, 1999) or increased GSH-Px activity in blood plasma at 24 and 48 h, in liver at 12, 24 and 36 h, and in kidney and spleen at 24 h (Bócsai *et al.*, 2016). It is interesting to note that a subtoxic dietary level of deoxynivalenol (DON) increased GSH-Px activity in chicken duodenal mucosa (Placha *et al.*, 2009) and dietary zearalenone (ZEA) increased GSH-Px in the duodenal mucosa and kidney tissues (Grešáková *et al.*, 2012). However, a mixture of DON and ZEA decreased GSH-Px activity in chicken liver, but did not affect it in duodenal mucosa (Borutova *et al.*, 2008).

2.5.12 Other toxicants and drugs

There is a range of toxic substances affecting GSH-Px activity in chickens. They mainly decreased GSH-Px activity, while some drugs were able to increase the enzymatic activity. For example, sodium nitroprusside reduced GSH-Px activity in chicken plasma and liver (Elzubeir and Davis, 1990). In chicken embryo fibroblasts paraquat decreased GSH-Px activity (Lawlor and O'Brien, 1997). Similarly P3,3',4,4',5-pentachlorobiphenyl decreased GSH-Px in chicken embryonic liver (Jin *et al.*, 2001), while 4-nitro-3-phenylphenol decreases GSH-Px in testicular cells (Mi *et al.*, 2010) and 2,3,7,8-tetrachlorodibenzo-p-dioxin treatment caused a decrease in GSH-Px activity in the liver of the hatchling chicken (Hilscherova *et al.*, 2003), chicken liver (Lim *et al.*, 2007) and kidney (Lim *et al.*, 2008). Tri-ortho-cresyl phosphate decreased GSH-Px activity in cerebrum, spinal cord and sciatic nerve of hens after 5, 10, 15 and 21 days post-dosing (Zhang *et al.*, 2007). Furthermore, thiram (Li *et al.*, 2007), endotoxin (Zhang *et al.*, 2008), cyclophosphamide (Yu *et al.*, 2015b), CCl_4 (Shah *et al.*, 2016) and flourine (Chen *et al.*, 2011b) also decreased GSH-Px activity in the serum, liver and other organs.

Some drugs used in the poultry industry are able to affect GSH-Px. For example, it was shown that salinomycin (Kamashi *et al.*, 2004) and deltamethrin (Jayasree *et al.*, 2003) increased GSH-Px in whole blood of chickens. However, in another paper it was shown that GSH-Px activity in the liver decreased rapidly as a result of salinomycin treatment (Mezes *et al.*, 1992). In fact, in acute monensin poisoning GSH-Px activity in liver and breast muscle initially decreased, then tended to rise (Sályi *et al.*, 1990). Furthermore, vitamin A in high doses decreased GSH-Px activity in the chicken liver and brain (Surai *et al.*, 2000a), while menadione did not affect GSH-Px in chicken liver (Marchionatti *et al.*, 2008).

2.5.13 Antioxidants

Dietary antioxidants can affect GSH-Px activity in chickens, but data on the relationship between dietary vitamin E and GSH-Px are not consistent. Most of cases dietary vitamin E activate GSH-Px activity in blood (Rama Rao *et al.*, 2011), liver (Hu *et al.*, 2015b), erythrocytes (Cinar *et al.*, 2014), egg yolk and serum (Jiang *et al.*, 2013). However, under different experimental conditions vitamin E supplementation decreased chicken plasma GSH-Px (Mahmoud and Hijazi, 2007) or did not affect GSH-Px activity in muscles (Voljc *et al.*, 2011; Young *et al.*, 2003).

A water soluble dietary antioxidant vitamin C was shown to increase the GSH-Px activity in chicken plasma (Combs and Pesti, 1976; Oztürk-Urek *et al.*, 2001) or erythrocytes (Cinar *et al.*, 2014). Similarly, GSH-Px activity in serum (Chen *et al.*, 2011a; Jia *et al.*, 2014), liver (Chen *et al.*, 2011a; Guo *et al.*, 2014b; Jia *et al.*, 2014) and breast muscle (Chen *et al.*, 2011a), significantly increased in chickens fed with lipoic acid. Melatonin also stimulated GSH-Px activity (Pablos *et al.*, 1995a). In fact, the increases in GSH-Px activity of melatonin-induced chickens varied with the tissue and were between 22 and 134%. These percentages in GSH-Px activity were directly correlated with tissue melatonin content (Pablos *et al.*, 1995a). It is interesting to note that HSP70 significantly elevated GSH-Px activity and inhibited lipid peroxidation to relieve intestinal mucosal oxidative injury (Gu *et al.*, 2012).

2.5.14 Plant material and plant extracts

Various plant materials or extracts have shown to increase GSH-Px activity in chicken tissues. For example, dietary polysavone (1.5 g/kg), a natural extract from alfalfa, increased GSH-Px in chicken serum and liver (Dong *et al.*, 2011). The aforementioned extract is known to be rich in carotenoids and flavonoids. Therefore, dietary lycopene (carotenoid) in the maternal diet increases GSH-Px activity in the liver of the newly hatched chicks (Sun *et al.*, 2015). Similarly, tomato pomace, a rich source of lycopene, increases serum GSH-Px (Hosseini-Vashan *et al.*, 2016). *Curcuma xanthorrhiza* and *Origanum compactum*, rich sources of flavonoids, increased GSH-Px in chicken heart (Akbarian *et al.*, 2014), while orange peel extract and *C. xanthorrhiza* essential oil increases GSH-Px in chicken erythrocytes (Akbarian *et al.*, 2015) and pure curcumin also increased GSH-Px in heat-stressed broiler muscles (Zhang *et al.*, 2015). Similarly, a mixture of thymol and carvacrol in the chicken diet was shown to increase GSH-Px in muscle, serum and liver (Hashemipour *et al.*, 2013). In cultured muscle cells of embryonic broilers a phytoestrogen equol caused an increase in GSH-Px activity (Wei *et al.*, 2011b) and a low dose of equol (20 μg) injected into fertile eggs increased GSH-Px activity in chickens at day 49 by 16% (Wei *et al.*, 2011a). Supplementation of ginger increased GSH-Px activity in chicken serum (Zhang *et al.*, 2009), while supplementation of *Astragalus membranaceus* root powder increased activity of GSH-Px in the serum of chickens at 21 and 42 days (Zhang *et al.*, 2013). Furthermore, 1.0% lemon verbena and vitamin C elevated the level of blood GSH-Px by 51.8 and 27.9%, respectively (Rafiee *et al.*, 2016).

However, plants and their extracts are not always effective in changing GSH-Px activity. For example, xanthophyll supplementation enhanced antioxidant capacity and reduced lipid peroxidation in different tissues of hens and chicks, but did not affect serum or liver GSH-Px activity (Gao *et al.*, 2013). Dietary supplementation of cloves and agrimony or cloves and lemon balm did not affect blood GSH-Px activity in chickens (Petrovic *et al.*, 2012). Turmeric rhizome powder did not affect serum GSH-Px (Daneshyar *et al.*, 2012).

2.5.15 Amino acids

It was shown that supplementary methionine (5 g/kg) stimulates GSH-Px activity in growing chickens in the first period of postnatal life (Németh *et al.*, 2004). However, in later publications similar doses of Met (5.4 g/kg; Zhao *et al.*, 2009) or 5.9 mg/kg (Chen *et al.*, 2013) were shown to reduce hepatic GSH-Px. Other amino acids tested were shown to have a positive effect of GSH-Px in chickens. In particular, arginine increased GSH-Px in the egg yolk (Duan *et al.*, 2015), while threonine (Min *et al.*, 2017) and histidine (Kopec *et al.*, 2013) increased GSH-Px activity in serum and plasma, respectively. Adding betaine (1 g/kg) to a diet deficient in methionine can significantly improve antioxidant defences by increasing GSH-Px activity in the breast muscles of broiler chickens (Alirezaei *et al.*, 2012). It also can increase GSH-Px in chicken serum (Akhavan-Salamat and Ghasemi, 2016).

2.5.16 Other supplements

There is a range of feed constituents and supplements increasing GSH-Px activity in chicken tissues. For example, phytate increased GSH-Px activity in blood, heart and muscle in the absence of supplementary selenium, but decreased the GSH-Px activity in kidney (Shan and Davis, 1994). Furthermore, GSH-Px activity in the liver was increased as a result of dietary clinoptiolite (Wu *et al.*, 2013a), conjugated linoleic acid (Qi *et al.*, 2011), DDGS (Heincinger *et al.*, 2011) and mushrooms (Giannenas *et al.*, 2010), while porcine blood cells meal and blood meal (Kopec *et al.*, 2013a), dietary grasshoppers (Sun *et al.*, 2012) and mushrooms (Giannenas *et al.*, 2010) increased GSH-Px activity in chicken breast muscle. Furthermore, menadione increased GSH-Px activity in chicken enterocytes (Marchionatti *et al.*, 2013) and γ-aminobutyric acid increased serum GSH-Px activity in layers (Zhang *et al.*, 2012c). Yeast probiotic increased serum GSH-Px activity in broiler chickens (Aluwong *et al.*, 2013). Similarly *Lactobacillus plantarum* (probiotic) dietary supplementation increases GSH-Px activities in chicken serum and liver at 21 days of age (Shen *et al.*, 2014). Stimulating effect of a probiotic on liver and serum GSH-Px was confirmed later (Bai *et al.*, 2017). It has been shown that lipopolysaccharide (LPS) can decrease GSH-Px activity in intestinal mucosa (Wu *et al.*, 2016) and downregulated GSH-Px mRNA expressions (Zheng *et al.*, 2016). At the same time in chicken bursal lymphocytes *Sargassum* polysaccharides increase GSH-Px activity (Zhang *et al.*, 2011) and bursopentine, a novel pentapeptide isolated from chicken bursa of Fabricius increased GSH-Px activity in dendritic cells (Qin *et al.*, 2015).

2.6 GSH-Px and their biological roles

Various GSH-Px are characterised by different tissue-specificity and are expressed by different genes. Initially, it was thought that the major function of these peroxidases is to remove and detoxify hydrogen peroxide and lipid hydroperoxides. However, it seems likely that emerging roles of GSH-Px in maintenance of cellular redox state have a much bigger impact on cell metabolism and stress resistance than expected, affecting physiological events, such as differentiation, signal transduction and regulation of pro-inflammatory cytokine production, etc. Peroxynitrite scavenging and participation of GSH-Px enzymes in regulating the biosynthesis of various eicosanoids (leukotrienes, thromboxanes and prostaglandins) can affect cell signalling and transcription factor activation responsible for the modulation of many important cellular pathways (Surai, 2006). Indeed, many different environmental factors and messengers modulate GSH-Px in a complex manner, providing physiological regulation of antioxidant defence in the cell under stress conditions.

One of the important applications of Se and GSH-Px activity applies to the meat industry. Indeed, meat quality deterioration during storage is a question of great economical value and the role of GSH-Px in this process deserves further investigation. It has been shown that GSH-Px with other selenoproteins are involved in the prevention of lipid peroxidation during meat storage (Surai, 2006). In fact, GSH-Px activity is negatively correlated with meat peroxidation (Utama *et al.*, 2016). It is interesting to note that duck meat oxidation is faster than chicken meat (Muhlisin *et al.,* 2016) and chicken breast muscle samples had lower GSH-Px activities than cattle and camel Longissimus dorsi samples (Gheisari and Motamedi, 2010). As one could expect GSH-Px in chicken meat gradually decreased during 4 days (Gheisari and Motamedi, 2010) or 12 days (Ahmad *et al.*, 2012) storage at 4 °C. Meat cooking also causes a loss of GSH-Px activity (Muhlisin *et al.*, 2016). The activity of GSH-Px was significantly decreased in pale, soft and exudative (PSE)-induced meat samples (Carvalho *et al.*, 2017). Dietary Se supplementation increased GSH-Px activity in chicken muscles (Ahmad *et al.*, 2012).

It is necessary to underline that different forms of GSH-Px perform their protective functions in concert, with each providing antioxidant protection at different sites of the body. For example, gastrointestinal glutathione peroxidase (GI-GSH-Px) could be considered to be a barrier against hydroperoxide resorption (Flohe and Brigelius-Flohe, 2016). In particular, it has been suggested that the digestive tract is a major site of antioxidant-prooxidant interaction in the body (Surai, 2000, 2006; Surai and Fisinin, 2015; Surai *et al.*, 2003, 2004). In this case, a specific GI-GSH-Px is considered to be a major protector against lipid hydroperoxides found in the food/feed. It is generally accepted that during feed production and storage some polyunsaturated lipids are oxidised and they can cause health-related problems, including decreased growth, productive and reproductive traits of animals and compromised immunocompetence (Kanazawa and Ashida, 1998). It seems likely that this detrimental effect of feed peroxides would ultimately depend on the activity of GI-GSH-Px. Indeed, oxidised lipids can react with transition metals that can be found in the feed as feed

supplements (usually in inorganic catalytic form) producing free radicals. Those free radicals can react with natural or synthetic antioxidants present in the feed causing formation of lipid hydroperoxides. Therefore, GI-GSH-Px could deal with those peroxides, preventing them to enter the blood circulation. Indeed, it has been shown that after inclusion of lipid peroxides into the diet of rats their concentration in plasma was extremely low (Kanazawa and Ashida, 1998, 1998a). Furthermore, there are other selenoproteins in the gastrointestinal tract, including plasma GSH-Px, selenoprotein P and thioredoxin reductase (Mork *et al.*, 1998). As mentioned above, GSH-Px is an important antioxidant in plasma, which together with selenoprotein P and other antioxidant compounds, maintain antioxidant protection. On the other hand, PH-GSH-Px is an important antioxidant inside biological membranes where lipid peroxidation occurs and lipid hydroperoxides are produced. It seems likely that interactions between vitamin E and GSH-Px4 are important elements in the antioxidant defence of biological membranes. Therefore, cGSH-Px and PH-GSH-Px are found in cytosol, while eGSH-Px and SeP are located in the extracellular fluids and by working together they can provide antioxidant defence to the biological molecules inside and outside the cell (Takebe *et al.*, 2002). The authors have shown that cGSH-Px reduced t-BuOOH and H_2O_2 effectively, but did not reduce PC-OOH. Specific conditions required for the maximal enzymatic activities were different for each enzyme, however, both PH-GSH-Px and eGSH-Px showed very similar specific activities to PH-OOH. These data suggest that in different cellular and extracellular conditions a combination of various selenoproteins can be effective in detoxifying H_2O_2 and lipid hydroperoxides. Clearly, the GSH-Px family is an important part of the antioxidant defence in animal/chicken bodies and specific roles of the enzymes in regulation of other important functions warrant further investigation.

2.6.1 GSH-Px regulation and proteasomes

It is well known that ATP- and ubiquitin-independent proteolysis by the 20S proteasome is responsible for the selective degradation of oxidised proteins. In particular, the 20S proteasome shows an increased proteolytic activity toward oxidised polypeptides. On the other hand, it seems likely that increased GSH-Px1 activity can downregulate basal 20S proteasome activity. For example, it was shown a 30% decreased activity of the chymotrypsin-like activity of the 20S proteasome in human cells overexpressing GSH-Px1 (Kretz-Remy and Arrigo, 2003). This phenomenon was associated with a 2-fold increase in IκB-α half-life, a protein whose basal turnover is 20S proteasome-dependent. The authors suggested that, the intracellular redox status is an important element activating or down-regulating the 20S proteasome chymotrypsin-like activity in living cells. This is a very important finding, which explains how Se-reserves in the body can be used to improve antioxidant defence under stress conditions. The most probable sequence of events is as follows:

- when organic Se is used in the diet, Se reserves in the form of SeMet are non-specifically incorporated in various proteins, for example in muscles;
- under stress conditions, the requirement for selenoproteins to prevent free-radical related damages is increased, but selenium bioavailability is decreased due to decreased feed consumption;

- redox status of muscle cells decreases due to depletion of antioxidants and probably some proteins are oxidised;
- proteosomes are activated and protein degradation is increased;
- SeMet is released from proteins and used for additional synthesis of selenoproteins;
- antioxidant defences are improved and redox status of the cells are changed;
- proteosome activity is decreased and antioxidant-prooxidant equilibrium is established.

2.7 Thioredoxin reductases as a major part of the thioredoxin system

The redox status of the cell is a major determinant of many different pathways including gene regulation (Reczek and Chandel, 2015). The thioredoxin system (thioredoxin/thioredoxin peroxidase (peroxiredoxins)/thioredoxin reductase) and the glutathione system (glutathione/glutathione reductase/glutaredoxin/glutathione peroxidase, Holmgren, 1989) are believed to be the major players in this regulation (Gromer *et al.*, 2004; Holmgren, 2000). Together they supply electrons for deoxyribonucleotide formation, antioxidant defence, protein and DNA synthesis and repair and redox regulation of signal transduction, transcription, cell growth, differentiation and apoptosis (Mustacich and Powis, 2000). Indeed, the thioredoxin system not only plays a crucial role as a thiol/disulfide redox controller, it is also essential for certain organisms as the only system ensuring redox homeostasis (Kováryová and Kollárová, 2015). Biological roles of the thioredoxin system are diverse and include (Gromer *et al.*, 2004), first of all, antioxidant defence by direct catalysis of several antioxidant reactions and by regeneration of other antioxidant enzymes, such as peroxiredoxins or methionine sulfoxide reductase inactivated by oxidative stress, recycling dehydroascorbate to ascorbate, and reduction of ubiquinone to ubiquinol. The thioredoxin system is also involved in redox regulation of gene expression, various cellular functions, synthesis deoxyribonucleotides (DNA synthesis and repair), protein biosynthesis, hormone and cytokine action, apoptosis, etc. (Lu and Holmgren, 2014). Some of the enzymatic reactions that appear to be modulated by thioredoxin similarly represent major control points for cellular metabolism (Figure 2.3). Therefore, as mentioned above, the thioredoxin system consists of thioredoxins, thioredoxin peroxidase (peroxiredoxins) and thioredoxin reductases.

Thioredoxin (Trx), an approximately 12 kDa thiol/disulfide oxidoreductase, was first characterised in 1964 in *Escherichia coli* and three years later it was described in rat hepatoma cells (see Powis *et al.*, 2000 for review). Thioredoxin with a redox-active dithiol/disulfide is an electron donor for essential enzymes, including ribonucleotide reductase and a general protein disulfide reductase (Holmgren, 2001). Furthermore, Trx represents an intracellular redox regulator that is important for the regulation of redox-sensitive transcription factors and maintaining them in active form during oxidative stress. In fact, most, if not all, of the functions of Trx depend on the activity of TrxR. The cDNA sequence for chicken Trx predicts a protein of 105 amino acids with a molecular weight of 11,700 (Jones and Luk, 1988). The authors showed that the sequence of chicken Trx is very similar to sequences of other thioredoxins.

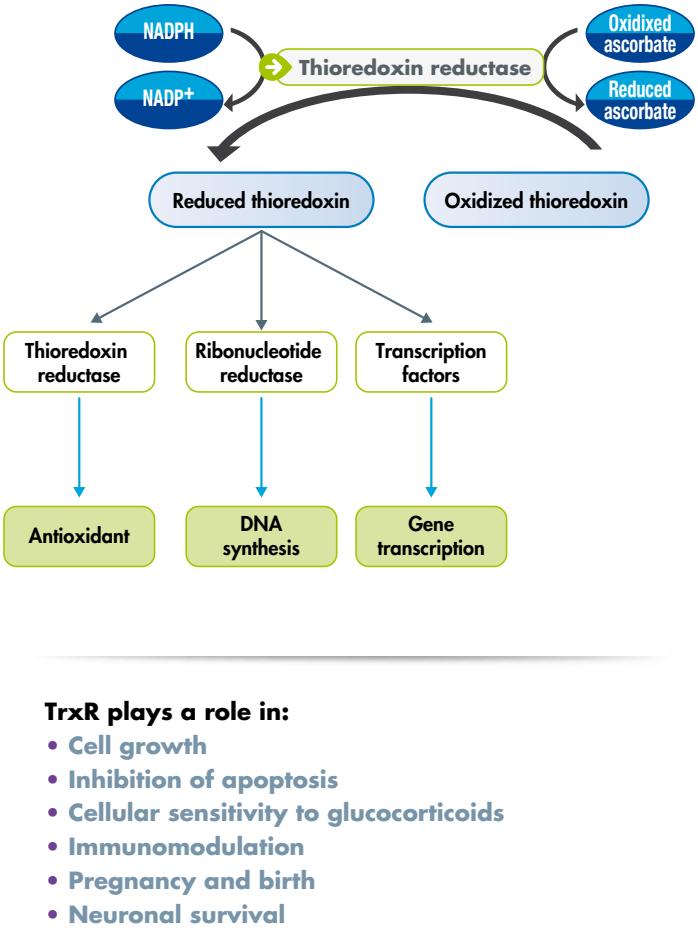

Figure 2.3 Thioredoxin system – a major player in redox regulation.

Comparison of the chicken Trx protein sequence with those from bacteria and plants indicate structural features that appear to be essential for activity. To investigate the biological significance of Trx2, chicken Trx2 cDNA was cloned and clones of the conditional Trx2-deficient cells were generated using a chicken B-cell line, DT40. It was shown that chicken Trx2 is an essential gene and that Trx2-deficient cells undergo apoptosis upon repression of the Trx2 transgene, showing an accumulation of intracellular ROS (Tanaka *et al.*, 2002; Wang *et al.*, 2006). Increased Trx expression in chicken ovarian follicles was associated with high rates of egg production (Yang *et al.*, 2008). Trx was found to be expressed in chicken jejunum (Xiao *et al.*, 2012) and was shown to be an important protein of the chicken seminal plasma (Marzoni *et al.*, 2013). Furthermore, chicken mitochondrial Trx2 was shown to possess disulfide

reductase activity in a concentration-dependent manner showing protective effects on LPS-induced oxidative stress in chicken hepatocytes (Hu *et al.*, 2015a).

Peroxiredoxins (Prxs) are a family of highly conserved antioxidative proteins (non-seleno peroxidases), currently comprising of six members in mammals and located in different parts of the cell. Indeed, Prxs have a wide subcellular distribution and perform divergent biological functions (Poynton and Hampton, 2014). Prx activity is based on a redox-active cysteine that is oxidised to a sulfenic acid by hydroperoxides, including hydrogen peroxide, organic peroxides, peptide and protein hydroperoxides, and peroxynitrite. These cysteine-dependent peroxidases play major roles not only in peroxide detoxification, but also in regulating peroxide-mediated cell signalling. Prx importance is unarguable, as knockouts of the most highly expressed Prxs are associated with increased oxidative stress and reduced genome stability. Prx1 is the most ubiquitously expressed member of the peroxiredoxin family involving in antioxidant defence, cell differentiation and proliferation, immune responses, regulation of apoptosis, and chaperone actions (Daly *et al.*, 2008). At least 4 different classes of Prx protein have been evolutionary conserved in chickens (Han *et al.*, 2005). Chicken Prx proteins demonstrate antioxidant activity similar to those of the mammalian enzymes and Prx expression in chickens are not tissue specific, indicating their essential role as a housekeeping gene in all tissues to protect against oxidative damage (Han *et al.*, 2005). Prx1 was shown to be expressed in chicken jejunum (Xiao *et al.*, 2012), chicken embryonic kidney (Cao *et al.*, 2011) and chicken macrophages (Lavric *et al.*, 2008), while Prx6 was shown to be expressed in chicken liver (Huang *et al.,* 2011a) and chicken gut (Lee *et al.*, 2014). Therefore, Trxs, Prdxs and TrxRs are important antioxidants participating in cellular/organismal adaptation to stress and their upregulation is considered to be an important approach to improve stress resistance of poultry. It seems likely that the entire thioredoxin system in mammals and birds is dependent on Se availability.

Thioredoxin reductase (TrxR) was first characterised by Holmgren in 1977 from calf liver and thymus and 5 years later it was purified from rat liver cytosol by Luthman and Holmgren (1982). Fourteen years later it was shown that human TrxR is a selenoenzyme (Gladyshev *et al.*, 1996; Tamura and Stadtman, 1996). It was shown that TrxR has a subunit molecular weight of 58,000 and a native molecular weight of 116,000. The enzyme is highly specific for NADPH with a Km of 6 μM. It contains an flavin adenine dinucleotide (FAD) prosthetic group and is sensitive to inhibition by arsenic. Selenocysteine is required for the activity of this enzyme, since the Cys mutant enzyme is inactive. Whereas H_2O_2 was a substrate for the wild-type enzyme, all mutant enzymes lack hydroperoxidase activity (Zhong and Holmgren, 2000). Furthermore, radiolabelling of proteins by incubation of the cDNA-transfected cells with sodium [^{75}Se] selenite showed that ^{75}Se was incorporated into the expressed TrxR protein (Fujiwara *et al.*, 1999) confirming a requirement for Se for the formation of functional TrxR. Therefore, mammalian TrxRs are a class of flavoproteins that use NADPH as an electron donor and belong to the family of oxidoreductases (Ganther, 1999) that share sequence identity and mechanistic similarity with glutathione reductases (Gasdaska *et al.*, 1995; Mustacich and Powis, 2000). These enzymes are

involved in linking the thioredoxin system to reduced glutathione and the nucleotide cofactors (Holmgren and Bjornstedt, 1995).

There are at least three forms of this enzyme (Table 2.4). TrxR1 is located predominantly in the cytosol, while TrxR2 is found in mitochondria (Miranda-Vizuete *et al.*, 2000; Powis *et al.*, 2000). Human mitochondrial TrxR consists of 521 amino acid residues with a calculated molecular mass of 56.2 KDa. It is highly homologous to the previously described cytosolic TrxR1. It is interesting that TrxR2 has an extra 33 amino acids in its molecule at the N-termins. It was shown that mRNA for TrxR2 is highly expressed in prostate, testis and liver. The TrxR2 gene consists of 18 exons spanning about 67 kb, with a chromosomal localisation at position 22q11.2 (Miranda-Vizuele *et al.*, 2000). Finally, TrxR3 is located in the testes (Sun *et al.*, 1999). Later, Sun *et al.* (2001) demonstrated that testes TrxR has a broad substrate specificity and can reduce several components of the thioredoxin and glutathione systems (Mustacich and Powis, 2000). Therefore, it has been called thioredoxin and glutathione reductase (TGR). It was shown that TrxR1 and TrxR2 are essential for embryonic development in mice (Gladyshev, 2016).

It has been established that TrxR is a homodimer. A selenenylsulfide was identified as the active site of TrxR and a structural model of the mechanism of the enzyme was proposed (Zhong *et al.*, 2000). The most striking feature of TrxR enzymes is their sensitivity to oxidising conditions that cause changes in conformation (Gorlatov

Table 2.4. Classification of human thioredoxins and thioredoxin reductases (adapted from Miranda-Vizuete *et al.*, 2004; Surai, 2006).

Name	Chromosomal localisation	Size, kDa	Tissue specificity	Subcellular localisation
Thioredoxins				
Trx-1	9q31	11.71	ubiquitous	mainly cytosolic, nuclear upon certain stimuli
Trx-2	22q13.1	11.87	ubiquitous	mitochondrial
Txl-1/Trp32	18q21.2	32.25	ubiquitous	cytosolic
Erdj5/JDPI	2p22.1-23.1	91.08	ubiquitous	endoplasmic reticulum
Sptrx-1	18p11.2-11.31	53.27	testis/spermatid	sperm fibrous sheath
Sptrx-2	7p14.1	67.27	testis/spermatid	sperm fibrous sheath
Sptrx-3	Not determined	14.57	testis/spermatid	Golgi
Txl-2	3q22.3-23	36.85	ubiquitous, especially in testis and lung	associated with microtubules in cilia and flagella
Thioredoxin reductases				
TrxR1	12q23-24.1	54.71	ubiquitous	cytosolic
TrxR2	22q11.21	53.06	ubiquitous	mitochondrial
TGR	3p13-q13.33	63.63	ubiquitous, but highly expressed in testis	cytosolic

and Stadtman, 1998). Such conformational changes are suggested as important with regard to triggering cell signalling in response to oxidative stress (Ganther, 1999). In addition to participation of TrxR in cell signalling and redox regulation of transcription factors, reactivation of oxidatively inactivated proteins (Ganther, 1999) could be of great importance in antioxidant defence in the cell. Therefore, TrxRs are involved in protein folding and critical protein-protein and protein-DNA interactions (Ebert-Dümig *et al.*, 1999).

TrxR can also directly reduce thioredoxin, hydrogen peroxide, lipid hydroperoxides, ascorbyl free radical, dehydroascorbic acid, lipoic acid and selenite (Holmgren, 2001) and may have a role in detoxification reactions (Holmgren and Bjornstedt, 1995). The ability of mammalian TrxR to reduce dehydroascorbic acid (May *et al.*, 1997) could be an important link between Se, ascorbic acid and vitamin E in general antioxidant recycling.

Mammalian TrxRs show increased activity with Se supplementation in the nutritional to supranutritional ranges (Ganther, 1999; Holmgren, 2000). An additional unique property of TrxR is its hydroperoxidase activity, which provides self-protection from inactivation by hydroxyl radicals (Zhong and Holmgren, 2000). A general scheme of reactions and functions of thioredoxin reductase in the cell is shown in Figure 2.4 and detailed information on this enzyme can be found in the review by Nordberg and Arner (2001).

TrxR activity in cells is modulated by an intricate interplay, involving:

- Regulation by Se availability. In rats, liver and kidney TrxR activity increased several fold as a result of Se supplementation of the deficient diet (Berggren *et al.*, 1999). However, there is a tissue-specificity in this regulation. For example, after 12 month low Se diet consumption by rats, TrxR activity was decreased in the heart, liver, and kidney, but increased in the arterial wall (Wu and Huang, 2004).
- Regulation of the promoter of TrxR. A housekeeping type promoter in combination with alternative splice variants and transcriptional start sites.
- Posttranscriptional regulation through AU-rich elements. Mammalian TrxR1 and TrxR2 exhibit alternative splicing around the first exon. Regulation via Au-rich elements enables quick expression responses to various stimuli.
- Posttranslational inactivation by ROS and electrophilic agents (prostaglandin derivatives, lipid aldehydes, iodoacetic acid, arsenicals, gold compounds, quinines, nitrosoureas, cisplantin, dinitrohalobenzenes).

Data on TrxR activity in various tissues are obtained mainly with mammals, including laboratory animals and humans. Smith *et al.* (2001) compared TrxR activity in mammals and chickens, finding chickens having extremely low TrxR activities, probably reflecting low TrxR protein expression or being a result of differences between mammalian and chicken TrxR. In fact, Gowdy (2004) used Western blots and found TrxR protein expression at relatively low levels, as well as some differences in molecular weight of the chicken TrxR in comparison to the mammalian enzyme. Data on TrxR activity in chicken tissues have been presented by Edens and Gowdy

(2004). They showed that when Se supplementation was low, the highest TrxR activity was found in kidney and brain and the lowest in the liver. After Se supplementation, TrxR activity increased in practically all tissues studied. Furthermore, organic Se supplemented at 0.3 mg/kg increased TrxR activity significantly more than selenite at the same dose in heart and thymus. There was a similar tendency of increased Se availability from organic Se for activation of TrxR in the brain, breast muscle, bursa, thymus and spleen. The authors also showed that the highest TrxR activity was found in the nuclear pellet and mitochondrial lysates, while the lowest activity was seen in mitochondrial pellets (Edens and Gowdy, 2004). Recently, TrxR activity has been detected in a range of tissues (liver, lung, heart, kidney, brain, breast muscle, bursa, thymus, spleen, RBC and plasma) in broiler chickens (Gowdy *et al.*, 2015). Activity of chicken TrxR was shown to be selenium dependent. Subcellular distribution of TrxR activity was found in association with the cytosolic, nuclear pellet and mitochondrial fractions. Compared with sodium selenite, Se-yeast or SeMet significantly increased the activity and mRNA of TrxR1 in the liver and kidney of broiler breeders and their offspring (Yuan *et al.*, 2012). Selenium dietary supplementation (0.4 mg/kg diet) increased TrxR activity in duodenal mucosa, liver and kidney in chickens (Placha *et al.*, 2014). Se deficiency was associated with a decreased expression of TrxR2 in chicken thyroids (Lin *et al.*, 2014). Similarly, Se deficiency in chickens was associated with a significant decrease in activity of TrxR1 (by 50%), TrxR2 (by 83%) and TrxR3 (by 36%) in pancreas at the 55th day of the experiment (Zhao *et al.*, 2014c). Furthermore, TrxR expression decreased in chicken adipose tissues due to Se deficiency (Liang *et al.*, 2014). Low Se diet (0.028 mg/kg) or high Se diet (3 mg/kg) significantly reduced TrxR activity in chicken kidney with changes in their mRNA levels. In particular, low Se diet downregulated the mRNA expression of TrxR3 (Xu *et al.*, 2016).

TrxRs are involved in protein folding and critical protein-protein and protein-DNA interactions and mammalian TrxRs show increased activity with Se supplementation in the nutritional to supranutritional ranges (Surai, 2006). TrxR activity in cells is modulated by an intricate interplay, involving regulation by Se availability, posttranscriptional regulation and posttranslational inactivation by ROS. Both *in vivo* and *in vitro* studies demonstrated that Trx and TrxR have protective roles against cytotoxicity mediated by the generation of ROS (Calabrese *et al.,* 2009).

Biological roles of the thioredoxin system are diverse and include (Das, 2004; Gromer *et al.*, 2004; Rundolf and Arner, 2004):

- Antioxidant defence. By direct catalysis of several antioxidant reactions and by regeneration of other antioxidant enzymes, such as peroxiredoxins or methionine sulfoxide reductase inactivated by oxidative stress, recycling dehydroascorbate to ascorbate and reduction of ubiquinone to ubiquinol. In fact, the thioredoxin system is a major line of cellular defence against oxygen damage (Hirt *et al.*, 2002). Indeed, cytochrome c is a substrate for both TrxR1 and TrxR2 and cells overexpressing TrxR2 are more resistant to impairment of complex III in the mitochondrial respiratory chain upon both antimycin A and myxothiazol treatments, suggesting a complex III bypassing function of TrxR2 (Nalvarte *et al.*, 2004).

- Redox regulation:
  - Gene regulation by modulating several transcriptional factors, including NF-κB, FOS, Jun, Ref-1 and p53. The reducing activity of Trx for transcriptional factors is more than 100-fold higher than that of GSH (Nakamura, 2004).
  - Modulation of protein phosphorylation, by affecting activity of mitogen activating protein kinases and phosphoprotein phosphatases.
  - Regulation of apoptosis, by controlling apoptosis signal-regulating kinase 1.
  - Redox regulation of various cellular functions including cell proliferation, differentiation and maintenance of viability.
- Regulation of the synthesis of deoxyribonucleotides (DNA synthesis and repair) by providing reducing equivalents to ribonucleotide reductase.
- Involvement in hormone action and cytokine function. Trx can act as an autocrine growth-factor synergising with interleukin (IL)-1 and IL-2; there is evidence that Trx can act as iodothyronine deiodinase (ID) activator.
- Protein biosynthesis. The Trx system is important to maintain high activity of protein biosynthesis machinery in the cell.

Recently, it has been proposed that TrxR1 is a potent regulator of Nrf2, playing a central role in redox homeostasis, defence against oxidative stress, and regulation of redox signalling pathways (Cebula *et al.*, 2015). Indeed, disruption of TrxR1 protects mice from acute acetaminophen-induced hepatotoxicity through enhanced Nrf2 activity (Patterson *et al.*, 2013). It seems likely that TrxR1 reduces the disulfide bonds in Keap1 to arrests Nrf2 in the cytoplasm. On the other hand, inactivation or decreased activity of TrxR1 is associated with disulfide bond formation in Keap1, leading to Nrf2 release and its transfer into the nucleus to drive the transcription of many cytoprotective genes (Cebula *et al.*, 2015). Furthermore, TrxR1 is shown to be an Nrf2 target gene. Therefore, interactions within the antioxidant system are key factors regulating many physiological functions.

2.8 Iodothyronine deiodinases

It is well known that thyroid hormones play important roles in animals and human by controlling growth, development, differentiation and general metabolism in the body. More than 50 years have passed since the first publication demonstrating presence of triiodothyronine (T3) in the tissues of animals and humans given labelled thyroxine (T4) (Gross and Pitt-Rivers, 1951). Synthesis of thyroid hormones takes place in the thyroid gland, mainly in the form of prohormone T4 (Kohrle, 2016). In peripheral tissues, particularly liver and kidney, T4 is converted to T3 (catalysed by iodothyronine deiodinase (Dio)) and it is believed that more than 80% of circulating T3 is derived from deiodination of T4 in nonthyroidal tissue (Kohrle, 2013). Thyroid hormone receptors preferentially bind 3,5,3'-triiodothyronine (T3). Therefore, the metabolism of T4 secreted by the thyroid gland in peripheral tissues, resulting in the production and degradation of receptor-active T3, plays a major role in thyroid function (Decoyer *et al.*, 2005). There are three forms of Dio. Activity of Dio1 was found to be the highest in liver and kidney; of Dio2 in brain, brown adipose tissue and

pituitary; and of Dio3 in brain, skin and placenta (Arthur and Beckett, 1994). Several comprehensive reviews characterise these enzymes in detail (Darras and Van Herck, 2012; Kohrle, 2013, 2016; Orozco *et al.*, 2012; Schweizer and Steegborn, 2015). Some important characteristics of these enzymes are shown in Table 2.5.

A major advancement in the understanding of biochemical mechanisms of Dio actions began with the discovery that the activity of type I Dio depends on selenium status and therefore it was suspected that Dio is a selenoenzyme. For example, Se deficiency for periods of 5 or 6 weeks in rats produced an inhibition of T3 production from added T4 in brain, liver and kidney homogenate (Beckett *et al.*, 1989). Plasma T4 and T3 concentrations increased and decreased, respectively, in selenium-deficient animals. Administration of selenium, as a single intraperitoneal injection of selenite at 200 μg/kg body weight completely reversed the effects of selenium deficiency on thyroid-hormone metabolism. However, selenium administration at 10 μg/kg body weight had no significant effect on thyroid-hormone metabolism (Beckett *et al.*, 1989). Next year, it was suggested that hepatic Dio-1 was a selenoprotein (Arthur *et al.*, 1990). Indeed, solubilised hepatic microsomes from rats injected with ^{75}Se-labelled Na_2SeO_3 four days before killing were found by chromatography on agarose gels to contain a ^{75}Se-containing fraction with Dio1 activity.

It was shown that Dio activity was related to a single ^{75}Se-containing protein with a molecular weight of 27,400 Da. This protein could also be labelled with ^{125}I-bromoacetyl reverse tri-iodothyronine, an affinity label for Dio1 (Arthur *et al.*, 1990). In the same year, in a different laboratory, the same conclusion that type I iodothyronine 5'-deiodinase is a selenoenzyme was made (Behne *et al.*, 1990). Here, a 27.8 kDa membrane selenoprotein was identified in rat thyroid, liver and kidney. This membrane enzyme catalysed the deiodination of L-thyroxine to the biologically active thyroid hormone 3,3',5-triiodothyronine.

The authors showed that a decrease in activity of this enzyme, observed in the liver of Se-deficient rats, was due to the absence of a selenium-dependent membrane-bound component. Furthermore, by using various biochemical techniques, the identity of the ^{75}Se-labeled selenoprotein and the 27 kDa type I 5'-deiodinase subunit was confirmed. It was shown that the deiodinase subunit contained one selenium atom per molecule in the form of selenocysteine (Behne *et al.*, 1990).

Next year, it was shown that the mRNA for this enzyme contained a UGA codon for selenocysteine, which is necessary for maximal enzyme activity (Berry *et al.*, 1991). Several years later, in 1994, it was shown that type III deiodinase is also selenoprotein (for details see Kohrle, 1999) and, finally, type II deiodinase was also proven to be a selenoprotein (Bianco *et al.*, 2002; Curcio *et al.*, 2001). Indeed, this explained why conversion of T4 to T3 is impaired in experimental selenium deficiency and an essential role for selenium in thyroid hormone action was identified. In fact, Se deficiency altered both thyroid hormone synthesis in the thyroid gland and conversion of T4 into T3 due to decreased (by 10 times) activity of Dio (Kohrle, 2013, 2016).

Table 2.5. Main mammalian deiodinases (adapted from Surai, 2006).

Property	Type I 5^{I}-deiodinase	Type II 5^{I}-deiodinase	Type III 5^{I}-deiodinase
Function	systemic > local T3 production, degradation of rT3 and sulfated iodothyronines; source of plasma T3	local > systemic T3 production; provides intracellular T3 in specific tissues; source of plasma T3 (50%)	inactivation of T3 and T4
Expression	liver, kidney, thyroid, intestine, pituitary, heart, brown adipose tissue, placenta	pituitary, brain, brown adipose tissue, skeletal muscle, heart, skin, placenta, thymus, pineal and harderian glands, glial cells and tanycytes	placenta, brain; skin, uterus, fetal tissues, many tissues; not pituitary, thyroid, kidney, adult liver
Subcellular location	endoplasmic reticulum in liver, inner plasma membrane in kidney and thyroid	inner plasma membrane; p29subunit is associated with F-actin and perinuclear vesicles respectively	endoplasmic reticulum
Molecular mass of monomer, Da	29,000	30,500	31,500
Cloned in species	human, rat, mouse, dog, chicken; not expressed in rainbow trout	human, rat, mouse, chicken, rainbow trout	human, rat, mouse, chicken
Essential amino acids residues	histidine, selenocysteine, cysteine, phenylalanine	selenocysteine	selenocysteine
Induction	T3, retinoids; TSH and cAMP in thyroid only; testosterone (liver), carbohydrate	cAMP, fibroblast growth factor, phorbolesters, atrial natriuretic peptide and C-type natriuretic peptide in glial cells	T3, fibroblast growth factor, epidermal growth factor
Se deficiency	decreased	decreased	decreased
Substrate specificity	RT3 >> T4 > T3	T4 > rT3	T3 > T4
Structure	homodimer of a 27-kDa subunit	heterotrimeric complex of appr. 200 kDa containing a 29-kDa subunit	whole structure is not known, contains a 32-kDa subunit

The cloning of the first deiodinase cDNA in the rat by Berry *et al.* (1991) led to sequencing deiodinase in other species, including the chicken (Gereben *et al.,* 1999; Van der Geyten *et al.,* 1997). The amino acid sequence shows a high homology of chicken Dio1 with those of rat (60%), human (62%), and dog (58%), including an in-frame TGA codon at position 127. Furthermore, RNA secondary structure prediction also revealed a putative SECIS element in the 39UTR of ECL1711M (Van der Geyten *et al.,* 1997). The deduced Dio2 protein was shown to be 31 kDa and contained two in frame UGA codons encoding selenocysteine (Gereben *et al.,* 1999). One of those is in the highly conserved active catalytic centre, while the other one is located near the carboxyl terminus. The deduced cDio2 protein sequence was shown to be highly similar to those of rat (83% identity), human (82%), *Rana* (80%), and *Fundulus* (67%). However, it was only ~40% similar to cDio1 and partially to cDio3 deduced protein sequences. The Km of Dio2 is ~1.0 nM for thyroxine, and the reaction is insensitive to inhibition by 6-n-propylthiouracil. It was also shown that chicken Dio2 is expressed as a single transcript of ~6 kb in different brain regions and in the thyroid and lung. Unlike in mammals, Dio2 mRNA and activity were shown to be expressed in the liver of the chicken, suggesting a role for Dio2 in the generation of plasma 3,5,3^{I}-triiodothyronine in this species (Gereben *et al.,* 1999). Like the majority of selenoproteins, deiodinases are thioredoxin-fold proteins (Gladyshev, 2016).

In birds, this hormone is essential for yolk sac retraction, functional maturation of the lungs, pipping and hatching (Decuypere *et al.*, 1990). It has been shown that plasma T3 increases dramatically at time of pipping, when the embryo switches from allantoic to lung respiration (Decuypere *et al.,* 1979, 1982*).* This is correlated with a decrease in hepatic Dio3 activity and the authors suggested that the peak in plasma T3 at the end of incubation is caused by a decrease in its hepatic breakdown (Darras *et al.*, 1992). Dio, especially hepatic Dio3, are acutely regulated during embryonic development. Whereas hepatic Dio1 activity gradually increases during embryonic development from E14 onward, the Dio1 mRNA level remains relatively constant and it seems likely that the regulation of hepatic Dio3 expression during embryonic development occurs predominantly at the pretranslational level (Van der Geyten *et al.,* 1997). The presence of type III deiodinase in the Purkinje cells of the chicken cerebellum has been demonstrated (Verhoelst *et al.,* 2002). Furthermore, the distribution of Dio1 and Dio3 protein in chicken liver and kidney was studied (Verhoelst *et al.,* 2004). Dio1 was detected in hepatocytes in general and the expression pattern was most intense in the area surrounding the blood vessels, while Dio3 was distributed throughout the whole liver. In the kidney, co-localisation of both enzymes was found in the epithelial cells of the renal tubuli and in several layers of the ureter (Verhoelst *et al.,* 2004).

Chicken Dio2 contains 2 Secs (Sec-132 and Sec-265) and the Dio2 mRNA contains a second UGA codon (Zhu *et al.*, 2016). The authors suggested that the first Sec, presented in the putative active centre of Dio2, played a central role in the deiodination process, while the second TGA codon might be a termination signal.

It was shown that Se deficiency in chickens caused a decrease in plasma T3 concentrations and impaired growth of birds (Jianhua *et al.*, 2000). The effects of different forms and concentrations of Se on the regulation of Dio1 mRNA levels in chicken hepatocytes were evaluated (Wu *et al.*, 2010). Primary cultured chicken hepatocyte monolayers were incubated for 24 h with 0 (control), 0.5, 1, 1.5, 2, 3, 4 or 5 μmol/l of Se supplied as DL-selenomethionine (SeMet) or sodium selenite (SS). The Dio1 mRNA levels were significantly increased in all the groups treated with Se (vs control), with maximal effects being observed at 1.5 μmol/l of SeMet and at 0.5 μmol/l of SS, respectively. SeMet at doses of 1.5-5 μmol/l had a greater effect on Doi1 mRNA than SS at equivalent doses. After resulting in a maximal effect, higher Se supplementation led to a dose-dependent reduction in Doi1 mRNA levels in all the hepatocytes treated with Se (Wu *et al.*, 2010). Recent results showed that Se deficiency inhibited the conversion of T4 to T3 and decreased the levels of the crucial metabolic enzymes of the thyroid hormones, Dio1, Dio2, and Dio3, in chickens. In addition, the decreased selenoproteins (Dio1, Dio2, Dio3, TxnR2, SelI, SelU, GSH-Px1, and GSH-Px2) induced by Se deficiency may indirectly limit the conversion of T4 to T3 in chicken thyroids (Lin *et al.*, 2014).

In general, Dio is ranked higher in priority for available Se supply than cytosolic GSH-Px and similar in ranking to that for PH-GSH-Px and SeP (Kohrle, 2013, 2016). In fact, selenium regulation of selenoproteins in the Se-deficient rat liver model has four different patterns (Sunde, 2001):

- GSH-Px is unique with a dramatic (90%) decrease of activity and mRNA level;
- GSH-Px4 represents a second pattern with modest (60%) decrease in activity and little change in mRNA;
- TrxR and SelP show a third pattern with dramatic decrease of activity and little change in mRNA;
- Dio could be classified as having a fourth pattern characterised by dramatic (95%) decrease in activity, but modest (50%) decrease in mRNA.

2.9 Other selenoproteins

Contrary to the well-characterised selenoproteins mentioned above, the other selenoproteins are less studied and their functions are less obvious. They described in the next paragraphs.

2.9.1 Selenophosphate synthetase-2

Selenophosphate synthetase-2 (SPS) is an enzyme involved in selenocysteine biosynthesis providing an autoregulatory mechanism for selenoprotein expression (Guimaraes *et al.*, 1996). Selenophosphate is synthesised from selenide and ATP by selenophosphate synthetase, and is required for selenocysteine synthesis and its subsequent incorporation into selenoproteins (Low *et al.*, 1995). There are two forms of SPS in mammals with SPS-2 being a selenoprotein. For example, SPS was

detected using immunoblotting in extracts of rat brain, liver, kidney, and lung (Kim and Stadtman, 1995). The reaction catalysed by SPS proceeds as follows:

$$ATP+selenide+H_2O \longrightarrow selenophosphate+P_i+AMP$$

Se deficiency in chickens was associated with decreased expression of SPS in liver (Liu *et al.*, 2014b), muscles (Yao *et al.*, 2014), muscle stomach (Huang *et al.*, 2017), thymus (Khoso *et al.*, 2015; Yang *et al.*, 2016), spleen and bursa of Fabricius (Yang *et al.*, 2016), aorta vessels (Du *et al.*, 2016), thyroid (Lin *et al.*, 2014), erythrocytes (Luan *et al.*, 2016), pancreas (Zhao *et al.*, 2014a) and adipose tissue (Liang *et al.*, 2014).

2.9.2 15-kDa selenoprotein

15-kDa selenoprotein (Sep15) was identified and characterised by Gladyshev *et al.* (1998) when it was purified from human T-cells. In fact, its mRNA showed all features known to be necessary in other eukaryotic selenoprotein mRNAs to promote selenocysteine insertion into proteins (Gladyshev *et al.*, 1998). It contains a single selenocysteine residue in the middle of a 162-amino acid open reading frame and has no detectable homology to known proteins (Gladyshev *et al.*, 2001). The Sep15 gene spans 51 kb of the human genome and is organised in five exons and four introns (Kumaraswamy *et al.*, 2002). In accordance with the incidence of human 15-kDa protein gene expression, tissues can be placed in the following descending order: thyroid > parathyroid tumour > prostate pre-cancerous cells > prostate > foetal lung > aorta ≥ foetal retina > retina > testis > foetal heart (Gladyshev *et al.*, 1998). Sep15 knockout mice are viable, but develop cataracts (Gladyshev, 2016). Sep15 is located in the endoplasmic reticulum being bound to UDP-glucose: glycoprotein glucosyltransferase (Gladyshev *et al.*, 2001) suggesting Sep15 to participate in the control of protein folding (Carlson *et al.*, 2016). Genes encoding Sep15 have also been detected in mice and rat (Kumaraswamy *et al.*, 2002). The role of this selenoprotein is not clearly defined, but it seems likely that Sep15 is involved in reduction or isomerisation of disulfide bonds, regulated by endoplasmic reticulum (ER) stress (Labunskyy *et al.*, 2009), and participates in the quality control of glycoprotein folding (Ferguson *et al.*, 2006). Sep15 might also be part of the antioxidant defence system under various stress conditions (Carlson *et al.*, 2016). For example, recently it has been shown that reduction of Sep15 expression aggravated tunicamycin (Tm)-induced cell apoptosis and caspase activation in human lens epithelial cells. Furthermore, the knockdown of Sep15 exacerbated Tm-induced oxidative stress while ER stress was not correspondingly elevated (Yin *et al.*, 2015).

It was shown that the chicken Sep15 SECIS element was located in the 3'-untranslated region (UTR). SECIS was classified as type I. The nucleotide identity of chicken Sep15 shared 65.6-91.5% identity with other species. Furthermore, the amino acid sequence of chicken Sep15 shares an 69.6-93.8% identity with other species. Chicken Sep15 reserved higher identity with bird homologies (nucleotide, 91.5%; amino acid, 93.8%) than with mammal homologies (nucleotide, 72.7-77.6%; amino acid, 75.8-79.5% (Sun *et al.*, 2014). The predicted secondary structure of chicken Sep15 reserved a C×U

motif between a predicted α-strand and a β-helix. Indeed, chicken Sep15 is shown to be a member of Rdx family and has a thioredoxin-like domain and a surface-accessible active site redox motif. Therefore, Sep15 can function as a thiol-disulphide isomerase involved in disulphide bond formation in the endoplasmic reticulum. Furthermore, chicken Sep15 is a thioredoxin-like fold protein and can participate in the preservation of cell redox potential (Sun *et al.*, 2014). Under common conditions, the expression of Sep15 was higher in the spleen followed by the bursa of Fabricius and thymus. Se deficiency decreased the levels of Sep15 in these immune tissues. The deficiency of Sep15 reduced the cell viability and increased the occurrence of oxidative stress in chicken splenocytes increasing the sensitivity of cell to H_2O_2. The silencing of Sep15 increased the GSH-Px activities and MDA levels (Sun *et al.*, 2014). Indeed, Sep15 can be involved in antioxidant protection in the cell. Sep15 is an ER-selenoprotein in birds and structural coordination has been described in the binding between Zn^{2+} and chicken Sep15. It seems likely that Sep15 is involved in signal pathway activation leading to the reduction of unfolded proteins in the ER (Zhu *et al.*, 2016).

When a low Se chicken diet containing 0.01 mg/kg Se was supplemented with sodium selenite (0.3 mg/kg Se), SelN concentration in chicken muscles increased by 33 and 46% at 2 and 4 weeks, respectively (Huang *et al.*, 2015b). It has been shown Se deficiency in chicken was associated with decreased mRNA expressions of Sep15 in the liver (Liu *et al.*, 2014b), muscles (Yao *et al.*, 2014), muscle stomach (Huang *et al.*, 2017), kidney (Zhang *et al.*, 2016), thymus (Khoso *et al.*, 2015; Yang *et al.*, 2016), spleen and bursa of Fabricius (Yang *et al.*, 2016), aorta vessels (Du *et al.*, 2016), thyroid (Lin *et al.*, 2014), erythrocytes (Luan *et al.*, 2016) and adipose tissue (Liang *et al.*, 2014).

2.9.3 Selenoprotein H

Selenoprotein H (SelH) is a 14 kDa, thioredoxin fold-like protein that contains a conserved Cys-X-X-Sec motif (X is any amino acid). Its expression is widely distributed throughout a variety of tissues and relatively high in early stages of embryonic development (Novoselov *et al.*, 2007). Levels of SelH mRNA are highly sensitive to adequate Se intake (Sunde *et al.*, 2009). SelH is nucleolar oxidoreductase and a redox-responsive DNA binding protein participating in regulation of expression of various genes involved in *de novo* antioxidant (GSH) synthesis and phase II detoxification in response to redox status changes (Novoselov *et al.*, 2007). It was shown that overexpression of SelH protects HT22 neuronal cells against superoxide formation after UVB irradiation (Ben Jilani *et al.*, 2007) and exposure to oxidants (H_2O_2) upregulated SelH expression in human cells, while SelH protects against cellular senescence to oxidative stress through a genome maintenance pathway involving ATM and p53 (Wu *et al.*, 2014a). It has been suggested to consider SelH as a sensor of oxidative stress in the nucleus, where it may play a dual role in redox maintenance as an antioxidant and regulation of gene expression as a transactivator (Zhang *et al.*, 2016a). Indeed, SelH was shown to regulate redox homeostasis and suppresses DNA damage. SepH deficiency impairs the redox balance by decreasing the levels of ascorbate and methionine, while increasing methionine sulfoxide formation. It was

also shown that SepH deficiency induces an inflammatory response and activates the p53 pathway, while loss of SepH increases susceptibility to oxidative stress and DNA damage (Cox *et al.*, 2016).

Decreased mRNA expression of SepH in chicken liver (Liu *et al.*, 2014b), kidney (Zhang *et al.*, 2016), muscles (Yao *et al.*, 2014), muscle stomach (Huang *et al.*, 2017), adipose tissue (Liang *et al.*, 2014), thymus, spleen and bursa of Fabricius (Yang *et al.*, 2016), pancreas (Zhao *et al.*, 2014a), erythrocytes (Luan *et al.*, 2016), thyroid (Lin *et al.*, 2014), testes (Gao *et al.*, 2017) and aorta vessels (Du *et al.*, 2016) was shown to be associated with Se deficiency.

2.9.4 Selenoprotein I

Selenoprotein I, first identified by Kryukov *et al.* (2003) in sequenced mammalian genomes by methods that rely on identification of selenocysteine insertion RNA structures, the coding potential of UGA codons, and the presence of cysteine-containing homologs, is a transmembrane protein belonging to the CDP-alcohol phosphatidyltransferase class-I family and it is involved in phospholipid synthesis. Human SelI is ubiquitously expressed in multiple tissues (Horibata and Hirabayashi, 2007). The SelI gene is conserved in human, chimpanzee, Rhesus monkey, dog, mouse, rat, chicken, fruit fly, mosquito, *Caenorhabditis elegans*, and frog. The amino acid sequence for SelI contains seven transmembrane helices (Reeves and Hoffmann, 2009). High-fat diet in pigs was shown to upregulate SelI in thyroid and downregulate it in pancreas, subcutaneous fat and pituitary (Zhao *et al.,* 2015). Further research is needed to address questions regarding this selenoenzyme in terms of its functions in avian species. Chicken SelI is shown to be located in the ER membrane residing entirely in the lipid bilayer (Zhu *et al.*, 2016). It was shown that SelI catalyses the formation of lipid phosphatidylethanolamine (Liu and Rozovsky, 2015), an important constituent of biomembranes. It was hypothesised that the physiological role of chicken SelI Sec was related to the composition of the membrane (Zhu *et al.*, 2016).

Se deficiency in chicken was shown to decrease mRNA expressions of SelI in kidney (Zhang *et al.*, 2016), muscle stomach (Huang *et al.*, 2017), adipose tissue (Liang *et al.*, 2014), thymus, spleen and bursa of Fabricius (Yang *et al.*, 2016), pancreas (Zhao *et al.*, 2014a), erythrocytes (Luan *et al.*, 2016), thyroid (Lin *et al.*, 2014) and aorta vessels (Du *et al.*, 2016). However expression of SelI in chicken testes increased due to Se deficiency (Gao *et al.*, 2017).

2.9.5 Selenoprotein K

SelK is a small (~12 kDa) protein and it was initially identified through bioinformatic analysis of the human genome (Carducci *et al.,* 2012). SelK ubiquitous expression in human and animal tissues, such as heart, liver, pancreas and skeletal muscles was clearly shown (Lu *et al.,* 2006). Chicken SelK was present as a homodimer containing unknown domains formed by 2-91 residues, but Sec was positioned within the C-terminal five residues facing the cytoplasm (Zhu *et al.,* 2016).

Recent results showed that GSH-Px1, GSH-Px2, GSH-Px4, Dio3, SepW1, SelH and SepP1 mRNA levels in the siRNA group were decreased, and TrxR1, TrxR3, SepN1, SelS, SelT, SelM, Sep15, SelI, SelO and SepX1 mRNA levels were increased after 24 h of siRNA treatment (Fan *et al.*, 2016). The GSH-Px1, GSH-Px2, GSH-Px4, TrxR1, TrxR2, TrxR3, Dio3, SepN1, SelS, SepW1, SelT, SelH, SelM, Sep15, SelI, SelU, SelP, SelO and SepX1 mRNA levels in the siRNA group were decreased and the GSH-Px2 and SPS2 mRNA levels in the siRNA group were increased after 48 h of siRNA treatment (Fan *et al.*, 2016). After 72 h of siRNA treatment, the GSH-Px3, Dio2, Dio3 and SelH mRNA levels were decreased while the mRNA levels of TrxR1, TrxR2, TrxR3, SepN1, SelT, SelM, SelI, SelO, SepX1 and SPS2 were increased (Fan *et al.*, 2016). Thus, selenoproteins show a different response to SelK silencing at different time points. Indeed, SelK has an ubiquitous expression in the chicken myoblast and plays a certain role in regulating the expressions of other selenoproteins. It seems likely that TrxR1, TrxR2, SelO, SepP1, Dio2 and SepB play special roles in response to silencing by SelK. Therefore, it could well be that SelK is associated with antioxidant function, ER protein folding, Ca regulation roles and hormone activity regulation (Fan *et al.*, 2016). SelK likely plays an important role in antioxidant defences of the cell. For example, overexpression of SelK attenuated the intracellular reactive oxygen species level and protected cells from oxidative stress-induced toxicity in neonatal rat cardiomyocytes (Lu *et al.*, 2006). Indeed, SelK is shown to be a stress-regulated protein protecting HepG2 cells from ER stress agent-induced apoptosis (Du *et al.*, 2010). Recently, a specific role of SelK was shown as a cofactor in the post-translational addition of palmitic acid to certain proteins (palmitoylation), a modification that stabilises the expression of various proteins (Hoffmanm, 2016) affecting immunity and possibly other physiological functions of poultry. Interestingly, gene silencing of SelK was shown to induce inflammatory response (inflammatory factors and inflammation-related cytokines), and to activate HSP expression in chicken myoblasts (Fan *et al.*, 2017). SelK shows a high response to Se deficiency in chicken kidney (Zhang *et al.*, 2016), adipose tissue (Liang *et al.*, 2014), thymus, spleen and bursa of Fabricius (Yang *et al.*, 2016), pancreas (Zhao *et al.*, 2014a), erythrocytes (Luan *et al.*, 2016), muscles (Yao *et al.*, 2014), muscle stomach (Huang *et al.*, 2017), thyroid (Lin *et al.*, 2014), testes (Gao *et al.*, 2017) and aorta vessels (Du *et al.*, 2016).

2.9.6 Selenoprotein M

SelM was first reported as a new selenoprotein using bioinformatic methods (Korotkov *et al.*, 2002). It was shown to be highly conserved from plants to humans. It is considered to be a distant homolog of SEP15 and, like SEP15, it resides in the endoplasmic reticulum (Gladyshev, 2016). In mammals, SelM is ubiquitously expressed in many tissues, including muscle, liver, kidney, cerebral cortex, pituitary, thyroid, and testis (Zhou *et al.*, 2011). SelM is an antioxidant enzyme participating in the promotion of cell growth and survival in a variety of tissues, although the exact mechanisms remain to be fully elucidated (Gong *et al.*, 2016). Furthermore, SelM positively regulates the ERK, NOTCH, and STAT3 signalling pathways and is involved in maintenance of Ca^{2+} homeostasis.

In chicken, the SelM coding sequence (CDS) has 459 bases with an in-frame TGA triplet. The sequence theoretically encodes 152 amino acid residues, with Sec the 37th residue. The chicken SelM had 78% CDS homology to human SelM and 82, 78, 94, 73 and 73% CDS homology to cow, duck, turkey, zebrafish and frog, respectively (Huang *et al.*, 2016). It was shown that a SECIS element, with the conserved adenosine (-AAA-) rather than the cytidine (-CC-) motif in the apical loop was found in the 3'-untranslated region of the cDNA (mRNA), which is similar to the majority of avian species. The chicken SelM protein was aligned with 16 species. It shows an overall 58% identity with a highly conserved thioredoxin-like domain containing a CXXU motif across all the species. Indeed, the chicken SelM protein is characterised by a β-α-β-β-β-α secondary structure pattern that forms a classical motif observed in thioredoxin-like fold proteins. The amino acid sequence of chicken SelM shares a 79, 84, 97, and 58% identity with SelM, in humans, duck, turkey, and zebrafish, respectively. In phylogeny, when SelM from the 20 species was genetically clustered, the *Phasianinae*, chicken, and turkey had the shortest distance (Huang *et al.*, 2016). The mRNA and protein levels of SelM in the brain of Se-deficient chickens were significantly lower than those in the brain of Se adequate chickens. The authors suggested that SelM is one of the major selenoperoxidases contributing to the prevention of brain injury possibly through the peroxide scavenging and antioxidant functions.

It has also been shown that Se deficiency in chicken caused decreased mRNA expressions of SepM in the liver (Liu *et al.*, 2014b), aorta vessels (Du *et al.*, 2016), thyroid (Lin *et al.*, 2014), muscles (Yao *et al.*, 2014), muscle stomach (Huang *et al.*, 2017), erythrocytes (Luan *et al.*, 2016), pancreas (Zhao *et al.*, 2014a), thymus, spleen and bursa of Fabricius (Yang *et al.*, 2016), adipose tissue (Liang *et al.*, 2014), but increased mRNA expression of SelM was evident in kidney (Zhang *et al.*, 2016).

2.9.7 Selenoprotein N

Selenoprotein N (SepN1, SelN) gene was first identified *in silico* and *in vivo* by using a conserved RNA structural motif by Lescure *et al.* (1999). As in other selenoproteins incorporation of SeCys takes place at a redefined UGA codon and requires the involvement of stem loop structure formed by the SECIS sequence. The human SEPN1 gene is located on chromosome 1p36 (RSMD1 locus) and contains 13 exons spanning 18.5 kb (Moghadaszadeh *et al.*, 2001) and it produces a 4.5 kb transcript encoding a 590 amino acid protein. The authors showed that mutations in SEPN1 caused congenital muscular dystrophy with spinal rigidity and restrictive respiratory syndrome. Therefore, it was the first description of a selenoprotein implicated in a human disease. Furthermore, a relationship between classical multiminicore disease (MmD; an autosomal recessive congenital myopathy characterised by the presence of multiple, short core lesions in most muscle fibers) and the selenoprotein N gene (SEPN1) was suggested (Ferreiro *et al.*, 2002). Therefore, desmin-related myopathy with mallory body-like inclusions was suggested to be caused by mutations of the selenoprotein N gene (Ferreiro *et al.*, 2004). The main SEPN1 gene product corresponds to a 70 kDa protein, containing a singly SeCys residue (Petit *et al.*, 2003). The authors also showed that SepN is a glycoprotein localised within the endoplasmic

reticulum. SepN1 was found to present in high levels in several human foetal tissues and at much lower levels in adult tissues, including skeletal muscle. This protein is also highly expressed in cultured myoblasts (Petit *et al.*, 2003). The authors suggested a role of SelN in regulation of early development and in cell proliferation or regeneration. Furthermore, SEPN1 zebrafish mutants exhibited strong developmental defects related to abnormalities in muscle development and architecture (Deniziak *et al.,* 2007) confirming an important role of SelN in muscle development.

SepN1 is thought to have a redox function, and this idea is supported by the observation of a motif similar to the catalytic site of thioredoxin reductases (TrxRs) on the ER side of the SEPN1 sequence (Marino *et al.*, 2015). In fact, it was shown that the promoter of the SepN1 gene contains CpG islands, predicted target sites for the transcription factor nuclear factor kappa beta (NF-κB), an ER stress response element, as well as binding sites for the redox-sensitive transcription factor AP1 (Castets *et al.*, 2012). Indeed, excessive protein oxidation in cells devoid of SepN1 (Arbogast *et al.*, 2009; Lescure *et al.*, 2009) confirms the antioxidant functions of SepN. Furthermore, it was shown that SEPN1 levels parallel those of endoplamic reticulum oxidoreductin 1 (ERO1), an ER protein thiol oxidase, and that SEPN1's redox activity defends the ER from ERO1-generated peroxides. Cells lacking SEPN1 are hypersensitive to ERO1 overexpression and conspicuously defective in ER calcium re-uptake (Marino *et al.*, 2015). Indeed, SelN is involved in maintenance of ER redox and calcium homeostasis. Chicken SelN was shown to contain a SCUG motif and a catalytic site similar to the GCUG motif in TrxR. Furthermore, the domain in SelN was shown to be similar to the classical one (Zhu *et al.,* 2016). The authors also indicated that the important FAD- and NADPH-binding domains of classical TrxRs were missing from SelN.

The expression of chicken SelN mRNA was studied in a range of chicken tissues of 90 day old chickens and it was shown that it was higher in the pituitary gland, craw, glandular stomach, rectum, spleen, lung, and testis (1.85-, 1.5-, 3.3-, 4.9-, 4.7-, 2- and 3.2-fold, respectively, compared with kidney) (Zhang *et al.*, 2012d). SelN mRNA was lower in the duodenum, small intestine, caecum, thymus, and pancreas (90, 89 and 73%, 1.25- and 1.11-fold, respectively, compared with the kidney). At the same time, it was shown that the expression of chicken SelN mRNA was comparatively low in the cerebrum, thalamus, spinal cord, tongue, esophagus, muscular stomach, bursa of Fabricius, liver, breast muscle, flight muscle, shank muscle, thigh muscle, and heart (Zhang *et al.*, 2012d). The authors showed that the expression of SelN mRNA in skeletal muscles is high in embryos and lower in postnatal stages indicating a potential role of SelN in chick embryo skeletal muscle development. In chickens it was shown that SelN mRNAs abundance was different for different muscles in the control. Levels of SelN mRNA were lower in breast muscle and flight muscle than in other tissues in the control. The effect of Se supplementation on SelN mRNA abundance was small in breast muscle and flight muscle, which was consistent with tissue Se content. Changes in SelN mRNA abundance in thigh and shank muscles were correlated to Se supply. Levels of SelN mRNA were increased by Se intake, while excessive Se supplementation led to dose-dependent decreasing trends of the mRNA level (Zhang *et al.*, 2012a). Dietary selenium supplementation was shown to increase mRNA and/or activities of

6 selenoprotein genes, including SelN in the liver of AFB_1-treated chickens (Sun *et al.*, 2016). Similarly, it was shown that Se supplementation significantly increased the expressions of selenoprotein K, selenoprotein N, selenoprotein S, and selenoprotein T, which were reduced by cadmium in chicken splenic lymphocytes (Zhao *et al.*, 2014c). The expression of SelN gene in cardiac muscle responded to dietary Se concentrations: It was downregulated in the Se deficiency group and upregulated in the Se excess group compared with the moderate Se group (Zhang *et al.*, 2014b). When a low Se chicken diet, containing 0.01 mg/kg Se, was supplemented with sodium selenite (0.3 mg/kg Se) SelN concentration in chicken muscles increased by 14 and 177% at 2 and 4 weeks, respectively (Huang *et al.*, 2015b).

Similar to other selenoproteins decreased mRNA expressions of SepN1 in the liver (Liu *et al.*, 2014b), thymus (Khoso *et al.*, 2015), aorta vessels (Du *et al.*, 2016), thyroid (Lin *et al.*, 2014), erythrocytes (Luan *et al.*, 2016), thymus, spleen and bursa of Fabricius (Yang *et al.*, 2016), kidney (Xu *et al.*, 2016; Zhang *et al.*, 2016), muscle stomach (Huang *et al.*, 2017), and adipose tissue (Liang *et al.*, 2014) was shown in Se deficient chickens. Interestingly, Se deficiency was shown to increase expression of SepN1 in chicken testes (Gao *et al.*, 2017).

2.9.8 Selenoprotein O

Selenoprotein O (SelO) has remained enigmatic since identification of its sequence in the human genome by Kryukov *et al.* (2003). While human Sel O is predicted to consist of 669 amino acids with a calculated molecular weigth of 73.4 kDa, there is no information regarding its physiological role and possible functions. Because of the presence of a Cys-X-X-Sec motif, it could well be that SelO has a redox function (Reeves and Hoffman, 2009). Using bioinformatics tools, it was predicted that SelO protein adopts a three-dimensional fold similar to protein kinases and the possibility of an oxidoreductase-regulated kinase function for SelO was suggested. Furthermore, expression data from bacteria and yeast suggest a role in oxidative stress response (Dudkiewicz *et al.*, 2012). Later it was shown that in mammals SelO is a redox-active mitochondrial selenoprotein. Indeed, CxxU motif was identified in the C-terminal region of SelO and SelO reversible oxidation by H_2O_2 was shown in HEK 293T cells. SelO was shown to be localised to mitochondria and expressed across mouse tissues with low response to Se deficiency, suggesting its high position in selenoprotein hierarchy (Han *et al.*, 2014). It was suggested that chicken SelO might have kinase activity and ligand binding sites containing Mg^{2+} was identified (Zhu *et al.*, 2016).

It has also been shown that Se deficiency in chicken downregulates mRNA expressions of SepO in liver (Liu *et al.*, 2014b), kidney (Zhang *et al.*, 2016), adipose tissue (Liang *et al.*, 2014), thymus, spleen and bursa of Fabricius (Yang *et al.*, 2016), pancreas (Zhao *et al.*, 2014a), erythrocytes (Luan *et al.*, 2016), muscles (Yao *et al.*, 2014), muscle stomach (Huang *et al.*, 2017), thyroid (Lin *et al.*, 2014) and aorta vessels (Du *et al.*, 2016).

2.9.9 Selenoprotein P

The history of selenoprotein P (SepP) discovery is related to characterisation of plasma proteins after injection with ^{75}Se-selenite. SeP was distinguished from GSH-Px in plasma in 1977 by Herrman, and was identified as a plasma selenoprotein in the early 1970s (Herrman, 1977). Indeed, known at that time selenoprotein (GSH-Px) was not the only one in the plasma. Finally, 9 years after GSH-Px characterisation as selenoprotein, Motsenbocker and Tappel (1982) described the same plasma protein in monkey and rat and called it selenoprotein P. Indeed, SepP was the second, after GSH-Px, selenoprotein identified in mammals and its name was given to this protein because of its plasma location. SepP is a glycoprotein representing from about one third of total plasma Se in humans (Allan *et al.*, 1999) to about 60-70% (Kohrle *et al.*, 2000) or 60-80% of total plasma Se in humans and rodents (Arthur and Beckett, 1994). In fact, SepP is the major plasma selenoprotein, which is synthesised primarily in the liver, that delivers Se to certain other organs and tissues (Gladyshev, 2016). Selective deletion of SepP in hepatocytes was associated with impairment in selenium supply to extra-hepatic tissues and worsens dietary selenium deficiency (Hill *et al.*, 2012). Receptors mediating cellular uptake of SepP have recently been identified. Indeed, Sertoli cell ApoER2 is a SepP receptor and a component of the selenium delivery pathway to spermatogenic cells (Olson *et al.*, 2007).

SepP contains up to 10 selenocysteine residues per 43 kDa polypeptide chain (Allan *et al.*, 1999). Indeed, the unique feature of SepP is that it is the only selenoprotein known to have more than one Se atom per polypeptide chain. The gene for human SeP is located on chromosome 5q31 (Arteel *et al.*, 2002). It was initially believed that this selenoprotein was a major transport form of Se (Motsenbocker and Tappel, 1982) owing to its comparatively high concentration of Se. In fact compared with other Se-containing proteins, the addition of purified SepP to the SepP-depleted serum or a serum-free medium was the most effective for the recovery of cellular GSH-Px activity (Saito and Takahashi, 2002) confirming an idea that SepP functions as Se-supply protein, delivering Se to the cells. Indeed, SepP participates in the distribution of Se from the liver to peripheral tissues, such as the brain (Schweizer *et al.*, 2016). Furthermore, it has been suggested that SepP might have an important role as an antioxidant in plasma (Steinbrenner *et al.*, 2006). In fact, SepP is shown to have two domains: the N-terminal domain appears to be an enzyme with redox properties, while the C-terminal domain contains selenium that is in transit. Selenoprotein P can contribute to the decomposition of peroxynitrite (Arteel *et al.*, 1998) and protection of endothelial cells against damage from peroxynitrite, having peroxidase activity *in vitro* (Sies *et al.*, 1998). SepP of bovine serum acts as a survival-promoting factor in neuronal cell culture (Mostert, 2000). Indeed, SepP reduces neither H_2O_2 nor tertiary butyl hydroperoxide, but reduces phospholipid hydroperoxides using tert-uni ping-pong mechanism, similar to those described for GSH-Px, therefore, SepP is considered to act as an extracellular PH-GSH-Px (Saito *et al.*, 1999). It was demonstrated in a cell-free system that SepP isolated from human plasma protects low-density lipoproteins against oxidation (Steinbrenner *et al.*, 2016) and knock-down of SepP1 was shown to sensitise the astrocytes to oxidative stress-mediated cell death (Steinbrenner *et*

al., 2006). Thioredoxin is shown to be the preferred electron donor for SeP (Takebe *et al.*, 2002). This protein has been shown to bind heavy metals, is considered as a negative acute phase protein and can promote neuron survival *in vitro* (Kohrle *et al.*, 2000). It has been suggested that SepP has specific roles in protecting against Se toxicity and sequesting Cd^{2+} and Hg^{2+} ions (Yoneda and Suzuki, 1997). Clearly the antioxidant-related functions of this protein could be a great advantage for animals. SepP-knockout mice were viable, but exhibited reduced growth and developed ataxia (Schomburg *et al.*, 2003).

SepP is expressed in various tissues including liver, heart, brain, kidney, testis and muscle in rats and also in placenta and uterus in the mouse and associated with cell membranes (Schweizer *et al.*, 2016). It is interesting that SepP can be synthesised in the brain (Yang *et al.*, 2000). It seems likely that tissues, such as the liver, can take up small-molecule forms of selenium whereas presence of the element in selenoprotein P facilitates uptake by tissues like the brain. Four isoforms of the protein have been identified in rat plasma, including the full length protein containing 10 SeCys, and three shortened isoforms (Chen and Berry, 2003). SepP genes in mammals include 10-12 UGA codons and two SECIS elements in the 3^{I} untranslated region (Chen and Berry, 2003). It is interesting to note that in Se deficiency SepP mRNA and protein expression are preferentially retained in comparison to other selenoproteins (Chen and Berry, 2003) showing high position of SepP in selenoprotein hierarchy.

SelPa contains 10 Sec residues in humans and mice while 13 Sec residues were evident in chicken SelPa and the SelPa gene was shown to contain two SECIS elements in *Gallus gallus*. (Zhu *et al.*, 2016). It seems likely that SelPa and GSH-Px3 are major secreted selenoproteins participating in Se transport functiins. Data on SepP in poultry are very limited (Yuan *et al.*, 2013). Indeed, an 8 wk experiment was conducted to investigate the effect of different sources (0.15 mg/kg) of Se on the concentration and gene expression of SepP in broiler breeders and their offspring. It was shown that organic Se sources (Se-yeast and SeMet) were more effective than SS in increasing SepP levels in the serum and liver of broiler breeders, but not in the kidney. Furthermore, the levels of SepP in the serum, liver, and kidney of offspring were significantly higher in SY or SeMet treatments than in the SS treatment. The authors also showed that the liver SEPP1 mRNA level of broiler breeders in SY or SeMet treatments was significantly higher than that in the SS treatment; however, no differences were observed in the kidney of broiler breeders. At the same time, day-old chicks from SY or SeMet breeder treatments had a significantly higher SEPP1 mRNA level than those from the SS treatment (Yuan *et al.*, 2013). In general in liver and kidney SepP concentration and expression were comparable. Furthermore, Se deficiency in chickens was associated with decreased expression of SepP in a liver (Liu *et al.*, 2014b), muscles (Yao *et al.*, 2014), muscle stomach (Huang *et al.*, 2017), kidney (Zhang *et al.*, 2016), erythrocytes (Luan *et al.*, 2016), thymus (Khoso *et al.*, 2015; Yang *et al.*, 2016), spleen and bursa of Fabricius (Yang *et al.*, 2016), aorta vessels (Du *et al.*, 2016), thyroid (Lin *et al.*, 2014), pancreas (Zhao *et al.*, 2014a) and adipose tissue (Liang *et al.*, 2014).

2.9.10 Selenoprotein Pb

SelPb is not found in placental mammals, but can be found in birds, fish and *Platypus* and *Marsupial* mammals (Mariotti *et al.*, 2012). It is still not well characterised, but generally speaking it is a modified SelP. In zebrafish it was shown that, while SelPa was similar to mammalian SelP containing multiple Sec residues and two SECIS elements in its mRNA, SelPb contained one Sec residue and retained a single SECIS element (Kryukov and Gladyshev, 2000). During embryogenesis of zebrafish SelPb was identified in the yolk syncytial layer and liver (Thisse *et al.*, 2003). The biological role and physiological functions of SelPb are not known but most likely they are not different from classical SelP.

It has also been proven that Se deficiency in chicken was associated with decreased mRNA expressions of SelPb in liver (Liu *et al.*, 2014b), muscles (Yao *et al.*, 2014), muscle stomach (Huang *et al.*, 2017), pancreas (Zhao *et al.*, 2014a), thyroid (Lin *et al.*, 2014), aorta vessels (Du *et al.*, 2016) and adipose tissue (Liang *et al.*, 2014), but increased mRNA expressions of SepPb was evident in chicken kidney (Zhang *et al.*, 2016) due to Se deficiency. Furthermore, Se deficiency was associated with increased expression of SelPb in chicken testes (Gao *et al.*, 2017).

2.9.11 Selenoprotein R

Selenoprotein R (MsrB) was identified as a zinc-containing stereo-specific methionine sulfoxide reductase B (Kryukov and Gladyshev, 2002; Moskovitz *et al.*, 2002). Furthermore, it has been shown that there was a loss of MsrB activity in the *MsrA*$^{-/-}$ mouse in parallel with losses in the levels of MsrB mRNA and MsrB protein (Moskovitz and Stadtman, 2003). Se deficiency in mouse was associated with a substantial decrease in the levels of MsrB-catalytic activity, MsrB protein, and MsrB mRNA in liver and kidney tissues (Moskovitz and Stadtman, 2003). It has been reported that human and mouse genomes possess three MsrB genes responsible for synthesis of the following protein products: MsrB1, MsrB2 and MsrB3 (Kim and Gladyshev, 2004). In particular, MsrB1 was present in the cytosol and nucleus and exhibited the highest methionine-R-sulfoxide reductase activity due to presence of selenocysteine (Sec) in its active site. Other mammalian MsrBs are not selenoproteins and contain cysteine in place of Sec and were less catalytically efficient (Kim and Gladyshev, 2004). Details of biochemical functions of MsrB were described in Chapter 1. Indeed, MsrB1participate in repairing oxidised proteins and support resistance of proteins, cells, tissues, and organisms to oxidative stress, both *in vitro* and *in vivo*. Furthermore, it seems likely that MsrB1 can also regulate protein functions through reversible Met sulfoxidation, thereby controlling various biological processes (Kaya *et al.*, 2015). Indeed, MsrB is an oxidoreductase that protect against the effects of oxidative stress by increasing oxidative stress resistance and repairing damaged proteins via cyclic methionine oxidation/reduction (Lee *et al.*, 2016). Chicken MsrB1 was shown to bind with Zn^{2+} through 4 Cys (-23, -26, -69 and -72) residues (Zhu *et al.*, 2016). In particular, Cys-26 and Cys-72 were shown to form a disulfide bond being potentially the catalytic centre

of the domain. It seems likely that chicken MsrB is an important player in protecting proteins against oxidative damage.

Decreased mRNA expressions of MsrB in the chicken liver (Liu *et al.*, 2014b), thymus (Khoso *et al.*, 2015; Yang *et al.*, 2016), spleen and bursa of Fabricius (Yang *et al.*, 2016), aorta vessels (Du *et al.*, 2016), thyroid (Lin *et al.*, 2014), muscles (Yao *et al.*, 2014), muscle stomach (Huang *et al.*, 2017), erythrocytes (Luan *et al.*, 2016), pancreas (Zhao *et al.*, 2014a), kidney (Xu *et al.*, 2016) and adipose tissue (Liang *et al.*, 2014) was shown due to Se deficiency. Furthermore, increased activity of MsrB in muscles as a result of improved Se status due to usage of OH-SeMet in the diet (Zhao *et al.*, 2017a), could help decrease protein oxidation and reduce drip loss.

2.9.12 Selenoprotein S

Selenoprotein S (SelS) has been recently identified by Kryukov *et al.* (2003). It is considered to be a novel member of the glucose-regulated protein family and its function is related to the regulation of cellular redox balance.

It was shown that chicken SelS contained an unknown domain. It is predicted that the 14-193 residues that form the α-helices and other principal parts of SelS might be responsible for its redox and structural properties (Zhu *et al.,* 2016). The authors suggested that chickens SelS can protect the cell against oxidative damage to regulate ER stress-induced apoptosis. Recently the induction of the SelS gene in HepG2 cells has been characterised and its function examined as an antioxidant (Gao *et al.*, 2004). SelS gene expression was shown to be up-regulated in the liver of *Psammomys obesus* after fasting. In fact, SelS was regulated by glucose deprivation and ER stress in HepG2 cells. For example, glucose deprivation and the ER stress inducers tunicamycin and thapsigargin increased SelS gene expression and protein content several-fold. The overexpression of SelS increased Min6 cell resistance to oxidative stress-induced toxicity (Gao *et al.*, 2004). SEPS1 was shown to protect RAW264.7 cells from pharmacological ER stress agent-induced apoptosis (Kim *et al.*, 2007). It has been shown Se deficiency in layer chickens caused a decrease of mRNA expressions of SepS in the liver (Liu *et al.*, 2014b), muscle stomach (Huang *et al.*, 2017), thymus (Khoso *et al.*, 2015; Yang *et al.*, 2016), spleen and bursa of Fabricius (Yang *et al.*, 2016), aorta vessels (Du *et al.*, 2016), thyroid (Lin *et al.*, 2014), erythrocytes (Luan *et al.*, 2016), adipose tissue (Liang *et al.*, 2014) and kidney (Zhang *et al.*, 2016). SeMet enhanced mRNA and protein expression of glutathione peroxidase 1 (GSH-Px1), selenoprotein S (SelS), and thioredoxin reductase 1 without and with AFB_1 treatments. Furthermore, knockdown of GSH-Px1 and SelS by GSH-Px1-specific siRNA and SelS-specific siRNA diminished the protective effects of SeMet against AFB_1-induced immune toxicity. It is concluded that SeMet diminishes AFB_1-induced immune toxicity through increasing antioxidant ability and improving GSH-Px1 and SelS expression in splenocytes (Hao *et al.*, 2016).

2.9.13 Selenoprotein T

Selenoprotein T (SelT) is ubiquitously expressed in many tissues, conserved from plants to humans, and found in the endoplasmic reticulum (Grumolato *et al.*, 2008). Interestingly, chicken SelT was shown to contain two thioredoxin-like folds with the Sec to be located in the first thioredoxin-like fold (Zhu *et al.*, 2016). The authors showed that the SelT helix was formed between residues 143 and 164, with both faces in the helix to be hydrophobic (Zhu *et al.*, 2016). SelT is expressed in the smooth muscle tissues of blood vessels, esophagus, bronchus, stomach, and intestine of rats, and the transcription of the SelT was very sensitive to dietary Se (Guo *et al.*, 2016). The amino acid sequence of chicken SelT shares 90.1% identity with that of *Macaca* and *Mus musculus*, while it shares 89.6 and 88.5% identity with *Sus scrofa* and *Cavia porcellus*, respectively. It was shown that SelT is derived from a common ancestor with other SelT family proteins and that it is a novel selenoprotein that differs from the SelT of mammals and aquatic invertebrates (You *et al.*, 2014). The complete nucleotide sequence of the gene encodes 199 amino acids. Chicken SelT has been identified as a member of the Rdx protein family, which shows a sequence similar to that of the thioredoxin-like fold and a conserved CxxU motif. Therefore, SelT participates in maintaining the redox balance of the cell and responds to changes in such a balance (You *et al.*, 2014). When SelT was knocked down in murine cells, the expression of several oxidoreductase genes increased, reflecting the involvement of SelT in redox regulation (Sengupta *et al.*, 2009). It has been shown that disruption of the *Selt* gene is lethal during embryogenesis, and its conditional knockout in the brain causes the increases the sensitivity of animals to stress conditions, in particulat to neurotoxin-induced neurodegeneration. It seems likely that SelT is involved in maintenance of the redox balance that control homeostasis, signalling and survival of cells with intense metabolic activity during development (Boukhzar *et al.*, 2016).

It has been shown that Se-deficiency decreased the expression levels of SelT in the bursa of Fabricius, thymus, and spleen (You *et al.*, 2014). Similarly, the level of SelT mRNA in the pectoral muscle (Huang *et al.*, 2011b; Yao *et al.*, 2013a), muscle stomach (Huang *et al.*, 2017), liver (Huang *et al.*, 2011b), aorta vessels (Du *et al.*, 2016), thyroid (Lin *et al.*, 2014), erythrocytes (Luan *et al.*, 2016), pancreas (Zhao *et al.*, 2014a), kidney (Zhang *et al.*, 2016) and adipose tissue (Liang *et al.*, 2014) was shown to be regulated by Se status.

2.9.14 Selenoprotein U

Selenoprotein U (SelU) was characterised by Castellano *et al.* (2004). They used a comparative genomics approach that relied on the genome-wide prediction of genes with in-frame TGA codons, and the subsequent comparison of predictions from different genomes. They applied this method to various genomes and identified a novel selenoprotein family, named SelU, which is present in fish, chicken and sea urchin. In particular, selenium incorporation into chicken SelU was demonstrated and the SelU expression pattern in zebrafish embryos characterised (Castellano *et al.*, 2004). In great contrast to chicken and fish, mammals, worms and land plants

contained cysteine homologues. Data of Castellano *et al.* (2004) indicate a scattered evolutionary distribution of selenoproteins in eukaryotes, and suggest that other taxa-specific selenoproteins probably exist.

In chicken, the coding sequence (CDS) of SelU was shown to contain 387 bases with a typical mammalian selenocysteine insertion sequence (SECIS) located in the 3'-untranslated region. The deduced amino acid sequence of chicken SelU contains 224 amino acids with UAA as the stop codon (Jiang *et al.*, 2015). Like all SelU genes identified in different species, chicken SelU contains one well conserved selenocysteine (Sec) at the 85th position encoded by the UGA codon. The SECIS element was with the conserved adenosine (-AAA-) rather than the motif cytidine (-CC-) motif. The abundance of SelU mRNA in muscle, liver, kidney, heart, spleen, and lung was shown to be downregulated by Se deficiency. However, it was not affected by dietary Se concentrations in testis and brain (Jiang *et al.*, 2015). Selenium deficiency in chicken was shown to be associated with decreased mRNA expressions of SepU in the liver (Liu *et al.*, 2014b), thymus (Khoso *et al.*, 2015; Yang *et al.*, 2016), spleen and bursa of Fabricius (Yang *et al.*, 2016) aorta vessels (Du *et al.*, 2016), thyroid (Lin *et al.*, 2014), muscles (Yao *et al.*, 2014), muscle stomach (Huang *et al.*, 2017), erythrocytes (Luan *et al.*, 2016), adipose tissue (Liang *et al.*, 2014) and kidney (Zhang *et al.*, 2016).

2.9.15 Selenoprotein W

Selenoprotein W (SeW) was purified and characterised by Vendeland *et al.* (1995) and it consists of 87 amino acids and contains about 1 g atom Se as selenocysteine per 1 g mol of protein (Ream *et al.*, 2001). Four different forms of this protein have been purified from rat muscle with molecular masses ranging from 9.5 to 10 kDa (Allan *et al.*, 1999). Therefore, SeW was originally isolated from muscle and now there is growing evidence indicating presence of this protein in other tissues.

SeW gene from Se-fed chicken brain was cloned and sequenced (Li *et al.*, 2011b). The complete cDNA sequence of chicken SelW was shown to comprise 834 bp. A TGA codon encoding Sec was observed at positions 113-115, while the selenocysteine (Sec) residue in chicken SeW is located at the 13th position encoded by the UGA codon, as all SelWs in other species are (Ou *et al.*, 2011). The calculated molecular weight of chicken SelW was shown to be 9.3217 kDa with a theoretical isoelectric point of 7.0. It was found that the SECIS element was located in the 3'-UTR of the chicken SelW mRNA. Homology analysis showed that there are close matches of chicken SelW with many other SelWs in animals. In particular, the homology of the coding nucleotide sequences and the deduced amino acid sequences of SelW from thirteen species of animals was determined. The chicken SelW protein contained 85 amino acids and was the shortest of the thirteen proteins (Ou *et al.*, 2011). Mice and rat SelW proteins are shown to be the longest, containing 88 amino acids, whereas the sheep, cattle, horse, dog, pig, human, monkey and orangutan SelW proteins contain 87 amino acids, and the zebrafish and frog SelW protein is one amino acid longer than chicken SelW. The amino acid sequence of chicken SelW shares 56.5 and 61.2% identity with fish and frog SelW and rodent SelW, respectively. The nucleotide identity range of CDS varied

from 55.8 to 60.5% between chicken and other animal SelW from mammals to aquatic invertebrates (Li *et al.*, 2011a). SeW expression in chicken tissue is regulated by Se status. This includes liver (Sun *et al.*, 2011), pancreatic tissue (Wang *et al.*, 2011b), chick embryo neurons (Li *et al.*, 2012), testes (Khalid *et al.*, 2016), erythrocytes (Luan *et al.*, 2016), pancreas (Zhao *et al.*, 2014a), thymus, spleen and bursa of Fabricius (Yang *et al.*, 2016), kidney (Xu *et al.*, 2016; Zhang *et al.*, 2016), muscle stomach (Huang *et al.*, 2017), adipose tissue (Liang *et al.*, 2014) and other tissues (Ou *et al.*, 2011).

On the one hand, it was shown that SeW mRNA expression is high in skeletal muscle followed by brain, but extremely low in other tissues from chickens fed a commercial unsupplemented maize-based diet containing about 0.1 mg/kg feed-derived selenium. On the other hand, the SeW gene is ubiquitously expressed in heart, skeletal muscle, brain, testis, spleen, kidney, lung, liver, stomach and pancreas in chickens fed a commercial diet supplemented with sodium selenite (4 mg/kg; Ou *et al.*, 2011). The expression pattern of SelW in 36 different tissues from 60 day old chickens has been determined. It was shown that expression of chicken SelW mRNA was higher in the pituitary, spinal cord, sciatic nerve, cerebral cortex, cerebral nuclei, thalamus, cerebellum, muscle, cartilage, trachea, gizzard and artery, and lower in the pancreas, testis, ovary, kidney and veins (Li *et al.*, 2011b). Furthermore, SelW is widely expressed in the gastrointestinal tract tissues of birds and the transcription of the SelW gene is very sensitive to dietary Se. In fact, a significant increase in SelW mRNA levels was observed in the gastrointestinal tract tissues of 90 day old male chickens fed the diets containing 1-3 mg/kg sodium selenite while decreased SelW mRNA levels were observed in the esophagus, crop, proventriculus, gizzard, duodenum and cecum in chickens fed the diet containing 5 mg/kg sodium selenite (Li *et al.*, 2011a). In the gut of chickens supplemented with 0.15 or 1.5 mg/kg selenium in the form of sodium selenite, the Se contents were found to be the highest in the duodenum and the lowest in the rectum, while the SelW mRNA expression was the highest in the gizzard and the lowest in the rectum. In addition, the SelW mRNA levels in the gut tissue were found to increase in a time-dependent manner with increasing feeding time. Furthermore, the expression of the SelW mRNA in the gut tissues of chickens was found to correlate with the dietary Se concentrations (Gao *et al.*, 2012).

SelW is shown to be widely expressed in immune organs of birds and Se-supplementation of the feed increases SelW expression in the thymus and the bursa of Fabricius (Yu *et al.*, 2011). SelW plays an important role in protection of splenic lymphocyte of birds from oxidative stress. In fact, mRNA expression of SelW was effectively increased after treatment with sodium selenite, and H_2O_2-induced cell apoptosis was significantly decreased and cell viability significantly increased (Yu *et al.*, 2014). Furthermore, H_2O_2 induced a significantly up-regulation of the Bax/Bcl-2 ratio, Bax, Bak-1, caspase-3 and p53 and down-regulation of Bcl-2. However, when lymphocytes were pretreated with Se before treatment with H_2O_2, the Bax/Bcl-2 ratio and mRNA expression of those genes were significantly decreased, and Bcl-2 was increased. SelW-silenced cells were shown to be more sensitive to oxidative stress induced by H_2O_2 than control cells and were characterised by up-regulation of apoptosis-related genes. In addition, silencing the lymphocyte SelW gene decreased

their cell viability, and increased their apoptosis rate and susceptibility to H_2O_2 (Yu *et al.*, 2014). Recent results suggested that SelW plays an important role in the protection of immune organs of birds from inflammatory injury by regulation of inflammation-related genes (Yu *et al.*, 2015c). In particular, it was shown that Se-deficient diets effectively decreased mRNA expression of SelW and induced a significantly up-regulation of cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), NF-κB, prostaglandin E (PTGEs) and tumour necrosis factor (TNF)-α mRNA levels. Furthermore, the histopathological analysis showed that immune tissues were injured in the low-Se groups. In addition, *in vitro*, H_2O_2 induced a significantly up-regulation of the mRNA levels of inflammation-related genes (iNOS, COX-2, NF-κB, PTGEs, and TNF-α) in cultured splenic lymphocyte. Furthermore, when lymphocytes were pretreated with Se before exposure to H_2O_2, the inflammation-related genes were significantly decreased. Indeed, silencing SelW significantly up-regulated the inflammation-related genes (iNOS, COX-2, NF-κB, PTGEs, and TNF-α) in cultured splenic lymphocyte (Yu *et al.*, 2015c).

It has been shown that Se plays important roles in the development of chicken skeletal muscles and highest SelW expression is associated with optimal Se status. In fact, SelW was detected in wing muscle, pectoral muscle, and thigh muscle and in myoblast in culture. It was shown that SelW both increased along with the growth of organism and the differentiation process of myoblasts. The thigh muscle is shown to be more responsive to Se intake than the other two skeletal muscle tissues (Ruan *et al.*, 2012). It seems likely that skeletal and cardiac muscles SelW mRNA levels are highly regulated by Se supplementation and different muscle tissues showed differential sensitivity. In fact, Se supplementation increased SelW mRNA abundance in thigh and shank muscles and SelW mRNA levels displayed a different expression pattern in different skeletal and cardiac muscles. However, Se excess was associated with a reduction in SelW mRNAs (Zhang *et al.,* 2012a). In chicken embryonic myoblasts a significant correlation between expressions of SelW and myogenic regulatory factors was shown (Wu *et al.*, 2012). In fact, chicken SelW protects embryonic myoblasts against cell apoptosis mediated by endogenous and exogenous H_2O_2 Silencing the myoblast SelW gene caused reduced cell viability, increasing their apoptosis rate and susceptibility to H_2O_2. Furthermore, knockout of SelW was associated with apoptosis promotion indicative by up-regulated Bax and caspase-3 and down-regulated Bcl-2 (Yao *et al.*, 2013b). SelW may play a role in the regulation of inflammation reaction in Se-deficiency myopathy (Wu *et al.*, 2014c). It was shown that dietary Se deficiency reduced the mRNA expression of SelW in chicken wing, pectorals, and thigh muscles, while the mRNA expression levels of inflammation-related genes in chicken skeletal muscle tissues at different time points were increased. Indeed, Se deficiency induced the inflammatory response in chicken skeletal muscle (Wu *et al.*, 2014c). In chicken myocardial cells Se treatment increased the expression of SelW and caused a downregulation of p53, NF-κB, and TNF-α (Liu *et al.*, 2016c) indicating the anti-inflammatory role of Se supplementation. When a low Se chicken diet containing 0.01 mg/kg Se was supplemented with sodium selenite (0.3 mg/kg Se) SelW concentration in chicken muscles increased by 85 and 109% at 2 and 4 weeks, respectively (Huang *et al.*, 2015b).

The exact function of SelW is not known, but recent research reports have indicated that avian SelW has an antioxidant function *in vivo* and *in vitro*. In fact, SelW could reduce the oxidative damage induced by H_2O_2 and had an important protective function against oxidative damage. Indeed SeW overexpression in chicken cells was associated with a markedly decrease in sensitivity to H_2O_2-induced oxidative stress and had a lower apoptotic cell death, increased cell viability and decreased levels of caspase-3, caspase-8, and fas mRNA than wild-type cells (Han *et al.,* 2012). The aforementioned data presented by Liu *et al.* (2016c), Yao *et al.* (2013b), Yu *et al.* (2014, 2015c) also confirm antioxidant and anti-apoptotic and anti-inflammatory role of SeW.

2.10 General conclusions

Avian species contain 25 known selenoprotein genes (Mariotti *et al.*, 2012). The chicken selenoproteome consists of 4 Sec-containing GSH-Px, including cytosolic GSH-Px1, gastrointestinal GSH-Px2, plasma GSH-Px3, and phospholipid hydroperoxide, GSH-Px4; three TrxRs, including cytosolic TrxR1, mitochondrial TrxR3 and thioredoxin/glutathione reductase TGR; and three deiodinases. Other selenoproteins are SelW, SPS2, SelP, SelPb, Sep15, SelM, MsrB1, and selenoproteins I, N, O, H, T, K, S and U. Recently, the family of chicken selenoproteins has been extended to include 26 genes responsible for selenoprotein synthesis (Lei, 2017; Zhao *et al.*, 2017).

As shown above the selenoprotein family is getting a lot of attention in recent years and it seems likely that selenoproteins are a key element in regulating antioxidant system of the body. In fact selenoproteins are involved in the regulation of (Surai, 2006):

- the vascular tone by affecting the superoxide/nitric oxide balance;
- cell adhesion by controlling cell adhesion molecule expression;
- apoptosis via regulation of apoptosis signal-regulating kinase-1;
- eicosanoid production by controlling the activity of cyclooxygenases and lipoxygenases;
- inflammatory processes and atherogenesis by above mentioned mechanisms.

Molecular mechanisms of selenoprotein action are diverse (Surai, 2006), including:

- scavenging hydroperoxides and regulating eicosanoid production and signalling events;
- repairing oxidised proteins (methionine sulfoxide reductase B) by reducing SH groups into active form;
- prevention of oxidative inactivation of enzymes;
- modulation of protein phosphorylation by thioylation;
- release of Trx from complexes with kinases;
- regulation of hydroperoxide-dependent protein/protein interaction;
- maintaining redox balance of the cell and participating in cell signalling.

Indeed, most of the functionally characterised selenoproteins are oxidoreductases that contribute to various redox functions in biological systems, including antioxidant defences, maintaining redox balance and cell signalling.

Recently, bioinformatics tools were used to characterise chicken selenoproteins in detail (Zhu *et al.*, 2016). The results of this comprehensive analysis are summarised in Table 2.6. Furthermore in chicken 16 selenoproteins SECIS elements were shown to be type II and six selenoproteins belonged to the type I, while the chicken SelI and SelPb SECIS elements were not confirmed (Zhu *et al.*, 2016).

Selenoprotein metabolism in poultry is complex and there are mechanisms providing a discrimination between different selenoproteins in the case of Se deficiency and supply. In fact, in accordance with selenoprotein expression decrease due to Se deficiency, chicken tissues can be placed in the following descending order: adipose tissue (25 selenoproteins (SP) decreased; Liang *et al.*, 2014) > erythrocytes (24 SP; Luan *et al.*, 2016) = thyroid (14 SP; Lin *et al.*, 2014) > muscles (19 SP; Yao *et al.*, 2014) = liver (19SP; Liu *et al.*, 2014b) > kidney (14 SP; Zhang *et al.*, 2016) > pancreas (12 SP; Zhao *et al.*, 2014a) = central nervous system (CNS) (12 SP; Jiang *et al.*, 2017) > testes (3 SP; Gao *et al.*, 2017). Indeed, there is tissue specificity in selenoprotein expression. For example, highly expressed selenoproteins differ substantially between various parts of the CNS including cerebral cortex (Sepp1 and SelO), bulbus cinereus (GSH-Px3), cerebellum (SelW and SelO), thalamus (SelT and Sep15) and marrow (GSH-Px2 and GSH-Px 3) (Jiang *et al.*, 2017).

Table 2.6. Selenoprotein location in chicken (adapted from Zhu *et al.*, 2016).

Location/feature	Selenoproteins
Inside the cell	GSH-Px1, GSH-Px2, GSH-Px4, DIO1, DIO2, DIO3, TrxR1, TrxR2, TrxR3, Sep15, SelH, SelI, SelK, SelM, SelN, SelO, SelT, SelU, SelW, MsrB1, SPS2
Outside the cell/secreted	SelPa, SelPb and GSH-Px3
Endoplasmic reticulum	DIO1, DIO2, DIO3, Sep15, SelI, SelK, SelM, SelN SelS and SelT
Mitochondria	GSH-Px1, GSH-Px2, GSH-Px4, TrxR1, TrxR2, TrxR3, SelM, SelO and SelU
Cytoplasm	GSH-Px1, GSH-Px2, GSH-Px4, TrxR1, TrxR2, TrxR3 and SelW
Nucleus	GSH-Px4, MsrB1 and SelH
Golgi apparatus	SelT
Membrane	SelI
Membrane-bound	SelI, SelK, SelS, SelT, DIO1 and DIO3
Zn-containing	Sep15, MsrB1, SelW and SelM
POP-containing	GSH-Px1, GSH-Px2, GSH-Px3 and GSH-Px4
Thioredoxin-like fold-containing	GSH-Px1, GSH-Px2, GSH-Px3, GSH-Px4, DIO1, DIO2, DIO3, TrxR3, SelT, SelH, SelW, Sep15, SelM, SelU and SelO
Flavin adenine dinucleotide-interacting	TrxR1, TrxR2 and TrxR

Established and suggested selenoprotein functions are shown in Figure 2.4. Today we probably know only a limited number of the mechanisms by which Se is involved in diverse systems, such as redox signalling, regulation of apoptosis, immunomodulation, spermatogenesis and embryonic development. Recent findings associated with many various roles of thioredoxin and glutathione systems in the cell could aid in explaining the effects of Se deficiency on farm animals and poultry. Indeed, lipid and protein oxidation are involved in the development of diseases, such as exudative diathesis, encephalomalacia, muscular and pancreatic dystrophy. Interestingly, recently it has been shown that Trx has the potential to interact with 19 selenoproteins (Liu *et al.*, 2017). Indeed, thioredoxin silencing in chicken cardiomyocytes was associated with inhibition of GSH-Px1, 2, 3, 4, TrxR 1, 2, 3, Dio 1, 2, SelT, SelW, SelK, SepX1, and over-expression of the rest of the selenoproteins (Yang *et al.*, 2017). The authors showed a negative correlation of Trx with Dio3, SelM, 15kDa, SelH, SelU, SelI, SelN, SelP1, SelO, SelS, SPS2, SelP, while the other selenoproteins showed a positive correlation with Trx.

However an understanding the molecular mechanisms of these well-known diseases is still lacking. It seems likely that a compromised glutathione/thioredoxin redox system in tissues as a result of Se and/or vitamin E deficiency could be responsible for structural changes (as a result of damage to membranes and important proteins) leading to metabolic changes and development of clinical deficiency symptoms.

Biological systems contain a range of regulatory mechanisms to prevent damaging effects of free radicals and toxic products of their metabolism. In particular, vitamin E recycling, gene redox regulation and anti/prooxidant redox signalling should be given

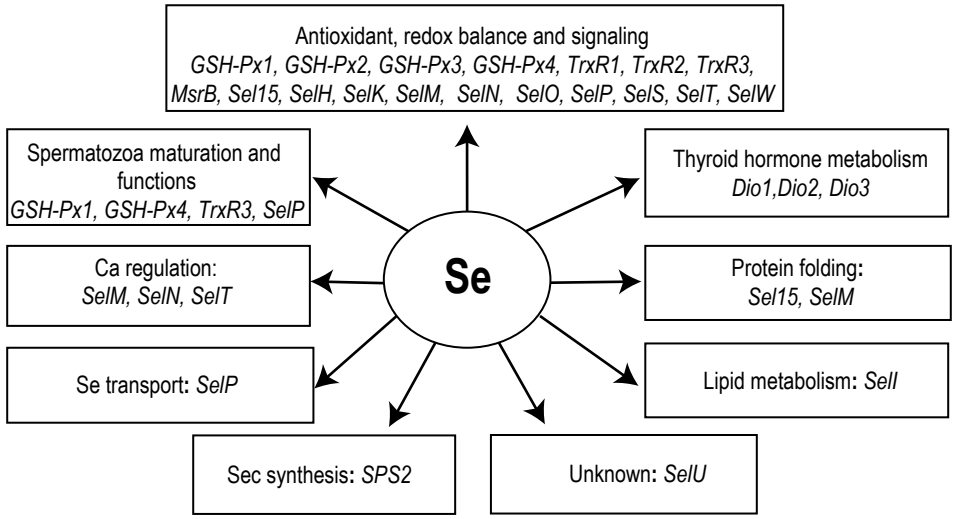

Figure 2.4. Established and suggested selenoprotein functions (adapted from Surai *et al.* (2018c).

increased attention in future studies. As mentioned previously, all antioxidants work together to create an integrated antioxidant system. For example, in Se deficiency redox status of the cell could be maintained by the glutathione system. However, the antioxidant system is dependent on the nutritional provision of antioxidants (vitamin E and carotenoids) and cofactors (Se, Mn, Cu, Zn, and Fe) and has a limited ability to overcome dramatic stress conditions (high levels of toxins or prooxidants in the feed, severe disease, etc.).

Taking a central role of selenoproteins in antioxidant system regulation into account, it would be appropriate to call selenium the 'chief-executive of the antioxidant system' (Surai, 2017). Recent information on the effect of antioxidant-prooxidant balance in the cell on various metabolic pathways, as well as an importance of ROS in cell signalling events, stimulate re-consideration of antioxidant system regulation by nutritional means. Indeed, excess of vitamin E or other antioxidants, such as carotenoids, was shown to have no immediate threat to animal/human health. However, in long term, changes in the antioxidant-prooxidant balance in the body/cell resulted from overconsumption of antioxidants could be detrimental. On the other hand, by providing body with optimal amounts of co-factors, such as Se in right form, and building Se reserves could be more safe option of affecting antioxidant defences. Indeed, this could give an opportunity to existing regulatory mechanisms to be in place and prevent possible damages. For example, under stress conditions, the body can respond properly by additional synthesis of selenoproteins to deal with the overproduction of free radicals.

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Chapter 3
Selenium in feed: organic selenium concept

Nature does nothing in vain

3.1 Introduction

Selenomethionine (SeMet) was first studied as a possible cause of toxicity of seleniferous wheat in the 1950s and was later proven to be synthesised from inorganic selenium sources by various plants, including yeast, marine algae, *Candida albicans*, as well as by *Escherichia coli* and rumen bacteria (for review see Schrauzer, 2000, 2003; Schrauzer and Surai, 2009). Detailed analysis of the literature suggested that organic selenium in the form of various selenoamino acids is a natural form of selenium in animal and human diets and that the digestive system adapted to this nutrient form during evolution, thereby explaining why there are principal differences in assimilation and metabolism between organic and inorganic forms of selenium (Surai, 2002, 2006). Due to this, an inconsistency exists in common practise of selenium supplementation of poultry diets. On one hand, naturally occurring organic selenium is represented by a mixture of selenoamino acids with SeMet comprising more than 50% of total selenium in many feed ingredients, including grains, oil seeds, etc. On the other hand, until recently the supplemental form of selenium for poultry has been inorganic, either selenite or selenate.

3.2 Selenium in soils and plants

Selenium (Se) is a chemical element with atomic number 34 and atomic weight 78.96 belonging to group VI of the periodic table of elements (Figure 3.1). This group also includes such non-metals as sulphur and oxygen.

In nature Se exists in two chemical forms, organic and inorganic. Elemental Se can be reduced to the Se^{-2} oxidation state (selenide) or oxidised to the Se^{+4} (SO_3^{-2}, selenite) or Se^{+6} (SO_4^{-2}, selenate). Therefore, inorganic Se can be found in different minerals in the form of selenite, selenate and selenide, as well as in the metallic (Se^0) form. In contrast, selenium in feed ingredients (forages, grains, oilseed meals, etc.) is an integral part of a range of Se-amino acids, including selenomethionine and selenocysteine, and exists in the Se^{-2} oxidation state. As a result, in nature, avian species receive Se mainly in the form of SeMet (Combs and Combs, 1986; Surai, 2006). Indeed, SeMet is considered a natural nutritional form of selenium for poultry (Table 3.1).

The selenium cycle in the food chain of poultry starts from soil and includes plant and animal sources ultimately dependent on its assimilation from the soil. Indeed, soils are

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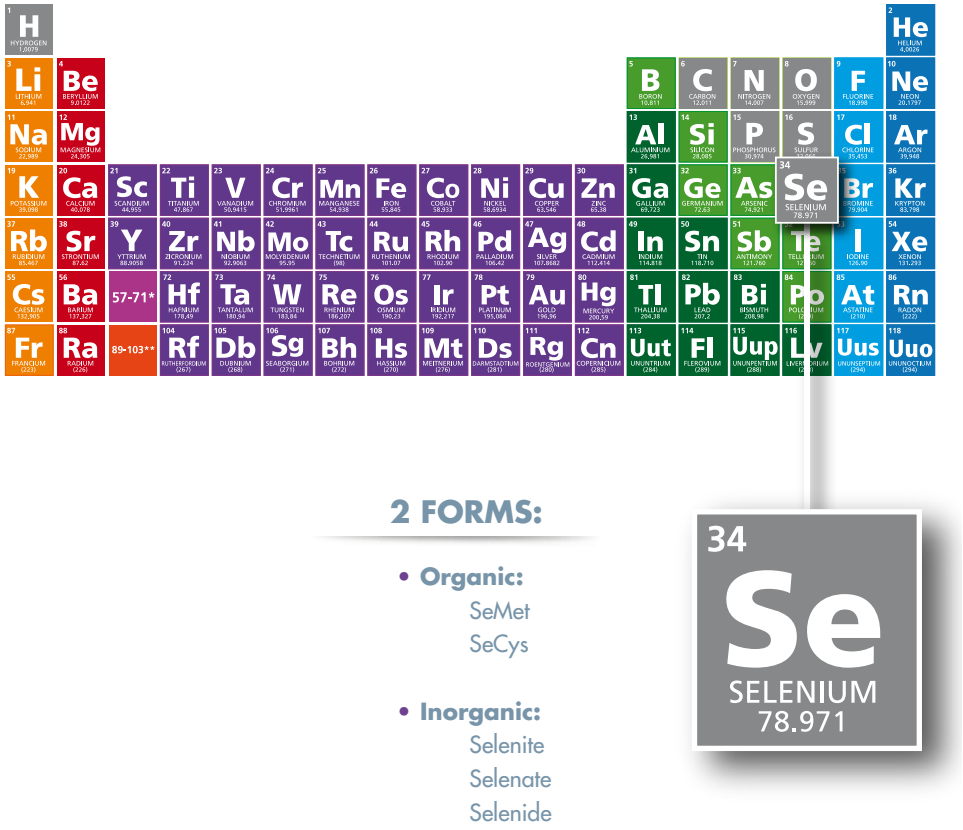

Figure 3.1 Selenium position in the periodic system of elements.

the major source of Se for plants and thereby for animals/poultry eating those plants and humans consuming plant and animal-derived foods. Selenium concentration in soils varies significantly (Reilly, 2006).

The Se content of most soils ranges between 0.1 and 2 mg/kg; and Se in soil exists in various forms, including selenides, elemental Se, selenites, selenates and organic Se compounds (NRC, 1983). High concentrations of Se are found mainly in sedimentary rocks and shales formed during the cretaceous period, while lower concentrations of Se are characteristic for igneous (volcanic) rock, sandstone, granite and limestone (Van Metre and Callan, 2001). Investigations conducted in China indicated that soils developed under tropic and subtropic conditions (laterite, yellow soil and red soil) are characterised by comparatively high Se levels (>0.3 mg/kg; Tan *et al.*, 2002). In contrast, soils developed under the temperate (warm) steppe and desert conditions (chernozem, chestnut soil, calcic brown soil, desert soil and solonchak) have moderate Se concentrations (0.14-0.30 mg/kg). Finally, such soils as brown earth, drab soil, dark

Table 3.1. SeMet in grains.

	Grains	SeMet proportion, % total Se	References
Wheat	wheat grain	56-83	Whanger, 2002
	wheat	50.4-81.4	Yang *et al.*, 1997
	wheat grain	72-85	Cubadda *et al.*, 2010
	spring wheat grains, Australia	90	Stadlober *et al.*, 2001
	spring wheat grain, India	66	Cubadda *et al.*, 2010
	durum wheat, Austria	62	Stadlober *et al.*, 2001
	winter wheat grain, India	58	Cubadda *et al.*, 2010
	wheat flour, Belgium	52	Moreno *et al.*, 2004
Barley	summer barley grains, Austria	77	Stadlober *et al.*, 2001
Maize	maize	61-64	Whanger, 2002
	maize	45.5-82.0	Yang *et al.*, 1997
Soybeans	soybeans	>80	Whanger, 2002
	soybeans	62.9-71.8	Yang *et al.*, 1997
Rice	rice	68-81	Whanger, 2002
	rice	54.9-86.5	Yang *et al.*, 1997
	basmati rice, India	93	Mar *et al.*, 2009
	jasmine rice, Thailand	96	Mar *et al.*, 2009
	white rice, USA	94	Mar *et al.*, 2009

brown soil, loessial soils, purple soil, red drab soil, developed under the temperate (warm) humid/sub-humid conditions are quite poor in Se (Tan *et al.*, 2002). In particular, low Se soils occur in the northeast to the southwest of China.

Furthermore, Se availability to plants depends on many factors including soil pH, the oxidation-reduction potential and mineral composition of the soil, rate of artificial fertilisation and rainfall. Therefore, the selenium soil to plant transfer depends on (Figure 3.2; Munier-Lamy *et al.*, 2007):

- plant species;
- physiological state;
- soil type;
- Se concentration in soil;
- form of Se in soil.

Furthermore, Se availability to plants depends on many factors, including soil pH, the oxidation-reduction potential and mineral composition of the soil, rate of artificial fertilisation and rainfall. In fact, the bioavailability of Se in soils for plants depends more on its form than on its total concentration:

- In the case of acidic soils or poor soil aeration, Se can form insoluble complexes with iron hydroxide and become poorly available. For example, at pH 6, only 47% of labelled Se was transferred from soil to ryegrass leaves. Increasing pH to 7

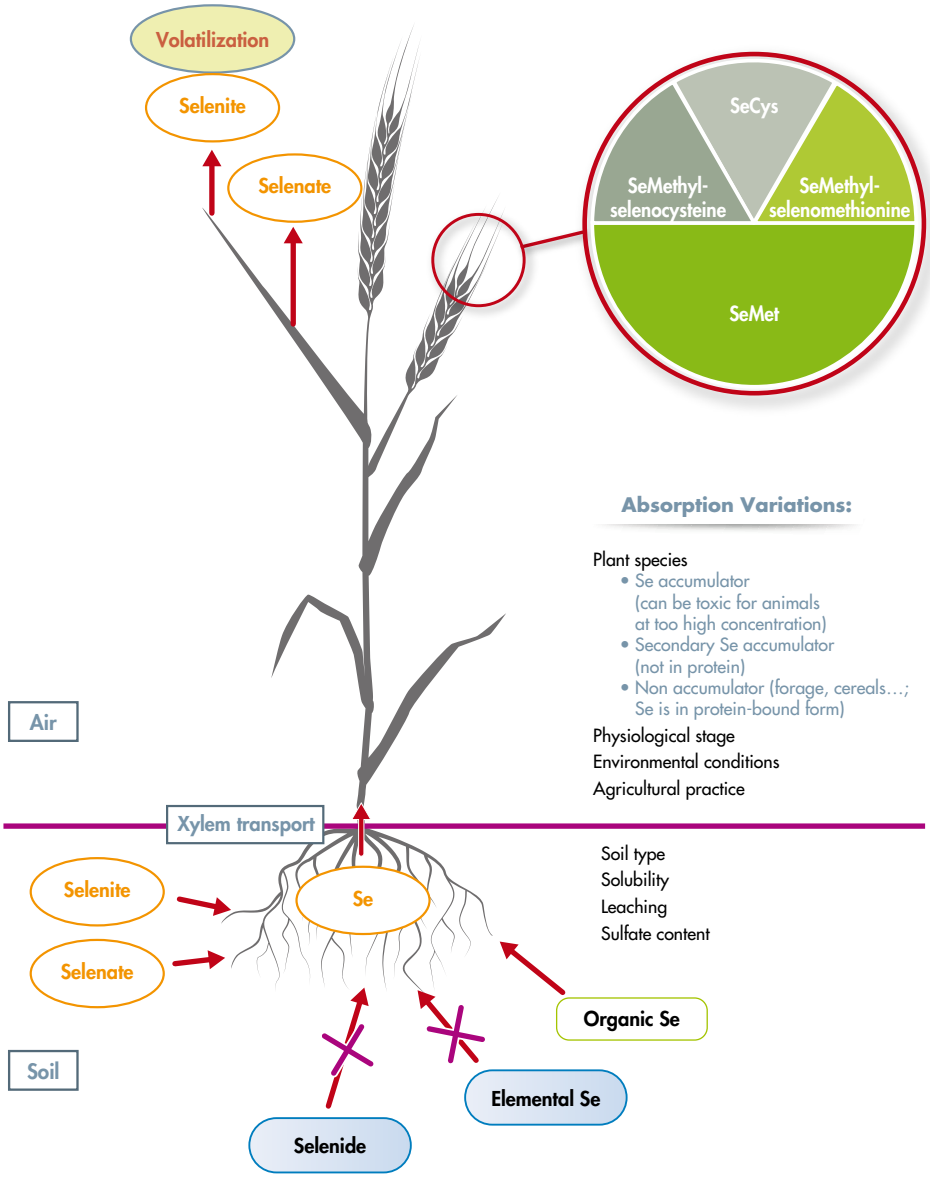

Figure 3.2 Selenium in soils and plants. [Se} = 0.1-2 ppm.

increased Se assimilation to 70% (Haygarth *et al.*, 1995). Indeed, Se in alkaline soils occurs in selenate form, where it is soluble and easily available to plants.
- Since sulphate competes with selenate for uptake by the sulphate transporter, high soil sulphate decreases Se uptake by plants (Terry *et al.*, 2000). This explains low Se availability from soils following application of certain types of fertilisers.
- Selenium can also be leached from the topsoil in areas of high rainfall. Therefore, areas with higher rainfall have lower forage selenium content.
- Solubility is the critical determinant of Se bioavailability to plants and the amount of water-soluble Se in soils varies substantially and does not correlate with total soil Se (Combs and Combs, 1986).
- Selenite is strongly absorbed by soils, while selenate is only weakly absorbed and leaches easily.
- Selenite and elemental Se are usually found in reducing environments and are unavailable to plants and animals. Indeed, selenite in soils undergoes a lot of microbially mediated transformations leading to the reduction of Se(IV) to insoluble Se(0) or to the production of volatile Se species (Février *et al.*, 2007).
- Selenite is present in mildly oxidising, neutral pH environments and typically humid regions, while selenate is the predominant form under ordinary alkaline and oxidised conditions (Goh and Lim, 2004). The authors also showed that the adsorption of selenite and selenate by soils appeared to be influenced by the variable pH-dependent charges on the soil particle surfaces. In particular, phosphate had more profound effects than sulphate on Se adsorption in the soil.
- Application of gypsum (calcium sulphate) to soils decreased Se availability for plants (NRC, 1983).
- Leaching occurs during the soil development process and irrigation water decreases the Se level in plants (NRC, 1983).
- Forage Se is reported to be low on sandy soils and lower on mineral upland soils than on organic moorland soils in the British Isles (MacPherson, 2000). The results show that trace metal concentrations in Libyan clay surface soil are higher than in sandy soil (El-Ghawi *et al.*, 2007).
- The main chemical changes under long-term waterlogged conditions are depletion of molecular oxygen, decrease of redox potential, and reduction of Fe (III) to Fe (II) and SeO_3^{-2} to Se^0. This leads to low availability of Se in soils, and subsequently low Se content (29 μg/kg) in brown rice grain produced in this Chinese region (Cao *et al.*, 2001). Indeed, selenite binds tightly to iron and aluminium oxides and thus is quite insoluble in soils (Jonnalagadda and Rao, 1993).

Selenium is transported via the xylem to chloroplasts in leaves where it is processed by the sulphur assimilation pathway into organic compounds. The selenate form is transported more easily from root to shoot than selenite or organic Se (Terry *et al.*, 2000). The uptake of selenium differs with the plant species. It ranges from 2 to 40% of initial selenium for lettuce, maize and radish, and reaches 4.8 and 17% for mycorrhizal and non-mycorrhizal ryegrass, respectively (Munier-Lamy *et al.*, 2007). Plants differ markedly in their ability to incorporate selenium from soil into tissues; and based on this ability, plants are divided into three major categories:

- Selenium accumulators. Some species hyperaccumulate Se in leaves and stems when grown on seleniferous soils. These plants stimulated the initial Se research, since they caused Se toxicity in animals grazing them. Selenium accumulator genera include species of *Astragalus, Stanleya, Morinda, Neptunia, Oonopsis,* and *Xylorhiza* (Terry *et al.*, 2000). These plants can accumulate up to several mg Se per g of dry weight and are ultimately toxic to animals. When these plants were grown hydroponically in the presence of selanate, Se in the older leaves was predominantly inorganic, while in young leaves and roots it was mainly (90-95%) in organic form (Ellis and Salt, 2003). A specific odour causes grazing animals to avoid them on pasture when other forages are available.
- Secondary Se accumulators accumulate high Se concentrations even when grown on soils with low or medium Se content. They include such species of the genera *Aster, Astragalus, Atriplex, Castilleja, Comandra, Grayia, Grindelia, Gutierrezia, Machaeranthera and Brassica* (Terry *et al.*, 2000). It is important to understand that in Se-accumulator plants, Se is not incorporated into proteins. This contrasts with non-accumulator plants, such as typical forage and cereal grains where Se is found predominantly in protein-bound form (Surai, 2006). This type of Se accumulator plant is more an exception than the rule of Se metabolism in plants.
- Non-accumulator plants. The third category includes most forage, cereal and oilmeal crop plants. Such plants contain less than 25 mg Se/kg dry weight and do not accumulate Se in access of 100 mg/kg, even when grown on seleniferous soils (Terry *et al.*, 2000). These plants typically have Se concentrations in a range of 0.01 to 1.0 mg/kg dry weight. However, there are species-specific differences in Se accumulation from the same soil. For example, lucerne is shown to accumulate more Se than other grasses under conditions of moderately low soil Se concentrations (Van Metre and Callan, 2001). Similarly, white clover (Davies and Watkinson, 1966) or tropical legumes (Long and Marshall, 1973) contains less Se than various grasses independently on its soil levels. In contrast, lucerne contains more selenium than in timothy, cocksfoot or brome grass (Ehlig *et al.*, 1968).

After absorption, the distribution of Se in various parts of the plant depends on species, phase of development and physiological conditions. For example, Se distribution was studied in *Astragalus bisulcatus*, an accumulator species capable of accumulating up to 0.65% of its shoot dry biomass as Se (Pickering *et al.*, 2000). It was shown that plants exposed to 5 µM selenate for 28 days contained predominantly selenate in the mature leaf tissue, whereas the young leaves and the roots contained exclusively organic Se. From this work it is clear that the fate of selenate is dependent on plant tissues and stage of growth. Therefore, chemical reduction of selenate to organic Se in plants is tissue-specific, inducible and developmentally dependent. It is likely that selenate reduction is a rate-limiting stage in the conversion of Se to organic forms (Pickering *et al.*, 2000).

The plant absorbs Se from the soil in the form of selenite or selenate and synthesises selenoamino acids with SeMet representing more than 50% of the Se in cereal grains (Olson and Palmer, 1976) with Se-methyl-selenomethionine, selenocysteine and Se-methyl-selenocysteine being the other major seleno-compounds found in

plants (Brody, 1994). In general, plants can also take up from the soil organic forms of selenium, such as SeMet. At present, Se in any form has not been scientifically demonstrated to be an essential nutrient for higher plants. Regardless, SeMet is the major seleno-compound in cereal grains, grassland legumes and soybeans (Whanger, 2002; Table 3.1). For example, in maize, rice, wheat and soybeans, SeMet comprises 45.5-82%, 54.9-86.5%, 50.4-81.4% and 62.9-71.8% of total Se, respectively (Yang *et al.*, 1997). SeMet accounted for 72-85% of the sum of the selenium species in wheat grain (Cubadda *et al.*, 2010). Even in wheat grown on seleniferous soils Se (up to 31 mg/kg Se), almost half occurred in the form of SeMet (Olson *et al.*, 1970). It is interesting to note that in three wheat cultivars, SeMet accounted for between 50 and 70% of the total grain Se, however, when N and/or S concentrations in soil were amended, SeMet only accounted for between 30 and 40% of total grain Se (Duncan *et al.*, 2017). Indeed, the majority of Se is present as SeMet in both rice and maize (Beilstein *et al.*, 1991). Similarly, SeMet was shown to be the main Se-containing amino acid identified in most of the extracts of Indian mustard (*Brassicaj uncea*), sunflower (*Helianthus annus*), and white lupine (*Lupinus albus*; Ximenez-Embun *et al.*, 2004).

The variability in the results of Se specification studies of plant material reflects analytical difficulties. For example, by using SeMet determination based on its reaction with CNBr, it has been shown that wheat samples, though having a 30-fold range in total Se content, all have about 45% of their total Se values in the form of SeMet (Wolf and Goldschmidt, 2004). However, the authors suggested that additional experiments are needed to verify that all SeMet in the wheat samples has been accounted for. SeMet is stored mainly in the grain and root, while lower concentrations of this amino acid are found in stems and leaves (Schrauzer, 2003).

It is generally accepted that environmental conditions and agricultural practises have a major effect on the Se content of various plant feeds. Water extractable Se accounted for 60.4-72.6% of the total Se in a plant (*Stanleya pinnata*) extract. Among the soluble Se compounds in the plant extract, Se-amino acids comprised 73-85.5%, Se [VI] ranged from 7.5 to 19.5% and non-amino acid organic Se was less than 7% (Zhang and Frankenberger, 2001). Selenium [IV] in most samples was below the detection limit (1 µg/g). This study showed that considerable amounts of the accumulated Se [VI] in the plant was metabolised to Se-amino acids during growth of the plant. The distribution of selenoamino acids in a Se-tolerant grassland legume species (*Melilotus indica* L.) grown in Se-laden soils was studied using high-resolution gas chromatography and gas chromatography/mass spectrometry (Guo and Wu, 1998). Five selenoamino acids including selenocystine, selenomethionine, selenocysteine, Se-methylselenocysteine, and gamma-glutamyl-Se-methylselenocysteine were identified and measured in plant tissues. SeMet constituted more than 50% of the total selenoamino acid in the plant. It seems likely that rate of Se accumulation in plants and its form depend on the Se form provided. For example, time-dependent kinetic studies in Indian mustard (*Brassica juncea*) showed that selenate was taken up 2-fold faster than selenite (De Souza *et al.*, 1998). For both selenate- and selenite-supplied plants, Se accumulation and volatilisation increased linearly with external Se concentration. It is important to note that Se-volatilisation rates were 2- to 3-fold higher in plants supplied with selenite

compared to selenate. In fact, the assimilation of selenate by plants appeared to be limited by its reduction, a step that is thought to be mediated by ATP sulfphurylase, which is rate limiting for selenate uptake and assimilation (Pilon-Smits *et al.*, 1999). Furthermore, there was a difference in Se metabolism among plants supplemented with various forms of Se. For example, selenite-supplied plants accumulated organic Se, most likely SeMet, whereas selenate-supplied plants accumulated selenate (De Souza *et al.*, 1998). It seems likely that Se volatilisation from selenate is limited by the rate of selenate reduction, as well as by the availability of Se in roots, as influenced by uptake and translocation. On the other hand, Se volatilisation from selenite may be limited by selenite uptake and by the conversion of SeMet to dimethylselenide (De Souza *et al.*, 1998).

3.3 Selenium absorption and metabolism

Recent advances in Se biochemistry have provided a deeper understanding of the principal differences in metabolism of the two forms of Se namely inorganic Se (sodium selenite or selenate) and organic Se (mainly SeMet). Organic Se, which can be found in grains, forages and other feed ingredients, is primarily in the form of SeMet and is metabolised in the same way as methionine (Wolfram, 1999). It is actively transported through intestinal membranes during absorption and actively accumulated in such tissues as liver and muscle. It is well known that methionine is not synthesised by animals/poultry and therefore it is an essential amino acid. The same is true for SeMet, which is not synthesised in animals/poultry and must be derived from feed sources (Schrauzer, 2000, 2003; Schrauzer and Surai, 2009). In contrast, inorganic Se is absorbed as a mineral and little is retained in tissue reserves (Figure 3.3). Therefore, a large part of inorganic Se is excreted with faeces in ruminants or with urine/urates in non-ruminants while little is stored in body proteins (Wolfram, 1999).

A number of factors influence the bioavailability and distribution of selenium in the body (Thomson, 1998), including:
- chemical form of Se;
- other dietary components;
- selenium status;
- physiological status;
- species.

Most of the research in Se biochemistry and metabolism has been using inorganic Se, namely selenite or selenate. For example, the concentration of ^{82}Se in organs and body fluids and the distributions of their constituents depending on the dose and time after the intravenous administration of ^{82}Se-selenite and -selenate to rats was investigated (Suzuki and Ogra, 2002). Selenite was taken up by red blood cells within several minutes, reduced to selenide by glutathione, and then transported to the plasma, bound selectively to albumin and transferred to the liver. In contrast to selenite, intact selenate was either taken up directly by the liver or excreted into the urine. In fact,

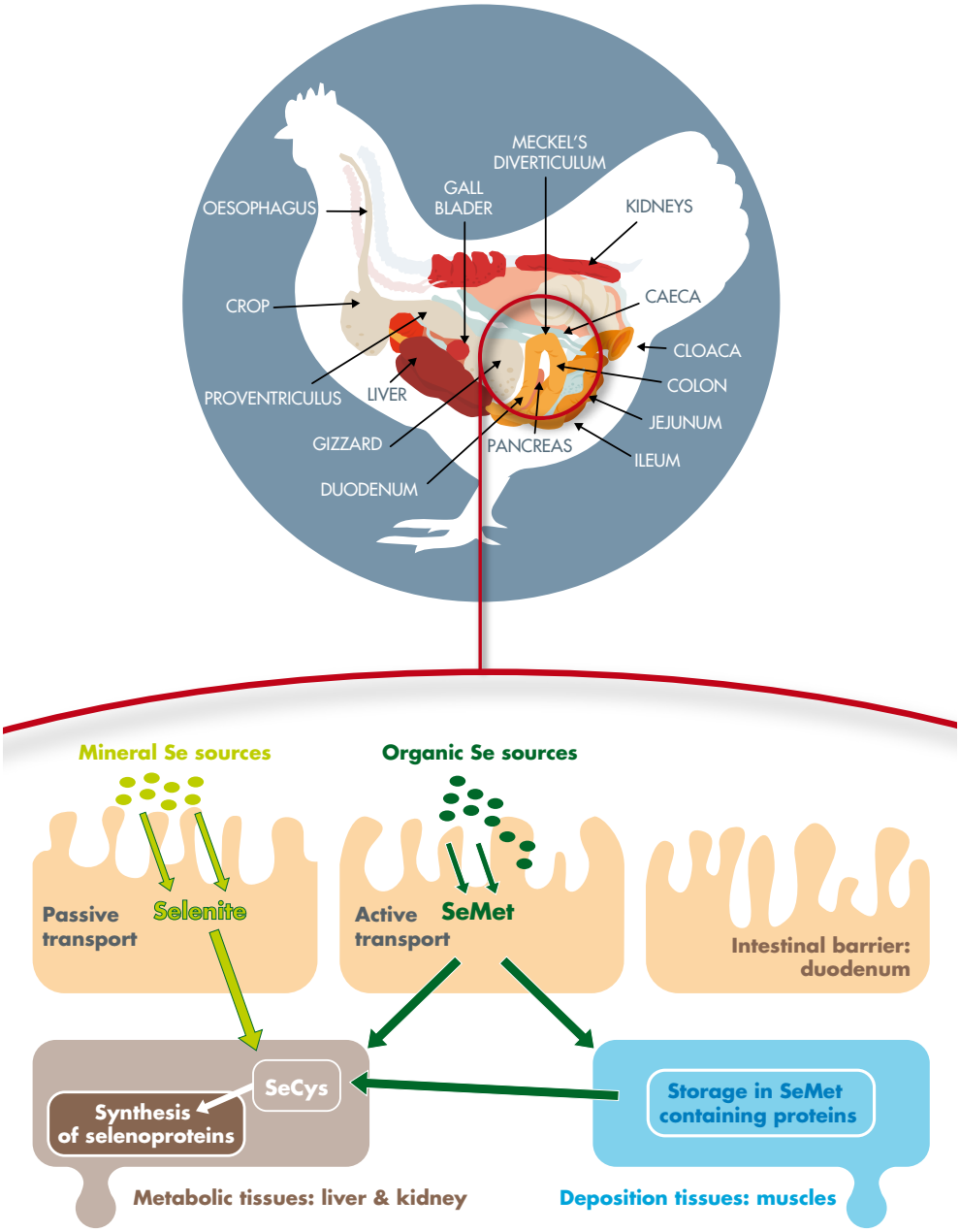

Figure 3.3 Selenium absorption.

the ^{82}Se of selenite origin and that of selenate origin were detected in the forms of the two Se peak materials (A and B) in the liver. The former was methylated to the latter *in vivo* and *in vitro*. The latter was identical with the major urinary metabolite and was identified as Se-methyl-N-acetyl-selenohexosamine (selenosugar). The chemical species-specific metabolic pathway for Se was explained by the metabolic regulation through selenide as the assumed common intermediate for the inorganic and organic Se sources and as the 'checkpoint metabolite' between utilisation for selenoprotein synthesis and methylation for the excretion of Se (Suzuki and Ogra, 2002).

Results of various *in vitro* and *in vivo* experiments with a variety of animal species and model systems have demonstrated that SeMet is readily absorbed through the gut. For example in dogs this process was two times faster than selenocysteine and four times faster than selenite absorption (Reasbeck *et al.*, 1985). Indeed, SeMet is better absorbed than selenite (Daniels, 1996). However, absorption is not a limiting factor to bioavailability, since organic and inorganic dietary Se are both well absorbed through the intestinal membrane (70-95%) (Finley, 2006).

The specific role of the chick duodenum in the assimilation of Se (selenate or selenite) was shown by Apsite *et al.* (1993). Selenite is passively absorbed in the intestine with highest concentrations found in duodenum, liver and kidneys (Apsite *et al.*, 1994). Absorption of Se was found to be greatest in the duodenum and anterior ileum of the chicken (Pesti and Combs, 1976). Absorption of ^{75}Se from selenite, selenate, and SeMet was determined in ligated loops from duodena, jejuna, and ilea of Se-deficient rats (0.009 mg/kg Se) or rats fed selenite-supplemented diets (0.20 mg/kg Se) (Vendeland *et al.*, 1992). Selenium deficiency had no effect on absorption of any selenocompound in any intestinal segment. SeMet was absorbed from all segments. In contrast, selenate and selenite were most efficiently absorbed from the ileum. In mice SeMet was absorbed in the entire intestinal tract, most rapidly from the duodenum, with decreasing rate of absorption in the following intestinal segments (Andersen *et al.*, 1994).

SeMet was more rapidly removed from the ligated intestinal segment of chicken and more efficiently retained after oral or parenteral administration (Humaloja and Mykkänen, 1986). The percentage absorption of both Se compounds was greatest from the duodenal segment of the small intestine. The transport of these Se compounds does not appear to depend on the dietary level of Se since the percentage absorption was not altered by feeding the birds diets supplemented with 0.4 or 4.0 mg/kg Se prior to the measurement of absorption (Humaloja and Mykkänen, 1986). In general, the absorption of Se-amino acids is accelerated by the specific amino acid active transport mechanisms in the gut mucosa. Sodium selenite is absorbed more slowly, possibly by simple diffusion through the intestinal mucosa, than the amino acid-bound Se compounds.

Growing male birds were given orally 4 muCi of ^{75}Se per kg live weight each in the form of DL-SeMet, and were bled (via the wing vein) at fixed intervals (Stanchev *et al.*, 1979). It was found that the resorption of ^{75}Se-SeMet through the digestive tract

of birds as registered by the radioactivity of the blood was most intensive between the 3rd and the 6th hour, after which a decline was observed. Most intensive was the metabolism of SeMet in the kidney, spleen, testes, and pancreas. Comparatively lower was the metabolism of this agent in the breast, bone and brain. Radioactivity of SeMet was lowest in the femoral muscle. As much as 18.2% of the introduced amount of SeMet was excreted after 72 hours through the faeces and urine.

The relative efficiency patterns for uptake of different seleno-compounds during *in vitro* perfusion and *in vivo* ligated segments was SeMet > selenate > selenite. In contrast, selenite was taken up most rapidly by brush border membrane vesicles, followed by SeMet and selenate in decreasing order. Selenate and SeMet appeared in the vascular effluent largely unchanged, but selenite was metabolised extensively during absorption (Whanger *et al.*, 1996). Selenomethionine as well as methionine were transported across the pig jejunal brush border membrane by a single, Na^+-dependent, carrier-mediated process common for both amino acids (Wolffram *et al.*, 1989).

The metabolism of ^{75}Se-selenite and ^{75}Se-SeMet in chick blood was studied *in vitro*. ^{75}Se from selenite was rapidly taken up by erythrocytes and then subsequently released into the plasma in a protein-bound form. However, ^{75}Se-SeMet showed a more gradual and continuous accumulation in erythrocytes over the 12-hour incubation period, according to a hyperbolic type function. ^{75}Se-selenite was incorporated into GSH-Px, whereas ^{75}Se from SeMet was mostly incorporated into haemoglobin (Ilian and Whanger, 1989). In the same experiment binding of ^{75}Se from either selenite or SeMet to plasma proteins was dependent on the presence of erythrocytes. Addition of reduced glutathione and glutathione reductase to plasma produced the same effects as erythrocytes on binding of ^{75}Se from selenite, but not from SeMet, to plasma proteins. It was also shown that ^{75}Se in these Se-containing proteins is bound in selenotrisulphide bond.

The distribution of Se in plasma fractions was investigated in guinea pigs fed various levels (basal, 0.5, 1.0, 2.0, 4.0, 6.0 and 8.0 mg Se/kg) of dietary SeMet (Gu *et al.*, 1998). There was a corresponding increase of Se concentration in liver, kidney, brain, testis, spleen, heart and muscle with each increase of dietary Se, but GSH-Px activity did not change in liver, brain, testis, heart or muscle in pigs fed any of the Se levels as compared to controls fed a basal commercial diet. There was a redistribution of Se between various fractions in the blood. For example, on a percentage distribution basis, the Se in selenoprotein P decreased, and that in the albumin fraction increased with increased dietary intakes of Se as SeMet. Similarly, the greatest percentage of Se was in the albumin fraction of Chinese people living in the high Se areas, whereas the greatest amount was in the selenoprotein P fraction in subjects living in deficient and Se-adequate areas of China (Gu *et al.*, 1998). Increases in the ratios of Se/albumin in either the plasma or the albumin fraction also occurred with increases of Se intake of these subjects.

It seems likely that ingested Se is firstly bound to albumin which transports the element to the liver, where Se is released and serves for the synthesis of selenoprotein P which is released into the bloodstream to become itself a Se transporter between the liver and other organs and tissues (Suzuki *et al.*, 2009). In fact, liver and kidney are considered to be the two main places of synthesis for most selenoproteins, including SeP and cellular GSH-Px (liver) and extracellular GSH-Px (kidneys) (Suzuki *et al.*, 2009). Okuno *et al.* (2001) indicated that in mouse liver SeMet was directly metabolised to CH_3SeH by an alpha,gamma-elimination enzyme analogous to bacterial L-methionine gamma-lyase, in addition to the generally acceptable pathway via selenocysteine. It has been suggested that L-selenohomocysteine generated from SeMet metabolism can be efficiently recycled to SeMet in mammals (Zhou *et al.*, 2000). When sodium selenite was administered to chickens, the kidney was the most responsive to additional dietary Se, while skeletal (thigh) muscle showed no response to supplemental Se (Guenter and Bragg, 1977). The total Se content in kidney and liver from rats treated with SeMet, selenocysteine or sodium selenite were markedly higher than those in other organs. The highest accumulation of the total Se was observed in these two organs when rats were given SeMet (Nakamuro *et al.*, 1997).

The number of published studies in animals/poultry suggested that the metabolic fate and physiological function of dietary selenite may differ from that of SeMet or of feed/food Se (Figure 3.4). It has been postulated that there are two distinct metabolic pools of Se in the body (Daniels, 1996). The main exchangeable metabolic pool includes all forms of Se derived from inorganic selenite/selenide, including endogenously synthesised selenoproteins (e.g. GSH-Px, selenoprotein P, etc.), excretory Se metabolites (trimethylselenium ion) and various other intermediary products of selenite metabolism. This is an active Se pool providing for synthesis of the primary functionally important selenocompounds (Daniels, 1996). The second Se pool consists of SeMet-containing proteins and potentially can contribute to the first pool via participation in selenoprotein synthesis. In fact, Burk *et al.* (2001) demonstrated that Se from SeMet, but not that from selenate or selenocysteine, can be incorporated into albumin, presumably as SeMet in the methionine pool. In another study, albumin was purified from plasma of a human before and after 28 days of supplementation with 400 μg Se/day as SeMet. It was shown that the albumin contained 1 Se atom, presumably as SeMet, per 8,000 methionine residues before supplementation and 1 per 2,800 after supplementation (Hondal *et al.*, 1999).

These findings support the view that SeMet is a non-specific form of Se that is metabolised as a constituent of the methionine pool, where it is randomly distributed, and unaffected by specific Se metabolic processes. Therefore, SeMet can be considered as a storage form of Se in animals and humans. In contrast, no evidence was obtained for non-specific incorporation of Se into plasma proteins when administered as selenate or as selenocysteine. These forms of the element appear to be metabolised by specific Se metabolic processes (Burk *et al.*, 2001).

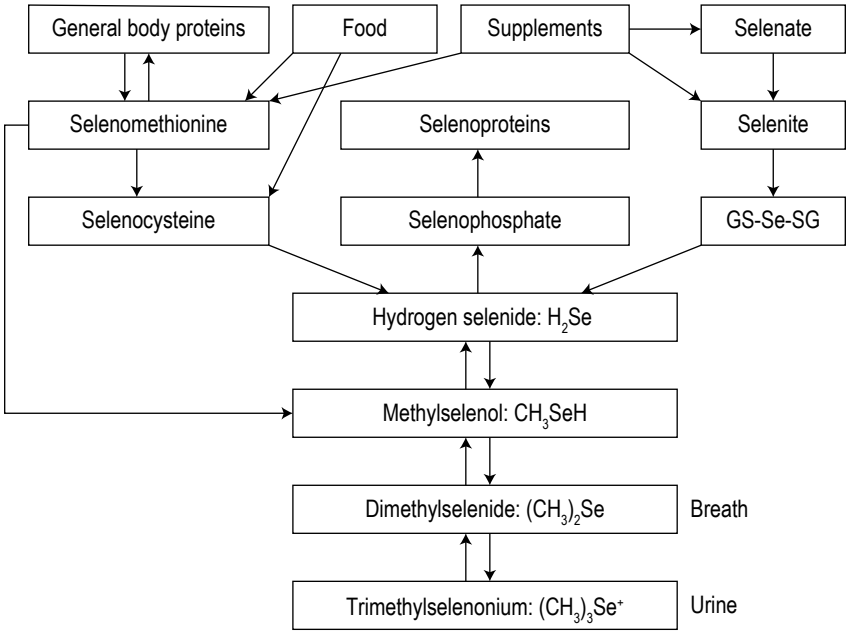

Figure 3.4. Metabolism of selenomethionine, selenite and selenite (adapted from Combs, 2001; Meuillet *et al.*, 2004; Schrauzer, 2000, 2003).

The skeletal muscles are the major Se-storage organ, accounting for about 46.9% of the total Se in the human body, while the kidney contains only 4% of the Se reserves (Oster *et al.*, 1988). In humans, whole body Se depends on the regional location and varies from 3-6 mg up to 13-20 mg (Daniels, 1996). GSH-Px activity and deposition of Se were examined in tissues of rats given dietary Se for 7 weeks as either selenite or SeMet with ^{75}Se radiotracer of the same chemical form (Beilstein and Whanger, 1988). The authors showed that the proportion of ^{75}Se as SeMet was higher in tissues of rats fed SeMet (highest in muscle and haemoglobin, 70%, and lowest in testes, 16%). In contrast, selenocysteine was the predominant form of Se present in tissues of rats given selenite.

In the chicken breast and leg muscles, SeMet comprises 66% and 56.1%, respectively, of total selenium (Bierla *et al.*, 2008), while after enrichment SeMet proportion went up to 99% indicating non-specific incorporation of SeMet into muscle proteins. The ratio of SeMet to Met is variable and dependent on the amount of SeMet and Met in the diet, with Met always present in large excess over SeMet. For example, in American adults, the SeMet:Met ratio in skeletal muscle is in the order of 1:7,000, as estimated from the selenium and sulphur contents of human skeletal muscle. A similar corresponding ratio of 1:>6,000 is found for chicken breast muscle (Schrauzer and Surai, 2009). It is interesting that in the basic starter chicken diet the ratio of SeMet:Met is about 1:60,000; in the growing diet it is 1:50,000 and this ratio is almost

the same for breeder birds. After dietary Se supplementation in the form of SeMet at 0.3 mg/kg, the ratio SeMet:Met in the diet changes to 1:15,000 at the start, down to 1:12-14,000 later in life. Furthermore, our calculation indicates that in egg yolk this ratio is approximately 1:160,000 and in egg white about 1:87,000, and this ratio can be changed substantially after enrichment of eggs with selenium. As mentioned above, SeMet comprised 53-71% of total Se in the egg albumen and 12-19% in the egg yolk (Lipiec *et al.*, 2010). These findings support the view that SeMet is a non-specific form of Se that is metabolised as a constituent of the methionine pool where it is randomly distributed. In addition, in chickens muscle organic selenium has the longest half-live (12 days), as well as in brain and lungs (13 days), while the shortest biological half-lives were observed in major metabolic organs: the liver, kidney and pancreas, with half-lives close to 4 days (Brandt-Kjelsen *et al.*, 2014). The half-life of ^{75}Se in the heart was 9 days and 7 days in blood. Therefore, SeMet that is non-specifically accumulated in muscle proteins can build Se reserves, which can be used in stress conditions, where Se requirement is increased, but feed consumption usually is decreased. Under stress conditions, protein catabolism by proteasomes can release SeMet, which can serve as a source of Se for newly synthesised selenoproteins, such as GSH-Px, thioredoxin reductase, methionine sulfoxide reductase, etc. Those enzymes can deal with overproduction of free radicals and prevent decrease in productive and reproductive performance of farm animals. It is important to mention that selenocysteine provided with feed had no advantage in comparison to sodium selenite (SS) in terms of Se concentrations in tissues (Deagen *et al.*, 1987) and it is not able to build any Se reserves in the body. It was proven that Se from both selenite and SeMet is readily available for synthesis of the selenoenzyme GSH-Px in rat tissues (Pierce and Tappel, 1977).

There are several lines of direct and indirect evidence confirming the idea that Se, accumulated in tissues in the form of SeMet, can be available for selenoprotein synthesis (Alfthan *et al.*, 2000; Ip and Hayes, 1989; Levander, 1983; Pappas *et al.*, 2005; Persson-Moschos *et al.*, 1998; Robinson *et al.*, 1985; Surai, 2000; Surai *et al.*, 2006):

- Studies in our laboratory (Surai, 2000) indicated that chicks hatched from eggs enriched with Se by means of dietary Se-yeast had higher liver GSH-Px activity, not only at hatching, but more importantly, even at 5 days post-hatch. More recent observations with quails (Surai *et al.*, 2006) and chickens (Pappas *et al.*, 2005) indicate that when organic Se was included in the maternal diet, Se concentration in the liver of the progeny was alleviated up to 2-4 weeks post-hatch.
- It was clearly demonstrated that the effects of maternal organic Se supplementation on the expression of GSH-Px in the offspring fed on low Se diet are sustained for several weeks after hatching (Pappas *et al.*, 2005). In fact, when the offspring from the two parental groups (high Se and low Se) were both maintained on a low-Se progeny diet, the tissue Se concentrations in chicks originating from the high-Se hens remained significantly higher for 3-4 weeks after hatching, compared to the values in chicks from the low-Se hens. Similarly, tissue GSH-Px activity remained significantly higher in chicks obtained from the high-Se hens for 2 (liver) or 4 (muscles) weeks post-hatch. It seems likely that maternal Se has an epigenetic effect on Se metabolism in progeny chicks, since significantly increased Se concentration

in the muscles of 4 week old chickens reflects better assimilation of Se from the diet or lower usage Se in the body.

- The bioavailability of the Se pool in maintaining liver GSH-Px activity during a period of Se deprivation, following excess selenite or SeMet loading was assessed in rats (Ip and Hayes, 1989). In this study half-life of decay of the enzyme was calculated to be 4.2 and 9.1 days, respectively, in rats that had already been exposed to 3 mg/kg Se as either selenite or SeMet.
- In a human study, Persson-Moschos *et al.* (1998) showed that in individuals who had been supplemented with organic Se, the decline in the level of selenoprotein P following a period of supplementation was slower than in individuals who had been supplemented with selenite.
- When wheat and selenate were used as Se sources in a supplementation study in Finnish men it was shown that once the supplements were withdrawn, platelet GSH-Px activity declined less in the group given wheat Se (Levander *et al.*, 1983).
- After several weeks' supplementation with high-Se bread, plasma Se of New Zealand subjects increased from 50-70 ng/ml to 120-175 ng/ml (Robinson *et al.*, 1985). Plasma Se remained elevated when supplementation ceased.
- In SeMet or Se-yeast supplemented mice, liver GSH-Px activities declined more slowly during Se depletion than in mice given selenite (Spallholz and Rafferty, 1987 cited by Schrauzer, 2003).
- In children the relative bioavailability of Se-yeast vs selenite measured as GSH-Px activity was similar in plasma, red blood cells, and platelets, however, Se-yeast provided a longer lasting body pool of Se (Alfthan *et al.*, 2000).
- A study with broiler chickens fed organic or mineral Se demonstrated that endogenous Se could be released from tissues, and, thus, organic Se sources were more efficient in maintaining the GSH-Px level (Payne and Southern, 2005). Aforementioned data indicate that the protective effect of organic selenium is more pronounced under stressful conditions.
- Recently, it has been shown that embryonic mortality in eggs laid by 23 week old broiler breeders was highest in the first and last week of incubation, but significantly reduced as the age of the flock increased. In the trial reported by Pappas *et al.* (2006), the mortality of 27 week old breeders in week 3 of egg incubation was 3.5% for the control group and 10.6% in the fish oil supplemented breeders. Inclusion of Se-yeast (0.4 mg/kg) in the diets decreased mortality to 3.0 and 6.2%, respectively. Similarly, fish oil (FO) inclusion in the breeder diet reduced both hatchability and 1 day old chick weight (Pappas *et al.*, 2006). The addition of organic Se to the FO diets ameliorated some of these adverse effects. Therefore, Se reserves in the body (mainly in the muscles) built in the form of SeMet non-specifically incorporated into the proteins in place of Met could be considered as an important element in increasing adaptive ability of chickens (animals) to various stresses. This could increase their productive and reproductive performance.

The mechanisms regulating a SeMet conversion to H_2Se and further to synthesis of SeCys and respective selenoproteins are not clear at present, but is seems likely that changes in redox status of the cells/tissues and activation of proteasomal protein degradation could be involved. Indeed, ATP- and ubiquitin-independent proteolysis

by the 20S proteasome is responsible for the selective degradation of oxidised proteins. *In vitro*, the 20S proteasome shows an increased proteolytic activity toward oxidised polypeptides. In fact, a 30% decreased activity of the chymotrypsin-like activity of proteasome in cells overexpressing GSH-Px1 was shown (Kretz-Remy and Arrigo, 2003). This observation correlated with a 2-fold increase in IκB alpha half-life, a protein whose basal turnover is 20S proteasome-dependent. Furthermore, following exposure to H_2O_2, human T47D cells overexpressing GSH-Px showed a seleno-dependently decreased accumulation of intracellular ROS and 20S proteasome chymotrypsin-like activity. Moreover, exposure of HeLa cells to antioxidant compounds reduced the proteasome 20S chymotrypsin-like activity. These results suggest that GSH-Px activity or pro-reducing conditions can downregulate basal 20S proteasome activity (Kretz-Remy and Arrigo, 2003). Indeed, the chymotrypsin activity of the proteasome is strongly attenuated by GSH-Px overexpression. This suggests that selenium is a key element that controls proteasome activity. This could be a feedback mechanism of recognition of SeMet as a source of Se for selenoprotein synthesis. Under stress conditions some amino acids inside muscle proteins would be oxidised and this will trigger an increase in proteasome activity to degrade such proteins and release SeMet to be available as an additional source of Se for selenoprotein synthesis. When antioxidant-prooxidant equilibrium is restored increased GSH-Px activity would decrease proteasome activity and protein degradation. Indeed, SeMet is the major selenocompound found initially in animals given this selenoamino acid, but is converted with time afterwards to selenocysteine when incorporated in functional selenoproteins (Whanger, 2002). For example, the chemical forms of Se were determined in erythrocyte and liver proteins after injection of ^{75}Se as either sodium selenite or SeMet in male weanling rats. Void volume proteins contained principally selenocysteine (^{75}SeCys) in [^{75}Se]selenite-injected animals. This material contained both ^{75}SeMet and ^{75}SeCys 1 d post-injection in ^{75}SeMet-injected animals, but primarily ^{75}SeCys at 20 d afterwards (Beilstein and Whanger, 1986). This means that SeCys was synthesised from SeMet and with time all SeMet was converted to SeCys. In acid hydrolysates of whole liver ^{75}Se was recovered principally as ^{75}SeCys from animals injected with [^{75}Se]selenite. However, for animals injected with ^{75}SeMet, liver ^{75}Se was present initially as ^{75}SeMet, but after five days the majority of liver ^{75}Se was as SeCys. The long-term fate in rats of an oral dose of [^{75}Se]selenocystine was compared with that of an oral dose of [^{75}Se]SeMet. It was shown that intestinal absorption of [^{75}Se]selenocystine was 81% of the administered dose and that of [^{75}Se]SeMet was 86% (Thomson *et al.*, 1975). The initial utilisation of [^{75}Se]selenocystine was different from that of [^{75}Se]SeMet. However, after the first week ^{75}Se from both sources appeared to be metabolised similarly, suggesting that dietary Se of both forms is ultimately incorporated into the same metabolic pool (Thomson *et al.*, 1975).

Weanling male rats were fed a basal Se-deficient diet or this diet plus 2 mg/kg Se as either selenite, SeCys or SeMet for nine weeks (Deagen *et al.*, 1987). Except for the kidney, the tissue Se concentrations were similar in rats fed selenite or SeCys, but the Se content in testis, muscle, pancreas, heart, spleen, whole blood, erythrocytes and plasma was significantly higher in rats fed SeMet than in those fed either selenite or SeCys. The greatest increase due to SeMet compared with the selenite and SeCys

treatments was about 10-fold in the muscle compared with 1.3- to 3.6-fold for the other tissues (Deagen *et al.*, 1987). In general, SeMet has a slower, whole body turnover in comparison to sodium selenite and there is greater efficiency in the re-utilisation of Se from SeMet (Swanson *et al.*, 1991). Indeed, the average whole body half-lives of SeMet and selenite in humans were shown to be 252 and 102 days, respectively, confirming re-utilisation of SeMet in the body (Patterson *et al.*, 1989). It should be noted that only a small proportion of the methionine pool can be replaced by SeMet, since only part of methionine could be replaced by SeMet in the diet. Furthermore, protein turnover prevents accumulation of SeMet to toxic levels in the organism (Schrauzer, 2003).

In fact, rapid turnover of various selenoproteins and dependence of this process on Se status were described. For example, the half-life of GSH-Px is approximately 3 days (Sunde *et al.*, 1989), and 2-iodothyronine deiodinase has a half-life of only 30-45 minutes (Botero *et al.*, 2002; Curcio *et al.*, 2001; Kim *et al.*, 2003), while that of selenoprotein P in plasma is 3-4 hours (Burk and Hill, 1994). In growth medium there was an increase in TrxR mRNA levels of 2-5-fold at 1 microM Se and an increase in the stability of TrxR mRNA with a half-life for degradation of 21 hours compared to 10 hours in the absence of Se (Gallegos *et al.*, 1997). Similarly, the selenoprotein W mRNA half-life in myoblasts is about 57 hours for cells grown in a low Se medium while Se treatment increased half-life by 2-fold (Gu *et al.*, 2002). Therefore, it is clear that Se reserve development could be an important regulatory mechanism for maintaining effective antioxidant defence during periods of increased demand. Therefore, from a nutritional viewpoint, SeMet is superior to selenite, especially with respect to maintenance of GSH-Px during periods of Se inadequacy (Ip and Hayes, 1989).

At physiological levels of Se intake, urine is the most important route of excretion and regulates Se homeostasis (Daniels, 1996). Various Se metabolites were found in urine, including trimethylselenium. There is a great body of evidence indicating that urinary Se is lower when organic Se is used in comparison to selenite.

3.4 Selenium status and bioavailability

To assess Se status of animals/poultry and humans various static or functional tests are used. Static tests measure the total quantity of Se in various accessible tissues and body fluids, such as hair, nails, blood or its components, and urine. Functional tests measure the activity of Se-dependent enzymes, or a physiological or behavioural function dependent on Se (Gibson, 1989). There is no single test that can describe Se status and a combination of various techniques is preferable and this explains a great discrepancy in results of comparative evaluation of Se bioavailability from various sources. The problem is that it is difficult to choose a proper end point for such evaluation.

Selenium availability from plant-based sources varies. For example, biological availability of Se for prevention of exudative diathesis (ED) was taken as 100% for

sodium selenite. It ranged in other forms of Se from 74% for sodium selenate to 7% for elemental Se; in plant feedstuffs from 210% for lucerne meal to 60% for soybean meal and in animal feedstuffs from 25% for herring meal to 8.5% for fish solubles (Table 3.2; Cantor and Scott, 1974; Cantor *et al.*, 1975).

However, SeMet was four times as effective as either selenite or selenocystine with respect to prevention of pancreatic degeneration, and increasing the relative weight and Se concentration of the pancreas (Cantor *et al.*, 1975). Cantor and Scott (1974a) observed that protection against ED was closely related to plasma GSH-Px. Miller *et al.* (1972) concluded that the retention of Se in fish meal or solubles was 43% relative to selenite, and 31% relative to SeMet.

Newly-hatched White Leghorn chicks with low-Se status were fed a low-Se basal diet for two weeks post-hatch followed by either continued depletion or a repletion period of four weeks with graded levels of Se (0.03, 0.06, 0.09 and 0.12 mg/kg) provided via sodium selenite, wheat or fish meal. The bioavailability of Se in wheat and fish meal in comparison to selenite for increasing the activity of GSH-Px was 78 and 58%, respectively; and for increasing whole blood Se concentration was 123 and 107%, respectively (Hassan *et al.*, 1993). Based on the activity of plasma GSH-Px, the biological availability of Se in soybean meal, lucerne, fish meal and SeMet was 33, 85, 82 and 92%, respectively (Ikumo and Yoshida, 1981). On the other hand, Se availability in fish meal, based on the prevention of incidence of ED, was 74%. In contrast to previous data, Se in fish meal was poorly able to prevent deficiency in

Table 3.2. Bioavailability of Se in feedstuffs (adapted from Cantor, 1997).[1]

Feedstuff	Biological availability, %
Dehydrated alfalfa meal	210
Brewer's yeast	89
Cottonseed meal	86
Corn	86
Brewer's grains	80
Wheat	71
Distiller's dried grains and solubles	65
Soybean meal	60
Herring meal	25
Tuna meal	22
Poultry by-product meal	18
Menhaden fish meal	16
Meat and bone meal	15
Fish solubles	9

[1] Sodium selenite was used as standard and prevention of exudative diathesis in chicks was used as an index of Se bioavailability.

chickens (Martello and Latshaw, 1982) and the results of Whitacre and Latshaw (1982) clearly showed that the commercial preparation of fish meal significantly decreased Se utilisation. Availability of Se in feeds was estimated in relation to restoring blood serum GSH-Px activity in Se-depleted chickens. The availability of the Se (relative to Se in selenite) in capelin fish meal was 48.0 (38.5-60.0), mackerel fish meal, 34.1 (32.3-35.8), soybean meal, 17.5, maize gluten meal, 25.7, and SeMet, 78.3% (Gabrielsen and Opstvedt, 1980). These data on Se availability from fish sources are in line with others studied, but data on soybean meal are somewhat low. It is possible that thermal treatment of processed soya could affect Se availability.

In Se-deficient chicks, when providing Se at 10 μg/kg diet, selenite and selenocystine were about equal in promoting weight gain and preventing ED, while SeMet was less effective. Tissues from chicks given the Se sources providing 60 μg/kg diet for four weeks were analysed for Se. The Se content of tissues from chicks given selenite or selenocystine was similar. Chicks given SeMet had higher concentrations of Se in pancreas and breast muscle than the others, but lower concentrations in kidney, liver and heart (Osman and Latshaw, 1976). Bioavailability of Se in oats, meat meal and SeMet based on efficacy in preventing ED in Se-depleted chicks was 41, 30 and 77%, respectively, while based on Se biopotency in elevating GSH-Px activity in plasma, it was 33, 21 and 77%, respectively. When the increase in Se concentration in cardiac muscle was used as the indicator of bioavailability, the Se bioavailabilities were 60, 42 and 114% for oats, meat meal and SeMet, respectively. Thus, SeMet was superior to sodium selenite in increasing Se concentration in tissues, but inferior to it in protecting chicks against ED (Hassan, 1987). By taking both the Se level and the GSH-Px activity into consideration, organic Se was judged to be more bioavailable than selenite and selenate for humans (Clausen and Nielsen, 1988). Therefore, Se from yeast, wheat and lucerne is highly available, while Se in most other plant sources is moderately available. Selenium in fish meal is poorly available (Cantor, 1997), despite the fact that Se content of fish meal can be quite high. Indeed Se availability depends not only on feed ingredient but also on the methods used for evaluation.

3.5 Effectors of selenium absorption, metabolism and bioavailability

Animal studies reveal that various factors influence Se bioavailability. Animals cannot synthesise SeMet or distinguish it from methionine, and as a result it is non-specifically incorporated into a wide range of proteins (Daniels, 1996). SeMet is retained in tissue proteins to a greater extent than selenocysteine and the inorganic forms. However, a number of other factors besides chemical form, may also influence the bioavailability and distribution of Se, including other dietary components, Se status, physiological status and species (Thomson, 1998). For example, Se is better absorbed from a high protein diet (Daniels, 1996). In particular, it has been suggested that SeMet is preferentially incorporated into body protein when dietary Met is limited (Tian *et al.*, 2001). Se bioavailability also depends on many other nutrients in the diet, including Cu, Zn, Mg, vitamins B2 and B6, etc. (Surai, 2006).

It seems likely that Se can affect absorption of other nutrients. For example, in studies investigating the relationship between vitamin E/Se and glucose absorption, Se plus vitamin E or Se alone modified jejunal glucose absorption, depending on the duration of feeding and age of chickens. In younger chickens, providing the supplements for 11 days depressed glucose absorption, but when given the supplements for 18 days glucose absorption was increased. In older chickens Se alone or with vitamin E given for 13 days increased glucose absorption (Giurgea and Roman, 1992).

Experiments were conducted to determine the initial effects of oral Se administration on Se-deficient chicks. Administration of 5 μg Se as seleno-DL-Met increased voluntary feed consumption within 2-3 hours, whereas selenite did not have a significant effect until 3-4 hours (Bunk and Combs, 1980). Spontaneous activity, body weight gain and plasma glucose concentration increased 6-8 hours after Se administration. The earliest response in the specific activity of Se-dependent GSH-Px occurred in plasma at 8 hours and in liver at 24 hours after Se administration. The onset of pancreatic atrophy, however, was not affected by the level of feed intake suggesting that the effect of Se upon appetite may be distinct from the involvement of Se in nutritional pancreatic atrophy and fibrosis. Inorganic Se compounds were shown to be anti-feed intake factors, whereas the organic Se compounds tested had little such activity.

An interesting study was conducted with an artificial diet to examine the feeding responses of a generalist herbivore, *Spodoptera exigua* (Hubner) (*Lepidoptera*: *Noctuidae*), to various forms and concentrations of Se (Vickerman and Trumble, 1999). Tests initiated with neonates showed larvae significantly preferred the control diet over one containing sodium selenate, sodium selenite, or selenocystine, but at most concentrations showed no preference between the SeMet-containing and control diets. Choice tests initiated with third instars demonstrated a preference for the control diet over sodium selenate and sodium selenite treatments. In contrast, no significant responses were found with third instars offered a choice between selenocystine or SeMet and untreated controls. Similarly, when Se-enriched yeast was used as a source of organic Se, young Se-deficient laying hens reduced their Se deficit by preferentially selecting the high-Se diet (Zuberbuehler *et al.*, 2002).

3.6 Selenium sources for poultry

As mentioned above, Se content of feed and food ingredients greatly varies (Table 3.3) depending on many different factors. For example, Se concentration in maize and rice grown in normal and high Se areas can vary 100-500-fold. Indeed average data on Se content in feedstuffs presented in various tables are not suitable for diet balancing and Se supplementation is a routing practice in commercial animal and poultry production. In fact, FDA approved Se supplements for poultry and swine in 1974 in the form of selenite or selenate.

While Se form was not rigorously considered in the initial research into Se nutrition, for the last 40 years information has accumulated indicating that the natural form of Se in

Table 3.3. Selenium concentrations in various feed ingredients, mg/kg (adapted from NRC, 1983).

Ingredient	USA	Canada
Alfalfa meal	0.01-2.00	0.02-0.27
Barley	0.05-0.5	0.02-0.99
Brewer's grains	0.15-1.00	0.29-1.10
Corn	0.01-1.00	0.01-0.33
Fish meal	1.0-5.0	1.3-3.4
Linseed meal	0.5-1.2	0.7-1.5
Meat meal	0.08-0.5	0.2-0.81
Oats	0.01-1.00	0.01-1.10
Poultry by-product	0.5-0.10	-
Soybean meal	0.06-1.00	0.04-0.78
Wheat	0.1-3.00	0.02-1.5
Wheat bran	0.1-3.0	0.24-1.3
Wheat middling	0.15-1.0	0.41-0.89
Whole soybeans	0.07-0.90	-

plant-based feed ingredients consists of various selenoanimo acids with SeMet being major form of Se in grains, oil seeds and other important feed ingredients. Therefore, organic Se is the natural form of Se to include in feed formulations. However, sodium selenite remains in use in many animal feeds. The limitations of using inorganic Se are well known and include toxicity, interactions with other minerals and vitamins (Figure 3.5), low efficiency of transfer to milk, meat and eggs and an inability to build and maintain Se reserves in the body. As a result, a high proportion of the element consumed in inorganic form is simply excreted. Further, a prooxidant effect of the selenite ion (Spallholz, 1997) is a great disadvantage as well, particularly when shelf life of food animal products is considered.

Ingredient interactions should be carefully considered. It has been shown that selenite can be dissolved when dispersed in feeds of relatively high water activity. When dissolved, it may form selenious acid and disperse as a vapour losing its biological activity and nutritional function (Eisenberg *et al.*, 2007). When the premix contains sodium selenite and ascorbic acid, the chemical reaction between them causes selenite reduction to elemental Se, which is not adsorbed in the digestive tract of animals, and ascorbic acid is also oxidised thereby loosing biological activity. Therefore, in such a situation, both nutrients are lost. Pink particles in the premix very often represent elemental Se produced in a way mentioned above. This could happen in the premix/feed during storage or in the digestive tract during absorption. In fact, when solutions of selenite and ascorbic acid (AA) were mixed (proportion between AA and selenite was similar to those in supplements) – after about 2 hours from the preparation it is present at about 50% of that expected and progressively decreases to practically disappearance after 24 hours (Gosetti *et al.*, 2007). Furthermore, transport of ^{75}Se was

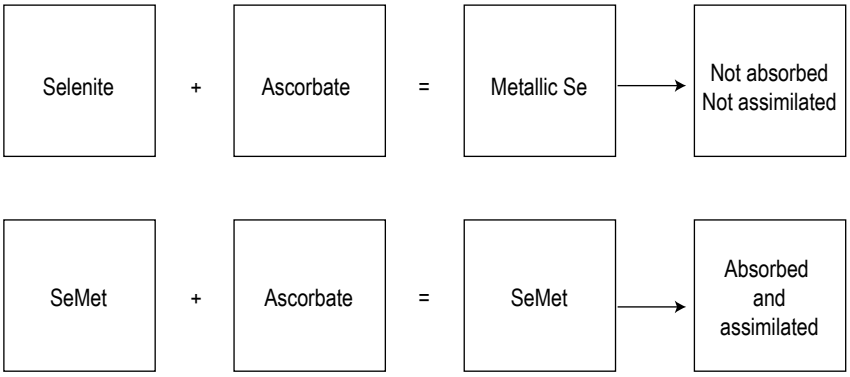

Figure 3.5. Selenium-ascorbate interactions in premixes and digestive tract.

inhibited when AA and selenite were injected directly into ligated duodenal loops of anaesthetised chickens. The mechanism of the inhibition seemed to be precipitation of Se within the intestinal lumen, since less ^{75}Se was found in the supernatant fraction of the luminal fluid when AA was present (Mykkänen and Mutanen, 1983). Therefore, vitamin C in premixes is compatible with organic Se, but incompatible with selenate or selenite. This issue is extremely important for anti-stress premixes containing increased levels of ascorbic acid. For example, an interaction of 0.5% vitamin C with either selenite or seleno-DL-Met (SeMet, 3 mg/kg) was studied in rats (Ip, 1986). Results showed that the protective effect of selenite in tumorigenesis was nullified by vitamin C, whereas the chemopreventive action of SeMet was not affected. The authors suggested that selenite is reduced by vitamin C to elemental Se and was not available for uptake by tissues. Similarly, the availability of Se was reduced almost to zero when selenite and 1 g AA were taken together well before the meal (Robinson *et al.*, 1985). Similarly, 0.5 or 0.25% of vitamin C in the diet completely negated the accumulation of Se in blood, liver and the mammary gland induced by 3 mg/kg selenite supplementation. Indeed, selenite is a hygroscopic compound and can be dissolved when dispersed in feeds of relatively high water activity. It is also interesting that other compounds in the premix can similarly reduce Se in selenite to produce elemental Se, which is not absorbed from the feed. For example, a reference from Michigan State University (Groce *et al.*, 1973) indicates changes in odour and/or colour, when premixes containing 200 mg/kg Se were prepared with glucose monohydrate, corn starch or sucrose and then stored at room temperature. The order was musty and sweetish in character, while the colour changes involved appearance of pink to dark red particles in the original white matrix. Indeed, Se retention was decreased and Se excretion was increased as a result of old premix inclusion in pig diets. In contrast, ascorbic acid enhances SeMet assimilation from the diet. Furthermore, SeMet itself is considered to possess antioxidant properties (Schrauzer, 2000).

Thus, the use of sodium selenite in animal diets has recently been questioned (Fisinin *et al.*, 2008; Mahan and Peters, 2004; Ortman and Pehrson, 1997, 1998; Surai, 2002,

2006; Surai and Fisinin, 2014, 2015, 2016, 2016a). Prooxidant properties of selenite and its interactions with other nutrients, including vitamin C, put pressure on feed manufactures to find new, more effective sources of supplemental Se. Therefore, the simplest idea was to use Se forms produced by plants.

3.7 Selenium-enriched yeast: pluses and minuses

It is well known that chemical and physical properties of Se and sulphur are very similar, reflecting similar outer-valence-shell electronic configurations and atomic sizes (Combs and Combs, 1984). Therefore, plants cannot distinguish between these two elements when synthesising amino acids. As a result they can synthesise SeMet when Se is available (Figure 3.6). This biological feature was the basis for development of the commercial technology of organic Se production from yeast. Indeed, various commercial forms of Se-yeast found their way to the market place and have shown to be effective sources of Se for poultry and animal production (for review see Fisinin *et al.*, 2008; Surai, 2006; Surai and Fisinin, 2014, 2015, 2016, 2016a; Surai *et al.*, 2010).

The legal definition of Se-yeast is as follows:

> Selenium yeast is a dried, non-viable yeast (*Saccharomyces cerevisiae*) cultivated in a fed-batch fermentation which provides incremental amounts of cane molasses and selenium salts in a manner which minimises the detrimental effects of selenium salts on the growth rate of the yeast and allows for optimal incorporation of inorganic selenium into cellular organic material. Residual inorganic selenium is eliminated in a rigorous washing process and must not exceed 2% of the total selenium content in the final selenium yeast product (LII, 2015).

Therefore, only total Se level and inorganic Se proportion in Se-yeast is officially regulated. However, there are several points to be addressed in relation to commercial usage of Se-yeast. First of all, it is necessary to mention that yeast is a live organism and its composition will depend on the genetics and conditions of growing, including temperature, pH, oxygen concentration, etc. It seems likely that selenoamino acid composition of the yeast depends on various factors, including yeast species, growth conditions, as well as analytical techniques used. For example, when three different commercial yeast products were analysed, results showed that the proportion of water-soluble Se varied from 11.5 up to 28.0% and water insoluble polysaccharide bound Se proportion varied from 15.5 up to 72% (Encinar *et al.*, 2003). In fact, Se-yeast has been reported to contain over 60 various selenium compounds (Arnaudguilhem *et al.*, 2012) and it is well established that SeMet is the major selenocompound in Se-enriched yeast. However, its proportion greatly varies. Rayman (2004) presented a literature review analysing Se-speciation in different Se-yeast supplements and the percentage of SeMet in 7 supplements were quite variable: 84, 69, 75, 81, 83, 61 and 60%. In particular, the author presented data on SeMet percentage of individual Se-yeast products. The values were 58-65% of total Se in LALMINe (Lallemand, Montreal,

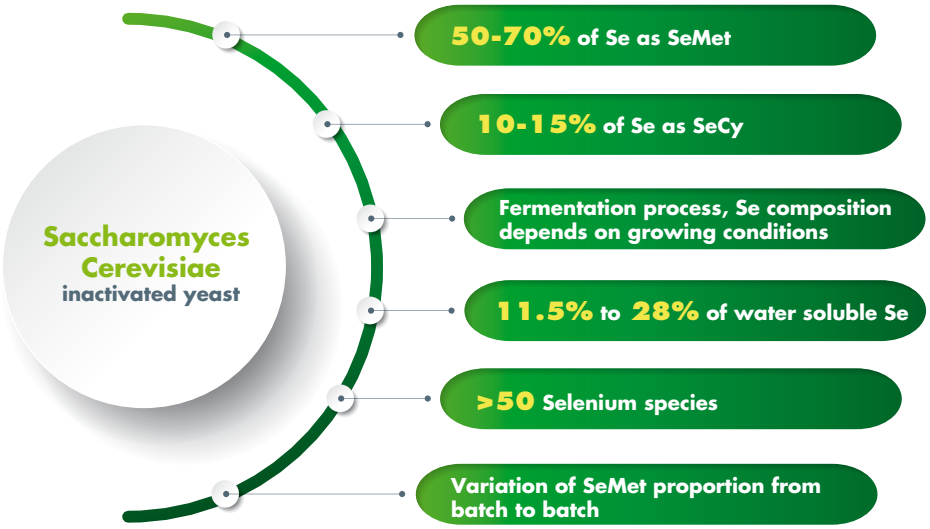

Figure 3.6. Selenium-enriched yeast.

Canada); 60-70% of total Se in SelenoExcelle (Cypress Systems, Fresno, CA, USA); 54-60% of total Se in Seleno Precisee (Pharma Nord, Vejle, Denmark) and 62-74% in Sel-Plex (Alltech, Nicholasville, KY, USA). Indeed, the presented data depend not only on the technology of Se-yeast production (strain of yeast, source of Se, temperature, oxygen concentration, etc.), but also on the extraction efficiency of the technique used by the analytical laboratory. The author concluded that commercial Se-yeast products found on the market contain almost all their Se in organic form and about 55-75% of it (allowing for extraction efficiency) was present as SeMet (Rayman, 2004). In general, Bierla *et al.* (2012) mentioned that the typical criteria for Se-yeast of industrial use is >60% SeMet and 2% could indicate a low quality Se-yeast (Bierla *et al.*, 2012).

A great variability in SeMet content of Se-yeast products has been reported. For example, 85% of Se contained in Se-yeast was found to be present in the form of SeMet and 91% was organic (Fan *et al.*, 2003). SeMet comprised 79% of the extracted selenium and 64% of total selenium in Se-yeast; the selenium levels were at the 2-3 mg/g (McSheehy *et al.*, 2005). SeMet in yeast and nuts comprised, respectively, 65 and 75% of total Se (Wrobel *et al.*, 2003). Similarly, a proteolytic enzyme extract of Se-yeast was found to contain Se as SeMet (74.8%), selenocystine (9.9%), selenite (5.1%) and as at least three unknown Se compounds (10.2%, Yoshida *et al.*, 2002). SeMet comprised about 85% of total Se compounds found in Se-yeast used for human trials (Ip *et al.*, 2000). Similarly, Se-yeast, which was used as a source of Se in the PRECISE and other trials, contained SeMet at 54-60% of the total selenium (Larsen *et al.*, 2004). A commercial source of Se-enriched yeast tablets containing 210 g Se/g was found to contain 73% of the total Se as SeMet (Wolf *et al.*, 2001). Samples of five leading

manufactures worldwide [Alltech(KY), Angel (China), Biorigin (Brazil), Lallemand (Canada), Lesaffre (France)] were investigated by Casal *et al.* (2010). The pro-portion of water-extractable selenium varied from 16 to 35% and proportion of SeMet varied from 44±7 to 93±12%. The authors also showed between batch variations. For example, one sample was analysed as two different production batches and showed 27 and 35% of water-extractable selenium, while SeMet comprised 93±12 and 86±25% of total Se. Recently, a considerable incorporation of selenocysteine (SeCys) in proteins of the yeast proteome despite the absence of the UGA codon was demonstrated (Bierla *et al.*, 2013). The authors concluded that 10-15% of selenium present in Se-enriched yeast is in the form of selenocysteine. This means, that if all Se in Se-yeast is accounted for, the maximum SeMet proportion would not exceed 85%, but in many cases will be lower than that.

It has been shown that the main advantage of organic Se in poultry and animal production is related to its ability to build Se reserves in the body in the form of SeMet, which can be recruited in times of stress (Fisinin *et al.*, 2008; Surai, 2006; Surai and Fisinin, 2014, 2015, 2016, 2016a; Surai *et al.*, 2010). Therefore, ideally, it would be necessary to specify the percentage of SeMet (active compound of the Se-yeast) in the yeast product and price would depend on the SeMet level in the product. This could be achievable if Se assimilation from all yeast products would be the same, but this is not the case judging on the Se location in different Se-yeast compounds. For example, the non-soluble protein fraction accounted for up to 40% of the total selenium in the yeast (Encinar *et al.*, 2003). It should be noted that absorption of dietary Se (organic Se) is generally believed to be good (about 80% absorption) (Reilly, 2006). Absorption and retention of Se from Se-yeast, measured in twelve volunteers fed ^{77}Se-labelled SelenoPrecise yeast (Pharmanord, Velje, Denmark), was between 75 and 90% (Sloth *et al.*, 2003). Other Se-yeasts gave different results (between 50 and 60%). The same Se-yeast was analysed by Zheng *et al.* (2003) using enzymatic digestion with an on column recovery of Se of up to 93% found SeMet to be the predominant species (78% of the total Se), while selenite (2%), selenocystine (1.9%), the major unknown species (10%) and the other unknowns (7.8%) accounted for the remaining selenium in the enzymatic extracts. Recently, two commercially-available Se-yeast preparations, containing SeMet at 63 and 56.7% have been tested in broilers (Simon *et al.*, 2013). It was shown that differences in nutritional efficacy of the preparations were proportional to the SeMet content.

Therefore, it is important to know under which form Se is consumed with regard to the bioavailability of the different forms of Se. Furthermore, at present the task to guarantee the SeMet level of a product is almost impossible to achieve. This relates to the variability of SeMet proportions in the yeast products due to different conditions of their production, as well as to analytical difficulties in detection of Se species in Se-yeast. In fact, a part of SeMet in the Se-yeast is bound in resistant membrane hydrophobic proteins and difficult to release during sample preparation. Se-yeast can be characterised by the selenium metabolic profile (selenometabolome) reflecting the yeast strain and fermentation parameters, and forms a precious fingerprint

of the origin of preparations available on the market and of the reproducibility of the production process (Lobinski *et al.*, 2000). Recently, a two-dimensional size-exclusion-strong cation-exchange HPLC with parallel ICP-MS and ESI-MS detection was developed as an advanced tool for selenometabolomics studies of yeast (Casal *et al.*, 2010).

3.8 SeMet and OH-SeMet

Another option to improve Se status of poultry and farm animals would be to use pure SeMet as a dietary supplement (Schrauzer, 2000, 2001, 2003; Schrauzer and Surai, 2009). There are some respectable publications showing beneficial effects of organic Se in the form of SeMet in the poultry diets (Wang and Xu, 2008; Wang *et al.*, 2011; Yuan *et al.*, 2011, 2013). Recently, it has been determined whether SeMet or Se-yeast acts with different potency on six bio-chemical markers, including intraprostatic dihydrotestosterone (DHT), testosterone (T), DHT:T, and epithelial cell DNA damage, proliferation, and apoptosis (Waters *et al.*, 2012). By analysing dogs supplemented with SeMet or Se-yeast that achieved equivalent intraprostatic selenium concentration after supplementation, there was no significant difference in potency of either selenium form on any of the six parameters over three different ranges of target tissue selenium concentration. However, SeMet in purified form is unstable and easily oxidised. For example, it has been shown that in the freeze-dried samples of oyster total Se and Se species evaluated are stable for at least 12 months under all the conditions tested. However, after purification of Se species, including SeMet, in the enzymatic extracts they are only stable for 10 days if stored at 4 °C in Pyrex containers (Moreno *et al.*, 2002). After storage of SeMet water solution for 30 days at 20 °C, less than 80% SeMet was recovered (Lindemann *et al.*, 2000). Potentially bioavailable selenium-containing compounds in Se-yeast were investigated using the candidate reference material SEAS (Reyes *et al.*, 2006). SeMet was the major compound identified in the gastrointestinal extract, while SeMet selenoxide was its main degradation product formed after medium and long-term sample storage, respectively. Indeed, pure SeMet is chemically oxidised to SeMetO under oxic conditions in the small intestine (Lavu *et al.*, 2016). The oxidability of SeMet during storage could explain different results in terms of gene expression between SeMet-supplemented and Se-yeast-supplemented groups of mice (Barger *et al.*, 2012). An alternate form of Se dietary supplementation could be algae biomass specially enriched with selenium. Indeed, when the algae are exposed to Se in the form of selenite, they are able to synthesise organic selenium species. The technology of heterotrophic fed-batch cultivation of the microalga *Chlorella* enriched by organically bound Se was developed. In fact, spray-dried Se-*Chlorella* biomass was shown to have a similar potency as a dietary Se source for poultry and farm animals as Se-yeast (Doucha *et al.*, 2009 and references therein).

Recently, a new stable organic Se source called Selisseo® (SO) has been developed which is a selenomethionine hydroxyanalogue, 2-hydroxy-4-methylselenobutanoic acid or HMSeBA (Briens *et al.*, 2013, 2014). Two experiments were conducted on broiler chickens to compare the effect of HMSeBA (SO), with two practical Se additives, SS and Se-yeast. The different Se sources and levels improved muscle Se concentration compared with the non-supplemented group (NC), with a significant source effect in the following order: SS, Se-yeast and SO ($P<0.05$). In fact, the relative muscle Se enrichment comparison, using a linear regression slope ratio, indicated an average of 1.48-fold (95% confidence interval: 1.38, 1.58) higher selenium deposition in muscle for SO compared to Se-yeast (Briens *et al.*, 2014). Seleno-amino acid speciation results for Se-yeast and SO at 0.3 mg Se/kg feed indicated that muscle Se was only present as SeMet or SeCys, showing a full conversion of Se by the bird. The results confirmed the higher bioavailability of organic Se sources compared with the mineral source and demonstrated a significantly better efficiency of HMSeBA compared with Se-yeast for muscle Se enrichment. In particular, the authors showed that Se muscle concentrations significantly improve with SO, increasing the relative bioavailability for total Se by 39% compared with Se-yeast. On the one hand this could be a reflection of a higher SeMet level in the diet (almost 100% SeMet in SO vs 60-70% SeMet in Se-yeast). On the other hand, there could be other biochemical differences in the Se metabolism, since SO increased the SeCys level in the muscle. It would be interesting to note that hens fed the diet with HMSeBA-0.2 accumulated more Se in their eggs (+28.8%) and muscles (+28%) than those fed the diet supplemented with Se-yeast-0.2 (Jlali *et al.*, 2013). After 21 days, organic Se sources maintained (Se-yeast) or increased (Hydroxy-SeMet) breast muscle Se concentration compared to hatch value whereas inorganic source (SS) or NC showed a significant decrease in tissue Se concentration (Couloinger *et al.*, 2015). Furthermore, HMSeBA in turkey diet improved GSH-Px activity in thigh muscles and decreased lipid peroxidation (Briens *et al.*, 2016). These results showed the greater ability of HMSeBA to increase Se deposition in eggs and breast muscle of laying hens, which could be of great importance for breeding birds and newly developing chicks (Figure 3.7).

Recently, the EU decided to limit the maximum supplementation with Se-yeast to 0.2 mg Se/kg complete feed for reasons of consumer safety (EC, 2013). At this comparatively low level of supplementation advantages of organic Se in the form of Se-yeast will be less pronounced, and alternative effective sources of organic selenium with higher efficiency of transfer to eggs and animal tissues would play a bigger role in poultry reproduction. Therefore, aforementioned results indicated that a new source of organic selenium in the form of 2-hydroxy-4-methylselenobutanoic acid, supplied in the same dose as Se-yeast in the chicken diet could provide additional benefit in terms of Se reserves in the muscles, as well as Se transfer to the egg and probably to the developing embryo. This potentially can be translated into better antioxidant protection in stress conditions of commercial poultry production. Major differences between organic selenium and sodium selenite are shown in Table 3.4.

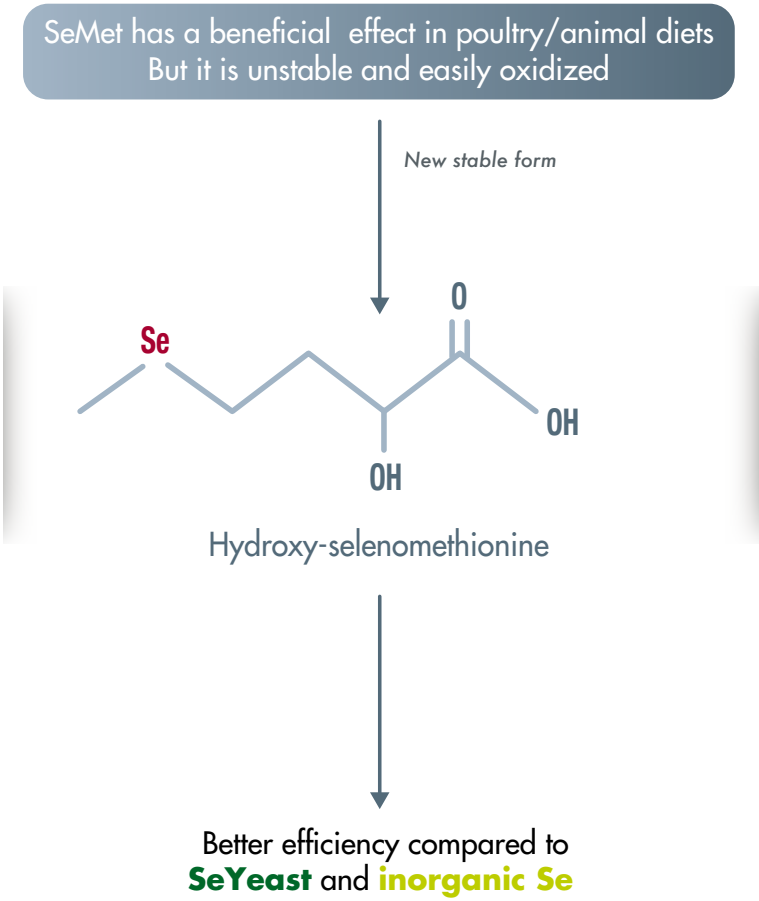

Figure 3.7. Selenomethionine.

3.9 Chelated Se products

There is a range of products on the market claiming to contain chelated Se (Se-glycinates, Se-proteinates, Se-amino acids complexes, etc.), however, the chemical position of Se in the periodic table of elements indicates that Se is not a true metal, and therefore its chelating ability is in question. Indeed, attempts to determine chelated Se in such products ended up with a detection of only inorganic Se (selenite and selenate; Amoako *et al.*, 2009; Kubachka *et al.*, 2017). This could explain the results of Givens *et al.* (2004) showing that a chelated selenium-amino acid complex in the cow diet is not different from sodium selenite when efficacy of Se transfer to the milk was assessed. Indeed, chelated Se products are not related to SeMet and, probably, should not be included into the organic Se category.

Table 3.4. Major differences between organic selenium and selenite.

	Organic selenium	Selenite
Absorption	similar to methionine with active transport in the gut	similar to other mineral with passive transport in the gut
Accumulation	building Se reserves by non-specific incorporation of SeMet into the proteins	not accumulated in the body
Toxicity	at least 3 times less toxic than selenite	highly toxic, can penetrate via skin causing problems
Bioavailability	higher bioavailability in comparison to selenite to animals/poultry	lower bioavailability in comparison to SeMet
Antioxidant activity	possesses antioxidant properties per se and could scavenge NO and other radicals	possesses prooxidant properties and could stimulate free radical production when reacting with GSH
Effect on DNA	stimulate DNA-repair enzymes	causes DNA damage
Transfer to eggs and muscles	transferred to egg and muscles giving an opportunity to produce Se-eggs and Se-meat	poorly transferred to eggs and muscles
Reactions with other elements	neutral, ascorbic acid promotes SeMet assimilation from the diet	highly reactive, reduced to metallic, unavailable selenium by ascorbic acid
Protective effect in stress conditions	provide additional protection due to Se reserves in the body	cannot provide additional protection due to absence Se reserves in the body
Effect on drip loss	decrease drip loss	does not affect drip loss
Environmental issues	better retention in tissues, less released with faeces and urine	low retention in tissues and high release with faeces and urine
Stability	stable	stable
Classification based on the mode of action	feed additive	drug

3.10 Nano-Se products

Recently, selenium nanoparticles (SeNPs, nano-Se) have received substantial attention as possible novel nutritional supplements, because of their lower toxicity and ability to gradually release selenium after ingestion (Skalickova *et al.*, 2017). It has been suggested that nano-Se can serve as an antioxidant with reduced risk of selenium toxicity and as a potential chemopreventive agent if the induction of GST by selenium is a crucial mechanism for its chemopreventive effect (Wang *et al.*, 2007). The authors also showed that nano-Se has an ability to increase selenoenzymes activity comparable to SeMet. However, the question still is how elemental Se can be involved in SeCys synthesis and selenoprotein expression. There are several reports of successful testing

of nano-Se in broiler nutrition (Cai *et al.*, 2012; Gulyas *et al.*, 2017; Wang, 2008; Zhou and Wang, 2011). In many cases low nano-Se toxicity is considered as its main advantage. However, one should also realise that Se toxicity is not a major problem in the poultry industry and Se in the form of sodium selenite or organic Se (SeMet, Se-yeast or other preparations) is an essential part of premixes produced worldwide. It seems likely that nano-Se could be a new chemopreventive agent for treatment of various diseases (Zhang *et al.*, 2008), including cancer, but its nutritional value as a feed supplement for poultry industry is questionable (Surai *et al.*, 2017).

3.11 Conclusions

Selenium is an essential element in animal/poultry nutrition and its dietary supplementation in optimal form and amount is a key for maintaining their health, productive and reproductive performance. It has been proven that the organic form of Se (mainly SeMet) in the animal/poultry diet has a range of advantages in comparison to traditional sodium selenite. In fact, the organic Se concept considers SeMet as a storage form of Se in chicken body. Since animals/poultry are not able to synthesise SeMet, its provision within a diet is a key element of a strategy to fight commercially-relevant stresses. Indeed, under stress conditions, when increased selenoprotein expression requires additional Se, its provision with feed usually decreased due to reduction in feed consumption, Se reserves in the body (mainly in muscles) could help to maintain an effective antioxidant defences. The animal/poultry industry is looking for most effective sources of organic Se to be commercially used. Se-yeast received substantial attention and commercial applications are a reliable source of organic Se. However, the active Se component of the yeast, SeMet, comprises only 50-70% of the total Se, and there is no proof that the rest (30-50%) of Se in the form of SeCys, MeSeCys, etc. has any additional benefits in comparison to sodium selenite. From the production point of view it is difficult to guarantee a certain percentage of SeMet in the Se-yeast, since there is a range of factors (yeast strain, medium composition and selenite concentration in it, temperature, etc.) affecting the aforementioned parameter. Analytical difficulties in precise determination of the SeMet amount further complicates the issue. Stability issues with pure SeMet in the poultry diets restrict its wide usage. Major sources of organic Se in the poultry market are shown in Table 3.5.

It seems likely that a recently developed product (hydroxyl-SeMet) combines advantages of both Se-yeast (SeMet stability) and pure SeMet (high proportion of SeMet), and could be considered as a next step in Se application in poultry nutrition. Indeed, the first generation of Se supplements for poultry included selenite and selenate. The second generation of organic Se for poultry included Se-yeast, SeMet and Zn-SeMet products. Their advantages and disadvantages are mentioned above. Indeed, OH-SeMet can be considered as the next, third generation of Se supplement for poultry combining advantages of previous Se supplements on the market. It is well appreciated that via selenoprotein expression Se is involved in regulation of cell growth, apoptosis and modifying the action of cell signalling systems and transcription factors

Table 3.5. Sources of organic Se on the market (adapted from Surai *et al.*, 2018).

Source	Comments	SeMet[1]
Se-yeast	well researched, SeMet 50-70%	yes
SeMet	not stable, >95% SeMet	yes
Zn-SeMet	not stable, >95% SeMet	yes
OH-SeMet (Selisseo)	stable, >95% OH-SeMet	yes
Se-proteinates	chemistry is not proven	no
Se-glycinate	chemistry is not proven	no
Se-chelates	chemistry is not proven	no
Selenohomolathionine	biochemistry of metabolism is not clear	no
Other (nano-Se, etc.)	under development	no

[1] Since only SeMet can be non-specifically incorporated into proteins in measurable amounts, it is an active component of organic selenium sources.

and therefore its adequate dietary supply is a crucial factor for many physiological processes in animal/poultry body.

Major advantages of organic Se in the poultry diet are related to building Se reserves in the body which can be used in stress conditions as additional sources of Se to upregulate selenoproteins and improve antioxidant defences (Figure 3.8).

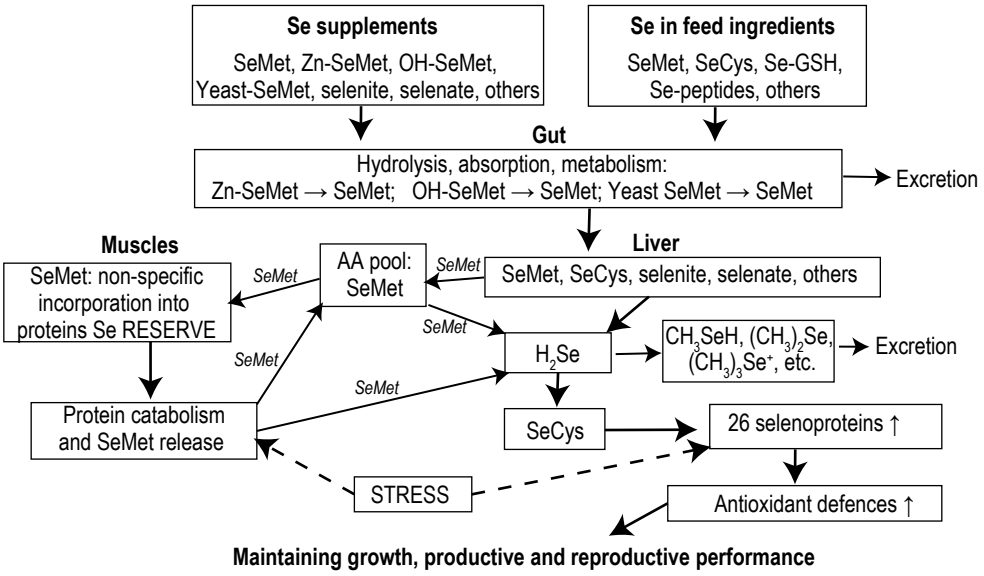

Figure 3.8. Organic selenium in action (adapted from Surai *et al.*, 2018).

There is a range of Se-containing compounds in the feed, including SeMet, SeCys, Se-GSH, Se-peptides, etc. and various Se supplements, including selenite, selenate, SeMet, Zn-SeMet, OH-SeMet, Se-yeast, etc. can be included into premix. All those Se forms come to the intestine where initial hydrolysis (SeMet will be released from Se-yeast or Zn-SeMet; OH-SeMet will be converted into SeMet) and some metabolic changes will take place. This includes excretion of Se metabolites via bile, faeces and urine. Furthermore, selenite, selenate, SeMet and some other Se forms will be delivered to the liver for metabolic changes and distribution. In parallel, some SeMet will go to the free amino acid pool and build Se reserves, mainly in muscles. The next step of Se assimilation and metabolism includes the conversion of all major forms of Se into H_2Se from which SeCys will be synthesised and incorporated into 25 newly synthesised selenoproteins that are an integral part of the antioxidant system of the body.

Under stress conditions, protein catabolism will take place, which will release some SeMet incorporated into those proteins and this SeMet will be converted into H_2Se and further into newly synthesised SeCys and 25 selenoproteins. In fact, this additional source of Se will be responsible for upregulation of selenoprotein genes, and the additional synthesis of selenoproteins will upregulate antioxidant defences and help the body to adapt and overcome stress with minimal negative consequences. In case selenite is used in the diet, Se reserves in the muscles will not be built and therefore the ability of the body to adapt to stress will be restricted.

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Chapter 4
Selenium deficiency in poultry

We never know the worth of water till the well is dry

4.1 Introduction

Se deficiency in the chicken, especially in combination with low vitamin E supply, is responsible for the development of a range of diseases (Table 4.1; Figure 4.1). In general, selenium and selenium + vitamin E deficiency are associated with development of a variety of pathological conditions, which target major body systems, including muscles, heart-vascular, reproductive and nervous systems. Various animal species are shown to have different susceptibility to selenium deficiency and different tissues show various degrees of severity upon a selenium deficient diet. In general, some signs

Table 4.1. Diseases associated with selenium and vitamin E deficiency in animals (adapted from Surai, 2006).

Syndrome	Tissue or organ affected	Species
Encephalomalacia	cerebellum	chick, turkey, emus, partridges, quail, pheasant and various zoo species
Exudative diathesis	vascular	chick, turkey, duck, salmon, catfish
Microtic anaemia	blood, bone marrow	chick, monkey, pig, rat, salmon, catfish
Liver necrosis	liver	pig, rat, mouse
Pancreatic fibrosis	pancreas	chick, salmon, mouse
Erythrocyte haemolysis	erythrocytes	chick, lamb, monkey, rat
Muscular degeneration	skeletal muscle	chick, duck, goose, ostrich, flamingo, monkey, dog, rabbit, guinea pig, horse, calf, lamb, kid, mink, antelope, pig, rodents, salmon, catfish
Microangiopathy	heart muscle	turkey, pigs, calf, lamb, rat, dog, rabbit, guinea pig, cow, sheep, goat, baboon, antelope, elephant, deer
Kidney degeneration	kidney tubules	monkey, rat, mouse
Embryonic degeneration	vascular system	pig, rat, mouse
Poor hatchability	egg embryo	chick, turkey
Steatitis	adipose tissue	pig, chick
Testicular degeneration	testes	pig, calf, chick, pig, monkey, rat, rabbit, guinea pig, hamster, dog
Retained placenta	placenta	cow
Impaired fertility	spermatozoa	sheep, cattle, poultry, pig, rat
Ill-thrift	thyroid, pituitary	lamb, calf

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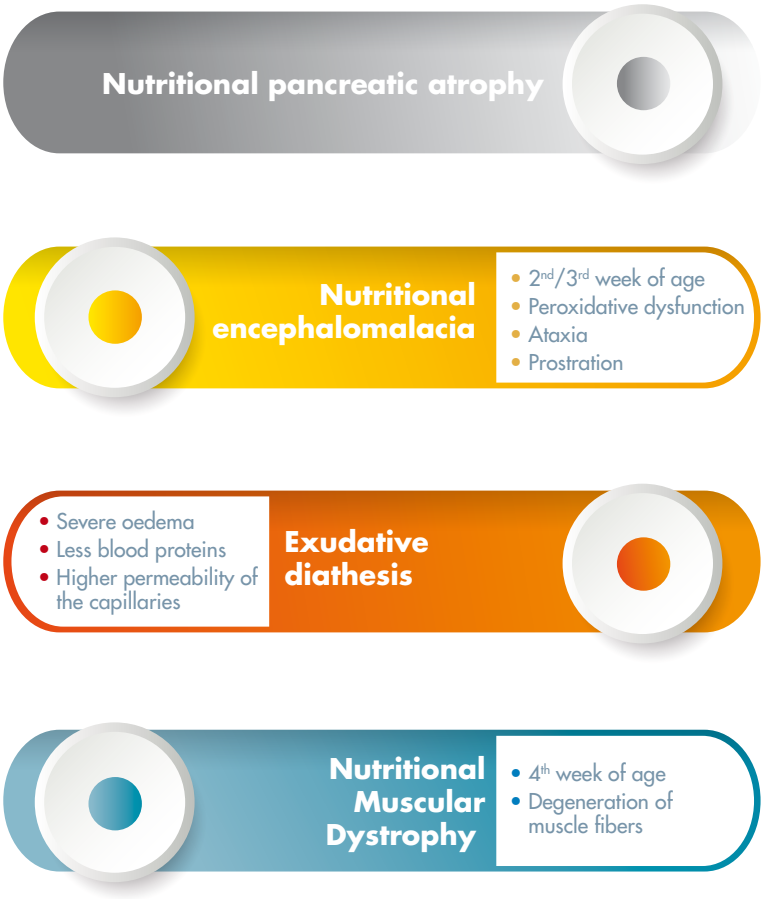

Figure 4.1. Selenium deficiency in poultry.

of selenium and Se + vitamin E deficiency (nutritional muscle dystrophy) are similar in various species; others (encephalomalacia) are species-specific.

4.2 Exudative diathesis

Among various selenium-related disorders and diseases exudative diathesis (ED) is the most studied one (Barthlomew *et al.*, 1998; Noguchi *et al.*, 1973). ED is the disease, which appears in chickens deficient in both vitamin E and Se and is characterised by severe oedema as a result of an increased permeability of the capillaries in combination with reduced levels of blood proteins (Kristiansen, 1973). As a result of leakage of blood fluids through the capillaries and from minor haemorrhages in muscles, in the area of the breast under the skin, an accumulation of an exudate with a protein pattern similar to blood serum or plasma can be seen.

Haemoglobin degeneration causes a bluish-green colour of the exudates, which can be seen through the skin. Autopsy findings and histopathological lesions were observed only in subcutaneous tissue and skeletal muscle. In particular, the subcutaneous tissue was oedematous with hyaline vascular lesions and haemorrhages (Hassan *et al*, 1990). The thigh muscles were more susceptible to deficiency lesions than were the breast muscles, and showed in acute stages degenerative processes of the muscle fibres, including calcium deposits, vascular lesions and haemorrhages. In subacute and chronic cases, reparative changes and muscle damage may develop independently of the hyaline vasculosis (Hassan *et al.*, 1990).

The condition can occur at any age but is most prevalent in young growing chickens and turkeys (Whitehead and Portsmouth, 1989). In the chicks, obtained from laying hens depleted of vitamin E and selenium exudative diathesis was observed at hatching, indicating that the deficiency lesions had developed during the embryonic period, whereas these signs were not observed in chicks obtained from commercial laying hens adequate in vitamin E and Se on the depletion diets until they were 2 weeks old (Hassan *et al.*, 1990). ED will only occur if the diet is deficient in selenium and Se is 200 times more effective than vitamin E in preventing this disease. Therefore, this symptom is primarily considered as a selenium deficiency (Machlin and Gordon, 1962). ED is also described in ducklings (Dean and Combs, 1981). ED develops in chickens at age of 3-6 weeks and chicken mortality can be as high as 80% (for review see Surai, 2002; Surai *et al.*, 1994). ED was associated with low levels of muscle Se, liver Se-GSH-Px and vitamin E and was also accompanied by a simultaneous increase in the liver non-Se-GSH-Px (Hassan *et al.*, 1990).

It has been suggested that the inflammatory response associated with a Se/vitamin E deficiency in a chick may be responsible for ED (Bartholomew *et al.*, 1998). The authors suggested that cytokines produced by leukocytes could be responsible for transparent fluid accumulation and haemorrhaging. It is well recognised that Se is the major protection against ED. The vitamin E supplemental level of 15 mg/kg was not adequate to provide a complete protection against ED (Hassan *et al.*, 1990). ED was inhibited by the rutin and silymarin treatments, but exacerbated by quercetin, morin, and ferulic acid. Changes in concentrations of vitamin E in plasma, liver, or muscle, caused by the various treatments (other than vitamin E), were not related to protection against ED (Jenkins *et al.*, 1992).

It was shown that alpha-tocopherol and Se deficiencies caused multiple alterations in the antioxidant system and adversely affected the redox state of chicken superficial pectoralis muscle (Avanzo *et al.,* 2001). In particular, chickens fed diets deficient in vitamin E and selenium displayed the lowest GSH level and GSH-Px activity. Indeed, vitamin E deficiency was linked to lowering mitochondrial thiol levels. GSH-Px/Cu,Zn-SOD ratio was 2.8 in animals fed selenium and vitamin E, and decreased to 0.13 in animals deficient in both nutrients as a result of oxidant-induced damage mediated by hydrogen peroxide (Avanzo *et al.,* 2001). In broilers ED was associated with decreased mRNA levels of 23 selenoproteins in the thymus, spleen, and bursa of the Fabricius tissues. In fact, the mRNA levels of Dio1 in the thymus, TrxR in

the spleen, and TrxR3 in the bursa of Fabricius decreased significantly (90.9, 83.3, and 96.8%, respectively; Yang *et al.*, 2016). In addition, Se deficiency mainly influenced the expression of antioxidative selenoproteins, especially GSH-Px, TrxR and iodothyronine deiodinases (Dios) in chicken immune organs (Yang *et al.,* 2016). In an earlier study, dietary Se deficiency associated with ED, significantly decreased mRNA levels of 7 common selenoprotein genes (GSH-Px1, GSH-Px4, SepW1, SepN1, SepP1, SelO, and SelK) in muscle and liver (Huang *et al.*, 2011). Whereas supplementing α-tocopherol acetate enhanced only the muscle SepX1 mRNA level, it actually decreased hepatic GSH-Px1, SelI, TrxR1, and TrxR2 mRNA levels. The authors concluded that dietary Se protected chicks from the Se deficiency disease ED, probably by upregulating selenoprotein genes coding for oxidation- and/or lesion-protective proteins. Furthermore, the protection by vitamin E might be mediated via other selenoproteins and/or Se-independent mechanisms (Huang *et al.*, 2011). Furthermore, Se deficiency with ED development was associated with a reduction of the mRNA expression of SelT in the immune organs (spleen, bursa of Fabricius and thymus) of broilers with exudative diathesis due to Se deficiency (Pan *et al.*, in press). The authors showed that Se deficiency was also associated with increased expression of proinflammatory cytokines (IL-1R and IL-1β) and increased oxidative stress as indicated by increased H_2O_2 and •OH concentration and decreased catalase activity in all three immune organs. Selenium deficiency was shown to reduce cell viability, increase intracellular ROS level, and stimulate cell apoptosis of vascular smooth muscle cells (VSMCs; Wang *et al.*, 2017). This could result in the destruction of the vascular structure and blood exudation. Interestingly, several selenoproteins, especially SelI, are shown to be correlated with apoptosis-related genes including caspase-3 and Bcl-2. Indeed Se deficiency is responsible for apoptosis of various cells including VSMC (Wang *et al.*, 2017).

4.3 Nutritional pancreatic atrophy

It seems likely that nutritional pancreatic atrophy (NPA) is the only clearly defined Se deficiency syndrome uncomplicated by deficiencies of other antioxidants (Cantor *et al.*, 1975; Combs, 1994; Thompson and Scott, 1969, 1970). For example, Se deficiency uncomplicated by vitamin E deficiency was produced in chickens by an amino acid diet complete in all known nutrients except Se (Gries and Scott, 1972; Noguchi *et al.*, 1973). In fact, disruption of endoplasmic reticulum is the primary ultrastructural lesion of the pancreas in the selenium-deficient chick (Root and Combs, 1988). It has been suggested that Se deficiency induced pancreatic injury by influencing nitric oxide (NO) and selenoproteins in pancreas of chickens. In particular, to clarify molecular mechanisms of pancreatic atrophy in chickens the effect of dietary Se deficiency on the expressions of 25 selenoproteins and the content of NO were studied (Zhao *et al.*, 2014). Se-deficient diet contained Se at the level of 0.033 mg/kg. The results showed that 25 selenoproteins were significantly decreased by Se deficiency. In particular, selenoproteins, such as TrxR1, SelS, SelU, SepX, and SPS2, were highly and more extensively expressed than other types of selenoproteins in pancreas of chickens (Zhao *et al.*, 2014). The most significant decreases in expression due to Se deficiency

were observed for TrxR2, GSH-Px1, GSH-Px3, SelI, Dio1, SepP, SepW1, SelO, SelT, SelM, SepX1, and SPS2. It is interesting to note that, NO content, inducible nitric oxide synthase (iNOS) activity, and mRNA level were increased strikingly compared to the control group (Zhao *et al.*, 2014). Furthermore, Se deficiency in chickens was associated with a global down-regulated expression of selenoprotein encoding genes, including 18 genes in the pancreas, 14 genes in the muscle and 9 genes in the liver. Similarly, insulin signalling-related genes were downregulated in the pancreas, liver and muscle, causing dyslipidaemia, hypoinsulinemia and hyperglycaemia in chicken (Xu *et al.*, 2017). Interestingly, TrxR1 and SelS genes were up-regulated in the liver of Se-deficient chickens and this phenomena needs further investigation. Probably, the expression of selenoproteins depends on the severity of Se deficiency. For example, recently, it has been shown that SepP1, GSH-Px3 and SelK in the pancreas were expressed at levels comparable to housekeeping transcripts and only 33, 25 and 50% of selenoprotein transcripts were down-regulated significantly by Se deficiency in the liver, gizzard and pancreas, respectively (Li and Sunde, 2016). Furthermore, when gradual levels of Se were supplemented to chickens fed on a Se-deficient diet, pancreas GSH-Px1 and GSH-Px4 activities lacked defined plateaus, with breakpoints at 0.3 μg Se/g (Li and Sunde, 2016). Furthermore, the shortest biological half-lives for Se in chickens were observed in the major metabolic organs, the liver, kidney and pancreas with half-lives close to 4 days (Brandt-Kjelsen *et al.*, 2014).

4.4 Nutritional encephalomalacia

It is widely believed that nutritional encephalomalacia (NE) is one of the most important signs of antioxidant (mainly vitamin E) deficiency in chickens, fed on a diet containing high amounts of linoleic or arachidonic acid (Century and Hurwitt, 1964; Combs and Hady, 1991; Machlin and Gordon, 1962; Marthedal, 1973; Pappenheimer and Goettsch, 1931). The role of selenium and particularly various selenoproteins in prevention of NE is not clear at present, however, it seems likely that improved antioxidant defences by optimisation of Se supply could have positive effects. The major symptoms of NE include ataxia, sudden prostration, with legs outstretched and toes flexed, and head retraction with lateral twisting. The head becomes twisted either backwards or forwards and uncoordinated muscular spasms affect the legs (Whitehead and Portsmouth, 1989). It is believed that NE is associated with peroxidative dysfunction (Fuhramann and Sallmann, 1995) leading to ataxia, prostration and death (Hassan *et al.*, 1990). Gross and histological lesions are primarily found in cerebellum and in chickens the lesions can be also seen in the cerebrum (NRC, 1977). In poultry, gross lesions include a friable, swollen cerebellum with necrotic reddish or brownish areas and microtrombosis (Jungherr *et al.*, 1956; Pappenheimer and Goettsch, 1931). Ischemic necrosis of the cerebellar cortex and white matter, capillary thrombi, haemorrhages, malacia and oedematous neutrophil are observed in the cerebellum (Wolf and Pappenheimer, 1931).

Encephalomalacia is mainly described for chickens (Pappenheimer and Goettsch, 1931), but also in turkeys (Rigdon *et al.*, 1961), emus (Aye *et al.*, 1998), game birds

(partridges, quails and pheasants; Mutarov, 1979; Swarbrick *et al.*, 1986) as well as in zoo species (Dierenfeld, 1993). Major histopathological alterations in turkey poults include congestion, haemorrhages, necrosis, and malacia associated with hyaline capillary thrombi affecting the cerebellar cortex and adjacent white matter (Klein *et al.*, 1994). NE in turkey poults leads to neurological, signs such as tremor, incoordination, and recumbency shortly after being moved to new quarters. Associated lesions included ischemic necrosis of the cerebellum and spinal cord (Frank and Bergeland, 1988; Jortner *et al.*, 1985).

The disease usually occurs during the second or third week after hatching, which may be related to the cerebellar polyunsaturated fatty acid (PUFA) accumulation that occurs at that time (Budowski *et al.*, 1987). In an experiment conducted by Fuhrmann and Sallmann (1996) NE started at day 9. In other experiments of the same authors (Fuhrmann *et al.*, 1994; Sallmann *et al.*, 1991) with similar type of linoleic acid-rich diet and commercial chicks, the disease began after day 16 reflecting differences in the initial levels of vitamin E in the tissues of newly hatched chicks. The lower the level of vitamin E in the incubation eggs and chick tissues the earlier the disease appears. The effect of the breeder diet on the severity of encephalopathy was observed in 4-week-old chicks (Bartov and Bornstein, 1980).

As mentioned above, experimentally NE in chicks can be induced by diets low in vitamin E and containing large amounts of linoleic acid (Bartov and Budowski, 1979). There are other nutritional and environmental factors predisposing chickens to encephalomalacia, including deficiency in animal protein and decreased temperature in hatcheries (Chen and Shi, 1985). However, outbreaks of this disease were registered in commercial conditions with diets containing sufficient levels of vitamin E and synthetic antioxidants (Hislop and Whitte, 1967a,b; Marthedal, 1973).

It is well recognised that NE developed on the diet rich in linoleic acid, but linolenic acid had a protective effect against this disease (Budowski and Crawford, 1985). Therefore it has been postulated that n-6 fatty acids, especially arachidonic acid degradation products including pentane (Fuhrmann and Sallmann, 1995) and hexanal (Hu *et al.*, 1989), have a prooxidative toxic effect which could lead to encephalomalacia development (Budowski *et al.*, 1979; Fuhrmann and Sallmann, 1996). Indeed, an overproduction of arachidonic acid-derived eicosanoids is considered as a factor in the aetiology of the cerebellar lesion and possibly a structural change due to a loss of docosahexaenoic acid and gain of arachidonic acid (Budowski *et al.*, 1987).

NE was induced in young chicks using a diet low in vitamin E and containing 8% ethyl esters derived from safflower oil fatty acids. Chicks receiving aerated linseed oil high in alpha-linolenic acid and low in α-tocopherol did not develop symptoms due to alterations in the arachidonic acid metabolism and thrombocyte function in young chicks (Vericel *et al.*, 1991). In that experiment tromboxane B2 synthesis was significantly decreased in the α-linolenic group in comparison to the ataxic chicks. Prostaglandin E2 production by aortal rings was also significantly influenced by the diet; ataxic chicks yielded the highest value and α-linolenic acid-supplemented chicks

had the lowest values. The lipoxygenase (LOX) oxygenation pathway of arachidonic acid metabolism was investigated in the cerebellum and cerebral hemispheres of young chicks (Greenberg-Levy *et al.*, 1993). Lipoxygenase products consisted mainly of 15-hydroxyeicosatetraenoic acid (15-HETE), accompanied by the 15-hydroperoxy analogue (15-HPETE) and the 5-HETE products. In the experiment, the yield of 15-HETE was 3 times greater in the cerebellum than in the cerebrum and phospolipase A2 activity of the cerebellum was 2-fold higher than that of the cerebrum. This could lead to increased vulnerability of the cerebellum to oxidative dysfunction.

In the liver of linoleic acid-fed vitamin E deficient chickens total aldehydes were increased (Fuhrmann and Sallmann, 2000). In plasma, vitamin E deficiency led to higher malondialdehyde and OH-nonenal concentrations. However, in brain of vitamin E deficient chicks, aldehyde concentration was not affected. Therefore, a direct role of unsaturated aldehydes for the development of NE in the cerebellum was not confirmed (Fuhrmann and Sallmann, 2000) and there was no increase in lipid peroxidation in the cerebellum of the chickens fed the NE producing diet (Fuhrmann *et al.*, 1996). Rather, the liver seemed to be affected by the oxidative stress. In this respect, our data (Surai *et al.*, 1996) showed that even low levels of vitamin E in the chicken embryonic brain are sufficient to prevent lipid peroxidation *in vivo*, probably due to efficient vitamin E recycling by other antioxidants including ascorbic acid. In comparison to other tissues studied chicken brain contains increased ascorbic acid concentrations. It is necessary to underline that newly hatched chick cerebellum contains higher vitamin E and lower ascorbic acids levels than cerebrum (Surai *et al.*, 1999).

For NE prevention and treatment, increased vitamin E doses and combinations with other antioxidants have shown to be effective (Surai *et al.*, 1994). Supplementation of selenium and methionine with or without simultaneous supplementation of a low level of dl-α-tocopheryl acetate had little effect on preventing NE. The preventive effect of other antioxidants, including ascorbic acid, methylene blue, ethoxyquin, 2,6-ditertiary-butyl-p-cresol and butylated hydroxyanisole was in proportion to their dietary level, and a high level of any of them could almost completely protect the chicks from NE, while diphenyl-p-phenylenediamine was not as effective and the effect was not proportional to the dose (Yoshida and Hoshi, 1977). When α-tocopherylacetate, short-chain α-tocopherylacetate, α-tocopherylquinone, short-chain α-tocopherylquinone and α-tocopheronolactone were added to vitamin E deficient diets (Kovalenko *et al.*, 1979), α-tocopherylacetate and α-tocopheronolactone showed the highest E-vitamin activity in preventing NE in chickens. The data confirmed a nonspecific function of vitamin E in preventing alimentary encephalomalacia.

High levels of lipid unsaturation and comparatively low antioxidant protection make the brain vulnerable to free radical attack; this is of particular importance in the chick with respect to the development of encephalomalacia, which is associated with an antioxidant system compromise (Dror *et al.*, 1976; Fuhrmann and Sallmann, 1995; Marthedal, 1973; Pappenheimer and Goettsch, 1931). Nevertheless, the precise mechanism of the development of the disorder remains unclear. For example, oxidative

stress can induce apoptosis in neurones (Ratan *et al.*, 1994), which may have some effect on the disease development. Involvement of Se deficiency and antioxidant-prooxidant balance in the brain cells in the development of NE still needs further clarification.

The molecular mechanisms of NE are still not clear. In the vitamin E-deficient cerebellum the cytosolic phospholipase A2 activity was increased (Fuhrmann and Sallmann, 1995a). Due to low content of vitamin E, the cerebellum is the most susceptible tissue to oxidative stress during vitamin E deficiency. Chicks hatched from eggs containing 16.5% linoleic acid of total yolk fatty acids were more susceptible to NE, than chicks hatched from eggs containing 7.5% linoleic acid. (Bartov and Bornstein, 1980). Indeed, nutritional encephalomalacia is closely related with dietary Se in avian species and it can be induced by Se deficiency.

It may be envisaged that the brain of the chick embryo may be at particular risk from peroxidative damage due to presence of very high levels of C20 and C22 polyunsaturated fatty acids in neuronal phospholipids (Surai *et al.*, 1999). The antioxidant system of the developing brain is poorly understood. It includes natural antioxidants, such as vitamins E and C, ubiquinols, glutathione, antioxidant enzymes – superoxide dismutase, glutathione peroxidase and catalase –and other elements, such as dopamine, noradrenaline, taurine and carnosine (Matsuo, 1993; Surai *et al.*, 1996). The chick embryonic brain is characterised by comparatively low level of vitamin E which is almost 100-fold lower compared to the liver and remains practically constant during the development (Surai *et al.*, 1996). This apparent deficiency in the brain's antioxidant defence capacity is further compounded by the relatively low levels of vitamin A, selenium, glutathione peroxidase and carotenoids (Surai, 2002, 2006). Our previous results (Surai *et al.*, 1993) also indicate that comparatively low levels of vitamin E persist in the brain of adult chicken. Thus it is a characteristic of the poultry brain to contain low levels of vitamin E. At the same time, the brain generates especially high levels of free radicals (Reiter, 1995) which are necessary for physiological responses (Westermarck *et al.*, 1993).

Our data (Surai, 1999) indicate that superoxide dismutase (SOD) is the main enzyme in antioxidant defence of the brain, especially during the final days of incubation when SOD activity in the brain is significantly higher compared to other tissues. However, GSH-Px and CAT activities in the brain were low, making it unlikely that they are the primary defence in this tissue. The antioxidant profile of the chicken brain is shown in Table 4.2. The cerebellum had some distinctive features in antioxidant concentration compared to the other brain regions, in particular the cerebrum. The cerebellum was characterised by significantly increased α-tocopherol concentration and catalase activity, but decreased ascorbic acid level and Mn-SOD activity compared to that in the cerebrum. In general, the brain was characterised by low level of vitamin E, low GSH-Px and catalase activity, but very high level of ascorbic acid and high SOD activity. Furthermore, the brain was characterised by comparatively low levels of Se (0.187±0.01 μg/g) and moderate levels of Zn (11.67±0.23 μg/g) and Cu (1.18±0.06 μg/g) (Surai, 2002).

Table 4.2. Antioxidant composition of the brain of a newly hatched chick (adapted from Surai *et al.*, 1999).

Antioxidants		Cerebrum	Cerebellum	Brain stem	Optic lobes
α-tocopherol	μg/g tissue	5.22	7.12	6.12	5.66
Ascorbic acid	μg/g tissue	889.4	711.3	747.72	850.53
Glutathione	nM/mg protein	41.12	38.12	40.13	39.56
Mn-SOD	U/mg protein	3.66	2.837	3.91	3.02
Cu-Zn-SOD	U/mg protein	6.910	6.985	7.52	8.03
Se-dependent GSH-Px	mU/mg protein	29.83	28.61	33.14	32.6
Se-independent GSH-Px	mU/mg protein	6.22	6.60	5.57	7.31
Catalase	U/mg protein	1.923	2.365	1.966	1.822

Although the basal levels of malondialdehyde (MDA) in the brain were low (Surai *et al.*, 1996), the susceptibility of the brain homogenates to lipid peroxidation during incubation in both the absence and the presence of Fe^{2+} was very high in comparison with other tissues. Thus, the low levels of endogenous vitamin E in the chick embryo brain may render the tissue homogenates highly susceptible to *in vitro* peroxidation (Kornbrust and Mavis, 1980). In embryonic brain antioxidant defence is allowed by high levels of ascorbic acid to enable the recycling of the low concentrations of vitamin E in order to maintain physiological requirements (Surai *et al.*, 1996). From our results (Surai, 1999) it is clear that SOD may be another important protective element in the embryonic brain.

Therefore, oxidative stress and the disorganised histological structure in chicken brain under conditions of Se-deficiency was shown to be main cause of nutritional encephalomalacia. Indeed, chicken brains are extremely susceptible to oxidative damage and selenium deficiency induces oxidative damage and disbalance of Ca homeostasis in the brain of chickens (Xu *et al.*, 2013). In fact, in Se-deficient chicken brains levels of Se and GSH, and activity of GSH-Px were significantly reduced. Furthermore, Se deficiency caused disorganised histological structures, damage to mitochondria, fusion of nuclear membranes and shrinkage of nuclei, a higher apoptosis rate and an increase of Ca homeostasis in the brain of chickens (Xu *et al.*, 2013). Recently, effects of a Se-deficient diet on transcription factor nuclear factor kappa beta (NF-κB) and four pro-inflammatory cytokines – tumour necrosis factor (TNF), cyclooxygenase2 (COX-2), iNOS and prostaglandin E synthase – mRNA expression in the chicken brain tissues associated encephalomalacia have been described (Sheng *et al.*, 2014). It has been shown that the production of pro-inflammatory mediators were increased following Se-deficiency. Therefore, the increased levels of NF-κB, COX-2, iNOS and TNF-α further confirmed the close relationship between inflammatory reaction and encephalomalacia. The most interesting findings of the study were the increased mRNA expression levels of NF-κB and its upstream regulator and downstream effectors in the Se-deficient chicken brain (Sheng *et al.*, 2014). Indeed, NF-κB is an important redox sensitive transcription factor regulating immune and stress responses and associated

with many disease states, such as chronic inflammation, cancer, neurodegenerative disorders, diabetes and stroke (see also Chapter 1). Similarly, it has been shown that a reduced supply of Se could up-regulate and activate NF-κB in testis (Shalini and Bansal, 2007), while Se supplementation was able to attenuate lipopolysaccharide-induced oxidative stress responses through NF-κB signalling pathway (Kim *et al.,* 2004). Indeed, NF-κB plays an important role in inflammation response, and the activation and inhibition of NF-κB in the chicken brain is closely related to the Se supply. In fact, it was shown that the expression of TNF-α and iNOS were increased in different areas of chicken brain along with Se-deficiency (Sheng *et al.*, 2014). These data indicate that inflammatory response is a key element in nutritional encephalomalacia and that pro-inflammatory cytokine-TNF-α may be activated by Se-deficiency. Furthermore, the expression levels of COX-2 were increased in different areas of chicken brain along with Se-deficiency at most of the time points (Sheng *et al.*, 2014). Indeed, the increased levels of NF-κB, COX-2, iNOS and TNF-α proved the close relationship between inflammatory reaction and encephalomalacia. Interestingly, it was shown that prolonged Se deficiency was responsible for Se content in chicken brain to be decreased indicating that the capability of Se retention in chicken brain was weaker than the mammals (Sheng *et al.*, 2014). Similarly, in aged rats Se induced protective effects against experimental dementia-induced brain, and blood oxidative injuries and apoptosis through regulation of cytokine production, vitamin E, glutathione concentrations, and glutathione peroxidase activity (Demirci *et al.*, 2017).

In general, it is well known that Se is essential for proper brain function (Cardoso *et al.,* 2015: Schweizer *et al.,* 2004; Solovyev, 2015). In mammals, it is shown that dietary Se deficiency is associated with phenotypes characterised by selenoprotein depletion in various organs, but the brain is usually well protected from Se loss (Zhang *et al.*, 2008). Therefore, it seems likely that in young chickens these mechanisms of Se preservation are not effective. The Se content of brain tissue, O-phosphoseryl-tRNA:selenocysteinyl-tRNA synthase (SepSecS; a critical enzyme for the biosynthesis and transformation of selenocysteine) gene expression levels and mRNA stability in the chicken brain and primary cultured chicken embryos neurons receiving Se supplements were studied by Li *et al.* (2012). The authors showed that Se content in the brain remains remarkably stable during Se supplementation. A significant increase in SepSecS mRNA levels was observed in all brain tissues of chickens fed diets containing 1-5 mg/kg sodium selenite. It is interesting to note that, significant changes in SepSecS mRNA levels were not observed in neurons treated with Se. However, Se altered the SepSecS mRNA half-life in cells. Therefore, Se could regulate SepSecS mRNA stability in the avian brain (Li *et al.*, 2012). Furthermore, the same authors showed earlier that SelW gene expression in the avian neural tissues was sensitive to dietary Se content (Li *et al.*, 2011) and suggested that the selenoprotein functions as an extracellular readily-available and reversible Se reservoir. Indeed, the SeW mRNA expression is high in skeletal muscle followed by the brain, but extremely low in other tissues from chickens fed a commercial maize-based diet (Ou *et al.*, 2011).

4.5 Nutritional muscular dystrophy

Nutritional muscular dystrophy (NMD) appears in chickens at about four weeks of age and is a result of simultaneous deficiency of vitamin E and sulphur amino acids (e.g. cysteine; Machlin and Gordon, 1962). It is characterised by degeneration of the muscle fibres of the breast and sometimes of the leg muscles. An exact role of selenium deficiency in the development of NMD in chickens needs further clarification. Histological examination revealed Zenker's degeneration, with perivascular infiltration, and marked accumulation of infiltrated eosinophils, lymphocytes and histocytes (Leeson and Summers, 2001). A light- and electron-microscopic study of affected muscles revealed fibres with hyaline and granular degeneration (Van Vleet and Ferrans, 1976). In hyalinised fibres, the initial ultrastructural alterations included increased density of the sarcoplasm and myofibrils, dilatation of sarcoplasmic reticulum, formation of subsarcolemmal vacuoles, and disruption of mitochondrial membranes. In later stages, alterations in these fibres included myofibrillar disruption and lysis, nuclear pyknosis and lysis, disruption of the plasma membrane with persistence of basal lamina and scattered adhering satellite cells, and eventual invasion by macrophages (Van Vleet and Ferrans, 1976). In fibres with granular degeneration, the ultrastructural observations included decreased density of the sarcoplasm, prominent mitochondrial swelling and distortion, and multiple foci of myofibrillar lysis that eventually coalesced to produce a generalised lysis (Van Vleet and Ferrans, 1976). Ultrastructural deterioration of the myopathic muscle included disintegration of blood vessel walls, transverse tubules and mitochondrial membranes, as well as the obvious disruption of the myofibrillar components (Gill *et al.*, 1980). NMD is often found in goslings and ducklings (Dvinskaya and Shubin, 1986). Usually it starts at 3-5 weeks of age, but sometimes it can be observed earlier. In chickens and turkey poults muscles of gizzard and crop are affected. As a result, feed remains in the crop and mortality is expected 3-10 days after the start of the disease. In turkey poults muscle degeneration is registered at 5-30 days of age and mortality can be as high as 70% (for review see Surai *et al.*, 1994). Myopathy of the gizzard and heart in turkeys is also a result of vitamin E and Se deficiency. NMD is most noticeable in the breast and thigh muscles, which develop pale streaks of dystrophic degenerated fibres. When ducklings were fed on the peroxidised diet from time of hatch, first signs of NMD were seen at day 10. At day 14 mortality started, which reached 22.5% at day 20 of the postnatal development (Shemet, 1977). In the gizzard and heart of birds that died from NMD degenerative changes were observed. After 30 days of the experimental period NMD was registered more than in a half of the ducklings. There is a possibility for the development of NMD during first 7-15 days of postnatal growth if chickens are hatched from eggs low in vitamin E and maternal diet was high in peroxides (German, 1981). Peroxidative damage to membranes has been hypothesised as a mechanism of tissue damage in muscular dystrophy.

Lameness associated with skeletal muscular degeneration was reported in ostrich chicks and flamingos (Dierenfeld and Traber, 1993; Vorster, 1984). It is interesting that skeletal muscle degeneration, encephalomalacia, exudative diathesis, and reduced hatchability and fertility have been reported in exotic birds with vitamin

E deficiency (Dierenfeld, 1989). Vitamin E deficiency among captive avifauna was mainly associated with piscovorous birds and raptors fed on meat or fish rich in PUFA and poor in vitamin E. In such birds pathological lesions of adipose tissues were described, including accumulation of ceroid pigment, necrosis and steatitis, as well as degeneration of cardiac and skeletal muscles, which are primarily signs of vitamin E deficiency (Dierenfeld, 1989). In zoo birds vitamin E deficiency can also cause degeneration of the so called hatching muscle (musculus complexus) in embryos that failed to pip (Dierenfeld and Traber, 1993).

The contents of α- and γ-tocopherol and their oxidation products, the tocopheryl quinones, were measured at 1 to 4 weeks after hatching in the muscle and other tissues of chickens with inherited muscular dystrophy (Murphy and Kehrer, 1989). The affected muscle (pectoralis major) of dystrophic birds contained significantly higher levels of α-tocopheryl quinone and a decreased ratio of α- to γ-tocopherol. These changes in the tocopherols are suggestive of oxidative stress in dystrophic muscle membranes. A disturbance of Ca^{2+} active transport and an increase in the membrane permeability for Ca^{2+} in the membranes of sarcoplasmic reticulum of the dystrophic muscles have been shown (Kurskii *et al.*, 1978). In particular, the level of ATP-dependent consumption of Ca^{2+} and activity of Mg^{2+},Ca^{2+}-ATPase decreased. Indeed, Se deficiency induces Ca^{2+} leak and calcification in skeletal muscles; however, the exact mechanism is still unclear.

At day 16 of incubation, muscles contain 2.4% of total lipids. The major lipid fractions of skeletal muscle of the embryo are phospholipids, triglycerides and free cholesterol. Phosphatidylcholine and phosphatidylethanolamine, high in C20 and C22 PUFAs, are by far the major components of the phospholipid fraction (Noble and Cocchi, 1990; Surai *et al.*, 1999). In the thigh muscle the activity of GSH-Px decreased on day 17 and then remained on that level up to the end of the development. There were no significant changes in the SOD activity throughout the development. In muscle a decrease in catalase (CAT) activity lasted from day 15 up to hatching time. Skeletal muscle is characterised by a comparatively low and stable vitamin E concentration. On the contrary, the ascorbic acid level significantly decreased during development (Surai *et al.*, 1996). Thus the antioxidant system of the muscle is not very potent and imbalance of the natural antioxidants in postnatal development may cause, such diseases as muscle degeneration (Machlin and Shalkop, 1956).

In a recent study, effect of Se-deficiency on chicken muscle has been studied (Yao *et al.*, 2016). The results showed that Se deficiency-induced typical muscular injuries accompanied by a Ca^{2+} leak, and oxidative stress injured the ultrastructure of the sarcoplasmic reticulum and mitochondria, decreased the levels of the Ca^{2+} channels, SERCA, SLC8A, CACNA1S, ORAI1, STIM1, TRPC1, and TRPC3; and increased the levels of Ca^{2+} channel PMCA. Furthermore, SelW knockdown closely correlated Se deficiency with a Ca^{2+} leak in muscles and therefore, the redox regulation role of SelW is crucial in Se deficiency-induced Ca^{2+} leak in muscles (Yao *et al.*, 2016). Dietary Se deficiency resulted in increased cell apoptosis associated with decreased GSH-Px activity and elevated lipid peroxidation in these muscles (Yao *et al.*, 2013). All the

responses were stronger in the pectoral muscle than in the thigh and wing muscles. Relative distribution of the four endoplasmatic reticulum (ER) resident selenoprotein gene mRNA (SelN1, SelK, SelS, and SelT) amounts and their responses to dietary Se deficiency were consistent with the resultant oxidative stress and cell apoptosis in the three muscles. Therefore, the pectoral muscle demonstrated an unique expression pattern of the ER resident selenoprotein genes and GSH-Px activity, along with elevated susceptibility to oxidative cell death, compared to the other skeletal muscles. These features might help explain why it is a primary target of Se-deficiency diseases in chicks (Yao *et al.*, 2013). It is interesting to note that Se-deficiency and excess (1.5 mg/kg) in chickens were associated with upregulated SelN mRNA levels in skeletal muscles compared with the control (0.15 mg/kg) Se group (Zhang *et al.*, 2014). In particular, it has been shown that Se deficiency mainly influences the gene expression of antioxidative selenoproteins in chicken muscles (Yao *et al.*, 2014). In fact, Se deficiency decreased the expression of 19 selenoproteins, 11 of which were antioxidative selenoproteins, and antioxidative selenoproteins, such as GSH-Px3, GSH-Px4 and Sepw1, may play crucial roles in chicken muscles. Furthermore, some other lower expressed selenoproteins (Dio1, SelU, SelPb, SepP1) were also excessively decreased (more than 60%) by Se deficiency (Yao *et al.*, 2014). It seems likely that SelW plays a crucial role in the regulation of inflammatory reactions in Se-deficiency myopathy. Indeed, dietary Se deficiency reduced the mRNA expression of SelW in chicken wing, pectorals, and thigh muscles. At the same time, Se deficiency increased the mRNA expression levels of inflammatory-related genes in chicken skeletal muscle tissues at different time points and the mRNA expression levels of inflammatory-related genes were significantly negative related to SelW (Wu *et al.*, 2014). Furthermore, the abundance of SelU mRNA in chicken muscle was downregulated by Se deficiency (Jiang *et al.*, 2015).

Dietary Se deficiency induced muscle fibre rupture and coagulation necrosis in the pectoral muscle of chicks at week 3. Thereafter, with increased MDA, it decreased total antioxidant capacity, and diminished GSH-Px activities in the muscle (Huang *et al.*, 2015). Furthermore, compared with the +Se chicks, the -Se chicks had lower muscle mRNA levels of GSH-Px1, GSH-Px3, GSH-Px4, SelP1, SelO, SelK, SelU, SelH, SelM, SelW1, and Sep15. The -Se chicks also had decreased production of 6 selenoproteins (long-form selenoprotein P (SelP-L), GSH-Px1, GSH-Px4, Sep15, SelW, and SelN), but increased levels of the short-form selenoprotein P in muscle at weeks 2 and 4 (Huang *et al.*, 2015). On the other hand, dietary Se deficiency elevated indexes of inflammation and apoptosis: muscle p53, cleaved caspase 3, cleaved caspase 9, COX-2, focal adhesion kinase (FAK), phosphatidylinositol 3-kinase (PI3K), phospho-Akt, NF-κB, p38 mitogen-activated protein kinase (p38 MAPK), phospho-p38 MAPK, phospho-JNK, and phospho-ERK and decreased muscle procaspase 3, procaspase 9, and NF-κB inhibitor α (Huang *et al.*, 2015). Therefore, the downregulation of antioxidative selenoproteins due to Se deficiency could cause oxidative stress and the subsequent peroxidative damage of chick muscle cells via redox/apoptotic signalling, including activation of the p53/caspase 9/caspase 3, COX-2/FAK/PI3K/Akt/NF-κB, and p38 MAPK/JNK/ERK signalling pathways. Clearly, effective metabolism of

peroxides and redox regulation are considered to be the mechanisms of selenoprotein involvement in prevention of the onset of NMD in chicks (Hung *et al.*, 2015).

It has been suggested that the oxidative deterioration of proteins in nutritional muscular dystrophy due to vitamin E deficiency is of great importance for the development of NMD (Shih *et al.*, 1977). In accordance with this suggestion, vitamin E plays a specific role in maintaining a proper redox state of the sulphur-containing amino acid in the proteins. In dystrophic muscles, the ratio of protein-bound disulphide to sulfhydryl content increased two- to three-fold as compared to that in the control muscle proteins. Proteins of low molecular weight, supposedly derived from proteolysis, were also found in the dystrophic muscles. Muscles of animals fed diets deficient in vitamin E and selenium displayed the lowest reduced glutathione level and GSH-Px activity (Avanzo *et al.*, 2001). The decreased levels of GSH were not due to a defective activity of glutathione reductase, which was increased in both mitochondria and cytosol. The absence of vitamin E was linked to lowering of mitochondrial thiol levels. Catalase activity increased in an attempt to counteract the decrease in glutathione peroxidase activity. The results obtained showed that α-tocopherol and Se deficiencies caused multiple alterations in the antioxidant system and adversely affected the redox state of chicken superficial pectoralis muscle. These data are not consistent with previous observations indicating that GSH concentration and GSH-Px activity in dystrophic muscles were significantly increased (Hull and Scott, 1976) suggesting the redistribution or impaired utilisation of various antioxidants in chicken tissues as a result of vitamin E deficiency. The specific need for cysteine in NMD can be explained as a result of reduction in transsulphuration of methionine to cysteine in the vitamin E deficient chicks (Hathcock and Scott, 1966). It seems likely that selenoprotein methionine sulphoxide reductase B could be also involved in regulation of thiol metabolism and prevention of NMD.

Vitamin E and its combination with other antioxidants is considered to be effective in prevention of NMD. For example a mixture of vitamin E (10 mg/kg), ethoxyquin (125 mg/kg), methionine (400 mg/kg), sodium selenite (1 mg/kg) and ascorbic acid (50 mg/kg) is shown (for review see Surai *et al.*, 1994) to be effective. Similarly for prevention of NMD in turkey poults and ducklings a mixture of vitamin E (40 mg/kg), ascorbic acid (100 mg/kg), vitamin B12 (30 μg/kg) and sodium selenite (0.5 mg/kg) is recommended (Shemet, 1977).

It is interesting that alpha-tocopheryl quinone was the most active substance in preventing chicken experimental muscular dystrophy (Donchenko *et al.*, 1981). Various flavonoids or simple phenolics at a dietary concentration of 1000 mg/kg completely prevented NMD, quercetin reduced its incidence, and quercetin, morin, and ferulic acid reduced the severity of the disorder (Jenkins *et al.*, 1992).

4.6 Impaired immunocompetence

Selenium is an essential element for the efficient and effective operation of the immune system and critical for the integrity of the cells and the receptors involved in the immune response (Surai, 2006). Selenium deficiency causes a decrease in phagocytic functions of macrophages and adversely affects interleukin production. In general, the immune system is very sensitive to Se-deficiency (see Chapter 7).

4.7 Impaired thyroid hormone metabolism

Se deficiency is associated with increased plasma T4 concentrations and decreased plasma T3 and thymulin concentrations (Chang *et al.*, 2005; Jianhua *et al.*, 2000). This could be related to impaired immunity and other detrimental consequences with respect to chicken growth and development. More recent results confirmed that Se deficiency influenced the conversion of T4 to T3 and induced the accumulation of T4 and FT4 (Lin *et al.*, 2014). In addition, the mRNA expression levels of the selenoproteins were generally decreased by Se deficiency. In addition, the decreased selenoproteins (Dio1, Dio2, Dio3, TrxR2, SelI, SelU, GSH-Px1, and GSH-Px2) induced by Se deficiency may indirectly limit the conversion of T4 to T3 in chicken thyroids.

4.8 Reduced fertility

Selenium has a special role in poultry male reproduction and its deficiency in cockerel diet can cause fertility-related problems (see Chapter 5). In fact, fertility and hatchability were low on the basal (low Se) diet and were corrected partly by vitamin E and completely by Se (Latshaw and Osman, 1974).

4.9 Reduced egg production and quality

Selenium deficiency is associated with impaired immunocompetence, reduced egg production and increased embryonic mortality (Figure 4.2; Combs and Combs, 1984). Egg production and fertility were maintained at about 77 and 92%, respectively, by the Se diet and fell to about 56% and almost 0 with the basal low Se diet (Cantor and Scott, 1974). Egg production was only 69% in Se-deficient birds against 81% in controls (Latshaw *et al.*, 1977). Weights of day-old chickens from hens given 0.05 and 0.1 mg/kg Se-supplemented diets were significantly heavier than those from hens given no Se. For more information see Chapter 5.2.

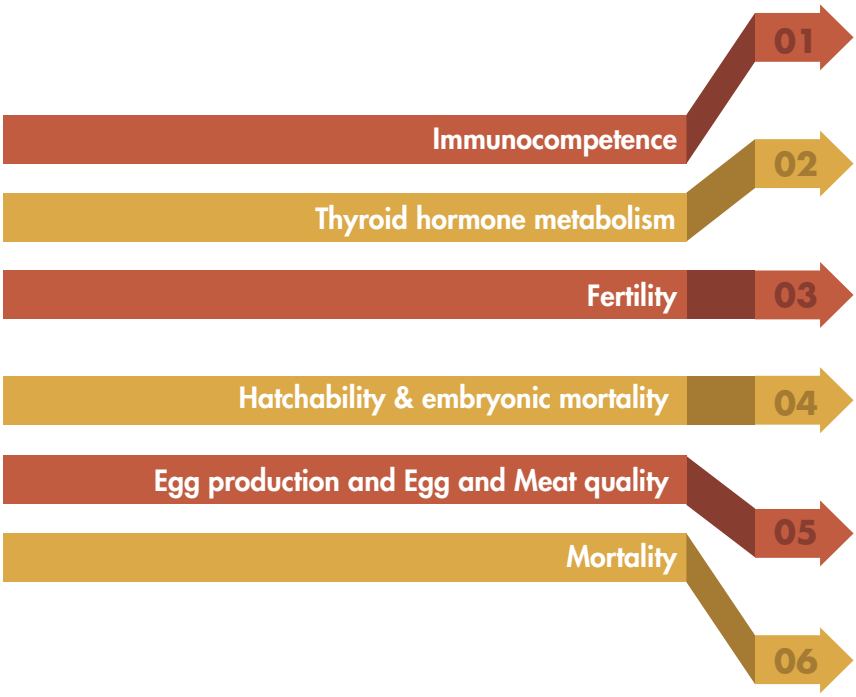

Figure 4.2. Impact of selenium deficiency on poultry production.

4.10 Decreased hatchability and increased embryonic mortality

Hatchability of eggs was depressed by the low-Se diet, further depressed by peroxidised fat and restored to normal level by supplementation of Se and vitamin E (Combs and Scott, 1977). Similar decrease in egg hatchability (from 92-93% down to 52%) as a result of Se deficiency in the breeder's diet was reported by Cantor and Scott (1974). Indeed, high incidence of dead embryos early in incubation was related to Se deficiency. In fact, eggs from hens fed very low levels of Se were more often infertile (12.6%), there were more dead embryos (29%) and lower hatchability of fertile eggs (15%). Mean respective values for controls were 4.1, 2.9 and 91% (Latshaw *et al.*, 1977). Moreover, Se is required in breeder turkey diets for optimum hatchability and viability of offspring (Cantor *et al.*, 1978).

4.11 Conclusions

As mentioned above, molecular mechanisms of the development of ED, as well as NE and NMD are still not clear. However, oxidative stress is a crucial factor in disease development and lipid peroxidation, disrupting membrane structure and function, is responsible for specific changes in the brain, muscles and vascular systems (Fraga

et al., 1987). For example, nutritional pancreatic atrophy in chicks may be overcome by feeding vitamin E at 15-20-fold excess over the levels normally regarded as nutritionally adequate (Whitacre *et al.*, 1987). Selenium supplementation can also decrease incidence of nutritional muscular dystrophy in the chick (Jonsson, 1993).

Recent understanding of the roles of various selenoproteins as integral elements of the antioxidant systems – with complex relationships among individual antioxidants in the biological system – could help explaining some clinical signs of diseases. For example, it was shown that liver cells can boost endogenous ubiquinone-dependent protective mechanisms in response to deficiency in vitamin E and Se (Navarro *et al.*, 1999). Therefore, in the absence of vitamin E and Se, enhancement of ubiquinone-dependent reductase systems can protect the membrane against peroxidation (Navarro *et al.*, 1998). Similarly, Se participation in a regulation of redox status of the cell can be crucial for explaining some signs of its deficiency. In this respect, the selenoenzymes TrxR and MsrB1, which are involved in regulation of many metabolic reactions in the cell, and provide protection against protein oxidation could be the major targets for the future research. Furthermore, it seems likely that apoptosis plays an important role in formation of clinical signs of Se deficiency (Nunes *et al.*, 2003).

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Chapter 5
Selenium in poultry nutrition

Do not count your chickens before they are hatched

5.1 Introduction

Se in poultry nutrition is related to its usage for breeders, including males and females, to improve antioxidant defences of breeder birds, male spermatozoa and developing embryo. This could give an extra protection in stress conditions of the commercial poultry production. Selenium in layer diet is associated with maintenance of high egg production peak, egg shell quality and internal egg quality (Haugh units), especially during hot summer weather. Selenium is also important for broilers, since it has immunomodulating properties and helps protecting immune cells from oxidative stress, it has a protective role in the gut participating in the maintenance of the antioxidant-prooxidant balance in the enterocytes and has a positive effect on meat quality, decreasing protein oxidation and preventing drip loss. Therefore, optimal supplementation of organic Se for broilers is associated with improved immunity (decreased mortality), feed conversion ratio (FCR) (via gut health) and meat quality.

5.2 Selenium for breeders

Two main areas should be considered in relation to the Se nutrition of breeders. Firstly, it has been proven that Se plays an important role in the maintenance of semen quality. Optimal Se status of poultry males is considered to be an important factor in ensuring the fertility of breeding stock (Surai, 2006). Secondly, Se status of the eggs from breeding birds is of great importance for the maintenance of the antioxidant system of the developing embryo. It is generally accepted that the hatching process is an oxidative stress and improvement in antioxidant defences of the embryo can increase hatchability (Fisinin *et al.*, 2008; Surai, 2002a, 2006).

5.2.1 Selenium and semen quality

The importance of Se nutrition of poultry males is related to the high proportion of polyunsaturated fatty acids (PUFAs) in avian semen and its susceptibility to lipid peroxidation (Figure 5.1 and 5.2; Surai *et al.*, 1998a).

It has been shown that during sperm storage, lipid peroxidation is associated with a significant decrease in PUFA concentration in spermatozoa. In particular, the main PUFA in the chicken semen (22:4n-6) was most susceptible to peroxidation. Its proportion in the phospholipid fraction was significantly decreased as a result of

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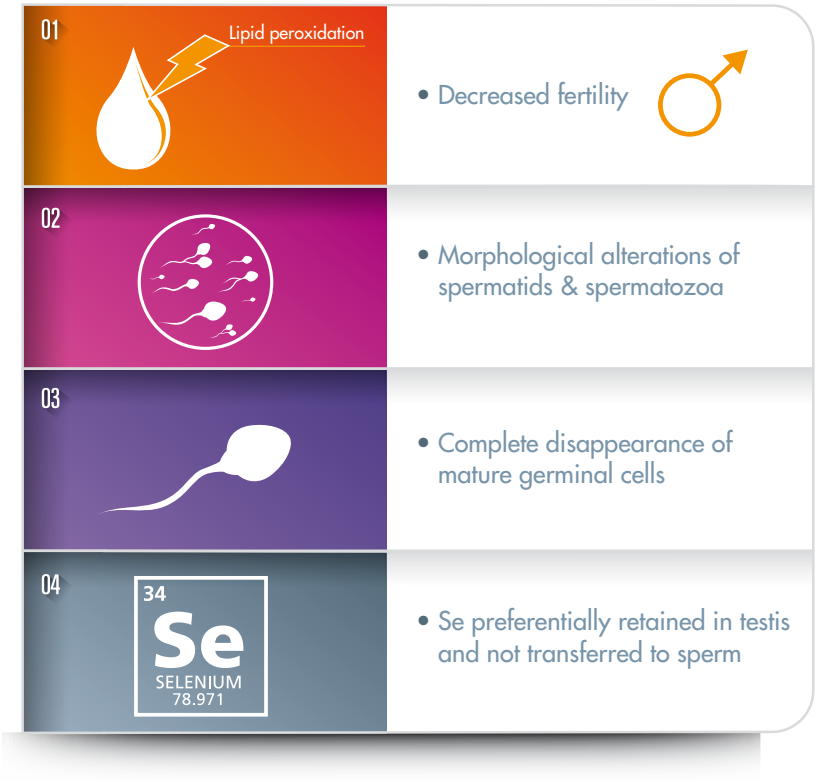

Figure 5.1. Semen quality and selenium deficiency.

incubation of chicken sperm for 12 h at 20 °C (Surai *et al.*, 1998b). The confirmation of the suggestion that the loss of PUFA was due to peroxidation came from the data showing simultaneous accumulation of thiobarbituric acid reactive substances (TBARS) in the semen (Surai *et al.*, 1998b). It has been shown that the total lipid content, the proportion of total phospholipids, and the levels of phosphatidylcholine, phosphatidylethanolamine and sphingomyelin were significantly decreased in chicken semen during *in vitro* storage and this was associated with a reduction in the proportion of motile, viable and morphologically normal cells (Blesbois *et al.*, 1999).

Similarly, in turkey a significant decrease in phosphatidylserine (by 47%) and phosphatidylethanolamine (by 35%), the two most unsaturated fractions of avian spermatozoa, was observed in spermatozoa incubated at 37 °C in the presence of exogenous Fe^{2+} (Maldjian *et al.*, 1998; Surai *et al.*, 1998c). Storage of diluted turkey semen for 48 h at 4 °C was also associated with a decrease in total phospholipid content and of phosphatidylcholine and, to lesser extent, of sphingomyelin, phosphatidylserine and phosphatidylinositol (Douard *et al.*, 2000). Interesting observations in female chickens have revealed the effectiveness of dietary supplementation with vitamin E, organic selenium or both, in sustaining fertility in ageing flocks (Breque *et al.*, 2003).

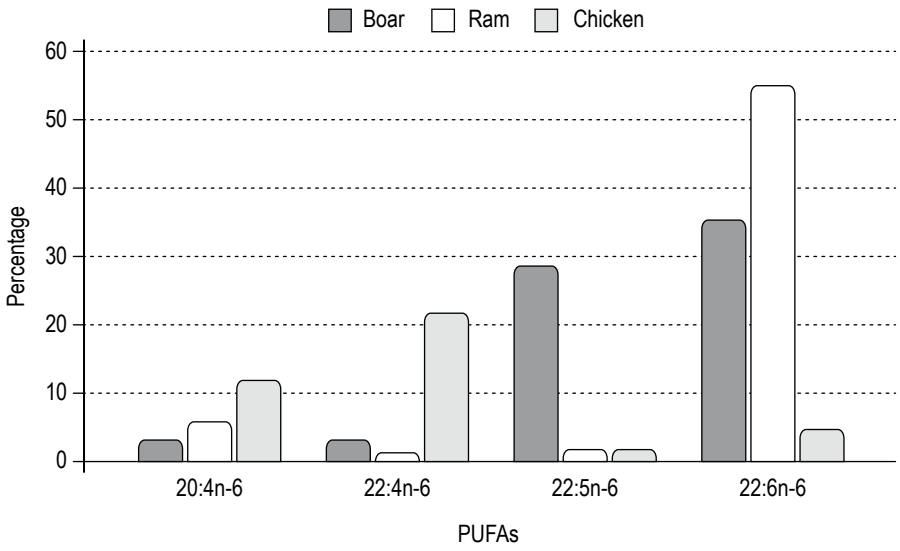

Figure 5.2. Polyunsaturated fatty acids (PUFAs) in spermatozoa phospholipids, % (adapted from Surai, 2002). 20:4n-6 =arachidonic acid; 22:4n-6 = docosatetraenoic acid; 22:5n-6 = docosapentaenoic acid; 22:6n = 3 docosahexaenoic acid.

This is probably due to the effect of organic selenium on the antioxidant system in the sperm storage tubular (Surai *et al.*, 1998a). One of the unique features of avian reproduction is the storage of spermatozoa within an oviductal sperm storage tubular, which enables the hen to produce fertile eggs during the 'fertile period' of 1-6 weeks, depending on the species. Thus, avian spermatozoa might be expected to have systems which maintain stability throughout this period. Indeed, recent results have confirmed the existence of a complex antioxidant system in the utero-vaginal portion of the fowl oviduct (Breque and Brillard, 2002). In particular glutathione peroxidase (GSH-Px) activity in the utero-vaginal junction was 12-fold higher than in the liver.

In our investigation, the average Se concentration in the whole chicken semen was shown to be 47±3 ng/g (Figure 5.3; Pappas *et al.*, 2005b). The Se was concentrated in the spermatozoa (cf. the seminal fluid) in the ratio of approximately 8:1, respectively. It was also shown that organic Se dietary supplementation of the cockerel's diet was associated with a substantial (more than double) increase in Se concentration in the semen (101±10 ng/g). GSH-Px has been found to be expressed in chicken seminal plasma and spermatozoa (Surai *et al.*, 1998a,b). There are species-specific differences in activity and distribution of GSH-Px in avian semen. For example, in seminal plasma total GSH-Px activity was the highest in turkey and lowest in duck and goose (Surai *et al.*, 1998a). In spermatozoa, on the other hand, the highest GSH-Px activities were found for goose and duck and much lower GSH-Px activity was recorded for guinea fowl, turkey or chicken. Recently, it has been shown that despite a high proportion of PUFAs and a low level of vitamin E, duck spermatozoa have the same susceptibility to lipid peroxidation as chicken spermatozoa (Surai *et al.*, 2000). It has been suggested

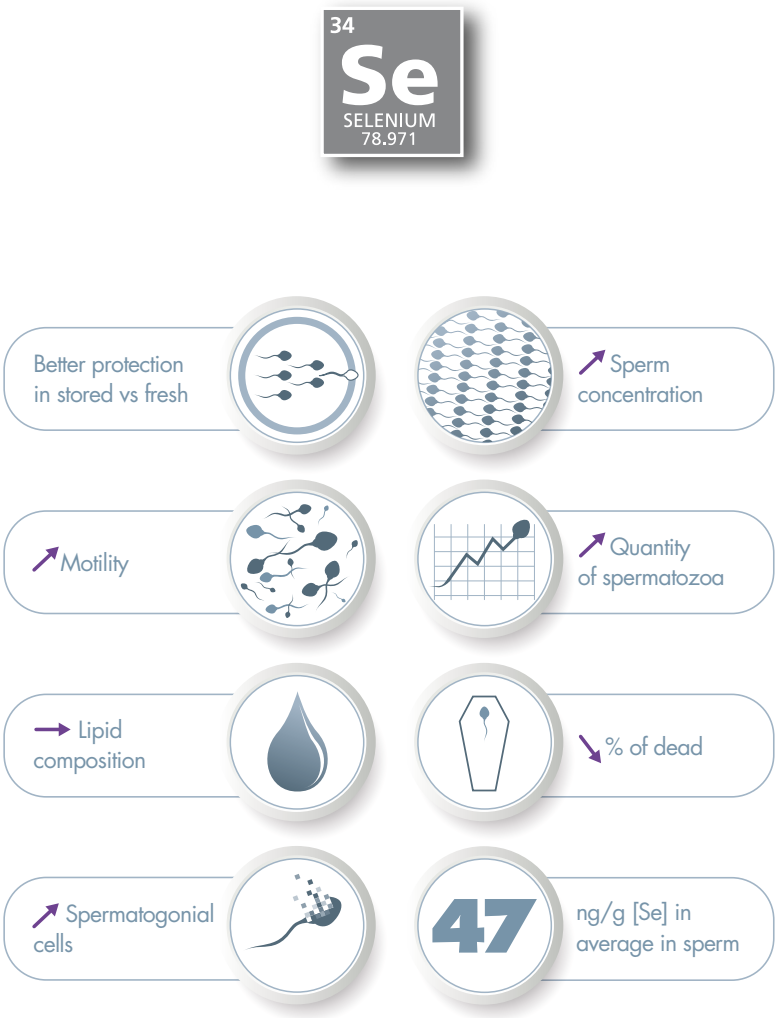

Figure 5.3. Semen quality and selenium supplementation.

that an increased activity of Se-GSH-Px in duck semen compensates for the relatively low concentrations of other antioxidants.

If selenium is limiting in the diet (which is the case in many countries in the world), then dietary supplementation of this trace element should have a beneficial effect on the antioxidant defence in various tissues including sperm. This was confirmed in our studies. Inclusion of Se in the diet of male chickens significantly increased Se-GSH-Px activity in the liver, testes, spermatozoa and seminal plasma (Surai *et al.*, 1998d). As a result, a significant decrease in the sperm's and tissue susceptibility to lipid peroxidation was observed. This protective effect was more expressed in

stored semen as compared to fresh. In this respect, it is extremely important that an inducible form of the enzyme (Se-GSH-Px) represents more than 75% of the total enzymatic activity in chicken spermatozoa and more than 60% in the testes and liver of cockerels. Indeed, Se-GSH-Px is an important element of the antioxidant system of the semen especially in stress conditions. For example, under heat stress conditions (33-36 °C in poultry house), inclusion of organic Se in the cockerel diets enhanced the semen quality traits, including the sperm count and motility and reduced the percentage of dead sperms in a dose-dependent manner (Ebeid, 2009). Furthermore, the inclusion of organic Se (0.3 mg/kg) in the cockerel diets doubled seminal plasma GSH-Px activity, compared to controls and reduced lipid peroxidation. Under heat stress conditions an effective combination of vitamin E (200 mg/kg) and organic selenium (0.3 mg/kg) in the cockerel's diet was associated with enhanced semen quality traits, including the spermatozoa count and motility, and reduced percentage of dead spermatozoa (Ebeid, 2012). Such a supplementation also substantially decreased TBARS in seminal plasma samples and enhanced the seminal plasma GSH-Px activity by 2-fold compared with controls. It seems likely that supplementation with organic selenium at 0.3 mg/kg could also be effective in turkey males. In one example, sodium selenite (0.3 mg/kg) was replaced by organic selenium (Se-yeast) in the diet of turkey males (Dimitrov *et al.*, 2007). After 30 weeks of feeding, semen samples were collected and analysed. After 6 h of semen storage, motility decreased in control group by 8.7%, while in Se-yeast-supplemented toms, motility decreased much less (3.95%). The positive effect of Se supplementation was observed on the lipid composition of stored semen: the concentration of total lipids and phospholipids in the seminal plasma from control group significantly increased, while in the experimental group it remained constant. In another study, dietary Se supplementation (0.3 mg/kg) enhanced the sperm concentration and the total number of sperm, and did not influence the antioxidative properties of turkey seminal plasma (Slowinska *et al.*, 2011). There was no difference between organic and inorganic Se sources. Dietary supplementation of the commercial gander diet with a combination of selenium (0.3 mg/kg) and vitamin E (100 mg/kg) increased the frequency and decreased the time interval of a complete ejaculatory response of the ganders to manual semen collections (82.7% supplement vs 73.5% control) (Jerysz and Lukaszewicz, 2013). Males from the supplemented group had significantly higher ejaculate volumes, sperm concentrations, and percentages of viable sperm and lower percentages of immature sperm (spermatids). Lipid peroxidation, expressed in terms of the malondialdehyde (MDA) concentration, was significantly lower in semen of the supplemented group as compared to the controls. Recently, it has been shown that Se dietary supplementation resulted in the upregulation of genes governing cell structure/morphology. In particular, the enrichment of such pathways was greater with organic selenium than with sodium selenite (SS) (Brennan *et al.*, 2012). The expression patterns suggested that Se in the organic form, is an important element for maintaining testicular cell structure. It seems likely that dietary Se can influence the population of spermatogonial stem cells (SSCs) of roosters during spermatogenesis and oxidative stress can potentially modulate SSCs during spermatogenesis. For example, in experiment conducted in China, 12-week-old Hy-Line roosters were fed with the basal diet (0.044 mg Se/kg DM) supplemented with 0 (control), 0.5, 1.0 or 2.0

mg Se/kg DM (from SS). The results show that Se concentration in blood and testis of the animals was progressively increased with the increasing Se level in diet. The highest GSH-Px activity and lowest MDA content in blood and testis was obtained in the treatment of 0.5 mg/kg. Furthermore, mRNA expression of (SSCs) markers were significantly lower in the control and 1.0 mg/kg groups when compared with the 0.5 mg/kg treatment (Shi *et al.*, 2014). The effects of dietary Se supplementation on the development of chicken testis and the expression of SelW and GSH-Px4 were studied by Khalid *et al.* (2016). Roosters were assigned randomly into the control group fed with a basic diet (containing 0.3 mg Se/kg) and the experimental group fed with a diet containing 0.6 mg Se/kg. The testes were collected individually at age of 6, 9, and 12 weeks. The results showed that dietary Se affected the number of cells in the seminiferous tubules and viability of Sertoli cells *in vitro* culture. Furthermore, SelW and GSH-Px4 expression in the testes increased significantly in the experimental group compared to that in the control group. Dietary Se deficiency exerts significant harmful effects on male reproductive organ and the intrinsic and extrinsic pathways and the upstream regulators, such as p53, Bax, and Bcl-2 are all involved in Se deficiency-induced testicular apoptosis in Hy-line cockerels (Huang *et al.*, 2016). In fact GSH-Px activity and Bcl-2 mRNA level in the testes and thyroidal triiodothyronine (T3) and free triiodothyronine (FT3) levels in serum by dietary Se deficiency (0.033 mg of Se/kg) were significantly decreased compared to the corresponding control groups. The Se deficiency-treated group showed a significant increase in MDA content, TUNEL-positive cells, and mRNA level of Bax, Caspase3, and p53 in the testes and thyroidal thyroxine (T4), free thyroxine (FT4), and thyroid-stimulating hormone (TSH) levels in serum. Histopathologically, Se deficiency caused impairments in the testes.

Therefore, taking together data on the effect of Se on male reproduction it could be concluded that Se dietary supplementation is an essential part of maintaining high semen quality and Se deficiency in roosters is associated with damages to the neck of spermatozoa (Figure 5.4).

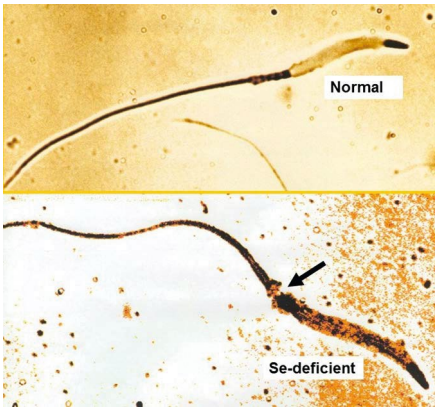

Figure 5.4. Image analysis of normal and Se-deficient chicken spermatozoa (adapted from Surai, 2002c).

Since organic Se is more efficiently transferred to the reproductive organs and semen it has an additional benefit on male reproduction, especially in various stress conditions. The general scheme of Se participation in chicken male reproduction is shown in Figure 5.5.

5.2.2 Selenium and chick embryo antioxidant defences

Chick embryo tissues contain a high proportion of highly PUFAs in the lipid fraction (Speake *et al.*, 1998) and therefore are sensitive to lipid peroxidation and need antioxidant defence (Surai, 2002a). It is well accepted that the maternal diet composition is a major determinant of antioxidant system development during embryogenesis and early postnatal development (Surai, 2002a). The antioxidant system of newly hatched chicks include the fat-soluble antioxidants vitamin E (Surai, 1999a; Surai *et al.*, 1996) and carotenoids (Surai, 2012a,b; Surai *et al.*, 2001a,b), water-

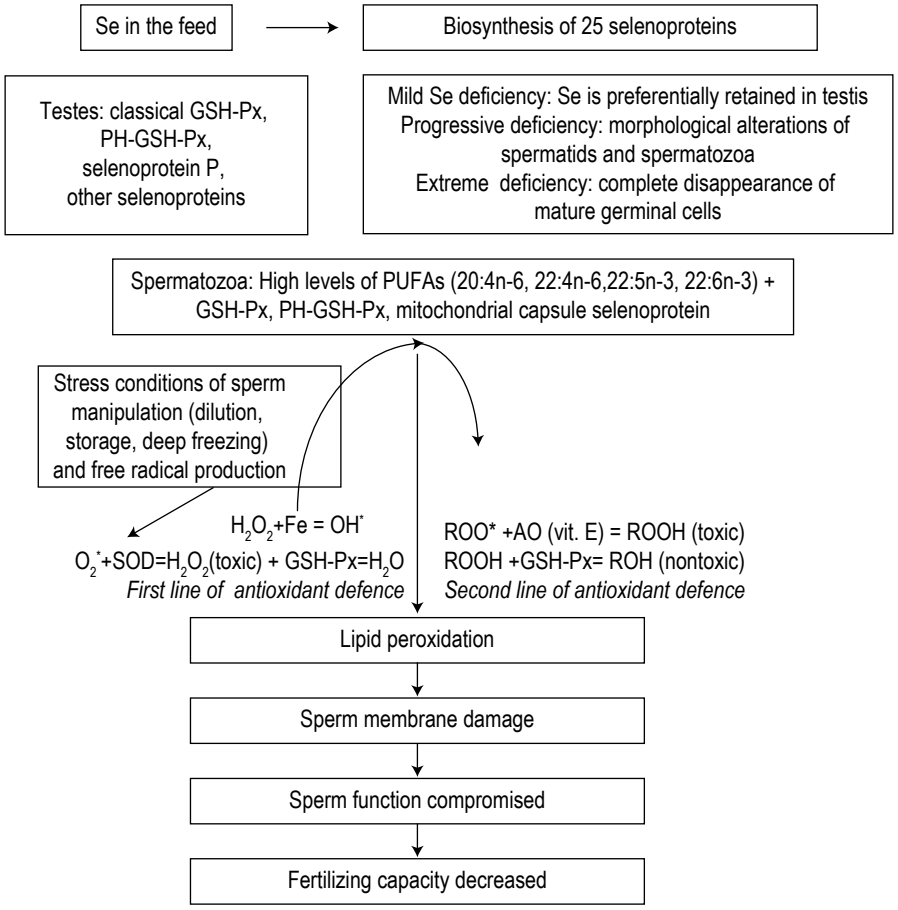

Figure 5.5. Selenium and male fertility (adapted from Surai, 2002c).

soluble antioxidants ascorbic acid (Surai *et al.*, 1996) and glutathione (Surai, 1999b; Surai *et al.*, 1999), as well as antioxidant enzymes superoxide dismutase (SOD), GSH-Px and catalase (Surai, 1999b), and selenium as a part of various selenoproteins (Pappas *et al.*, 2008; Surai, 2006). Vitamin E (Surai, 1999a; Surai and Speake, 1998a) and carotenoids (Surai, 2012a,b; Surai and Speake, 1998b; Surai *et al.*, 2001a,b) are transferred from feed into egg yolk and further to embryonic tissues. The Se content of the egg also depends on its concentration in the hen's diet, and also on the form of dietary Se used, since organic Se is more efficiently deposited in the egg albumen and at higher supplementation rate (0.3 mg/kg) in the egg yolk (Table 5.1; Cantor, 1997; Paton *et al.*, 2002).

Therefore, during egg incubation fat-soluble antioxidants are transferred to the developing embryonic tissues mainly during the last week of incubation (Surai, 1999a, 2002a; Surai *et al.*, 1996). It seems likely that Se from the albumen is transferred to the embryo during first 2 weeks of the embryonic development, while Se from egg yolk is delivered to the embryo during last week of incubation (Surai, 2006). In tissues of newly hatched chicks GSH-Px activity is represented by Se-dependent and Se-independent forms of the enzyme (Surai *et al.*, 1999). There is a tissue-specificity in distribution of these enzymes. In particular, in the liver Se-GSH-Px activity is higher than non-Se-GSH-Px as well as in all other tissues studies, including 177 vs 115; 160 vs 59; 99 vs 12; 100 vs 53; 46 vs 13 and 103 vs 38 U/g protein for liver, kidney, heart, lung, thigh muscle and yolk sac membrane, respectively. It should be noted that the specific activity of Se-GSH-Px in the embryonic liver increases continuously during the 2nd half of the *in vivo* developmental period so that the activity at hatching was 3.0 times greater (P<0.001) than that at day 10. The most rapid increase occurred between days 11 and 15 with a much more gradual increase thereafter (Surai, 1999b).

Antioxidant enzymes are a major cell defence against acute oxygen toxicity. They are responsible for the detoxification of reactive oxygen species and prevention of lipid peroxidation. The expression of their activities is regulated at gene level with tissue-specific features. They are considered as important embryo-protective enzymes during organogenesis when increased exposure to oxidant-derived free radicals or inadequate antioxidant defence could alter cellular response at critical points in development (Surai, 2006).

The results of our studies (Surai, 1999b; Surai *et al.*, 1999) indicate that different tissues of the embryo display distinct development strategies with regard to the acquisition of antioxidant capacity. A low oxygen pressure in the environment surrounding embryos during development seems to have been retained in the course of evolution to protect the vulnerable developing tissues from the damage caused by the action of reactive oxygen species (Ar and Mover, 1994) as far as the rate of free radical generation in cells is influenced by ambient oxygen concentration (Turrens *et al.*, 1982). The reactivity of oxygen radicals and their derivatives implicate them as possible effectors of oxygen-mediated changes in gene expression (Allen and Venkatraj, 1992).

Table 5.1. Effect of dietary Se on its accumulation in the egg (adapted from Surai and Fisinin, 2014).[1]

Bird type[2]	Experiment duration	Diet				Se accumulation in egg					Reference
		Se sources used	Unit	Se dose	BD Se level expression	Egg fraction	BD	SS	OS	OS:SS increase %	
Broiler breeders											
Cobb Breeders	8 weeks	BD, SY	µg/g	0.2	0.17	Y	0.298		0.605		Surai, 2000
						W	0.051		0.194		
Cobb Breeders	8 weeks	BD, SY	µg/g	0.4	0.17	Y	0.298		0.854		Surai, 2000
						W	0.051		0.403		
Hubbard ISA	8 weeks	BD, SY	µg/g	0.4	≤0.1	Y	0.108		0.490		Pappas *et al.*, 2005a
						W	0.029		0.186		
Hubbard ISA	7 weeks	BD, SY	µg/g	0.4	0.03	Y	0.0835		0.516		Pappas *et al.*, 2005c
						W	0.0299		0.249		
Ross 308	45 days	SS, SY	µg/whole egg	0.1	0.1	Y		7.16	7.22	1	Leeson *et al.*, 2008
						W		2.43	3.32	37	
						T		9.58	10.44	9	
Ross 308	45 days	SS, SY	µg/whole egg	0.3	0.1	Y		7.68	7.80	1.6	Leeson *et al.*, 2008
						W		2.81	4.81	71	
						T		10.44	13.01	26	
Chinese Broiler Breeders 48W	8 weeks	SS, SM, SY	µg/g	0.15	0.04	Y	0.492SM	0.469	0.476	1.5	Yuan *et al.*, 2011
						W	0.104SM	0.095	0.099	4.2	
Chinese Broiler Breeders 48W	8 weeks	SS, SM, SY	µg/g	0.30	0.04	Y	0.541SM	0.495	0.519	4.8	Yuan *et al.*, 2011
						W	0.127SM	0.110	0.121	10	
Chinese Broiler Breeders 39W	8 weeks	SS, SM	µg/g	0.3	0.04	Y		0.089	0.119SM	34	Wang *et al.*, 2011
						W		0.459	0.590SM	29	

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Table 5.1. Continued.

Bird type[2]	Experiment duration	Diet				Se accumulation in egg					Reference
		Se sources used	Unit	Se dose	BD Se level expression	Egg fraction	BD	SS	OS	OS:SS increase %	
Egg breeders and layers											
White Leghorn	6 weeks	SS, SM	µg/g	0.1	0.06	Y	0.1	0.33	0.32	-3	Paton *et al.*, 2002
						W	0.04	0.07	0.08	14	
						T	0.06	0.14	0.15	7	
White Leghorn	6 weeks	SS, SM	µg/g	0.2	0.06	Y	0.1	0.37	0.42	14	Paton *et al.*, 2002
						W	0.04	0.07	0.13	86	
						T	0.06	0.16	0.22	38	
White Leghorn	6 weeks	SS, SM	µg/g	0.3	0.06	Y	0.1	0.38	0.48	26	Paton *et al.*, 2002
						W	0.04	0.07	0.15	102	
						T	0.06	0.16	0.25	56	
Hy-Line 70W	4 weeks	SS, SY	µg/g	0.15	0.1	T	0.249	0.284	0.366	29	Payne *et al.*, 2005
Hy-Line 70W	4 weeks	SS, SY	µg/g	0.30	0.1	T	0.249	0.299	0.495	66	Payne *et al.*, 2005
Leghorn hens	3.5 weeks	SS, SM	µg/g DM	0.5	0.23	Y	1.34	2.15	2.26SM	5	Jiakui and Xiaolong, 2004
						W	0.75	1.67	1.59SM	-5	
ISA Brown 24W	27 weeks	SS, SY	µg/g DM	0.3	0.07	Y	0.62	0.93	1.48	59	Skrivan *et al.*, 2006
						W	0.58	1.36	2.05	51	
Rohman Laying hens	4 weeks	SS, SY	µg/g	0.2	0.15	T	0.193	0.256	0.268	5	Pan *et al.*, 2007
Lohman Laying hens	4 weeks	SS, SY	µg/g	0.5	0.15	T	0.193	0.295	0.329	12	Pan *et al.*, 2007
Lohman White	5.5 weeks	SS, SY	µg/whole egg	0.3	n/a	Y		4.89	6.04	24	Leeson *et al.*, 2008
						W		1.66	3.66	21	
						T		6.55	9.70	48	
CP Brown 71W	6 weeks	SS, ZSM	µg/g DM	0.3	0.3	Y	0.70	1.27	0.65ZSM	-48	Chantiratikul *et al.*, 2008
						W	0.71	0.68	1.54ZSM	126	
						T	0.40	0.73	0.84ZSM	15	
Shaver	16 weeks	SS, SY	µg/g	0.4	0.124	T	0.135	0.218	0.375	72	Pavlovic *et al.*, 2009
ISA Brown	11 weeks	SS, SM	µg/g DM	0.3	0.11	Y		0.98	1.40SM	43	Skřivan *et al.*, 2010
						W		0.62	1.22SM	97	
Hy-Line Brown	9 months	SS, SY	µg/g DM	0.4	0.13	Y	0.4	1.3	1.0	-23	Cobanova *et al.*, 2011
						W	0.5	0.9	2.7	200	
ISA Brown	24 weeks	SS, SY	µg/g DM	0.3	0.09	Y	603	1,073	1,066	-1	Skřivan *et al.*, 2013
						W	574	769	1,545	101	

[1] BD = basal diet; SS = sodium selenite; SY = Se-enriched yeast; SM = selenomethionine; ZSM = Zn-SeMet; OS = organic selenium; W = egg white; Y = egg yolk; T = total egg.
[2] Age of the birds: 24W = 24 weeks; 39W = 39 weeks; 48W = 48 weeks; 70W = 70 weeks; 71W = 71 weeks.

Embryonic gene expression of antioxidant enzymes develops in proportion to the degree of exposure to the reactive oxygen species in their environment and in preparation for the oxygen-rich environment after birth (Ar and Mover, 1994). Exposure of rat embryos with an immature antioxidant system to a high concentration of oxygen (20%) during early neurulation significantly increased the incidence of neural tube defects compared with control embryos exposed to a low O_2 concentration. The concentration of GSH in 20% O_2 exposed embryos was significantly reduced compared with that of control embryos and it was suggested that impaired responsiveness of the GSH dependent antioxidant system against oxidative stress plays a crucial role in oxygen-induced embryopathy (Ishibashi *et al.*, 1997). Yuan *et al.* (1995) studied species differences in the resistance of human, rabbit, rat, mouse and chick embryo cells against oxygen-induced growth inhibition. The extent of the resistance of the cells was in the following order: chick >rat >human >rabbit = mouse. Chick embryo cells, having the highest resistance against oxygen-induced growth inhibition, were at the lowest activity levels of antioxidant enzymes and at the highest concentration level of reduced glutathione.

5.2.3 Selenium in eggs

It has been shown that Se concentration in the egg depends on its dietary provision and the form of Se in the diet (for review see Surai, 2006). In general Se almost equally distributed between egg yolk (58%) and egg albumin (42%; Pappas *et al.*, 2005a). The idea is that if Se is deficient in the chicken diet, its level in the egg and in the developing embryo tissues would be not optimal for maximal expression of selenoproteins and effective antioxidant defence. Recently it has been shown that SeMet comprised 53-71% of total Se in the egg albumen and 12-19% in the egg yolk (Figure 5.6; Lipiec *et al.*, 2010). SeMet is non-specifically incorporated into the egg proteins in place of methionine and its level depends on the ratio SeMet/Met in the feed. Since SeMet is not synthesised by animals, only plant material or supplements can provide this amino-acid in the chicken diet. Based on aforementioned results one could expect that an inclusion of SeMet sources would mainly affect the Se level in the albumen and to lesser extent in the egg yolk. In fact, efficiency of Se deposition in the egg yolk was shown to be 13-14%, while in egg white it was 8-9% (Pappas *et al.*, 2005a).

Our results (Surai, 2000, 2006) indicated that the inclusion of organic Se into the commercial diet significantly increased the Se concentration in the egg yolk and albumen. The correlation between dietary organic Se and egg yolk Se content was found to be very high. In the case when dietary Se was higher than 44 μg/kg, this relationship was quantified through a regression equation:

$$y = 1.01x + 179.4 \ (r^2 = 0.96, P<0.01),$$

where y is Se concentration in the egg yolk (μg/kg); and x is the Se concentration in the feed (μg/kg).

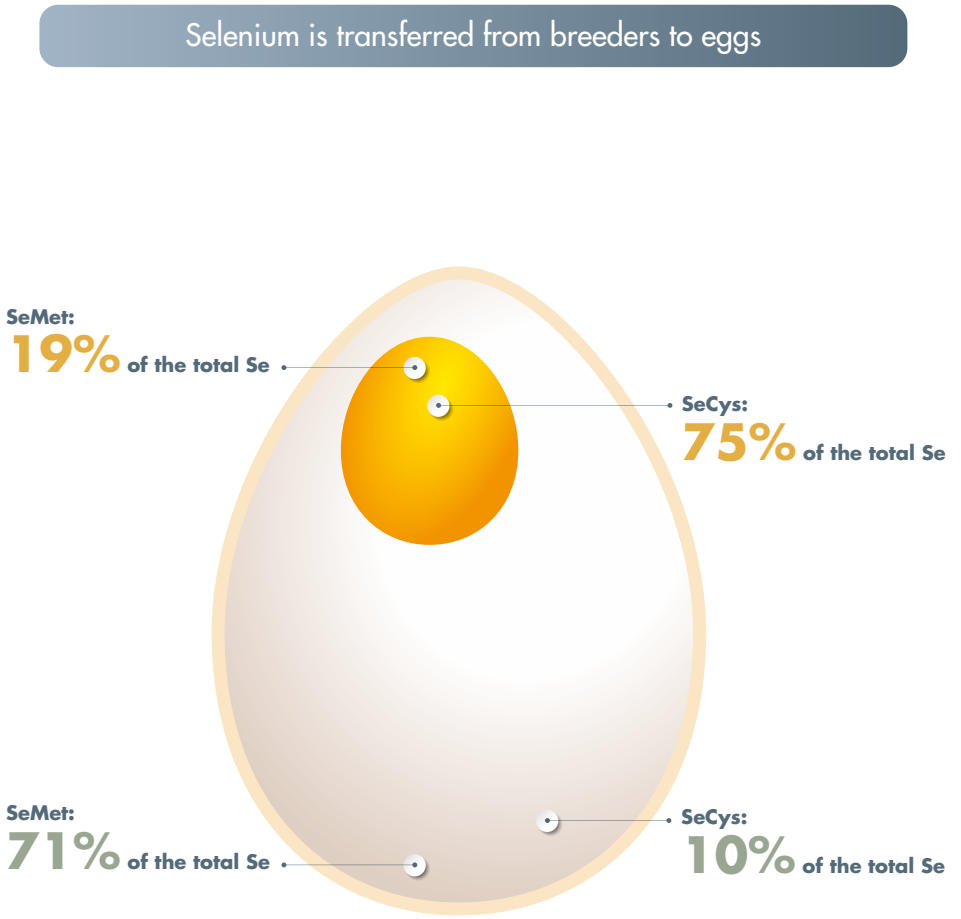

Figure 5.6. Selenium in eggs.

Similarly, a significant correlation between Se in the egg white and feed was found and an equation describing Se concentration in the egg white (y) is as follows:

$$y = 0.68x - 24.8 \ (r^2 = 0.98; P<0.01).$$

Using these equations it is easy to predict Se concentration in the egg when organic Se is used in the diet, because the Se content in the egg yolk and egg white reflects its concentration in the feed. The analysis of main results on the effect of dietary Se on its incorporation into the egg yolk and egg albumen (Table 5.1) confirms the idea: dietary SeMet affected mainly its level in egg white, while its effect on the Se concentration in the egg yolk was less pronounced. As can be seen from the data presented in the table, dietary Se supplementation increased Se level in both egg yolk and egg white.

It is clear that the organic form of Se is more effective in transferring Se to the egg, however, the effect of dietary organic Se was very much dependent on the background Se level.

The presented data indicated that there is a trend reflecting higher Se transfer to the egg when SeMet is used in comparison to Se-yeast. Furthermore, it was proven that a new Se source (2-hydroxy-4-methylselenobutanoic acid) was significantly more effective than Se-yeast in transferring Se to chicken muscles (Briens *et al.*, 2013, 2014; Figure 5.7) or egg (Jlali *et al.*, 2013).

In fact, the Se transfer efficiency values determined in relation to Se egg output and daily Se intake showed that the Se transfer efficiency was greater ($P<0.01$) in birds supplemented with Se as selenomethionine hydroxyanalogue,2-hydroxy-4-methylselenobutanoic acid (HMSeBA) at 0.2 mg/kg of diet (76.26%) than those provided the same amount of Se-yeast (56%). Furthermore, bioavailability of Se from HMSeBA was 28.8% higher ($P<0.01$) than from Se-yeast. Since, in major feed ingredients (grains) Se is found mainly in the form of SeMet (Whanger, 2002) high background Se would mask effects of low (0.1 mg/kg or less) organic Se supplementation. On the other hand, low background dietary Se is favourable for observations of dietary Se effects on its accumulation in the egg.

In an experiment conducted in China (Yuan *et al.*, 2012) broiler breeders were fed corn-soy-based diets supplemented with 0.15 mg/kg Se from sodium selenite (SS), Se-enriched yeast (Se-yeast) or SeMet. The results showed that, compared with

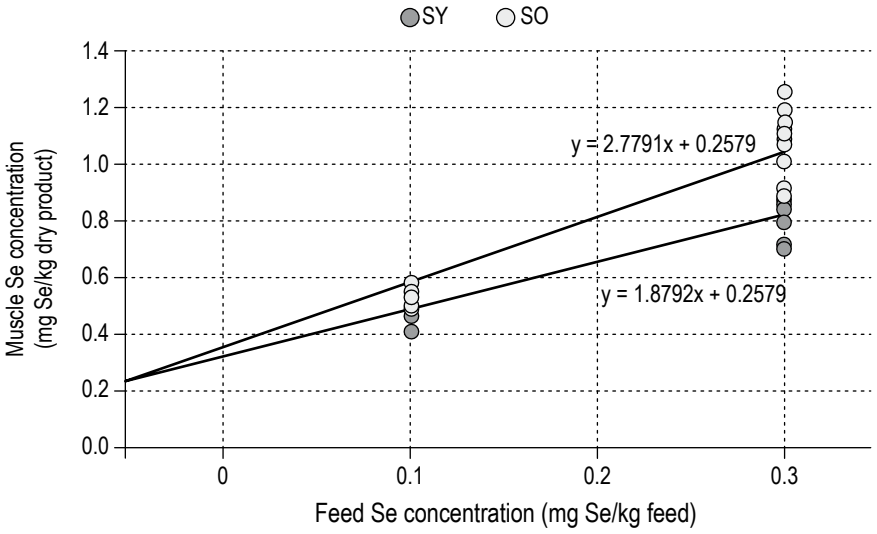

Figure 5.7. Linear regression model for muscle Se concentration of tissues from animals receiving a seleno-yeast (SY) or Selisseo (SO) selenium source, day 42 (adapted from Briens *et al.*, 2014).

sodium selenite, Se-yeast or SeMet significantly increased the activity of thioredoxin reductase-1 (TrxR1) in the liver and kidney of broiler breeders and their offspring, but not the GSH-Px1 activity. The liver GSH-Px1 and TrxR1 mRNA levels in Se-yeast or SeMet groups were higher than that in the sodium selenite group. Furthermore, compared with SS, both Se-yeast and SeMet significantly increased the concentration and mRNA level of selenoprotein P1 (SEPP1), the main selenoprotein ensuring transport of Se from liver to other tissues, in 1-day old chicks (Yuan *et al.*, 2013). In the study, no differences between Se-yeast and SeMet were observed.

An 8 week experiment was conducted in China to evaluate the effect of the sources and levels of Se on reproduction and Se retention (Yuan *et al.*, 2011). After receiving basal diet containing 0.042 mg/kg Se for 8 weeks, breeders were randomly assigned to six dietary treatments and fed diets supplemented with 0.15 or 0.30 mg/kg of Se from sodium selenite (SS) or from Se-yeast or from SeMet. Irrespective of the Se level, the Se concentrations in liver, kidney, pancreas, and breast muscle were greater in breeders fed organic Se compared with breeders fed SS. In general, SeMet was more effective in increasing Se concentrations in tissues in comparison to Se-yeast: kidney from breeders fed SeMet had a greater Se concentration than that from breeders fed Se-yeast. Yolk and albumen from SeMet treatments also had the greatest Se concentrations. There was also a more significant increase in Se concentrations in kidney and breast muscle of 1 day old chicks from SeMet treatments than those from Se-yeast treatments. It could well be that the advantage in Se accumulation in tissues due to SeMet supplementation in comparison to Se-yeast resulted from a difference in actual SeMet levels in the diet. Indeed, SeMet comprises only part (50-70%) of the total Se in the Se-yeast, while dietary SeMet preparations contain pure selenomethionine. It is necessary to note that in the study the increase in Se concentration in the breeder tissues, egg yolk and albumen, as well as in the tissues of the newly hatched chicks due to organic Se supplementation of the maternal diet was somewhat lower than one can expect based on the previous published results. Dietary supplementation of the basal diet for Hy-Line Brown containing Se 0.13 mg/kg with 0.4 mg/kg Se in the form of SS or Se-yeast for 9 months increased Se content of the egg from 5.1 µg in the basal diet group to 14.4 and 22.7 µg respectively (Cobanová *et al.*, 2011). When Se-yeast was used as a supplement, Se was mainly accumulated in the egg albumen and surprisingly Se concentration in egg yolk of SS-supplemented hens was higher than that in Se-yeast-group. When a commercial diet containing 0.11 mg/kg Se was supplemented with 0.4 mg/kg Se in the form of SS or Se-yeast it was shown that eggs from SS and Se-yeast had greater Se content than those from hens fed the basal diet (0.94 and 1.38 mg/kg vs 0.54 mg/kg, respectively) (Invernizzi *et al.*, 2013). Therefore Se-yeast eggs had 46.8% higher Se content than those from the SS group. Furthermore, organic Se supplementation was associated with a significant increase in eggshell breaking strength. It has been shown that Se dietary supplementation (0.3, 1.0 or 3.0 mg/kg) in the form of SS or Zn-L-selenomethionine did not affect hen performance or egg quality (Chantiratikul *et al.*, 2008). It was shown that the efficiency of Se transfer to the egg yolk from SS and SeMet at 0.3 mg/kg, was equal and Se concentrations in the egg yolk were 0.67, 0.98 and 0.98 mg/kg in the control, SS and SeMet groups, respectively. SeMet was much more effective in transferring

Se to the albumen than SS and Se albumen concentrations were 0.94, 1.35 and 1.92 mg/kg in the same groups, respectively. In most of trials comparing sodium selenite with organic selenium, no significant differences were observed among treatments for any performance parameters or mortality measurements made over the entire trial period.

It seems likely that organic Se supplementation to breeders is 'an insurance policy'; an investment into the egg quality to make sure that in stress conditions an optimal protection against over-production of free radicals would be expected. For example, breeders were fed with basal diet (BD) containing 0.04 mg/kg Se or BD supplemented with SS or SeMet at a level of 0.15 mg Se/kg. The rearing experiment lasted for 8 weeks after an 8-week pre-test. Eggs were collected during the last 10 days and incubated in a commercial incubator. On embryonic day 17, fertile eggs were treated with increased temperature (39.5 °C) for 6 h. Afterwards, chick embryos were collected and antioxidant defences were studied (Xiao *et al.*, 2016). The results showed that Se supplementation in the diet of breeders decreased ROS, HSP70, MDA, carbonyl and 8-hydroxydeoxyguanosine (8-OHdG) concentrations and increased GSH-Px, total superoxide dismutase (T-SOD), and catalase (CAT) activities in heat stress treated chick embryo ($P<0.05$). It was also shown that reactive oxygen species (ROS), malondialdehyde (MDA), carbonyl, 8-OHdG concentrations in the SeMet treatment group were lower than those in the SS treatment ($P<0.05$). Furthermore, Se supplementation elevated cellular GSH-Px1 mRNA level and activity, cytoplasmic thioredoxin reductase (TrxR1) activity and selenoprotein P (SelP) mRNA and protein level. Again, maternal organic selenium showed a higher value than maternal SS in upregulating GSH-Px1, TrxR1, and SelP mRNA levels, as well as GSH-Px1 and TrxR1 activities or SelP protein level (Xiao *et al.*, 2016).

Some aspects of egg quality such as Haugh units are adversely affected by length of storage. Inclusion of high levels of PUFA in the broiler breeder diets can also adversely affect the weight of the egg components. It is interesting to note that addition of organic Se to the broiler breeder diets can ameliorate some of the adverse effects of these factors (Pappas *et al.*, 2005a). Supplementation of laying hen diets with organic Se could benefit hatching egg quality, particularly where it may be necessary, as in the case of some pedigree or exotic lines, to store eggs for more than 14 d prior to incubation. In another experiment conducted at the Scottish Agricultural College, the diets were designed to contain <0.1 mg/kg of Se and about 0.5 mg/kg of Se for the non-supplemented (no added Se) and the organic selenium supplemented diets, respectively (Pappas *et al.*, 2006a). It was shown that the Se concentration in tissues (the eggshell and the brain and liver of 1 day old chicks) was higher in the high-Se treatments group compared to the low-Se group. As expected, fish oil (FO) inclusion in the breeder diet (a dietary stress factor) increased embryonic mortality in week 3 of incubation and reduced both hatchability and 1 day old chick weight in hens of both ages. In such conditions organic Se showed a protective effect. Indeed, the addition of Se to the FO diets ameliorated some of these adverse effects, because chicks hatched from eggs laid by 23 week old breeders of the FO + Se treatment were heavier than those receiving the FO treatment (Pappas *et al.*, 2006a).

A 10 week experiment was conducted with Ross 308 broiler breeder chickens in cages to evaluate the influence of organic and inorganic sources of supplementation (Rajashree *et al.*, 2014). A total of 600 birds at 29 weeks of age were divided at random into 4 groups and fed on a maize-soya basal diet supplemented with different forms of Se. The first (control) group was given the basal diet without Se supplementation, whereas the second, third and fourth groups were given, respectively, the basal diet with 0.3 mg/kg of inorganic Se in the form of sodium selenite or 0.3 and 0.5 mg/kg of organic Se in the form of Se enriched yeast (Se-yeast) for 10 weeks. At the end of the experiment (39 weeks), there was a reduction in mortality in breeders fed on the diet supplemented with 0.5 mg/kg of organic selenium. In addition, organic Se at 0.5 mg/kg increased egg production, percentage of settable eggs and hatchability (Rajashree *et al.*, 2014).

In physiological conditions or in well-balanced experiments it is difficult to see advantages of organic Se in the breeder diets, since in low stress-conditions Se requirement is quite low and can be met by small amount of Se in feed ingredients as well as supplemented SS. For example, the effect of different dietary vitamin E levels and different selenium sources on the productive and reproductive performance of broiler breeders was evaluated by Urso *et al.* (2015). Treatments consisted of 2 vitamin E levels (30 and 120 mg/kg) and two selenium sources (SS and zinc-L-SeMet). There was no influence of dietary vitamin E levels or selenium sources on egg production, while mature breeders (47 weeks old) fed zinc-L-SeMet and 120 mg vitamin E/kg feed produced heavier eggs and albumen. This increase in egg weight at this age could be a rather disadvantage from the point of hatching egg quality. On the other hand, the dietary inclusion of organic selenium promoted heavier hatchling weight until egg production peak (33 weeks), but did not influence hatchling quality or hatching window (Urso *et al.*, 2015). In general, improved body weight of hatchlings at the beginning of the reproduction could be considered to be an advantage in terms their further growth and development.

5.2.4 Maternal effects of selenium

It is generally accepted that the quality of newly hatched chicks depends on the egg composition. However, recent developments in the areas of maternal programming and gene expression, indicate that maternal affects can be seen further into the postnatal development of chicks, than previously thought. The prevailing nutritional environment during foetal development exerts powerful and long-lasting effects upon physiology and metabolism (Langley-Evans, 2009). For example, the concept of Developmental Origins of Health and Disease has been widely accepted and it brings new insights into the molecular pathogenesis of various diseases (Yan and Yang, 2014). The underlying mechanisms are still under discussion and epigenetic mechanisms may provide an explanation for the phenomenon (Pinney and Simmons, 2012). Transgenerational epigenetics is the phenomenon that the information of the environment of a female animal is translated into memory-like responses into the developing offspring. As a consequence, individuals of the next generation may show different phenotypic traits depending whether their mothers were kept under

different environmental conditions (Berghof *et al.*, 2013). The transfer and deposition of hormones, immunological factors and various nutrients of maternal origin into the egg could influence offspring morphology, physiology and behaviour in an epigenetic fashion (Biard *et al.*, 2007; Groothuis and Schwabl, 2008; Ho and Burggren, 2010; Royle *et al.*, 2001). In fact, a complex synergistic effect of breed-specific genotype and yolk environment exists early in chicken development, with yolk thyroid hormone and yolk testosterone as potential mediators of the physiological and morphological effects (Ho *et al.*, 2011). Taking an existence of specific selenoproteins (iodothyronine deiodinases), responsible for thyroid hormone metabolism (Surai, 2006) and effects of Se on gene expression (Barger *et al.*, 2012; Brennan *et al.*, 2011, 2012) into account, one can expect specific effects of maternal Se on progeny.

Maternal programming in birds would be related to the egg composition changes and it seems likely that there is an effect of egg composition on postnatal development of chicks. Indeed, dietary supplementation of the hen with an organic source of Se readily elevates the concentration of this element in the egg and consequently in the tissues of the day-old chick (Paton *et al.*, 2002; Payne *et al.*, 2005; Surai, 2000). Previous work has also shown that maternal Se supplementation increases the activity of GSH-Px in the tissues of the offspring at hatch (Combs and Scott, 1979; Surai, 2000), and that supplementary Se in the starter diet of newly hatched birds enhances the post-hatch expression of this enzyme (Cantor and Tarino, 1982; Mahmoud and Edens, 2003). An initial indication that the effects of maternal Se intake persist into the post-hatch life of the offspring was provided by our study showing that chicks originating from high-Se eggs continue to express elevated activities of hepatic GSH-Px for at least 5 days after hatching (Surai, 2000). Furthermore, increased Se concentration in the quail egg was associated with increased Se concentration in the liver, brain, breast and leg muscles of newly hatched quail (Surai *et al.*, 2006). This difference was shown to be significant for 2 weeks post-hatch. It has therefore been suggested, that the maternal effect of dietary selenium can be seen beyond the time of hatching and more attention should be given to this effect. Similar effects of maternal Se were seen in broiler breeders. Indeed, chicks hatched from breeders fed diets high in Se had higher concentrations of Se in the brain and liver than chicks hatched from breeders fed diets low in Se. Even after 14 days post-hatch, chicks that hatched from parents fed the high Se diets had higher tissue Se concentrations than those hatched from parents fed the diets low in Se, irrespective of tissue type (Pappas *et al.*, 2006). This study indicated that the Se levels of the hen diet can increase the Se reserves of the progeny, which could be important in periods of increased demand, such as during hatching or disease challenge. Indeed, during various stresses, including disease challenge, feed intake and thus gut absorption of minerals are usually depressed; therefore, the reserves present in the tissues become increasingly important to maintain selenoprotein synthesis in response to upregulation of selenoprotein gene expression (Surai, 2006).

Similar results have been seen with broiler breeders in our study conducted at the Scottish Agricultural College that measured the extent to which the effect of dietary supplementation of breeder diets with Se, continue onto the next generation (Pappas *et al.*, 2005c). Hens were maintained on control or Se-supplemented diets, containing

0.027 and 0.419 μg Se/g of feed, respectively. The high-Se diet elevated the Se content of the hens' eggs by 7.1 times. At hatch, the concentrations of Se in the liver, breast muscle and whole blood of chicks originating from the high-Se parents were 5.4, 4.3 and 7.7 times higher, respectively, than the values in the chicks of the low-Se parents. When the offspring from the two parental groups were maintained on the low-Se progeny diet, the tissue Se concentrations in chicks originating from the high-Se hens remained significantly higher for 3-4 weeks after hatching, compared to the values found in chicks from the low-Se hens. It should be noted that the Se found in the muscles of 4 week old chickens was not the Se that was transferred from the egg, but the Se assimilated from the diet. This means that there are changes in Se metabolism in chickens due to maternal Se supplementation. Similarly, tissue GSH-Px activity remained significantly higher in chicks from the high-Se hens for 2-4 weeks post-hatch. Thus, it was concluded that the effects of maternal Se supplementation persisted in the progeny for several weeks after hatching (Pappas *et al.*, 2005c).

Dietary supplementation of the breeder diet with Se-yeast for 4 weeks was associated with a significant increase in Se content of the muscles of 21 day old progeny chicks. Those changes were associated with decreased lipid and protein oxidation and muscle drip loss in comparison to chickens obtained from the breeders fed a control diet without Se supplementation and containing feed-derived Se at 0.13 mg/kg (Wang *et al.*, 2009). The study was conducted to investigate the effects of dietary maternal SeMet or SS supplementation at 0.3 mg/kg on performance and selenium status of broiler breeders and their progeny (Wang *et al.*, 2011). Pre-treatment period was 2 weeks, and the experiment lasted 8 weeks. All the offspring chicks were fed the same diet containing 0.04 mg Se/kg, and the experiment also lasted 8 weeks. It was shown that SeMet supplementation improved hatchability (90.8 vs 85.1). The Se concentration in serum, liver, kidney, and breast muscle of broiler breeders, selenium deposition in the yolk, and albumen and tissues' (liver, kidney, and breast muscle) selenium concentrations of 1 day old chicks were significantly increased by maternal SeMet supplementation compared to maternal SS supplementation. It is interesting to note that the antioxidant status of 1 day old chicks was also improved by maternal SeMet intake in comparison with maternal SS intake: increased GSH-Px and SOD in breast muscle, GSH concentration in kidney, total antioxidant capability in breast muscle and liver, and decreased MDA concentration in liver and pancreas of 1 day old chicks. Furthermore, SeMet supplementation of the maternal diet was associated with a significant improvement of FCR and decreased mortality of the progeny chicks.

The results of the study conducted in Poland by Jankowski *et al.* (2011) showed that the dietary supplementation of organic selenium in turkey parent flocks reduced the rate of oxidation processes in the egg and tissues of newly-hatched poults, yet it had no effect on the analysed parameters of cell-mediated immunity and growth performance of birds during the first five weeks of their life. However, the authors were not able to find any difference in Se concentration in the eggs produced by turkeys fed SS or Se-yeast which could indicate some problems with the experiment or analytical techniques. In another study with turkeys conducted in Poland it was confirmed that

replacement of SS by organic selenium in the form of Se-yeast did not affect hatching egg quality and egg hatchability or hatch from the eggs set (Stepinska *et al.*, 2012). A small increase in egg weight due to organic Se supplementation seems not of any benefit for the embryonic development. In an experiment conducted in China, the influence of mineral sources on broiler breeders and their offspring was investigated. Broiler breeder hens were fed with diets containing either organic or inorganic trace minerals (Cu, Mn, Zn and Se) at equal levels. Supplementations of organic minerals in breeders' diets were observed to have protective effects on breeders via increasing cholesterol and triglyceride clearance from plasma and decreasing plasma lipid peroxidation. Furthermore, increased body weight (2.247 vs 2.099, $P<0.05$) and feed efficiency (1.671 vs 1.704, $P<0.01$) at 42 days were observed in chicks from breeders fed organic mineral diets (Sun *et al.*, 2012).

In a recent experiment conducted in China, yellow broiler breeders were randomly distributed into 2 treatments, with a 14 day pretreatment and 56 day trial period. The treatments were fed a basal corn-soybean diet (0.04 mg/kg Se) supplemented with 0.3 mg/kg SS or SeMet. Fertile eggs were incubated and healthy chicks were collected and fed the same basal diet (0.04 mg/kg Se) for 56 days (Zhang *et al.*, 2014). It was shown that the Se concentrations in serum and tissues (liver, kidney, and breast muscle) of the 56 day old offspring were significantly increased by maternal SeMet intake compared with maternal SS intake. Furthermore, the antioxidant status of the 56 day old offspring was significantly improved by maternal SeMet supplementation in contrast with maternal SS supplementation. It was evidenced by increased GSH-Px activity in serum and breast muscle, GSH concentration in serum, and total antioxidant capability in pancreas, as well as cytosolic GSH-Px mRNA abundance in breast muscle, liver, and pancreas. It is important to mention that the maternal SeMet treatment had significantly reduced the 48-h drip loss of 56 day old offspring in comparison with maternal SS treatment (Zhang *et al.*, 2014). Aforementioned results clearly indicated that maternal effects in poultry in general and Se maternal effects warrants further investigation (Figure 5.8).

The effect of fortifying breeder diet with vitamins and minerals on gene expression in the intestine of progeny has been investigated (Rebel *et al.*, 2006). Unfortunately, the authors did not report the egg composition before and after dietary fortification, but from the data reported one could expect substantially increased levels of vitamin E in the egg and slightly increased levels of vitamins A, D, B1 and B2, as well as selenium. Gene expression patterns in the intestine were measured at 3 and 14 days of age with an intestinal cDNA-microarray. Between the two groups, 11 genes were found to be differently expressed at both 3 and 14 days of age. Genes that were expressed differently affected intestinal turnover, cell proliferation and development, metabolism and feed absorption. Indeed, this hypothesis needs further clarification; however, it is clear that maternal effect is seen beyond newly hatched chicks. In general this could be an example of maternal nutritional programming responsible for various changes in postnatal life of chicken. In fact, the chicken egg could be an ideal model to study this phenomenon and more research should be carried out in this area. When broiler

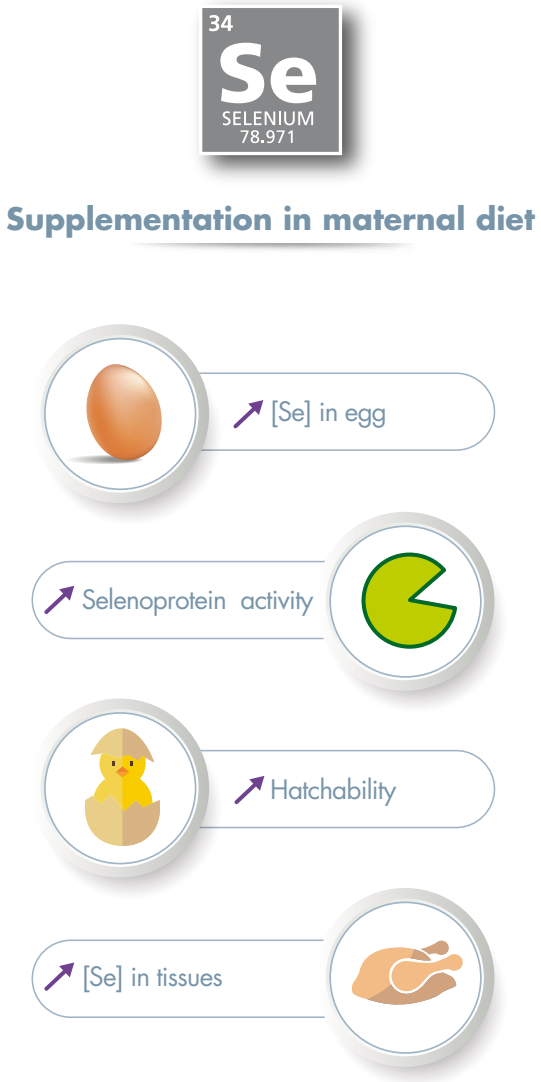

Figure 5.8. Maternal effect of selenium.

breeder diets were supplemented with Se-yeast, gene expression analysis revealed that the quantity of gene transcripts associated with energy production and protein translation were greater in the oviduct of the experimental birds in comparison to control breeders. Targets up-regulated by Se-yeast, included genes encoding several subunits of the mitochondrial respiratory complexes, ubiquinone production and ribosomal subunits (Brennan *et al.*, 2011).

5.2.5 The optimal selenium concentration in eggs: lessons from wild birds

There is no data available on testing optimal Se levels in the egg providing best protection for the developing embryo. However, our data on Se concentrations in eggs of free living wild birds in the UK and New Zealand indicate that they are similar or slightly higher than those obtained with laying hens supplemented 0.3 mg/kg Se in organic form (Pappas *et al.*, 2006b). It has been suggested that whilst for the past 150 years our diet has changed substantially, our genes have not. In particular, animal-derived food composition has changed dramatically, as a result of using cheap feed ingredients. The meat from animals in the wild and chicken eggs produced under completely natural conditions has quite different composition, when compared to commercially produced meat and eggs. Indeed, in many cases decreased Se levels in feeds and foods are the consequences of our agricultural practices, including soil acidification and the use of synthetic fertilisers containing sulphur and phosphorus. Therefore, eggs or meat produced 100-200 years ago, using free-range methods and fed on natural feed sources, grown on well-balanced soils, would have much higher concentration of Se than we currently find in animal products from many European and Asian countries.

Eggs of domestic chickens were studied because they are most consumed around the world (i.e. of most commercial interest) and easily available for analysis. In the absence of comparative data on eggs of other species, there is often a tendency to assume that results obtained for the chicken can be applied to avian eggs in general. While such generalisations may have some validity when considering the macronutrients of the egg such as total protein and total lipids, they can be totally misleading with regard to many of the egg's micronutrients. For example, it has recently become clear that the levels of omega-3 PUFAs, vitamin E and carotenoids in eggs not only show profound interspecies variations, but also are markedly higher for free-living species compared with their domesticated or captive counterparts (Surai, 2002a).

The Se concentrations of the yolks of 14 free-living avian species were measured, including; the black coot, common moorhen, lesser black-backed gull, American coot, yellow headed blackbird, Brewer's blackbird, house sparrow, barn swallow, tree swallow, Canada goose, American crow, blackbird, song thrush, and starling (3 from UK, 8 from Canada, 3 from New Zealand) (Pappas *et al.*, 2006b). The results varied over a 6-fold range, from 394 to 2,238 ng Se/g wet yolk, with a mean value of 1,040±524. UK species had a mean concentration of 522±192 (ng/g), compared to mean values of 1,194±584 and 1,147±200 respectively for the eggs collected in Canada and New Zealand. Therefore, Se concentration in the eggs from Canadian and New Zealand birds were respectively 2.3- and 2.2-fold greater than the mean value for the eggs from UK birds. The mean value from all the free-living species studied was nearly 10-fold higher than the concentration achieved by hens on the control diet. Even the species with the lowest level of yolk Se (the black coot) incorporated nearly 4 times more Se into its yolk when compared with the domestic chicken. Supplementation of the hens' diet with organic Se, only raised the Se concentration of the eggs into the lower end of the range observed for the wild birds. This data raises the question of

the adequacy of the egg Se concentrations currently achieved in commercial poultry production.

These free-living species of bird consume a variety of diets, which may include; earthworms, insects, seeds, grasses and aquatic plants and invertebrates. Nevertheless, each of these very different dietary strategies seems able to support the incorporation of Se into eggs to a much greater extent than is achieved by the basal ingredients of a standard chicken diet. It would be interesting to compare the Se content of eggs of housed chickens with those of free range chickens or of the wild jungle fowl (*Gallus gallus*, the ancestor of the domestic breeds). However, the possible effects of genetic selection for rapid growth or high egg output on the ability of the domesticated chicken to incorporate Se into eggs should also be considered. The mean Se concentration in yolks of the Canadian species was more than twice that of the UK birds. This may seem consistent with the higher concentrations/bioavailability of Se in North American compared with European soils, with consequent repercussions on Se intake throughout the food chain. However, it should be stressed that the UK data was derived from only three species, with no direct comparisons between the same species from the two locations, where eggs were sampled. Also, the three species of birds from New Zealand are not native to that country but were introduced from Europe.

Similar evidence of high Se concentrations in wild water fowl, were seen in the eggs of little egrets, black-crowned night herons and bridled terns from coastal areas of Hong Kong (Lam *et al.*, 2005). In tissues of the seabirds from the Barents Sea (Savinov *et al.*, 2003), Alaska and arctic Russia (Stout *et al.*, 2002) as well as in bald eagles from Adak Island, Alaska (Stout and Trust, 2002) selenium levels were several-fold higher in comparison to domestic chickens. Furthermore, high Se concentrations were reported in eggs from tree swallow, bank swallow and house wren (Dickerson *et al.*, 2002) and comparatively high Se levels were reported in wild turtle eggs (Lam *et al.*, 2006).

5.3 Selenium for commercial layers

An optimal Se status of layers is an important factor responsible for their antioxidant defences and increased organic Se supplementation (from 0.15 to 0.30 mg/kg) was associated with improved antioxidant system evidenced by increased concentrations of ascorbic acid, retinol and alpha-tocopherol, activities of catalase and SOD as well as the total antioxidant status (TAS) of serum (Zdunczyk *et al.*, 2013). Similarly, organic Se in turkey diets enhanced SOD activity, but not affect the antioxidant capacity of blood (Jankowski *et al.,* 2011; Mikulski *et al.,* 2009).

5.3.1 Selenium transfer to the egg and its protective effects

The supplementation of the diet with Se is an effective way to increase Se concentrations in whole egg (Čobanová *et al.*, 2011; Jiakui and Xiaolong, 2004; Jlali *et al.*, 2013; Kralik *et al.*, 2009; Paton *et al.*, 2002; Payne *et al.*, 2005; Scheideler *et al.*, 2010; Surai,

2000; Tufarelli *et al.*, 2016b; Utterback *et al.*, 2005). In fact, it is well appreciated that organic Se is transferred to the egg with higher efficacy than sodium selenite (Bennett and Cheng, 2010; Čobanová *et al.*, 2011; Jlali *et al.*, 2013; Kralik *et al.*, 2009; Pan *et al.*, 2007; Paton *et al.*, 2002; Payne *et al.*, 2005; Tufarelli *et al.*, 2016b; Utterback *et al.*, 2005;). Indeed, as mentioned earlier, the main form of Se in the egg is SeMet (Lipiec *et al.*, 2010) which cannot be synthesised by chickens/animals and therefore Se concentration in the egg is, to much extent, is a reflection of the SeMet concentration in the diet. SeMet is nonspecifically incorporated into the structural proteins when synthesised (Navarro-Alarcon and Cabrera-Vique, 2008; Schrauzer, 2001, 2003; Schrauzer and Surai, 2009) and thus increases the Se deposit in all tissues (Surai, 2002c, 2006; Surai and Fisinin, 2016e). Moreover, the absorption mode of both Se forms appeared different, leading to lower apparent digestibility of inorganic sources than organic sources as reported in various studies (Briens *et al.*, 2013; Choct *et al.*, 2004; Yoon *et al.*, 2007). Therefore, the amount of Se in eggs depends on source/form and level of Se added (Bennett and Cheng, 2010; Latshaw and Biggert, 1981; Payne *et al.*, 2005; Surai, 2006). In layers Se availability from a basal diet containing 0.18 mg Se/kg was shown to be 85.5%, while in the same diet supplemented with 0.3 mg/kg sodium selenite or Se-yeast the Se bioavailability is indicated to be 51.6 and 67,2% respectively (Chantiratikul *et al.*, 2018).

In an experiment conducted in Italy 48 ISA brown laying hens were divided into 3 treatment groups: a control group fed a basal diet containing 0.11 mg Se/kg of feed; a group fed a basal diet plus 0.4 mg/kg of feed of Se from SS; and a group fed a basal diet plus 0.4 mg/kg of feed of Se from SY (Invernizzi *et al.*, 2013). It was found that feed intake, egg mass ratio, and production performance were not affected by Se supplementation, regardless of the Se source. However, egg weight (+3.61 and +2.95%), eggshell weight (+4.26 and +5.38%), and eggshell surface (+2.43 and +1.96%) were significantly higher in the SS and SY groups than in the control, whereas breaking strength was significantly increased in the SY group. Furthermore, eggs from the SY group had higher Se levels than the SS group (Invernizzi *et al.*, 2013).

In a recent experiment the effect of adding Se-fertiliser during cereal (feed) production on the Se concentration in laying hen eggs was investigated. In particular, *Tritordeum*, a new cereal from a cross between durum wheat and wild barley species was produced using selenate as Se-fertiliser (Tufarelli *et al.*, 2016a). No difference was observed among dietary treatments on feed consumption and efficiency, egg mass, and laying rate, whereas egg yolk Se and vitamin E contents as well as liver and plasma Se levels were significantly increased as a result of replacement of sodium selenite with a natural organic Se from Se-enriched grains.

Indeed, Se from organic sources is more bioavailable than inorganic Se and it seems likely that L-selenomethionine shows higher Se transfer to eggs than Se-enriched yeast. For example, the effect of source and dosage of Se supplementation on Se in eggs and blood variables has been recently investigated (Delezie *et al.,* 2014). Ten treatments were used with 18 laying hens per group. The control diet was supplemented for 8 weeks with L-selenomethionine, Se-enriched yeast, or sodium selenite at 0.1, 0.3,

or 0.5 mg/kg of Se. Significantly higher Se levels in serum and egg contents were reached for the Se-supplemented groups compared to the control. No effect of Se source or dosage was observed on serum GSH-Px activity. The Se supplementation level in feed was reflected in the eggs, and the dose response was most pronounced for L-selenomethionine, followed by Se-enriched yeast, and was least when Se was added as sodium selenite. Similar results in terms of different efficacy of transfer of Se from various Se sources to the egg were reported by other authors as well. For example, in an experiment conducted in China, 630 131-day old brown laying hens were randomly assigned to 7 treatments for 168 days (24 wks) with 6 replicates of 15 hens per replicate. The SS and SY animals received a supplemented cornmeal and soybean diet that supplied a total Se 0.3 mg/kg, whereas SeMet was added at 4 different levels to the total Se at 0.1, 0.3, 0.5 and 0.7 mg/kg (Jing *et al.*, 2015). There were no differences in the performance of laying hens fed with organic and inorganic Se sources at the same level of supplementation. However, all hens fed the Se-supplemented diet showed improved antioxidant defences indicative by higher GSH-Px activity, higher SOD activity and lower MDA in plasma. Furthermore, Se supplementation was associated with greater Se contents in egg yolks, albumen, leg muscle, breast muscle, liver, and plasma compared to hens fed the control diet. In addition, the organic sources (SY and SeMet) exhibited a greater ability to increase the GSH-Px activity and Se content in albumen, leg, and breast muscles in comparison to the SS that was added at 0.3 mg Se/kg. In addition, hens fed a diet with SeMet accumulated more Se in albumen, leg, and breast muscle than those fed diets with SY (Jing *et al.*, 2015). It seems likely that pure SeMet is a preferable Se source for layer diet supplementation providing a maximum efficacy of Se transfer to the egg, however, as mentioned earlier commercial usage of such Se source is restricted due to its stability issues.

It seems likely that a new stable source of Se in the form of OH-SeMet could be a next step in the development of effective Se sources for poultry and animal diets. In fact, it was shown that the eggs from hens fed OH-SeMet at 0.2 mg/kg exhibited greater Se concentrations than those fed SY at 0.2 mg/kg, indicating a better efficiency of OH-SeMet to deposit Se into egg than SY. In addition, bioavailability of OH-SeMet was found to be 28.8% greater than SY (Jlali *et al.*, 2013). Interestingly, the better bioavailability of OH-SeMet compared to SY appeared as early as the eighth day of supplementation. After the first week of experiment, the supplementation of OH-SeMet at 0.2 mg Se/kg of diet was sufficient to demonstrate 18% greater egg Se deposition as compared to SY at same level of addition. The total Se deposited in breast muscle also confirmed the greater availability of Se from HMSeBA than SY. Indeed, when comparing the organic Se sources (OH-SeMet vs SY), the muscles of hens fed OH-SeMet at 0.2 mg/kg showed 28.05% more Se deposited than those fed SYat 0.2 mg/kg treatment (Jlali *et al.*, 2013). Indeed, the chemical form of Se in the feed additives can strongly determine the amount of Se uptake and its deposition in egg and muscle of laying hens. About 40 years ago, Cantor *et al.* (1975) have suggested that biological availability of dietary Se depended primarily on its chemical nature rather than on its digestion or absorption characteristics in the intestine. In a recent trial, laying hens were fed two corn-soybean meal-based diets comprising a control basal diet without Se supplementation and a test diet supplemented with Se at 0.2 mg/kg from OH-SeMet.

No difference was observed among dietary treatments on feed intake, egg weight and laying rate, whereas egg yolk fatty acid profile (percentage of PUFAs, including linoleic acid, arachidonic acid and docosahexaenoic acid) and vitamin E content were significantly increased due to hydroxyl-SeMet supplementation. Furthermore, hens fed Se-supplemented diet exhibited greater egg yolk total Se contents (Figure 5.9; Tufarelli *et al.*, 2016b).

The advantages of using organic selenium in commercial laying hens are related to:
- improved egg shell quality;
- maintenance of egg freshness in commercial stressful conditions;
- maintenance of egg production in commercial stressful conditions;
- possibilities of producing Se-enriched eggs.

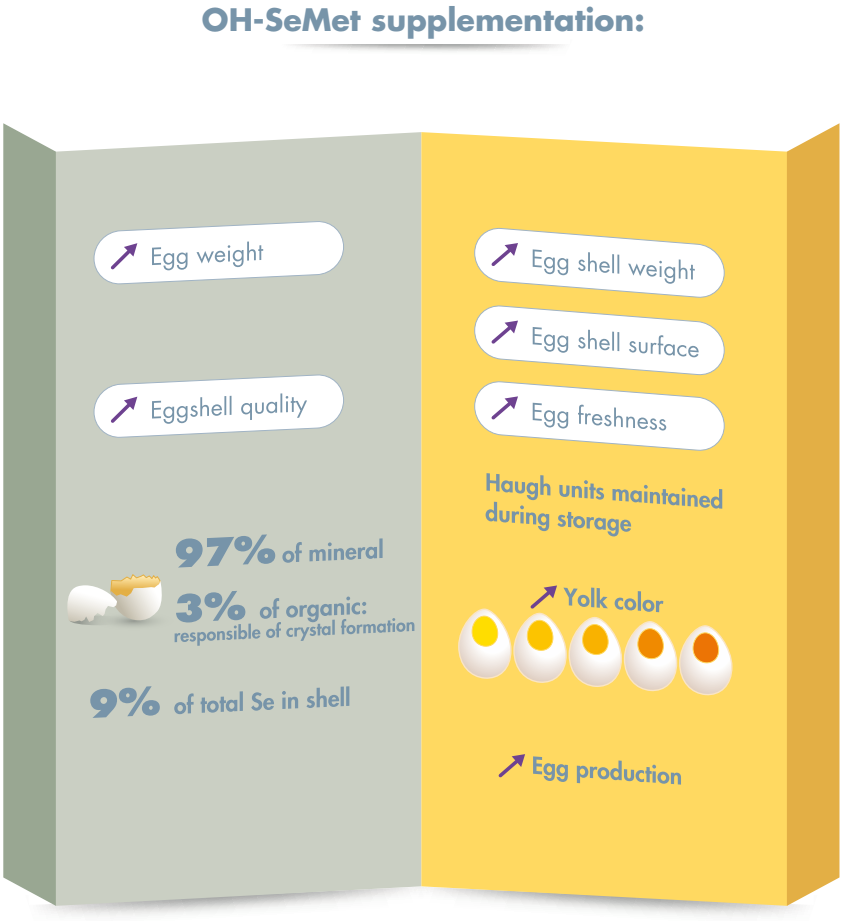

Figure 5.9. Organic selenium is better transferred to egg than mineral selenium.

5.3.2 Selenium and egg shell quality

It is well recognised that eggshells consists of about 95% of minerals and 5% of organic matrix. Recent evidence indicates that this organic matrix is responsible for the regulation of crystal formation in the developing shell. This means that this small part of the egg shell can substantially affect its quality. Since organic Se is an integral part of this matrix, it has been suggested that the form in which Se is supplied could affect shell quality and information is accumulating to substantiate this claim. For example, Paton and Cantor (2000a) showed an increase in shell breaking strength, when organic Se was fed to Babcock laying hens at 80 weeks of age. Dietary supplementation with organic selenium (0.3 or 0.5 mg/kg) was also shown to significantly increase the thickness of the shell of fresh eggs at 34 weeks, as well as increasing egg production and yolk weight (Pourreza and Pishnamzi, 2006). Similarly, replacement of 50% selenite (total supplemental Se 0.4 mg/kg) in the laying hen diet by organic Se was associated with a significant increase in egg shell weight and thickness (Klecker *et al.*, 1997, 2001). Trials in other poultry species showed that including high selenium (5.7 mg/kg) wheat at the level of 60% into quail diets was associated with increased Se content in tissues by 3- to 13-fold and significant increases in eggshell thickness (Stoewsand *et al.*, 1978). Replacing sodium selenite (0.2 mg/kg) in the diet with the same amount of organic selenium significantly increased egg specific gravity after 9 weeks (Renema, 2004). Furthermore, improvements in egg shell quality (weight, thickness and specific gravity), Haugh units and increased yolk and albumin weight were observed.

Details of Se accumulation in different parts of the egg were studied at the Scottish Agricultural College, UK. The diets were designed to contain less than 0.1 mg/kg for the non-supplemented group (no added Se) and about 0.5 mg/kg for the supplemented diets (Karadas *et al.*, 2005). Se supplementation significantly increased the Se concentration in all parts of the egg. In detail, Se concentration in shell, albumen, perivitelline membrane, yolk and shell membrane increased from 13, 30, 66, 85 and 131 ng/g for the non-supplemented treatments to 77, 249, 262, 516 and 854 ng/g for the Se supplemented treatments, respectively (Figure 5.10).

These data show that selenium is found in all parts of the egg, including shell and membranes. In fact, the highest Se concentration was detected in the shell membrane and Se concentration in the shell was comparable to that in the albumin. Selenium concentration in quail shells ranges from 122.5 to 186.5 ng/g, which represent about 12% of total egg Se and could be an additional source of Se during embryonic development (Surai *et al.*, 2004). This was demonstrated by Golubkina and Papazyan (2006); there was a significant decrease of Se concentration in eggshell (by 26%) and shell membrane (by 39%) as a result of egg incubation and embryo development. Organic Se dietary supplementation was associated with an increase Se concentration in the egg shell (by 36%) and shell membrane (by 24%). It is interesting to note that Se concentration in the shell of the eggs enriched with organic Se decreased during egg incubation only by13%, while in the shell membrane Se concentration did not change (Golubkina and Papazyan, 2006). This could mean that an increased Se concentration

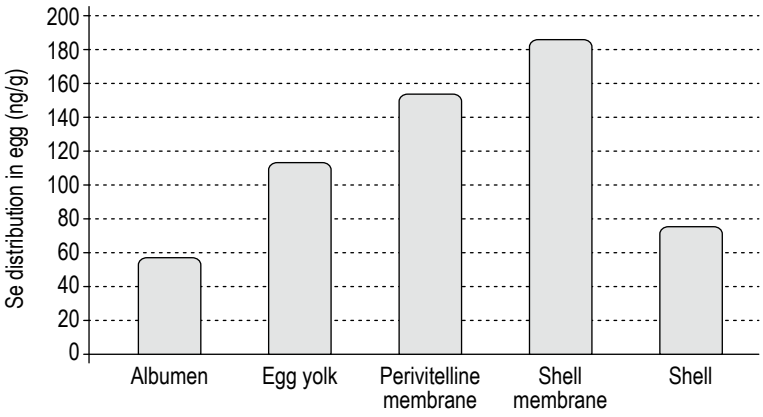

Figure 5.10. Selenium distribution in egg, ng/g (adapted from Surai, 2006).

in the egg yolk and egg albumin provided enough Se for the developing embryo and less Se is used from other sources (e.g. egg shell and shell membrane).

In comparison, shells of eggs of roseate terns (*Sterna dougallii*) and herring gulls (*Larus argentatus*) in the wild contain about 1-5% of the total egg Se (Burger, 1994). Inclusion of organic Se in quail diets at a level of 0.5 mg/kg, significantly increased Se concentration in the shell (231.2-458.3 ng/g). Se concentration in egg shells from organic Se was almost double that of the control birds, 307.6±40.5 ng/g compared to 152.6±11.4 ng/g. Furthermore, in Se-enriched eggs the shell contained about 9.2% of the total egg selenium (Surai *et al.*, 2004a,b). This change could possibly affect shell structure and could potentially be an additional source of Se for the developing embryo. However, selenium excess could be detrimental for egg production and shell quality. For example, in Japanese quail hens 7 mg/kg of dietary Se depressed egg weights with no effect on eggshell thickness (Stoewsand *et al.*, 1978a).

Additionally, it is possible that medullar bone in laying hens could, to some extent, accumulate Se and be available for shell formation. It is interesting that both Se deficiency and Se excess significantly decreased the biomechanical strength of rabbit bones, while bones belonging to the Se-adequate control group always had the largest modulus of elasticity (Turan *et al.*, 1997). Molecular mechanisms responsible for the Se effect on shell and bone formation are not yet fully known but the presence of Se in bones has been demonstrated. For example, in humans about 16% of total body Se was found in bones (Zachara *et al.*, 2001) and a relationship between, active vitamin D metabolites and the Se-dependent enzyme thioredoxin reductase (TrxR), was established (Schutze *et al.*, 1998, 1999). In fact TrxR was identified as a $1,25(OH)_2D_3$-responsive gene, providing a link between the induction of a differentiation program by $1,25(OH)_2D_3$ and the expression of the Se-responsive TrxR system in human osteoblasts. Similarly, the expression of TrxR and its regulation by $1,25(OH)_2D_3$ and selenite in monocytes, might be important for the induction of differentiation and

maintenance of function. An effect of TrxR activity on $1,25(OH)_2D_3$ would be expected and this could be an important link between Se and bone metabolism. On the other hand, antioxidant properties of various selenoproteins could also be important in maintaining antioxidant protection of the oviduct during egg shell formation. Effects of organic Se supplementation (0.25 mg/kg vs unsupplemented diet) of commercial layers diets were studied in Brazil using 5,000 Lohmann Selected Leghorn layers (Evencio *et al.*, 2006). Improvements in the ciliated epithelium of magnum, isthmus and egg shell gland, were seen in the birds supplemented with organic Se compared to control. Furthermore, organic Se supplemented layers also had higher secretory activity of many tubular glands. In the supplemented birds, a greater uniformity of the ciliated epithelium, mucous and secretory cells was observed, along with greater secretion in the lamina propria glands (Takata *et al.*, 2006).

5.3.3 Selenium and egg freshness

Organic selenium is transferred from the diet to the egg, stimulating GSH-Px in the egg yolk, white and probably in the perivitelline membrane (Figure 5.11). This probably leads to decreased lipid and protein oxidation and helping to maintain Haugh units at a high level during egg storage. Egg freshness is one of the most important parameters determining consumer perception and demand. During storage, egg freshness decreases and this process is associated with biochemical changes in composition and structure of egg membranes. In Brazil, organic Se (0.1-0.3 mg/kg) was added on top of a commercial premix, containing 0.15 mg/kg Se from an inorganic source (Pan *et al.*, 2004). A significant improvement in yolk colour and Haugh units, was observed as a result of organic selenium supplementation (Rutz *et al.*, 2003). These results are in agreement with other data from the same authors (Pan and Rutz, 2003). These data are commercially important, indicating the possibility of improved maintenance of egg quality during storage. The effect of Se in the form of sodium selenite or Se-yeast (0.3 or 0.5 mg/kg) in diets for laying hens, was studied (Boruta *et al.*, 2007). Both forms

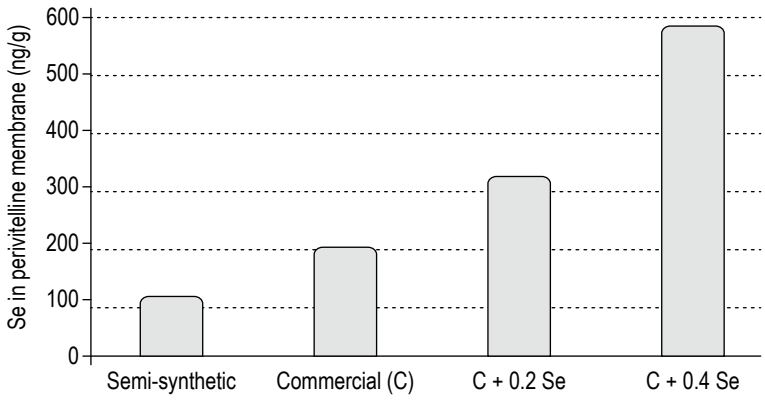

Figure 5.11. Selenium in perivitelline membrane, ng/g (adapted from Surai, 2006).

of Se improved egg freshness reflected by an increase in Haugh units. The greatest concentration of Se (565 ng/g) was found in eggs from laying hens fed 0.5 mg/kg Se in the form of Se-yeast, giving approximately 28-30 μg Se/egg which is about 50% recommended daily allowance for adults.

There are also implications for the incubation of eggs, since a long period of storage is associated with decreased hatchability. Indeed, organic selenium in the form of Se-yeast added to breeder diets improved Haugh units in eggs stored for 2 weeks (Pappas *et al.*, 2004, 2004a). Therefore, inclusion of organic Se in the diets of laying hens can be used to maintain egg quality during storage. Inclusion of organic selenium in chicken diets can also increase Se concentration in the perivitelline membrane (Surai *et al.*, 2004c), which could be an additional mechanism explaining how organic Se affects egg freshness during storage. The effect of Se on egg freshness probably depends on the age of the hens, composition of the diet and conditions of egg storage. Therefore, not all trials show benefits of organic Se on egg freshness, for example Paton and Cantor (2000) were not able to show an effect of dietary Se on Haugh units.

When considering molecular mechanisms of positive effects of Se on egg freshness initially most of attention has been paid to prevention of lipid oxidation of the perivitelline membrane. Indeed, as a part of a range of selenoproteins (GSH-Px, TrxR, and others) Se can decrease lipid peroxidation, however, it seems likely that water-holding capacity of proteins plays an important role in maintaining albumin quality during storage. In fact, protein oxidation has received tremendous attention due of its detrimental changes in meat, and, probably, in eggs during storage (Estevez, 2011, 2015; Huff-Lonergan and Lonergan, 2005; Lund *et al.*, 2011). Furthermore, a selenoprotein MsrB is responsible for prevention of protein oxidation (Surai, 2006) and its increased expression due to organic Se supplementation could be an important mechanism of maintaining Haugh units in eggs during storage.

5.3.4 Selenium and maintenance of egg production in commercial stressful conditions

An important advantage of organic selenium for laying hens is related to the maintenance of egg production during the peak production period. The problem is that even mild stress in a commercial egg production facility can affect peak egg production. Once egg production is decreased it is almost impossible to return it to the original level. Since Se provides additional antioxidant protection, it could help to overcome stress at this time and maintain high egg production at the peak of the production cycle. The effect of organic Se (0.3 mg/kg) on performance and egg quality of laying hens, in comparison to control birds without Se supplementation, was studied from 17 up to 38 weeks of age (Sara *et al.*, 2007). Organic Se supplementation was associated with increased egg production by 3.27%, decreased feed consumption per egg produced by 2.89%, increased egg yolk (15.0 vs 13.98 g) and increased egg white (38.95 vs 36.89 g). Furthermore, a partial or full replacement of sodium selenite by organic selenium was shown to increase egg production, egg weight and weight of egg parts including shell, yolk and albumin (Rutz *et al.*, 2003). Organic Se increased

yolk and white weights relative to control and there were trends towards improved egg production, weight and FCR (Pan and Rutz, 2003). Payne *et al.* (2005) also observed a linear increase in egg weight as dietary Se levels increased.

In an experiment where the diet contained 15% cottonseed meal, organic Se supplementation increased egg weight. Egg weight was 62.5 g compared to 60.6 g in eggs from hens fed SS or 60.2 g in the unsupplemented control group (Stanley *et al.*, 2006). Egg yolks were also largest from the organic Se group, 17.4 g compared to 15.9 g in the control group. Organic Se also improved specific gravity in eggs. Attia *et al.* (2010) indicated that addition of organic and inorganic Se to the diet of layers, accommodated in a semi-open house in Egypt, improved the productive and reproductive performance of Gimmizah breeding hens. In fact, egg weight and egg mass significantly increased and FCR significantly improved due to Se supplementation (0.15 or 0.30 mg/kg) compared with hens fed the control diet containing 0.1 mg Se/kg. However, there was no difference between sodium selenite and Se-yeast groups. It is interesting to mention that in a study conducted in Czech Republic (Skřivan *et al.*, 2013) replacement of sodium selenite (0.15 mg/kg) with Se-yeast improved the laying performance but it was not different from the unsupplemented control group containing 0.09 mg Se/kg. In addition, Arpášová *et al.* (2009) showed egg weight improvement with addition of Se at 0.4 and 0.9 mg/kg of diet as SY compared to the control group or SS supplemented group during a long (9-month) study, but there were negative effects of SS or Se-yeast on some shell quality traits. Similarly, in a study by Stępińska *et al.* (2012), the average egg weight was higher in turkey hens fed a diet with organic selenium than in layers receiving inorganic selenium, while an increase (from 0.15 to 0.3 mg/kg) in organic Se supplementation of layers contributed to a significant increase in egg weight (Zdunczyk *et al.*, 2013). Furthermore, Mohiti-Asli *et al.* (2008) have shown that supplementation of hens' diets with Se can improve the internal egg quality, including yolk and albumen weight and quality, as well as decrease the susceptibility of egg yolk to lipid peroxidation during storage but without any effect on shell breaking strength. In a recent study it was shown that dietary supplementation of organic Se could improve performance parameters and egg oxidative stability in laying hens, with the highest impact in diets containing oxidised (high peroxide values) fat sources (Laika and Jahanian, 2015). Furthermore, the effect of Se depended on the environmental conditions and background Se level in the diet. In an experiment conducted in Pakistan experimental birds (native Aseel chicken) were maintained in battery cages from 22 to 42 weeks. It was shown that the layers which received a diet supplemented with organic Se in the form of Se-enriched yeast had a range of advantages in comparison to sodium supplemented or unsupplemented birds. They gained sexual maturity earlier, and showed increased body weight, egg production, egg mass, FCR/dozen eggs, FCR/kg egg mass, and selenium contents in the whole egg, egg yolk and egg albumen (Zia *et al.*, 2016).

It is necessary to underline that when a balanced diet is used, in most cases the form of Se does not affect layer performance (Bennett and Cheng, 2010; Chantiratikul *et al.*, 2008; Jiakui and Xialong, 2004; Jlali *et al.*, 2013; Mohiti-Asli *et al.*, 2010; Pan *et al.*, 2011; Pavlovic *et al.*, 2010; Payne *et al.*, 2005; Scheideler *et al.*, 2010; Tufarelli *et al.*,

2016b). However, in stress conditions one can expect a protective effect of organic Se and probably some of the aforementioned results showing a positive response from replacing sodium selenite by organic Se sources can be related to various stresses. Indeed, if an experiment is conducted under ideal conditions Se requirement is very low and clearly can be met by the Se in the basic diet. On the other hand, commercial poultry production is associated with a range of environmental and technological (Surai and Fisinin, 2016a), nutritional and internal stresses (Surai and Fisinin, 2016b) and in such conditions organic Se providing SeMet can help building Se reserves in the body, an important element of the effective strategy of fighting stresses (Surai and Fisinin, 2014, 2016c,d). Furthermore, organic Se can have positive protective effects on the avian gut (for details see Chapter 8) and immunity (for details see Chapter 7). By preventing damages to the intestinal villi and maintaining immune cell communications in stress conditions of commercial poultry production, organic Se can provide beneficial effects for layers, as well as for breeders and broilers.

5.3.5 Opportunities for producing Se-enriched eggs

Another advantage of organic selenium for commercial layers is related to possibility of producing Se-enriched eggs, which will be described in details in Chapter 6 of this book. In an experiment conducted in the Netherlands, Hy-Line brown laying hens aged 40-50 weeks were fed on a diet containing 0.05 mg/kg feed-derived Se (De Lange and Oude Elferink, 2005). This diet was supplemented with 0.45 mg/kg Se in the form of Se-yeast, selenite or selenate and it was shown that Se-yeast improved FCR compared to selenite by 2% (P=0.02). After 8 weeks of feeding, results showed that the diets containing organic Se increased the Se content of eggs more than the two inorganic sources, 0.52 vs 0.33 mg/kg egg. There were no significant difference between selenate and selenite on their effect on egg Se concentration, 0.34 vs 0.31 mg/kg eggs. It was shown that feeding selenium for 2 weeks is not long enough to achieve the maximum Se level in eggs. However, after feeding extra Se for eight weeks, the utilisation of Se, as shown by output in eggs/input by feed, was significantly higher (49%) in eggs from the Se-yeast group, compared to selenate (30%) or selenite (28%). It was concluded that when using 0.5 mg/kg Se in organic form, it is possible to produce eggs containing about 30 μg Se/egg. In another experiment conducted in the USA, the basal diet contained 0.1 mg/kg Se and it was supplemented with 0.15, 0.3. 0.6 and 3 mg/kg Se in the form of sodium selenite or Se-yeast. Se concentrations in eggs of hens fed on selenite treatments were 0.249, 0.284, 0.299, 0.327 and 0.641 mg/kg, whilst the eggs from Se-yeast fed hens contained 0.366, 0.495, 0.670 and 2.207 mg/kg Se, respectively (Payne *et al.*, 2005). Clearly, inclusion of organic Se into the layers diets is an effective way to produce Se-enriched eggs.

5.4 Selenium for broilers

Selenium is essential element for growing chickens as an integral part of the antioxidant system of various tissues and the whole body. Therefore, an optimal Se status provides adequate synthesis of selenoproteins, responsible for protection against oxidative

stress imposed by various stress-factors of commercial chicken production. There is a range of publications indicating a positive response in antioxidant defences in growing chickens due to Se supplementation with a specific emphasis to organic Se as the most effective source of dietary Se supplementation (Surai, 2006; Surai and Fisinin, 2016e).

5.4.1 Selenium and antioxidant defences in broilers

Indeed, recent results clearly showed that activities of serum GSH-Px, total SOD, the abilities to inhibit hydroxyl radical ($OH^{\cdot}$) and total antioxidant capacity (T-AOC) were significantly higher in selenium yeast fed chickens than in sodium selenite groups, while lipid peroxidation expressed as the contents of MDA in selenium yeast groups were significantly lower than that of sodium selenite-fed chickens (Chen *et al.*, 2014). Similarly, whole blood GSH-PX activity was significantly higher due to the addition of 0.35 mg/kg organic selenium than that of inorganic selenium (Guo and Yuan, 1998) and the highest tissue GSH-Px activity was shown to be a result of organic Se supplementation (Wang and Xu, 2008). In plasma, supplemental SeMet at 0.225 mg/kg increased T-AOC, GSH-Px, T-SOD, CAT activities, GSH concentration and decreased MDA production, compared with the unsupplemented control and broilers fed SS diet (Jiang *et al.*, 2009). In breast muscle, the addition of SeMet significantly elevated T-AOC, GSH-Px, T-SOD, CAT activities, contents of metallothionein and GSH, and reduced carbonyl protein content. While compared with broilers fed SS diet, supplemental 0.225 mg/kg SeMet increased T-AOC, GSH-Px, CAT activities, and GSH content (Jiang *et al.*, 2009). Similarly, the antioxidant status was shown to be greatly improved in broilers of L-SeMet-treated group in comparison with the SS-treated group and was illuminated by significantly increased GSH concentration in serum, liver, and breast muscle, SOD activity in liver; total antioxidant capability (T-AOC) in kidney, pancreas, and breast muscle and decreased MDA concentration in kidney and breast muscle of broilers (Wang *et al.*, 2011a). Recently, an experiment has been conducted to study possible effects of supplementing various concentrations (0, 100, 200, 300, or 400 μg/kg diet) of organic Se on growth performance, carcass traits, oxidative stress, and immune responses in commercial broiler chickens reared in open-sided poultry house under tropical climatic conditions. It has been shown that lipid peroxidation in plasma decreased, while activities of GSH-Px and glutathione reductase in plasma increased by Se supplementation to broiler diets. Furthermore, the cell-mediated immunity (lymphocyte proliferation ratio) increased linearly with dietary Se concentration (Rao *et al.*, 2013).

The current National Research Council (NRC) dietary selenium (Se) requirement for broiler chickens is set at 0.15 mg Se/kg diet (NRC, 1994). It is based on results of studies in 1980-1990 and there is a need to assess Se requirements in today's poultry strains using biochemical and molecular biomarkers. For example, based on enzyme activities in liver, gizzard, and plasma, the minimum Se requirement in today's broiler chick is 0.15 μg Se/g diet; pancreas data indicate that the Se requirement should be raised to 0.2 μg Se/g diet to provide a margin of safety (Li and Sunde, 2016). Furthermore, similar to ideas mentioned above, in commercial stressful condition

this requirement could be substantially higher. Therefore, higher Se concentrations in chicken muscles due to organic Se dietary supplementation are responsible for Se reserves essential in stress conditions when Se requirement is increased while feed consumption and Se provision are decreased (Surai and Fisinin, 2016e). Indeed, in an excellent research trial Payne and Southern (2005a) showed that Se accumulated in chicken muscles due to organic selenium supplementation can be used to support selenoprotein expression (GSH-Px activity) in the case of low Se dietary availability when Se supplementation was stopped.

In a recent meta-analysis, a range of factors affecting Se accumulation in tissues has been examined, including Se source (12 different sources examined), type of chicken (laying hens or broilers), age of birds at the beginning of supplementation, duration of supplementation, year during which the study was conducted, sex of birds, number of chickens per treatment, method of analysis, tissue type, concentration of Se determined and Se added to feed (Zoidis *et al.*, 2014). It was shown that several factors including type of chicken, source of Se, type of tissue and analytical method used affect tissue Se concentration in chicken. It seems likely that several other factors may also affect Se absorption, distribution and retention in chicken tissues. The authors clearly have shown that Se concentration in plasma was positively correlated with that in the liver (r=0.845) and kidney (r=0.953), while Se concentration in blood was positively correlated with that of the liver (r=0.929), kidney (r=0.802) and yolk (r=0.999). Furthermore, in liver Se was positively correlated with that of the breast muscle (r=0.790), leg muscle (r =0.938) and kidney (r=0.988). There were different rates of Se accumulation across the various tissues. In fact, highest accumulation rates were estimated in the leg muscle (b=0.727), liver (b=0.570 and kidney (b=0.499), and lowest or nil in blood (b=0.326) and plasma (b=0.106; Zoidis *et al.*, 2014). It is interesting to note that supplementation with organic selenium not only increased Se concentration, but also reduced Cd concentration in the tissues. Se was negatively correlated with Cd and positively correlated with Zn, Cu and Fe (Pappas *et al.*, 2011). In a study conducted in Turkey the supplementation of organic selenium to diets containing adequate selenium (due to Se in premix) increased plasma, liver, femoral muscle, kidney and heart tissue GSH-Px activity in broilers (Salman *et al.*, 2009).

In an experiment with chickens, from day (d) 0 to d21, three Se sources namely SS, Se-yeast and a new organic Se source, OH-SeMet (SO) were tested at different supplementation levels and compared with an unsupplemented diet (NC; Briens *et al.*, 2013). The different Se sources and levels improved muscle Se concentration compared with the NC, with a significant source effect in the following order: SO>SY>SS. The digestibility of total Se measured between day 20 and 23 was shown to be 24, 46 and 49% for SS, SY and SO, respectively, with significant differences between the organic and mineral Se sources (Figure 5.12, 5.13 and 5.14).

These results confirmed the higher bioavailability of organic Se sources compared to the mineral source and demonstrated a significantly better efficiency of OH-SeMet compared to SY for muscle Se enrichment. Selenium deposition kinetics in muscles and feathers of broilers were determined in order to develop a rapid method to

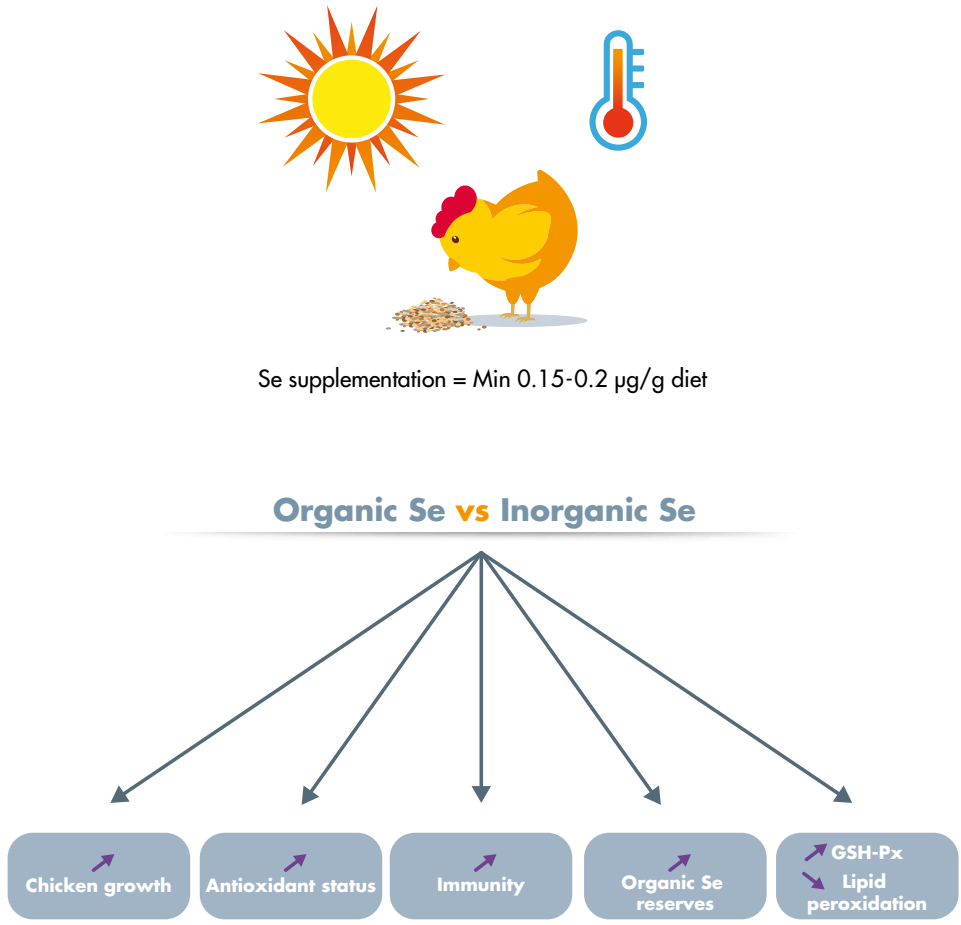

Figure 5.12. Selenium and antioxidant defenses for broilers.

compare bioavailability of selenium sources. Different Se sources, such as SO, SS and SY were compared in broiler chicks from 0 to 21 days of age (Couloigner *et al.*, 2015).

The diets used in the experiment were a negative control, not supplemented with Se and 3 diets supplemented with 0.2 mg Se/kg as SS, SY or SO. At 7 days of age, SO increased muscle Se content compared to day 0, whereas for the other treatments, muscle Se concentration decreased. After 21 days, organic Se sources maintained (SY) or increased (SO) breast muscle Se concentration compared to hatch value, whereas inorganic source (SS) or the non-supplemented group showed a significant decrease in tissue Se concentration. At day 21, Se contents of muscle and feathers were highly correlated (R^2=0.927) (Couloigner *et al.*, 2015). In addition, when compared to SS or Se-yeast, SO demonstrated an unique ability to enrich SeMet and total selenium

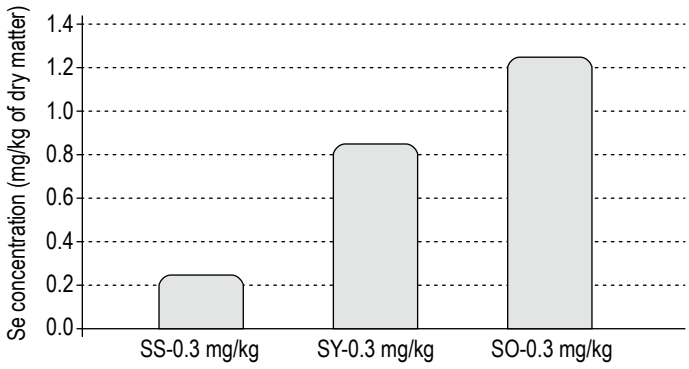

Figure 5.13. Selenium in chicken muscles at day 24 of broiler chicken fed different Se sources and levels (adapted from Briens *et al.*, 2013). SS = sodium selenite; SY = seleno-yeast; SO = OH-SeMet.

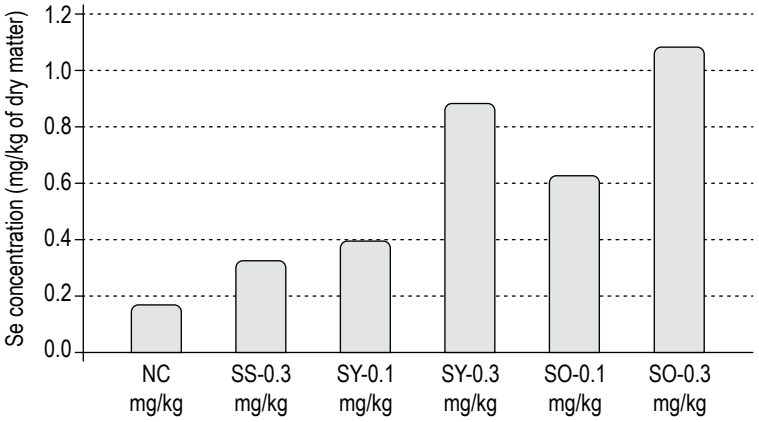

Figure 5.14. Total Se measurements in muscle at day 42 of broiler chicken fed different Se sources and levels (adapted from Briens *et al.*, 2014). NC = unsupplemented control; SS = sodium selenite; SY = seleno-yeast; SO = OH-SeMet.

depositions, to induce the early expression of selenoprotein S and methionine sulfoxide reductase B mRNA and thioredoxin reductase activity, and to enhance the protein production of GSH-Px4, selenoprotein P and selenoprotein U in the tissues of chicks (Zhao *et al.*, 2017).

The advantages of organic Se for broilers include (Fisinin *et al.*, 2008; Surai, 2006):
- increased in growth rate and improvement in FCR;
- decreased mortality and decreased drip loss during meat storage;
- an opportunity to produce Se-enriched chicken meat.

5.4.2 Selenium and chicken growth and development

Positive effects of dietary Se supplementation on chicken growth, development and health could be related to the antioxidant action of Se via various selenoproteins, the activation of thyroid hormones, as well as an improvement in gut health and immunity. It is metabolically very expensive to maintain an activated immune system, with many nutrients re-directed from growth and development, in times of challenge. Therefore, the immunomodulating properties of Se (Song *et al.*, 2006; Surai, 2006) could help the broiler utilise other nutrients efficiently and avoid wasting them by unnecessarily stimulating the immune system. Secondly, the antiviral effects of Se, including protection from damages to intestinal integrity caused by reovirus (Edens *et al.*, 2007), could also contribute to a better FCR. Furthermore, it was shown that in comparison to SS supplemented chickens, feed supplementation with organic selenium results in a more proficient conversion of T4 to T3 (Valcić *et al.*, 2011). The effect of Se supplementation with organic selenium or selenite (0.2 and 0.4 mg/kg), on chick development for the first 2 weeks post-hatch were studied (Papazyan and Surai, 2007). It was shown that level and form of selenium may alter intestinal morphology in broiler chicks. Both feed consumption and FCR were improved in chickens supplemented with 0.2 mg/kg Se in the form of Se-yeast and the relative mass of duodenum was also highest in this group. The addition of selenium from selenomethionine to the feed mixture increased body weight in chickens in comparison to SS fed or unsupplemented chickens (Skřivan *et al.*, 2008a).

The requirement of growing chickens for Se could substantially differ depending on production conditions. Laboratory studies indicate that biochemical indexes of Se status (e.g. GSH-Px activity) could be positively affected by supplementing the diet with a variety of levels of selenium. For example, the inclusion of 0.3 mg/kg Se from baker's yeast to chickens from hatch to day 35 significantly increased GSH-Px activity in erythrocytes, plasma and liver (Arai *et al.*, 1994). On the other hand, commercial results clearly show that sodium selenite is not the best form of selenium, with which to supplement chicken diets. Indeed, for the last few years a great body of evidence has been accumulated indicating that replacement of sodium selenite by organic selenium, beneficially affects broiler performance. In a trial, the body weight of broilers at 42 days of age was 2.38 kg in the control group (diet with basal Se level of 0.28 mg/kg), 2.43 kg after selenite (0.2 mg/kg) and 2.45 kg after Se-yeast (0.2 mg/kg) supplementation (Edens, 2001). Naylor *et al.* (2000) fed diets containing inorganic or organic Se at 0.1 and 0.25 mg/kg, finding that increasing dietary Se markedly improved feed efficiency. These positive effects of organic selenium on FCR were confirmed by Edens *et al.* (2001). In Serbia unsupplemented feed and feed containing organic selenium or SS were fed to broiler chicks. It was shown that organic selenium increased broiler chick performance, while selenium deficient feeds decreased performance and increased chick mortality (Stolic *et al.*, 2002). Broiler diets containing 0.15 mg/kg feed-derived Se were supplemented with 0.15 mg/kg Se in the form of sodium selenite or Se-yeast. Chickens fed diets containing organic selenium had the best production performance, in contrast to selenite-supplemented birds, weight gain was improved by 4.2% and feed efficiency by 9.8%. At the same time mortality was markedly decreased from 6.7%

in selenite-supplemented group to 0.84% in Se-yeast supplemented birds (Vlahovic *et al.*, 1998). Improved growth rate of broilers fed an organic selenium supplemented diet could be related to the increased concentration of the active form of thyroid hormone in serum of chickens supplemented with organic Se (Edens, 2001; Edens and Gowdy, 2004a; Jianhua *et al.*, 2000). An experiment was conducted on 810 broiler cockerels randomly divided into three groups: (1) control – no selenium supplement; (2) 0.3 mg Se/kg supplied in the form of Se-enriched yeast; and (3) 0.3 mg Se/kg, using enriched alga *Chlorella* as an Se source (Ševčikova *et al.*, 2006). The broiler chickens were slaughtered at 42 days of age and higher performance traits were seen in the experimental groups. The live weight of the broiler chickens was 2,431 and 2,425 g for the Se-yeast and *Chlorella* supplemented groups, respectively, in comparison to 2,319 g for the control birds. There were no significant differences between the groups in feed conversion or mortality, but Se-yeast supplementation improved FCR numerically (1.68 vs 1.79). Se concentration in breast muscle increased from 52.1 μg/g in the control to 217.4 μg/g in the Se-yeast group, and similar increases in Se were reported in the thigh muscle (247.9 vs 71 μg/g). Indeed, 100 g of such meat would deliver about 50% of the recommended daily allowance and could be called Se-meat.

A trial involving 2,400 male Ross broiler chicks (1 to 42 days of age) was carried out in Brazil (Arruda *et al.*, 2004). The addition of 0.1 mg/kg as Se-yeast in combination with 0.2 mg/kg Se as SS resulted in an improvement of body weight gain and FCR, when compared to 0.3 mg/kg selenite. In another experiment, the addition of 0.2 mg/kg Se as Se-yeast, in combination with 0.1 mg/kg Se as SS, improved the growth of broilers (Anciuti *et al.*, 2004). It is interesting to note that in the experiment all doses of organic selenium (0.1, 0.2 or 0.3 mg/kg) improved FCR in comparison to birds fed only inorganic selenium. In a study, conducted in Thailand, a total of 1,920 day-old male chicks (Cobb 500) were allocated to 6 treatments, including SS or Se-yeast at 0.2 mg/kg and no Se supplementation (Srimongkol *et al.*, 2004).

Furthermore, Wang and Xu (2008) found that both Se sources (SS and SY at 0.20 mg/kg) had a positive effect on FCR in broiler chickens after 21 days of feeding. The positive effects of organic Se on feed efficiency could be attributed to better feathering in chickens fed diets supplemented with organic Se, particularly during cold stress conditions (Edens, 1996, 1997, 2001). A group of 720 day-old Cobb 500 chicks were allotted, according to a 3×2×2 factorial design, to one of 3 environmental temperatures, 2 levels of Se and 2 sources of Se, and grown for 42 days (Dajhlke *et al.*, 2004). Irrespective of the level of Se in the initial diet, organic selenium improved feathering during the critical phase of broiler chicken thermal homeostasis, up to 21 days of age. Organic selenium induced more rapid whole body feathering in the slow-feathering males as well as in the normal-feathering females (Edens *et al.*, 2001a). It is interesting to note, that in the study the influence of organic selenium was still evident in the feathering from 21 to 42 days of age. Organic selenium at 0.1 mg/kg; improved feather score, decreased 24 h drip-loss, showed markedly higher deposition rate in breast muscle and a lower Se excretion rate compared to sodium selenite (Choct *et al.*, 2004).

In commercial poultry production stress conditions are a common factor decreasing production and reproductive performance of birds. Under these conditions, increased antioxidant supplementation would be beneficial in preventing detrimental effects of such stresses (Surai, 2002a, 2006). Among the natural antioxidants found in poultry feed, Se has a special place as a part of the first, second and third levels of antioxidant defence. Under stress conditions (challenge with *Escherichia coli*), inclusion of organic Se in the feed resulted in improved body weight (2,291 vs 2,004 g), better feed conversion ratio (1.84 vs 1.98) and reduced mortality (5.7 vs 18.3%; Edens, 2001). Challenging birds with pathogenic *E. coli* resulted in increased mortality and decreased body weight, but at the same time, replacement of SS by organic selenium substantially decreased mortality and improved chicken growth. In an experiment conducted in Russia three different feeds were used: a standard feed, a feed in which 1% oil was replaced with rancid oil, and a feed containing 20% of corn naturally contaminated with 37 μg/kg aflatoxin B_1 (AFB_1) (Egorov *et al.*, 2006). To each of feed, 0.3 mg/kg Se either as SS or Se-yeast was added. It was shown that supplementation of Se-yeast instead of SS to the standard, rancid oil or AFB_1-containing feed improved growth by 39, 58 and 68 g, respectively, whilst FCR was reduced by 0.02, 0.06 and 0.05 points. Indeed, in stress conditions the protective effect of organic selenium is more visible. Replacing SS with Se-yeast at 0.2 mg/kg in the diets of growing chicks was associated with significantly increased GSH-Px activity in chicken blood at 2, 3 and 4 weeks of age (Mahmoud and Edens, 2003). This difference in the GSH-Px activity in blood was maintained after heat stress and the response of heat shock protein (HSP70) to heat stress was different between these two groups. In the selenite-fed group it increased but in the Se-yeast fed group it decreased (Mahmoud and Edens, 2003), indicating that the experimentally supplemented birds overcame stress more easily. Furthermore, when chickens were subjected to cold stress and fed aflatoxin-contaminated feed, a combination of 0.1 mg/kg organic Se with 500 mg/kg vitamin E decreased the incidence of pulmonary hypertension syndrome and mortality (Stanley *et al.*, 1998). Similarly, substitution of inorganic Se by organic Se significantly reduced mortality associated with ascites in cold stressed broiler chickens. A combination of organic Se (0.3 mg/kg) with vitamin E (250 mg/kg) in the diet gave the best protection by decreasing ascites-related mortality 10-fold (Roch *et al.*, 2000). Inclusion of organic Se (0.3 mg/kg) into the diet of cold-stressed chickens increased their ability to adapt to stress, shown by increased GSH-Px activity in the liver (Ozkan *et al.*, 2007). It was also shown that Se supplementation of heat-stressed broilers increased the average daily feed intake, Se concentrations in liver and breast muscle, liver GSH-Px activity, serum antibody titers against H5N1 (Re-4 and Re-5 strain) and decreased mortality (Liao *et al.*, 2012). In the experiment, Se-yeast was more effective than SS or Se-proteinate in increasing tissue Se retention. Furthermore, it was shown that GSH-Px activity remained relatively constant, while SOD activity and MDA levels in skeletal muscle were enhanced on exposure to heat stress. In fact, the heat-stressed chicks that received the combined supplementary level of vitamin E and Se had the lowest levels of lipid peroxidation and the highest activity of SOD in the skeletal muscle and dietary Se also caused a significant increase in GSH-Px activity in the skeletal muscle (Ghazi Harsini *et al.*, 2012).

In general, analysis of the published data indicates that selenium supplementation at 0.3 mg/kg in the organic form provides optimum Se status for chicken growth and development (Figure 5.15). However, for immunomodulation higher doses of Se could be required and this needs further investigation.

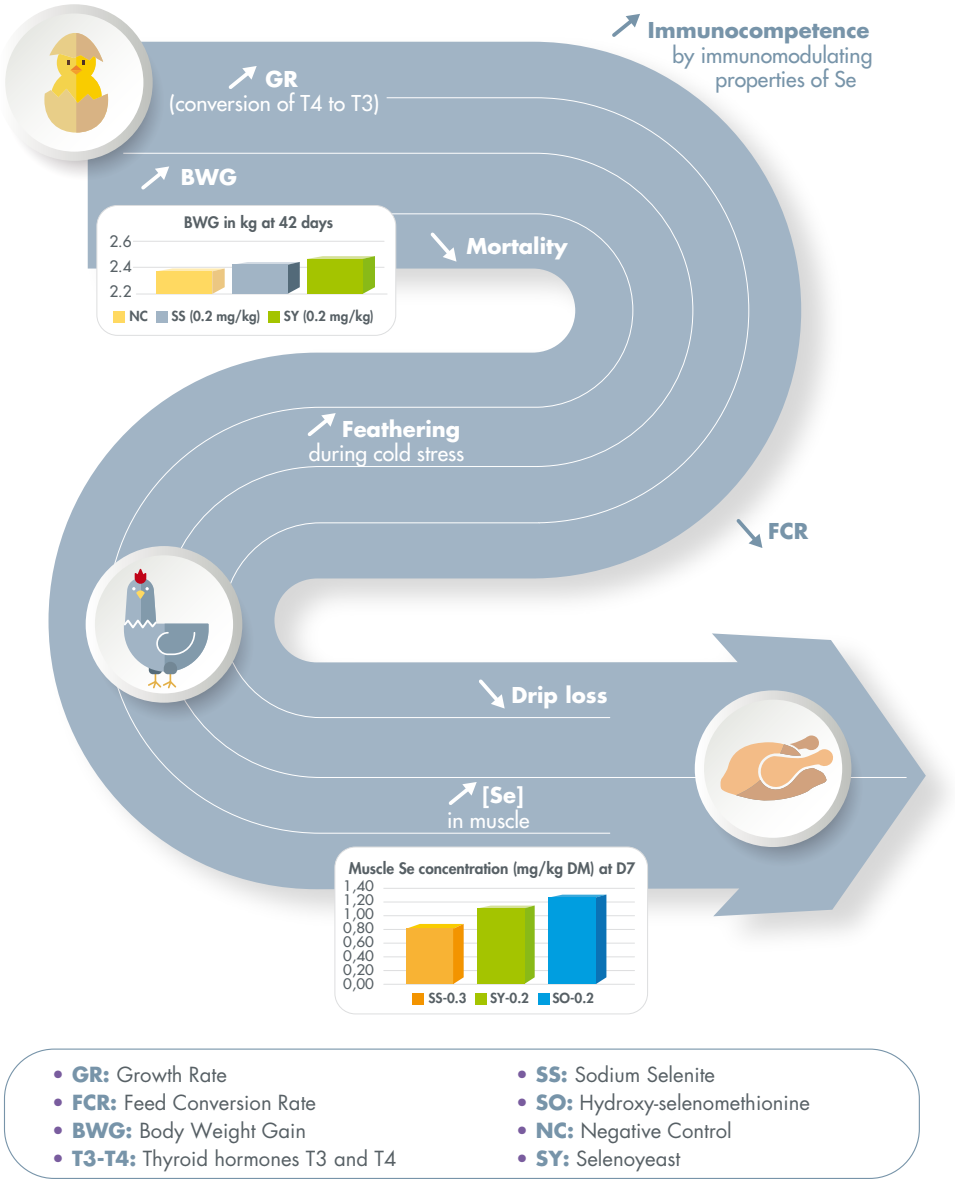

Figure 5.15. Effect of selenium on chicken growth and development.

5.4.3 Selenium and meat quality

It has been shown that only SeMet can be effectively transferred to the muscles (Surai, 2006) and there are species-specific differences in Se levels in muscles (Figure 5.16; Daun and Akesson, 2004). Indeed, an important advantage of organic selenium in the chicken diet is its ability to improve meat quality by decreasing drip loss and lipid peroxidation during meat storage. In an experiment conducted with broilers, their diets were supplemented with 0.05, 0.1 or 0.3 mg/kg organic Se with or without 100 IU vitamin E (Pesut, 2005). It was shown that all levels of supplementation with organic selenium or vitamin E, significantly reduced plasma TBARS at 28 days of age. GSH-Px activity in plasma was also increased in all Se-treatments. It was shown that Se-yeast had similar effects as vitamin E on plasma TBARS, but a combination of Se and vitamin E had an additive effect. In another experiment with a similar design, it was shown that based on TBARS in breast and thigh muscles after freezer storage (-20 °C for 16 weeks), 0.1-0.3 mg/kg organic Se had a similar antioxidant effect as 100 IU/kg vitamin E (Pesut *et al.*, 2005a).

These data are in line with the earlier results of Surai and Dvorska (2002a,b). Lipid peroxidation (TBARS) in frozen-raw chicken meat stored for 6 months were inhibited by dietary SeMet at 0.3 mg/kg (Perez *et al.*, 2010). Indeed, chicken breast meat had lower MDA content with diets supplemented with the organic Se sources, than with SS at 0.15 mg Se/kg (Ahmad *et al.*, 2012; Wang *et al.*, 2010).

Similarly, lipid oxidation as indicated by TBARS was affected by the dietary Se addition (Pappas *et al.*, 2012). More specifically, at day 1, a linear decrease in lipid oxidation (P=0.019) was observed with organic Se addition. Oxidation was lower in the breast of chickens supplemented with 0.3 or 3 mg/kg Se in the form of Se-yeast compared

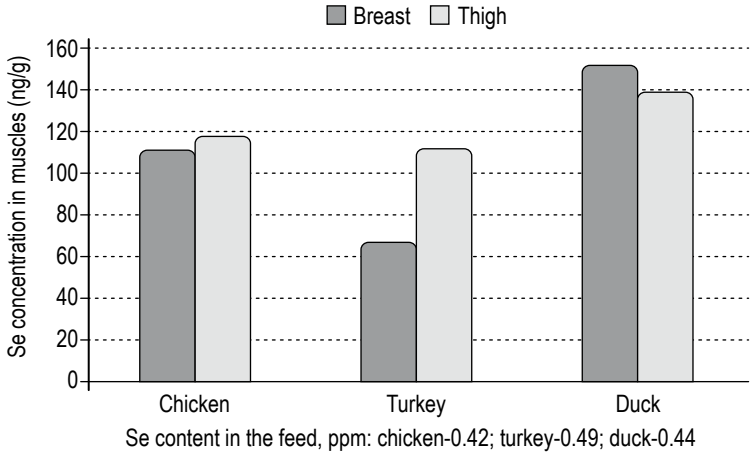

Figure 5.16. Species-specific differences in Se content in muscles, ng/g (adapted from Daun and Akesson, 2004).

to unsupplemented broilers by 30 and 38%, respectively. No effect of dietary Se on TBARS was observed at day 7. Replacing SS with organic Se in the broiler diet was associated with reduced lipid peroxidation (MDA) in breast samples after 0, 3 and 5 days of chilled storage at 4-6 °C (Chekani-Azar *et al.*, 2010). A similar protective effect of dietary SeMet vs SS (0.15 mg/kg) was seen in terms of decreased lipid peroxidation (MDA, 0.44 vs 0.78 nM/mg protein) in broiler fresh breast muscle (Wang *et al.*, 2011a). Clearly, organic Se is more effective in prevention of lipid peroxidation in meat. For example, Dlouhá *et al.* (2008) reported that the MDA value was lower in chicken breast meat over 5 days of storage at 3-5 °C after supplementation with Se-enriched *Chlorella* as compared to SS supplementation. It seems likely that a stabilising effect of Se is associated with maintaining muscle membrane integrity. In a further trial, broiler chickens were fed for 42 days on either a control diet containing <0.15 mg/kg Se as sodium selenite or the same diet top-dressed with 0.3 mg/kg organic Se. GSH-Px activity was higher in the thigh muscles of broilers fed organic selenium than in chickens fed with standard diet (Milincovic Tur *et al.*, 2006). In addition, thigh muscle from chicken fed organic selenium showed higher Mn-SOD activity at 2 weeks of age (Pirsljin *et al.*, 2006). It seems likely that SeMet can increase α-tocopherol contents of broiler meat in comparison to SS-fed chickens (Skřivan *et al.*, 2008a,b). This means that organic Se supplementation may not only affect expression of Se-proteins but can also potentiate other antioxidant enzymes and non-enzymatic antioxidants, improving the effectiveness of the antioxidant defence mechanisms. However, efficacy of organic Se in prevention of lipid peroxidation in the meat depends on many factors, including Se content in the basal unsupplemeted diet (mainly in the form of SeMet) and quality of supplements. For example, Mikulski *et al.* (2009) did not find any difference in TBARS content between SS and SY sources of Se; however, when compared to the control, both SS and SY groups had low TBARS values in turkey breast meat after 70 days of storage at -20 °C. Furthermore, in comparison to SS dietary supplementation, organic Se can increase the total antioxidant potential of the chicken meat (Ahmad *et al.*, 2012; Jiang *et al.*, 2009; Wang *et al.*, 2011).

Furthermore, Edens (1996) showed that drip loss decreased when organic Se was fed to broilers. Using a model system based on red blood cell membrane stability, Edens (2001) confirmed the membrane-stabilising effect of organic Se. In particular, Edens (1997) reported that in a trial, carrying out during spring, organic Se improved feathering and reduced drip loss. More conclusive results have been obtained at the University of New England in Australia (Naylor *et al.*, 2000). In this experiment, meat from birds receiving dietary organic Se had less drip loss. The effect of different levels and sources of Se in chicken feed, on meat quality has also been studied (Periae *et al.*, 2007). It was shown that Se supplementation did not affect meat pH but it significantly decreased drip loss, after 24 and 48 h of storage. In an experiment conducted in Turkey, dietary supplementation of Se in the form of selenite or Se-yeast were compared in groups of broiler chicks to those fed an unsupplemented diet (Deniz *et al.*, 2005). Weight gain over 42 days was shown to be 1,858, 1,921 and 1,948 g in the unsupplemented group, selenite and Se-yeast groups, respectively, while FCR was 1.96; 1.95 and 1.90, respectively. The whole carcass drip loss was also compared in this trial and found to be 1.06; 1.08 and 0.69%, respectively. Replacement of SS with

organic Se significantly decreased breast muscle drip loss at 24 h (2.77 vs 3.26, $P<0.05$) and 48 h (4.33 vs 5.22, $P<0.05$; Wang *et al.*, 2011a). Recent results have indicated that the odour, flavour, and overall acceptability were not changed due to different Se sources, levels, and storage days in chicken breast meat but showed significant influence on colour and juiciness during the 12 days of storage (Figure 5.17; Ahmad *et al.*, 2012).

Protective effect of organic selenium acting to decrease drip loss from poultry meat is a common phenomenon and has also been shown for pork and beef (Surai, 2006). The effect of Se supplementation as selenite or Se-yeast in turkey diets was studied by Acamovic and Bertin (2007). The basal diet contained less than 0.1 mg/kg Se and experimental diets were supplemented with 0.3, 0.4 or 0.5 mg/kg Se, in the form of Se-yeast or selenite, from day old to 16 or 20 weeks. There was no effect of Se treatment on turkey performance however, there was a trend ($P=0.085$) towards a decreased drip

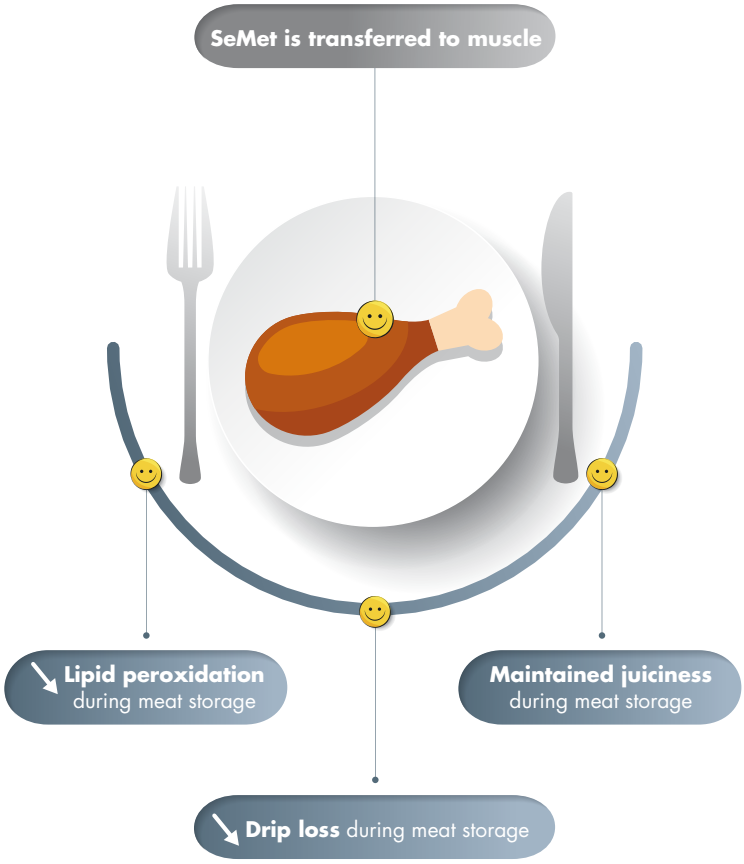

Figure 5.17. Meat pproduction and quality.

loss from leg muscle in the Se-yeast treatments (4.79%, 3.91% and 2.78% in the control, 0.5 mg/kg selenite and 0.5 mg/kg Se-yeast groups respectively). Organic selenium was also more effective than selenite in increasing GSH-Px activity in the blood. These data suggest that meat quality during storage can be improved by inclusion of organic Se in the diet. Recent developments in biochemistry of selenoproteins, have directed more attention towards enzymes, such as methionine sulphoxide reductase B (MSR_B), an enzyme responsible for the prevention of protein oxidation (Surai, 2006). Indeed, the role of protein oxidation in drip loss in meat and maintenance of egg freshness (Haugh units), deserve more attention. The activation of MSR by dietary selenium could be a mechanism to decreasing drip loss and improve the Haugh units of eggs, during storage.

5.4.4 Se-enriched meat production

An additional advantage of organic selenium in broiler nutrition is related to the possibility of producing Se-enriched chicken and this question will be addressed in the Chapter 6. Indeed, replacing SS with organic selenium was shown to double Se concentration in chicken meat. For example, when organic selenium was fed to growing broilers at 0.2 mg/kg, selenium concentration in the breast muscle increased over two-fold, compared to the same dose of selenite (Kuricova *et al.*, 2003). The effect of replacing SS with organic selenium (0.3 vs 0.3 mg/kg of Se) in broiler diets for 42 days was studied by Nollet *et al.* (2007). It has been shown many times that the organic selenium supplementation doubles the Se concentration in breast muscle. In this trial, the levels of selenium at 42 days were; 0.21-0.27 mg/kg in thigh meat for the organic selenium consuming birds compared to 0.15 mg/kg from those fed selenite. Similarly, birds given organic selenium contained levels of 0.25-0.30 mg/kg Se in breast meat compared to 0.12 mg/kg in broilers fed inorganic selenium. It was also shown that Se incorporation into meat is time-dependent and higher levels are found at 42 than at 28 days. The effect of dietary fat source, zinc (Zn) and Se supplementation on the composition and consumer acceptability of chicken meat was studied by Bou *et al.* (2007). It was shown that SS at dose of 1.2 mg/kg did not affect Se content of the meat whilst organic selenium supplementation at 0.2 mg/kg significantly increased Se content of the meat. When commercial broiler diets, containing a basal level of 0.12 mg/kg Se, were supplemented with either 0.3 mg/kg Se in the form of SS or Se-yeast, Se concentrations in the breast muscle were measured. The levels found in broilers at 50 days of age (mg/kg on a dry matter basis) were 0.472, 0.545 and 1.170, respectively (Payne and Southern, 2005b). This indicates that the replacement of SS with organic selenium in broiler diets, doubles the Se concentration in muscle tissue. It seems likely that the main form of Se found in muscle is SeMet and Se-yeast is an excellent source of this amino acid complex. Strong correlations were found between breast Se concentrations and the level of organic selenium supplementation of the broiler diet (r=0.992; Krstić *et al.*, 2012).

Therefore, since chickens cannot synthesise SeMet, when organic selenium is used as a Se source in chicken diets SeMet can be transferred directly from the feed to muscle, thereby increasing the Se content of the meat. In this example, Ross chickens were

fed diets containing no supplementary Se or 5 mg/kg of Se, either in the form of SS or Se-yeast. The chicken diets supplemented with organic selenium increased the total Se content of muscle tissue by approximately 25-fold (Acamovic and Bertin, 2006). When Se-yeast was fed, SeMet accounted for virtually all of the Se stored in both leg and breast muscles. The level of SeCys in tissues was not affected by supplementation.

Based on the aforementioned data it is possible to conclude that Se supplementation is an important element of chicken nutrition and the effect of Se depends on many factors and clearly can be seen when the basic diet is Se deficient. This is why most studies on broilers conclude that the Se source did not influence the growth parameters under controlled conditions (Ahmad *et al.*, 2012; Briens *et al.*, 2013, 2014; Chadio *et al.*, 2015; Chen *et al.*, 2014; Couloigner *et al.*, 2015; Jiang *et al.*, 2009; Krstić *et al.*, 2012; Marta del Puerto *et al.*, 2016; Payne and Southern, 2005a; Upton *et al.*, 2008; Yoon *et al.*, 2007), while chickens in an unsupplemented group were reported to have a decreased final body weight and eviscerated weight (Kristic *et al.*, 2012). In addition, increased body weight in 28-day old turkey poults compared to those fed a diet without supplements, was reported by Cantor *et al.* (1982). Similarly, independently of Se source, an increase of feed Se level (from 0.15 to 0.45 mg/kg) resulted in higher body weight of broilers (Funari Junior *et al.*, 2010). Growth of broilers was also positively affected by SS at 0.5 mg/kg (Jianhua *et al.*, 2000) or Se-yeast at 0.2-0.3 mg/kg (El-Sheikh *et al.*, 2010; Ševčikova *et al.*, 2006).

Therefore, Se supplementation can be considered as 'an insurance policy' giving a producer extra insurance that in stress conditions, if something in the technology goes wrong, the negative consequences in terms of chicken growth, development and health will be minimal and economical losses will be substantially decreased or prevented.

5.5 Conclusions

As can be seen from the aforementioned data selenium dietary supplementation in an optimal form and concentration is a key for maintenance of antioxidant defences of breeders, layers, rearing birds and growing broilers. Organic selenium has a range of advantages in comparison to a traditional SS. Firstly, SeMet accumulation in muscles of breeders/layers builds Se reserves which can be used in stress conditions when there is a need for increased Se supply, but feed consumption and Se provision are usually decreased. The Se reserves gives a chance to birds to overcome oxidative stress, the main mechanism of major commercially relevant stresses, Secondly, since the main form of Se in egg is SeMet and animals/poultry cannot synthesise this amino acid, only organic selenium can be effectively transferred to the egg and developing embryo providing additional protection in stress conditions of commercial poultry production. Indeed, improved Se status of the incubating eggs is considered to be an important element of the strategy to decrease negative consequences of hatching stress and to provide the best start for the developing chicken in early postnatal development. Furthermore, recent data clearly showed that increased Se status of

the hatching egg can affect chickens up to the end of growing period, e.g. up to 42-56 days. Thirdly, organic Se in the layer diet can effect egg shell quality and internal egg quality, including Haugh units during egg storage. In fact, new understanding of the role of protein oxidation in maintaining Haugh units in eggs gives a clear view that specific selenoproteins, namely MsrB1 and thioredoxin reductase, can be involved in the prevention of protein oxidation and maintaining egg freshness during storage. Fourthly, organic Se in broiler nutrition can help to build Se reserves in the body and improve antioxidant defences of the growing birds, including positive protective effects on the chicken immunity and gut health. It is necessary to underline that in many cases Se-related research has been done in well-maintained laboratory conditions providing almost ideal environments for birds. In such conditions Se requirements of the birds can be easily met by background Se in the feed and as a result most of productive and reproductive performance parameters are not affected. In those cases where some challenges/stresses were evident (low Se diet, high temperature, etc.) there were positive effects of organic Se, in comparison to traditional sodium selenite, on fertility, hatchability and chick viability in breeders, on egg shell and internal egg quality in layers and on growth rate, FCR, immunity and chick mortality in broilers. Furthermore, protein oxidation and its prevention by the aforementioned selenoproteins is suggested to be an important approach to decrease meat drip loss after storage. Indeed, it is a great challenge for researchers to establish optimal levels of Se supplementation of poultry providing best protection under stress conditions. However, accumulating evidence clearly indicates that organic selenium is the best option for the poultry producer/feed mill to meet the Se requirement of poultry in stressful conditions of commercial poultry production.

There are three generations of Se feed supplements. The first generation includes inorganic Se, mainly in the form of SS or selenate. Over the last 50 years these supplements did a good job in solving global Se deficiency problems. Indeed, Se-related diseases are not the case in industrial poultry production anymore. However, in order to meet the exact requirement of new, high productive breeds of egg- and meat-type of poultry organic Se has been shown to be a better option. The second generation of Se supplements for poultry includes organic Se, mainly in the form of Se-yeast or pure SeMet/Zn-SeMet. These supplements moved Se supplementation further and a range of research publications have shown the advantages of the aforementioned supplements. However, it has been shown that the proportions of the active component of Se-yeast, namely SeMet, varies from 50 up to 75% of total Se and it is practically impossible to guarantee the SeMet proportion in the Se-yeast, since it is dependent on many varied factors, including yeast strain, conditions of Se-yeast production, such as temperature, oxygen and nutrient concentrations in yeast growth medium, etc. Furthermore, to determine SeMet in the Se-enriched yeast is a difficult analytical task and only a few labs in the world can do it properly. Therefore, while Se-yeast is a good source of organic Se in poultry/animal nutrition the aforementioned problems decrease its commercial value. The usage of pure SeMet could be considered as an effective alternative to Se-yeast and there are a range publications claiming that SeMet is more effective in transferring Se to the egg and to the muscles than Se-yeast. Indeed, Se transfer to the egg and building Se reserves in muscle are the function

of SeMet availability from the feed/feed supplements. However, pure SeMet as a chemical is not stable and can be oxidised losing its biological activity. Therefore, in short, in well-balanced research trials pure SeMet can show a good efficacy as well as an excellent source of organic Se, but in commercial poultry feed production associated with feed storage, pelleting and other harsh treatments it can be oxidised and lose its biological activity. The third generation of the Se supplements for poultry/animal nutrition includes OH-SeMet, which combines the advantages of both Se-yeast (stability) and pure SeMet (high proportion of SeMet), and showed advantages in comparison to other organic Se supplements. It seems likely that a group of Se supplements called Se-proteinates, Se-glycinates, Se-chelates, etc. are not real sources of organic Se (SeMet) and in many cases they are not different from SS in terms of their transfer to the egg and building Se reserves in the body in the form of SeMet.

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Chapter 6
Selenium-enriched eggs and meat

An egg a day keeps diseases at bay

6.1 Introduction

The relationship between diet and human health has received substantial attention in the last few years, with the realisation that unbalanced diets can cause serious health-related problems. However, not everyone consumes the same food, and people meet their nutritional needs in many and varied ways. From the many food ingredients commonly present in our diet, natural antioxidants are considered particularly important. It is well known that free radicals produced under both normal physiological conditions and under stress conditions can have damaging effects on polyunsaturated fatty acids, DNA and proteins in the body. Antioxidant protection is vital for prevention or substantial reduction in damage caused by free radicals and products of their metabolism.

Food provides a major source of natural antioxidants for humans, including vitamin E, carotenoids, flavonoids and selenium (Se). Particular interest in Se has been generated as a result of clinical studies showing that dietary supplementation with organic Se, in the form of yeast grown on a media enriched with this trace element, decreased cancer mortality 2-fold (Clark *et al.*, 1996). Additionally, there is data indicating that inadequate selenium consumption is associated with poor health, genetic defects, decreased fertility and defence against various viral and bacterial diseases (Surai, 2006). Unfortunately, in many countries all over the world human food ingredients can contain inadequate levels of Se, and Se deficiency in human nutrition is a global problem. As a result, finding solutions to this problem is now on the agenda of many government health bodies.

Results derived from various research studies conducted over the last few years have indicated that the enrichment of animal-derived foods (mainly meat, milk and eggs) with Se via supplementation of animal feeds can be an effective way of increasing human Se status in countries where Se consumption falls below the recommended daily allowances (RDA), e.g. consumption in the UK is shown to be about 50% of the RDA.

6.2 Selenium and human health

There is a great body of evidence that shows the health-promoting properties of Se. Deficiency in the human population is associated with two diseases (Keshan disease

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and Kaschin-Beck disease) reported in areas of China and other countries characterised by an extremely low Se content in the soil and in the food chain. Furthermore, in humans, Se deficiency is associated with a compromised immune system and increased susceptibility to various diseases, including arthritis, cancer, cardiovascular disease, cataracts, cholestasis, cystic fibrosis, diabetes, immunodeficiency, lymphoblastic anaemia, macular degeneration, muscular dystrophy, stroke and some others. Adequate Se is also essential for immune function and can protect the immune system from oxidative damage. The results clearly indicate that selenium plays an important role in human health and disease prevention (Figure 6.1; Surai, 2006).

Clearly, the results of clinical studies suggest that an increase in the intake of selenium is associated with health benefits. However, the present focus should be on diagnosing and treating selenium deficiency resulting from a poor diet or disease. Data are being

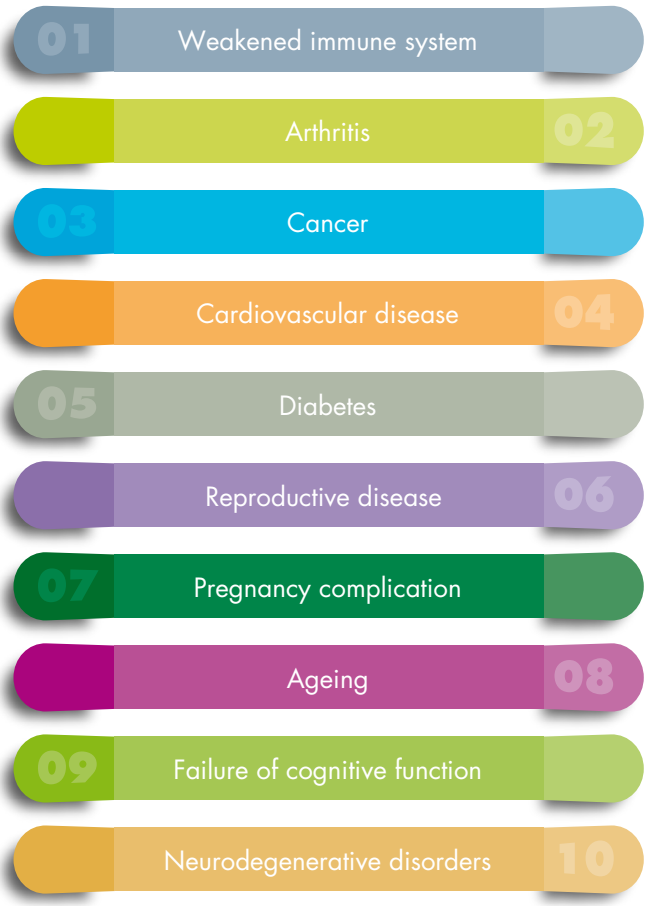

Figure 6.1. Selenium deficiency in humans.

actively accumulated to indicate that selenium deficiency is related to reproductive disorders in man, including poor semen quality and pregnancy complications, and that selenium dietary supplementation may potentially prevent these changes. In addition, selenium supplementation during pregnancy and in the postpartum period reduced thyroid inflammatory activity and the incidence of hypothyroidism (Negro *et al.*, 2007). Optimal selenium status has been shown to be beneficial in asthma, rheumatoid arthritis, cystic fibrosis, HIV, pancreatitis, brain and neurodegenerative disorders. Recently, it was shown that low serum selenium is independently associated with anaemia among older women (Semba *et al.*, 2009). Increased selenium status may also substantially decrease the negative effects of ingested heavy metals (Watanabe, 2002). Selenium is protective against oxidising radiation (e.g. UV) and can be considered as an anti-ageing agent. For example, low plasma selenium was independently associated with poor skeletal muscle strength in community-dwelling older adults in Tuscany (Lauretani *et al.*, 2007). Similarly, low serum selenium concentrations were associated with poor grip strength among older women (Beck *et al.*, 2007). Furthermore, sub-optimal selenium status may worsen muscle functional decrements subsequent to eccentric muscle contractions (Milias *et al.*, 2006). In elderly people in Spain, serum selenium was associated with self-perceived health, chewing ability and physical activity. In particular, subjects in the upper tertile of serum selenium had more than twice as much probability of reporting good health status, chewing ability and of doing more than 60 minutes of exercise/day.

Low serum concentrations of selenium can be used as a predictor of subsequent disabilities associated with ageing (Bartali *et al.*, 2006). Improved selenium status has been associated with a reduced risk of osteoporotic hip fracture in elderly subjects (Zhang *et al.*, 2006). A positive association between selenium supply and bone density was observed in a cohort study (Hoeg *et al.*, 2012). Plasma selenoprotein P concentrations have been shown to be positively correlated with bone mineral density in elderly women (Zhang *et al.*, 2014). In the elderly population, those with the lowest selenium levels had a significantly higher risk of mortality over a period of five years (Walston *et al.*, 2006). Similar conclusions were drawn from another study (a nine-year longitudinal study with six periods of follow-up). During the two-year period from 1991 to 1993, 1,389 men and women born between 1922 and 1932 were recruited. The effects of plasma selenium at baseline on mortality were determined. During the nine year follow-up, 101 study participants died. Baseline plasma selenium was higher in individuals who were alive at the end of the follow-up period than in those who died before this time point (Akbaraly *et al.*, 2005). It was also shown that elderly women living independently in the community who have higher serum selenium are at a lower risk of death (Ray *et al.*, 2006). It seems likely that low plasma selenium may be an independent predictor of mortality among older adults living in the community. For example, 1,042 men and women of 65 years or older were investigated in the Chianti region of Tuscany, in Italy (Lauretani *et al.*, 2008). Plasma selenium was measured at enrolment (1998-2000), and vital status was ascertained until May 2006. During follow-up, 237 participants (22.7%) died. At enrolment, mean plasma selenium concentrations among participants who survived or died were 0.96 and 0.87 μmol/l ($P<0.0001$), respectively. The proportion of participants who died,

from the lowest to the highest quartile of selenium, was 41.3, 27.0, 18.1 and 13.5% ($P<0.0001$). After adjusting for age, sex, education and chronic diseases, adults in the lowest quartile of plasma selenium at enrolment had significantly higher mortality compared to those in the highest quartile. When all-cause mortality was determined in a representative sample of the US population (13,887 individuals), followed up for mortality over 12 years, increasing serum Se levels up to 130 ng/ml were shown to be associated with decreased mortality (Sanmartin *et al.*, 2011).

The selenium status of the elderly is related to quality of life. For example, recent results of a cross-sectional survey of 2,000 rural Chinese, aged 65 years or older from two provinces in the People's Republic of China, support the hypothesis that a life-long low selenium level is associated with lower cognitive function (Gao *et al.*, 2007). Indeed, in the elderly, cognitive decline was associated with decreases of plasma selenium over time. Among subjects who had a decrease in their plasma selenium levels, the greater the decrease in plasma selenium, the higher the probability of cognitive decline (Akbaraly *et al.*, 2007). Indeed, evidence from human studies suggests a role for Se and selenoproteins in protection against cognitive decline (Aaseth *et al.*, 2016). Selenium has also been identified as playing a role in several neurodegenerative disorders, including Alzheimer's and Parkinson's disease (Cardoso *et al.*, 2015). A recent meta-analysis of the results from 33 studies demonstrated the association between poor selenium status and increased risk of spontaneous miscarriage, preeclampsia, pre-term labour and gestational diabetes (Mariath *et al.*, 2011). Evidence from observational studies indicates an inverse association of blood selenium level and the risk of preeclampsia, while Se supplementation was shown to significantly reduce the incidence of preeclampsia (Xu *et al.*, 2016). Furthermore, anti-inflammatory, antirheumatic and antiviral effects of selenium could be of great value for health maintenance (Rayman, 2012). Indeed, Se is considered to be essential for human well-being largely due to its potent antioxidant, anti-inflammatory and antiviral properties (Wrobel *et al.*, 2016). Furthermore, an inadequate Se intake is considered a risk factor for many ageing-related diseases, such as cancer, cardiovascular and immune disorders (Roman *et al.*, 2014).

EU RDA for Se is 55 μg/day, while USA RDA for various categories of people varies from 55 up to 70 μg/day. Since the Se content in plant-based food depends on its availability from soil, the level of this element in human foods varies among regions. In fact, Europe and many other countries worldwide are considered to be Se-deficient areas (Surai, 2006; Surai *et al.*, 2010; Table 6.1). In fact, in Europe, the average daily Se intake has been shown to be 40 μg/day (32-62 μg/day), as while in the US it is 2-3 fold higher: 93 μg/day for women and 134 μg/day for men (Waegeneers *et al.*, 2013).

Table 6.1. Low daily selenium intakes in selected countries, µg/day (adapted from Surai *et al.*, 2008).

Country	µg/day	Year
China, Keshan disease area	2-36	1985
China, Keshan disease area	7-11	2001
New Zealand, low-Se area	11	1984
Saudi Arabia	15	1997
Czech republic	10-25	1996
Poland	11-	2000, 2003
UK	12-43	1995, 1997, 1998, 2003
Libya	13-44	2005
New Guinea	20	1992
Czech republic	15-50	2003
Nepal	23	1988
Finland before selenium fertilisation	26	1987, 1984, 1985
India, vegan low income	27	1997
Croatia	27	1998
Belgium	28-61	1994
Egypt	29	1972, 1996
Serbia	30	2001
Slovenia	30	1998
China	26.0-37.2	2002, 2000
Croatia	27.3-33.9	1998, 2000
Slovakia	27-38.2	1996, 1998
Belgium	28.4-61.1	1989, 1994
Brazil	28.4-37.0	2004
Egypt	29	1972
New Zealand	29-38	1999, 2001, 2004
Sweden	29-44	1991, 2000, 2003
France	29-48	1994, 1994
Serbia	30	1995
Belgium	30	1993
Turkey	30-36.5	1996, 1997, 2004
Poland	30-40	2003
Sweden	31	1989
UK, 1994	32	1997
Turkey	32	1991
UK, 1995	33	1997
England	35	2000
Spain	35	1996
Germany	35-48	1989, 2000
Portugal	37	1990
Slovakia	38	1998
Sweden	38	1993

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Table 6.1. Continued.

Country	µg/day	Year
Denmark	38-47	2000
Germany	38-48	1989
Greece	39.3	2006
Sweden	40	1989
Denmark	40	1998
France	42	1988
Italy	43	1985
UK, 1985	43	1997
Belgium	45	1994
France	47	1991
Germany	47	1989
France	48	1994
India, conventional diet	48	1997
Austria	48	2001
Italy	49	1991
Egypt	49	1999
Ireland	50	2002
UK, 1974	60	1997

6.3 Strategies to deal with Se deficiency in human diet

When considering ways to improve human selenium intake, there are several potential options (Figure 6.2). These include:
- direct supplementation;
- soil fertilisation;
- supplementation of food staples such as flour; and
- production of Se-enriched functional food.

In some countries selenium supplements in tablet form are available to health conscious consumers. Some supplemental Se is supplied as inorganic salts, but the most common supplement uses high-Se yeast that contains SeMet and some other organic selenocompounds (Finley, 1999). Many prominent scientists have championed use of such natural antioxidants. For example, Professor Schrauzer, known for his pioneering work related to medical application of organic selenium, regularly consumed Se supplements. In fact, various dietary supplements are used by more than one-half (more than 100 million people) of the adult US population (Halsted, 2003). However, there are many people who either do not like to swallow capsules, cannot afford them or are simply unaware of the need to increase daily selenium intake.

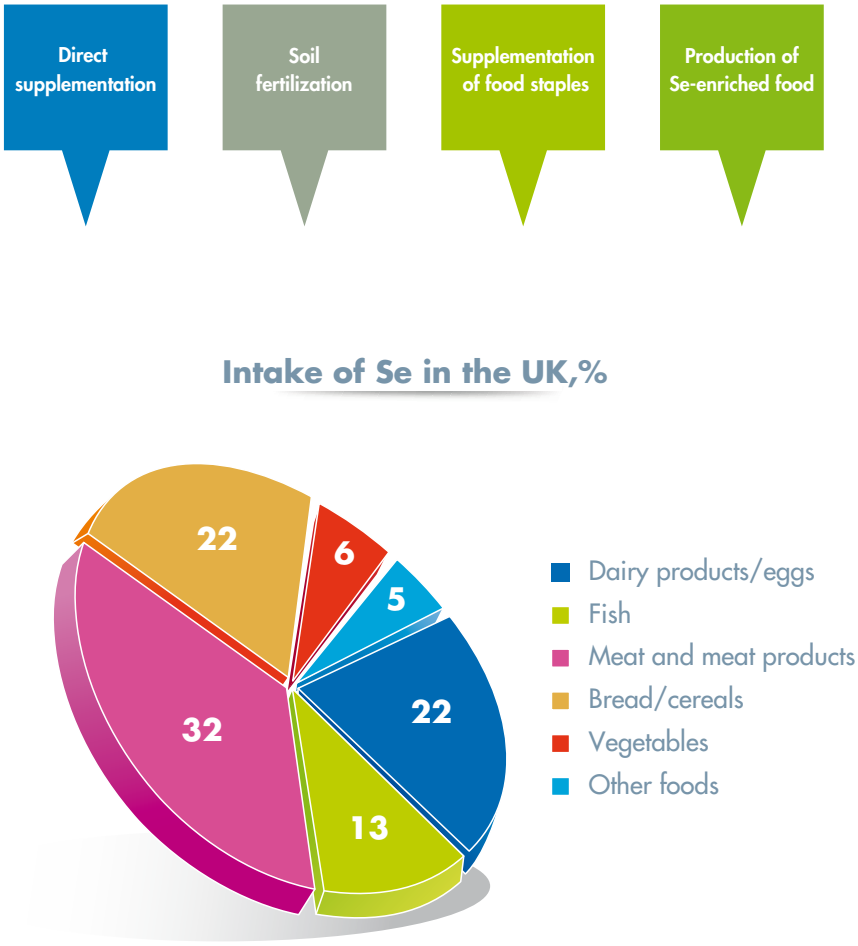

Figure 6.2. Ways of improving human selenium status.

Soil selenium availability is dependent on both selenium content and soil pH. Low crop selenium content owing to low soil pH is quite common situation in various countries all over the world. For example, in Finland the availability of soil Se for plants is poor owing to the relatively low Se concentration, low pH and high iron content of the soil. In areas where soil selenium content is low (Finland and New Zealand) sodium selenate was added to fertilisers used for both grain and forage production (Surai, 2006). In general, agronomic biofortification of crops with Se-containing fertilisers, which is practised in some countries, provides an important short-term solution for improving Se content of wheat (Hawkesford and Zhao, 2007; Lyons *et al.*, 2003).

Indeed, due to an extremely low Se intake in the 1970s in Finland, comprising about 25 µg/day, an official decision was made in 1984 to supplement commercial fertilisers with Se in the form of sodium selenate. As a result, almost all fertilisers used in Finland since 1985 have contained Se (15-16 mg/kg fertilisers for grain production and 6 mg/kg fertilisers for fodder production). As a result of implementation of this programme the selenium concentration of spring cereals has increased on average 15-fold compared with the level before the Se fertilisation. The mean increase in the Se concentration in beef, pork and milk was 6-, 2- and 3-fold (Alfthan *et al.*, 2015). This resulted in an increase of the average dietary human Se intake from 40 µg Se/day/10 MJ in 1985 to a present plateau of 80 µg Se/day/10 MJ. It is interesting to note that foods of animal origin, enriched with Se contribute over 70% of the total daily Se intake. The mean Se concentration in young adults and children in Finland increased from 1.04 and 0.87 µmol/l in 1985 to 1.59 and 1.31 µmol/l in 1990, respectively (Wang *et al.*, 1998). The Se level in human maternal milk was also significantly increased (Kantol and Vartiainen, 2001). Furthermore, the mean human plasma Se concentration increased from 0.89 µmol/l to a general level of 1.40 µmol/l (Alfthan *et al.*, 2015).

In fact, three different methods of Se application were tested: seed pre-treatment, fertiliser enrichment, and foliar application. Seed pre-treatment had some disadvantages, while the two other methods proved to be efficient in a series of experiments and in tests on a large number of farms all over Denmark. In general, foliar application of selenium provided a higher efficiency for increasing the selenium content of soybean than soil application and is successfully used for production of Se-enriched rice in China (Hu *et al.*, 2002).

This approach has been successful in Finland and New Zealand, but has had limited interest in other countries because of environmental issues. For example, in the USA, the use of Se fertilisers caused run-off of the element, resulting in its accumulation in the aquatic biota (Maier *et al.*, 1998). Furthermore, even in Finland, simultaneous increase of total nitrogen, phosphorus, and selenium levels in consecutive samples from some ground water pools indicated leaching of selenium from the fertilisers into the ground water in certain areas (Makela *et al.*, 1995).

Supplementation of staple foods, such as bread flour, is another approach to improving selenium status of the human population (Rayman, 1997). Alternatively, selenium-enriched yeast may be used to produce bread. For example, in Hungary bread produced with selenium-enriched yeast was given to ten volunteers with the daily Se dose being approximately 100 µg. After two weeks of supplementation, the subjects' mean whole blood selenium level increased significantly (from 52.2 to 66.1µg/l; Rumi *et al.*, 1994). Four New Zealand women were supplemented with 200 µg Se in the form of high-Se wheat bread daily for 8-13 weeks. GSH-Px activities increased in whole blood, erythrocytes, plasma and platelets of all subjects but increases were considerably less than those of Se concentrations in whole blood, plasma and erythrocytes (Thomson *et al.*, 1985). It is interesting, that during the post-dosing period Se concentrations and GSH-Px activities fell to levels which

were in most cases somewhat higher than baseline values, probably reflecting Se reserves built during organic selenium consumption. In the Netherlands six healthy subjects were supplemented with 200 μg Se as Se-rich bread for 6 weeks and another six subjects received low-Se bread and served as controls (Van Dokkum *et al.*, 1992). It seems likely that wheat Se has high bioavailability and is the main determinant of blood Se levels in Norway. For example, 18 healthy, Norwegian women were given Se-rich bread providing 100, 200 and 300 μg Se daily for 6 weeks. Serum Se increased by 20, 37 and 53 μg/l, respectively, in the three groups (Meltzer *et al.*, 1992). This approach deserves close consideration owing to its practical ability to reach wide segments of the population and previous success with other trace element deficiencies, such as iron. For example, in China Se-enriched wheat flour is produced by fortification with a Se-enriched mushroom extract (Combs, 2000). The UK supermarket chain ASDA sells bread produced from Canadian high Se flour and Brazil-nut bread is also available on the market (Rayman, 2002).

Table salt fortified with 15 mg/kg sodium selenite is used as a daily Se supplement to reduce the incidence of primary liver cancer in Se-deficient areas of China (Ning *et al.*, 2015; Surai, 2006). However, since Se in high doses could be toxic and selenite is not the optimal form of Se dietary supplementation, this approach is limited to the specific areas of China and did not find a great support in other countries. A fourth strategy is production of 'functional foods' enriched with selenium (Dvorska *et al.*, 2006; Fisinin *et al.*, 2008, 2009; Papazyan *et al.*, 2008; Surai, 2001, 2002a,b, 2006; Surai and Fisinin, 2015; Surai and Sparks, 2001; Surai *et al.*, 2000, 2006, 2007, 2008, 2010; Yaroshenko *et al.*, 2003). Common foods fortified with Se are shown in Table 6.2.

6.4 Addressing Se deficiency in humans via Se-enriched eggs

For the last three decades, designer egg production has made a substantial progress in many countries of the world. In particular, omega-3 enriched eggs can be found on supermarket shelves in Europe, the United States, Australia, Malaysia, and Thailand, among others. However, the omega-3 eggs comprise only the first step in the manipulation of egg composition. In particular, natural antioxidants, as well as vitamin D and folic acid have attracted substantial attention in relation to egg quality and nutritive value (Figure 6.3; Surai, 2002). When considering possibilities of egg

Table 6.2. Common foods fortified with selenium.

Selenium-enriched products by direct fortification	table salt, margarine, cereal gruel, beverages
Plant-derived foods, enriched with Se	Brussels sprouts, broccoli, brassica vegetables, garlic, onions, celery, mint, chamomile, tea, vinegar, beer, yeast, mushrooms and bread
Animal-derived foods enriched with Se	eggs, beef, lamb, pork, chicken, turkey, milk and milk products

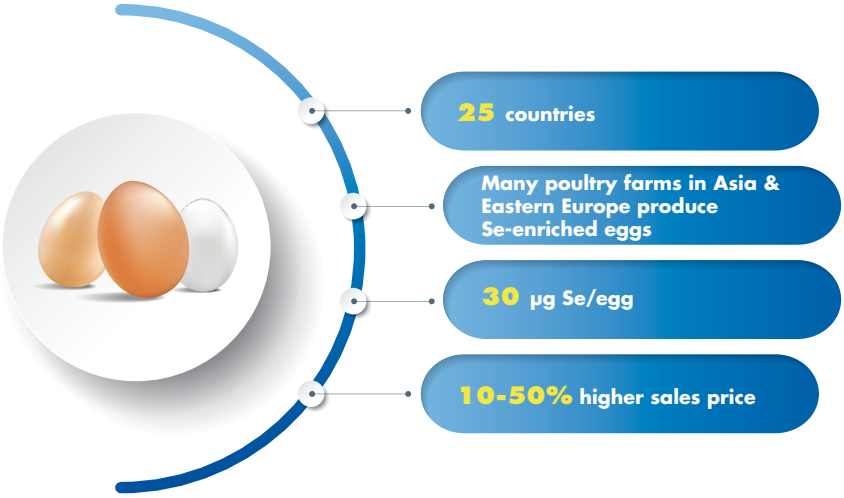

Figure 6.3. Selenium-enriched eggs.

enrichment with antioxidants it is necessary to take into account several important points:

- Efficiency of nutrient transfer from the feed to the egg. For example, vitamin E and lutein are effectively transferred to the egg, efficiency of vitamin A transfer is much lower and ascorbic acid is not transferred to the egg at all (Surai, 2002). There are no data available on the flavonoid transfer to the egg.
- Form of nutrient in the diet. Inorganic selenium (Se) in the form of selenite or selenate is characterised by a relatively low efficiency of transfer to the egg. However, organic Se in the form of Se-methionine is transferred to the egg much more effectively. This makes it viable to enrich eggs with Se. Either the oil or dry forms of vitamin E are suitable for inclusion into the chicken diet.
- Availability of commercial sources of effective feed forms of antioxidants. Vitamin E and other vitamins, organic Se, lutein and other carotenoids are commercially available.
- Possible toxic effects of nutrients for the laying hens. For example, vitamin A in high doses in the chicken diet can be detrimental for their health, it is not effectively transferred to the egg yolk and the elevations that are achieved are still far away from the daily requirement in this vitamin. There is a suggestion that vitamin D enriched eggs could be useful for diet improvement for senior citizens. Very high Se doses in the form of sodium selenite could be detrimental for chicken health and therefore there are legal limits on the amounts of Se that can be included into poultry diets.
- Amount of nutrient delivered with an egg in comparison with RDA. If enriched eggs are going to make a significant contribution to human health then arguably they should be capable of delivering an amount of a nutrient comparable with RDA. Our data showed that with a single egg it is possible to deliver all the daily

requirement of vitamin E (15 mg) and more than 50% of the RDA for Se (30 μg). This became an important marketing tool: 'add an egg to the diet and the requirement will be met'.

- Established health-promoting properties of nutrients and their shortage in a modern diet. The justification for inclusion of vitamin E into the egg is clear. It is an important component of the antioxidant defences, in many cases, diets are deficient in this antioxidant and consumption of high doses of vitamin E (higher than RDA) is beneficial. The same is true for lutein, a carotenoid that has well-established health-promoting properties (Surai, 2002) but is often deficient in the modern diet. Furthermore, the health-promoting properties of Se for maintaining health of general public are increasingly being recognised (Surai, 2006). Thus, in most of developed countries Se deficiency is a common feature, while on the other hand, the cancer-preventive properties of Se, and the importance of maintaining the optimal Se status for promoting health, are emerging from recent research.
- Possible interactions with assimilation of other nutrients from the egg. When an egg is enriched simultaneously with vitamin E and/or lutein, the lipids of egg yolk could help antioxidant assimilation. In fact, the amount of lipids in the egg yolk (about 5 g) and their composition (i.e. saturated, monounsaturated and polyunsaturated fatty acids) could provide an ideal milieu for vitamin E and/or lutein absorption by the human intestine. On the other hand, vitamin E, lutein and Se can prevent omega-3 peroxidation during absorption.
- Stability during egg cooking. Vitamin E, lutein and Se are quite stable during egg boiling or frying.
- Effect on appearance and taste. Vitamin E, carotenoids and Se do not affect the organoleptic characteristics of an egg other than helping to prevent the development of a 'fishy taint' in omega-3 eggs. Egg enrichment with lutein could be beneficial (in some countries) in terms of consumer preference of deep coloured egg yolk.
- Health claim regulations. Health claim regulations differ substantially from country to country. For example, in Malaysia there are no restrictions on health claims and such claims as 'delays the onset of aging' or 'increases fertility' can be found on egg boxes. However, in other countries, such as North America and those in the European Community health claims need to be substantiated. There are also important points in relation to the choice of products for antioxidant enrichment and eggs seem to be an ideal product for this purpose.
- Additional cost incurred by the technology. Eggs are one of the cheapest protein sources on the market and price sensitive. Therefore, it is necessary to calculate what would be a reasonable increase in egg price for the technology to be profitable. For example, by using high quality fish oil in the chicken diet the cost of egg production could easily be doubled.
- Possibilities to patent the technology. In order to commercially produce new type of eggs that will be more expensive than traditional eggs, but also more profitable, it is necessary to have a patent protection against copying of the technology.

Indeed, when considering ways to improve human Se intake, there are several potential options, including the production of Se-enriched eggs and meat (Surai, 2000, 2002, 2006; Surai and Fisinin, 2015). Several important factors must be considered when

choosing the best food supplementation strategy for a given population. In general, the main sources of dietary Se differ between different countries. For example, in the UK meat and meat products currently provide 32% daily Se consumption and dairy products and eggs are responsible for 22% Se consumption (BNF, 2001; Figure 6.4).

In contrast, in Russia about 50% Se in the diet originates from bread and cereals, while meat, milk and eggs provide about 20, 10 and 5% daily Se consumption, respectively (Golunkina *et al.*, 2002, Figure 6.5).

In the USA beef, white bread, pork, chicken and eggs account for half of the Se in the diet (Schubert *et al.*, 1987). In Ireland, meat and meat products (30%), bread

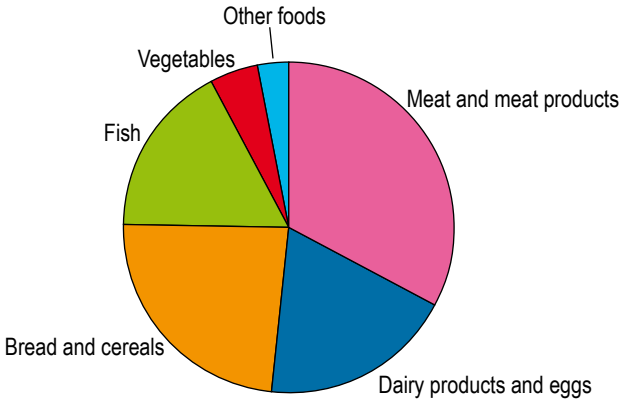

Figure 6.4. Estimated intake of Se from different food in the UK in 1997 (adapted from BNF, 2001).

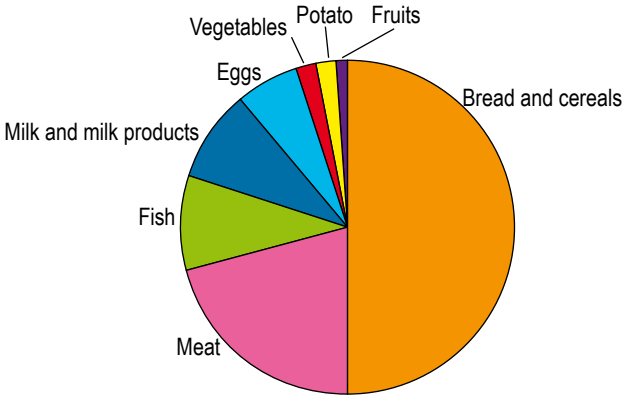

Figure 6.5. Estimated intake of Se from different food in the Russia (Ural region) (adapted from Golunkina *et al.*, 2002).

and rolls (24%), fish/fish products (11%), and milk and yoghurt (9%) were the main contributors to mean daily Se intake (Murphy *et al.*, 2002). In Japan, in the alpine communities, fish makes the largest contribution to dietary Se intake (48% of daily total), followed by eggs (24%), and meat (17%). In the coastal community, fish accounted for 58% of daily total Se intake, followed by meat (18%), and eggs (16%). In both districts, the total contribution of rice and wheat products was around 10% (Miyazaki *et al.*, 2004).

Among animal-derived products, the egg is ideally suited to meet the requirements mentioned in Table 6.3. The egg is a traditional and affordable food in most countries and is consumed by people of all ages more or less regularly, and in moderation. It is also a very safe vehicle for supplementation given that a toxic dose of Se from eggs would require consumption of more than 25 eggs per day over time, an unlikely situation. There is an option for simultaneous enrichment of eggs with several important nutrients, including omega-3 fatty acids, vitamin E, carotenoids (Surai, 2002; Surai and Sparks, 2001) and with a single egg it is possible to deliver around 50% of the human RDA for Se. It appears that pork, beef and chicken meat, as well as milk, can also be enriched with Se.

Before the advent of commercially available organic selenium for animal diets, the main problem as regards the enrichment of eggs with selenium was the low efficiency of transfer of inorganic selenium (the selenite or selenate forms) to the egg. In fact,

Table 6.3. Some characteristics of food choice for Se-enrichment (adapted from Surai, 2006).

The food should be	Comments
A part of traditional meals for the population	It would be counter-productive to attempt a change in culturally-based food habits by introducing a new type of food. Emphasis should be given to the possibilities of changing composition of existing foods such as by selenium enrichment.
Consumed regularly in a moderate amount	Since the objective is to deliver the amount of selenium needed to meet RDA it is necessary to choose food which is consumed regularly in moderate amount. Over-supplementation is unnecessary and undesirable.
Consumed by the majority of the population	This is particularly important given that immune function is more likely to be compromised in groups such as children and the elderly.
Affordable	Affordability of food would play an important role in the consumer choice.
Enriched with other health-promoting nutrients that are in short supply in the same population	Examples of minerals critical to health that are frequently deficient include iron and iodine. Vitamin E and lutein are also in short supply in the human diet. This can give a greater improvement in the diet.
Supplying a meaningful amount of the nutrient (e.g. at least 50% RDA)	This is an important point that distinguishes true functional foods from products that include 'tag-dressing' amounts of nutrients for advertising purposes.

even high doses of selenite in the diet of laying hens were not able to substantially enrich eggs with this trace element (as reviewed by Surai, 2002, 2006).

The concept of producing Se-enriched eggs first originated at the Scottish Agricultural College in 1998 (Surai, 2000, 2001). Indeed, a wide introduction of organic Se in the form of Se-enriched yeast into poultry diets was a revolutionary decision, making it possible to produce eggs with an increased Se concentration. Since the main form of Se in the egg is seleno-methionine (SeMet) and chickens cannot synthesise this amino acid, inclusion of sodium selenite into the chicken diet has limited ability to produce enriched eggs. However, SeMet from the feed or supplements is effectively transferred to egg yolks and albumin, providing the opportunity to produce Se-eggs.

At the same time as these developments, many media channels around the world have taken the first step in promotion of the commercial production of Se-enriched eggs (Table 6.4). Later it was proven that the consumption of such eggs could provide a good source of Se for humans (Surai *et al.*, 2004) and may provide a solution for global Se deficiencies in humans.

Table 6.4. Some examples of Se-enriched eggs produced in various countries.

Trade name	Countries
Origin	Northern Ireland
Mega-Eggs	Ireland
Vita-eggs	UK
NutriPlus	Malaysia
Selen Egg	Thailand
Organic Selenium Egg	Singapore
Heart Beat eggs	New Zealand
Tavas Yumurta	Turkey
Seker Yumurta	Turkey
Selenyum eggs	Turkey
Nutriplus	Portugal
Bounty Eggs	Philippines
Bag of Life (Koshik zhitja)	Ukraine
Rejuvenating (Molodiljnije)	Russia
Aksais' sun (Aksaiskoye solnishko)	Russia
Spring of cheerfulness (Rodnik bodrosti)	Russia
Universal (vSELENskoye)	Russia
Mettlesome eggs (Molodetskoye)	Belarus

6.5 Se-enriched eggs in a global context

Today, Se-enriched eggs are produced in more than 25 countries world-wide, with the Eastern European countries progressing the furthest in this regard. Russia is currently the most advanced country in this business, generating around 38 billion eggs, with 40% of poultry farms producing various modified eggs with increased levels of Se, vitamins, PUFAs and other functional compounds (Fisinin, 2007). There are more than 20 poultry businesses in Russia producing Se-eggs commercially. They are situated in various regions of the country ranging from St. Petersburg up to Siberia and the Far East. Generally they are not competing with each other in the local markets. In most instances, these eggs are sold with distinguishable names and brands including 'Rejuvenating', 'Aksais's sun', 'Spring of Cheerfulness', 'Universal', 'Cossack Village Eggs', 'Oval Wonder', 'Strong eggs', 'Activita', 'Selena' and 'Healthy Selenium'.

The level of Se delivered in a single Russian enriched egg varies from 20 up to 35 μg. In many cases eggs are simultaneously enriched with vitamin E, however, as a rule, the amount of vitamin E delivered from a single egg is less than 30% RDA. Prices for Se-enriched eggs vary and are usually higher by 10-50% in comparison with normal table eggs. The level of production of Se-eggs as a percentage of total egg production on these farms varies from 1 to 20%.

Much more advanced Se-egg production has been developed by Langut Ukraine, a company located in the Kiev region. The egg under the brand 'Bag of Life' and a trade mark 'Eggs from a good hen' were produced at the level of 1.2 million eggs daily and sold all over the Ukraine. In fact, all of the eggs which were produced by the company in 2006-2012 were Se-enriched. A single egg delivers about 30-35 μg Se (50% RDA), about 15-20 mg vitamin E (100% RDA) and also enriched with natural carotenoids. It is interesting to note that practically all of the aforementioned Se-eggs are produced using organic selenium as a major source of Se for laying hens at the level of 0.3-0.5 mg/kg in feed. One important advantage for Russia and the Ukraine in terms of Se-egg production is that they do not need to comply with EU feed additive legislation for local use, they have to follow their local regulations and they have strong marketing support.

6.6 Safety of Se-enriched eggs

Se-enriched eggs, as a rule, contain up to 30 μg Se per egg. Since the maximum safe dietary Se intake (average NOAEL: 'no observed adverse effect level') is 819 μg (Whanger, 2004), to have any detrimental effect from Se overdose one must consume more than 25 eggs a day for a long period of time. If we take into account maximum safe dietary intake of Se identified by the Food and Nutrition Board (2000) to be 400 μg, one can consume 13 eggs a day for a long period of time, a situation difficult to imagine. In most European and other developed countries, egg consumption is less than one per day, therefore, the safety margin here is more than 10-fold.

Observations with Se-egg production in various countries indicate the following:

- Costs involved in Se-egg production do not normally exceed 2-5% of the total feed costs.
- Organic Se supplementation of laying hens is associated with increased egg production, better shell quality, internal egg quality (Hough Units) and improved feed conversion ratio (FCR). These parameters pay money back and give profit at the level of 1:3-5. Therefore, producing marketing for 'Se-enriched' products is usually free of charge and can be used as an effective tool for promotion.
- Additional inclusion of Se to already existing modified eggs (omega-3, vitamin E-enriched, iodine-enriched, etc.) can further enhance their quality and marketing potential without substantial increase in price.
- Labelling regulations differ substantially from country to country, however, a two-fold increase in Se content of the egg would fit the 'Se-enriched' category of the most countries.
- Some countries (e.g. Eastern European and Asian) allow claims for the health benefit of Se, but in most areas it is only possible to put the level of Se on the label and give a comparison to the RDA. Even such limited labelling would be a great advantage for producers.

The prospects for increased production of Se-eggs worldwide are great and a major limitation in Se-egg production is a lack of public knowledge concerning the beneficial effects of Se in relation to human health. Indeed, the companies producing Se-eggs should invest more into public education to widen the market for Se-eggs.

6.7 Se-enriched meat

Various types of meat are important natural sources of Se in human nutrition. For example, in 2003 world pig meat production reached 95.8 million metric tons, while poultry meat rose to 75.2 million tons and the beef volume was 61.9 million tons (Best, 2004). In particular in 2000, an estimated £56 billion was spent on household food in the UK (Buttriss, 2002). In general, meat is a good source of Se. However, Se concentration in the meat varies substantially depending on geographical origin of the country and the Se supplements used. For example, pork produced in the UK, Australia and USA contains Se at the levels of 14, 9.4-20.5 and 14.4-45.0 μg/100 g respectively (McNaughton and Marks, 2002). In Sweden, pork contained Se at the level of 11.3 μg/100 g (Daun *et al.*, 2001). Indeed, it is well established selenite or selenate dietary supplementation is not effective in increasing Se concentration in the meat. The main form of Se in muscles of animals fed on grain-based diet is SeMet. For example, the Se-amino acids accounted for 91 (±8%) of the total selenium (mean of 95 samples of seven tissues analysed over a period of 18 months; Bierla *et al.*, 2008) with SeMet comprising more than 60% of total Se. When high doses of organic Se supplementation were used more than 95% of the Se in chicken breast and leg muscles was found in the form of SeMet. It is known that animals cannot produce SeMet, it must come with the food. This means that only organic Se, in the form of SeMet in the chicken, pig or cattle diet, can substantially increase the Se concentration in the meat.

Se concentration in commercial chicken meat varies substantially. Inclusion of selenite in the chicken diet, even in high concentrations (up to 8 mg/kg), only moderately increased the Se level (up to 23-26 μg/100 g) in chicken meat (Arnold *et al.*, 1973). On the other hand, as in the case with pork and beef, there is an opportunity to increase Se content in chicken meat by inclusion into the diet of organic selenium. For example, under commercial conditions in the Ukraine, the RozDon company produced chicken meat enriched with Se and vitamin E by dietary inclusion of organic Se and increased doses (250-500 mg/kg) of vitamin E (Yaroshenko *et al.*, 2004; Figure 6.6). The results indicated that dietary inclusion of organic Se from day old to slaughter significantly increased Se level in the breast (from 85.2 to 284.3 ng/g) and leg (from 72.2 to 274.2 ng/g) muscle in comparison to the chickens fed a commercial diet supplemented with selenite. Increased dietary vitamin E (250-500 mg/kg) during the last four weeks of growth also significantly increased vitamin E concentration in muscle tissue. A combination of increased concentrations of Se and vitamin E was responsible for substantial decreases in lipid peroxidation in the meat during storage at 4 and -20 °C.

In fact, concentration of malondialdehyde in the experimental meat was significantly decreased as a result of increased antioxidant concentrations in the meat. Data indicates that consumption of approximately 100 g Se-enriched chicken meat, which can be produced commercially, could deliver about 50% of the RDA for Se and could help in solving problems of Se deficiency (Surai, 2006) Furthermore, the combination of increased Se and vitamin E concentration in chicken meat could improve meat quality during storage. There is also a valuable option to produce Se-turkey, where the growth period is substantially longer. Indeed, commercial turkey meat produced in the USA contained Se at a level of 34 μg/100 g (Schubert *et al.,* 1987) reflecting high Se soils in the USA and showing a possibility of increasing Se content of turkey in other regions by using organic selenium in the diet.

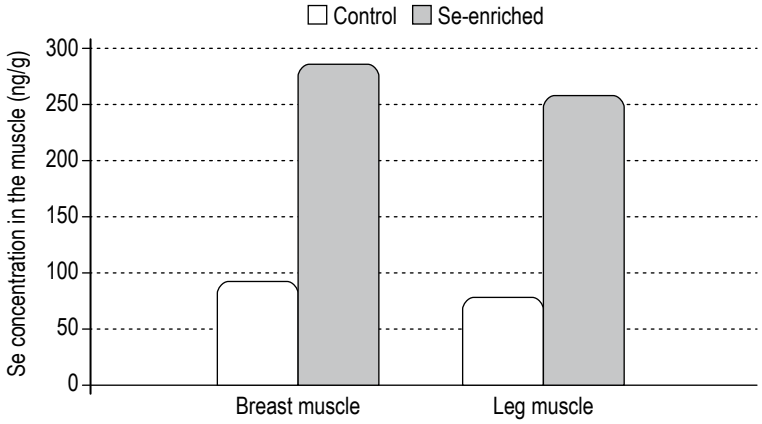

Figure 6.6. Se-enriched chicken meat (adapted from Yaroshenko *et al.*, 2004).

In the experiment with rats, after 9 weeks of the dietary Se repletion and feeding various types of meat as Se sources, it has been shown that relative Se availability (based on liver GSH-Px) was as follows: pork 86%, sodium selenite 81%, SeMet 80%, beef 80%, chicken 77%, veal 77%, and lamb 58% (Wen *et al.*, 1997). Similarly, it has been shown that beef is highly bioavailable sources of dietary Se when compared with selenite or selenomethionine (Shi and Spallholz, 1994). Using similar tests, the authors found that after Se depletion, the recovery of liver GSH-Px activity compared to the control animals (set at 100%), were as follows: selenite -98%, selenate -117%, raw beef -127% and cooked ground beef -139%, respectively. The data suggested that the bioavailability of Se from ground beef (139%) was greater than that from either selenite (100%) or selenate (117%).

It is necessary to underline that the consumption of Se-meat is a safe option, since in order to reach a level of 400 μg/day (maximum safe dietary intake of Se) one would have to consume more than one kg of meat every day, for a long period of time.

6.8 Optimal selenium forms in the diets for Se-egg and Se-meat production

Se transfer to the egg depends on many different factors; analytical techniques used for Se analysis and form of Se in the diet could be important variables. For example, in experiments conducted in Croatia it was shown that provision of organic selenium in concentrations ranging from 0.3 to 0.5 mg/kg in feed for hens, tended to accumulate only 30% more selenium in the eggs when compared with the same concentrations of inorganic selenium (Valentic *et al.*, 2003). These results contradict many other trials previously described, which showed that the Se levels in eggs were twice as high when feed was supplemented with Se-yeast, in comparison to inorganic forms of Se. Therefore, it seems likely that the effect of organic Se on poultry depends on the preparations used and various forms of organic Se could give completely different results. A selenised yeast, produced in Poland under laboratory conditions, was included in the chicken diet for 11 days at 0.5 mg/kg and a comparison with the same amount of sodium selenite was made (Dobrzanski *et al.*, 2003). There was no difference in Se availability from both sources and the Se concentration in the egg content increased only slightly, by 10.5%. Similarly, a Se-malt was produced in China under laboratory conditions. It was claimed to be organic Se and was fed to laying hens at 0.51 mg/kg for 24 days and it did not show any advantage over sodium selenite, in terms of Se content of the egg (Jiakui and Xialong, 2004). Furthermore, a study conducted in China reported no difference in Se concentration in the muscle of chickens fed either sodium selenite or Se-yeast at 0.2 mg/kg (Wang and Xu, 2008). Another paper from China reported only marginal differences in Se content in muscles of laying hens (0.182 vs 0.149 mg/kg) even after 1 mg/kg supplementation with Se-yeast, when compared to sodium selenite supplementation (Pan *et al.*, 2007). When organic Se in the form of SeMet was used in the chicken diet at 0.3 mg/kg there was no difference in the Se content of the egg compared to those eggs laid by hens fed on the diet supplemented with sodium selenite (Chantiraticul *et al.*, 2008). Indeed, it has

been proven that the efficiency of transfer to the egg of sodium selenite from the diet is comparatively low (Surai, 2006). For example, even high dietary supplementation of the chicken diet with Se in the form of sodium selenite (1 mg/kg) increased Se content in the egg only by 39% (Bargellini *et al.*, 2008).

The best efficacy of Se transfer to the egg and meat was shown to be with OH-SeMet (SO). Indeed, two experiments were conducted on broiler chickens to compare the effect of OH-SeMet with two practical Se additives, sodium selenite (SS) and Se-yeast (SY). The different Se sources and levels improved muscle Se concentration compared to the non-supplemented diet (NC), with a significant source effect in the following order: SS, SY and SO ($P<0.05$). In fact, the relative muscle Se-enrichment comparison, using linear regression slope ratio, indicated an average of 1.48-fold higher selenium deposition in muscle for SO compared to SY (Briens *et al.*, 2013, 2014). Seleno-amino acid speciation results for SY and SO at 0.3 mg Se/kg feed indicated that muscle Se was only present as SeMet or SeCys, showing a full conversion of Se by the bird. The results confirmed the higher bioavailability of organic Se sources compared to the mineral source and demonstrated a significantly better efficiency of SO compared to SY for muscle Se enrichment. In particular, the authors showed that Se muscle concentrations significantly improved with SO, increasing the relative bioavailability for total Se by 39% compared with SY. From one hand this could be a reflection of higher SeMet level in the diet (almost 100% SeMet in SO vs 60-70% SeMet in SY). On the other hand, there could be other biochemical differences in the Se metabolism, since SO increased SeCys level in the muscle. Furthermore, it would be interesting to note that hens fed the diet with SO accumulated more Se in their eggs (+28.8%; $P<0.01$) and muscles (+28%; $P<0.01$) than those fed the diet supplemented with SY (Jlali *et al.*, 2013). These results showed the greater ability of SO to increase Se deposition in eggs and breast muscle of laying hens, which could be of great importance for production of Se-enriched eggs and meat. Recently, EU decided to limit the maximum supplementation with selenised yeast to 0.2 mg Se/kg complete feed for reasons of consumer safety (Commission Implementing Regulation No. 427/2013 of 8 May 2013). At this comparatively low level of supplementation advantages of organic Se in the form of SY will be less pronounced, and alternative effective sources of organic selenium with higher efficiency of transfer to the egg and animal tissues would play a bigger role in poultry reproduction. Indeed, the idea of using SO to produce Se-enriched eggs has recently been successfully tested (Tufarelly *et al.*, 2016). The authors also showed a positive effect of organic Se in the layers diet on shell thickness and a significant decrease in percentage of broken and shell-less eggs was observed in the SO supplemented group.

6.9 Se-enriched eggs and meat as functional food

The concept of healthy food additives arrived from Japan in the 1970s and the term 'functional foods' appeared in 1984 (Harris, 2000). At this time consumers began to view food from a radically different point. This 'changing face' of food led to the development of a new area in the food and nutrition sciences known as functional

foods (Hasler, 2000). The Food and Nutrition Board of the National Academy of Sciences defines a functional food as one that encompasses potentially healthy products providing health benefits beyond that of the traditional nutrients it contains (Milner, 2000). This is in agreement with the data of the 1998 USA study from written questionnaires, completed by 2,074 respondents indicating that most shoppers believe foods can offer benefits beyond basic nutrition to functional nutrition for disease prevention and health enhancement (Gilbert, 2000). However, a recent USA survey reported that taste is the primary influence on food choice, followed by cost (Glanz *et al.*, 1998). Similarly, in a survey in Ireland, 'quality/freshness' of food was the most frequently selected food choice factor (51%) followed by 'taste' (43%) and 'trying to eat a healthy diet' (36%) (Kearney *et al.*, 2000).

Today, functional foods have received substantial attention (Hirai *et al.*, 2010; Ryan *et al.*, 2015; Siro *et al.*, 2008) and represent one of the fastest growing segments of the world food industry. For example, dairy products and other processed foods, including mayonnaise, margarine, dressings containing docosahexaenoic acid (DHA) (Takahata *et al.*, 1998), as well as n-3 enriched eggs (Surai, 2006) are already on the market in different countries. Antioxidant-fortified margarine is shown to be effective in the delivery of vitamins E and C as well as α- and β-carotene to human (Van het Hof *et al.*, 1998). In the USA, annual sales of functional food products comprise around $50 billion (Harris, 2000). In total, functional foods have a market share of around 2% in the US food market and are quickly growing (Menrad, 2003).

There are three major reasons for the increased interest in functional foods (Milner, 2000):
- increased health care costs;
- recent legislation; and
- scientific discoveries.

Recently, six major targets in relation to functional food science have been identified (Roberfroid, 2000):
- gastrointestinal functions;
- redox and antioxidant systems;
- metabolism of the macronutrients;
- development in foetal and early life;
- xenobiotic metabolism and its modulation;
- mood and behaviour or cognition and physical performance.

In the same review, the author has stated that the 'health benefit of a functional food will be limited if the food is not part of the diet'. Therefore, functional foods must remain foods and they must achieve their effects in amounts normally consumed in a diet (Contor, 2001). Eggs have not traditionally been regarded as a functional food, primarily due to concerns about their adverse effects on serum cholesterol levels (Hasler, 2000). However, recent finding described above indicating that there is little if any connection between dietary cholesterol and blood cholesterol levels, as well as between moderate egg consumption and heart diseases, could help changing a bad image of eggs. In this

respect, eggs enriched with selenium as well as with a combination of Se, DHA, vitamin E and lutein, ideally fit in the category of functional food enabling to substantially improve the diet (Surai, 2001; Surai *et al.*, 2000). Eggs are consumed regularly by the most of the population and are served as an integral part of traditional English or Mexican breakfast. The development of 'super egg' enriched with these 4 nutrients received a lot of publicity through mass media including radio, TV and newspapers. Some of newspaper titles are shown in Table 6.5. They clearly show great public interest in improvement of egg quality and in creation 'healthy' eggs.

The data indicate that a designer egg enriched in vitamin E, lutein, DHA and Se can be not only a good nutritional product but also a good vector for the delivery of four essential nutrients vital for human health. A crucial feature of these designer eggs is the synergistic combination of n-3 fatty acids with major antioxidants, vitamin E, lutein and Se, as an important approach to the improvement of the human diet. These eggs will not be able to replace vegetable and fruits as a major source of natural antioxidants and fish products as a source of DHA. However, they can substantially improve the diet, especially in countries like Scotland, significantly contributing to the recommended daily intake of vitamin E, lutein, DHA and Se. Commercially, it is possible to produce designer eggs enriched with 4 nutrients as mentioned above or with 3, 2 or 1 nutrient depending on the consumer demand. It seems likely, that egg yolk can also be enriched with vitamin D (Surai and Fisinin, 2015). As a result, price for the production of such eggs could substantially vary. Therefore, the way of egg to the functional food category started successfully and now it is consumer education which is needed to fulfil the idea of using eggs as functional food. Indeed, many categories of people will benefit from using designer eggs as part of their everyday diet.

Table 6.5. Some newspaper titles related to the super egg development (adapted from Surai, 2002).

Title	Newspaper	Date
Super egg that could poach the vitamin pill market	Daily Mail	June 12, 1998
Science goes to work on an egg	The HERALD	June 12, 1998
Super egg	Cambridge Evening News	June 12, 1998
Good health is no yolk	The EXPRESS	June 12, 1998
Scientists go to work on creating 'super-egg'	The TIMES	June 12, 1998
Experts crack the super eggs's secret	The EXPRESS	August 2, 1998
Scientists develop a natural panacea: New super egg bid to allay killer diseases	The HERALD	April 5, 1999
Scientists go to work on a super-egg	The GUARDIAN	April 7, 1999
Scots to market 'life-saving' eggs	The SUNDAY TIMES	April 5, 1999
Super-eggs to help fight against cancer	METRO	April 7, 1999
Could SUPEREGG save your life?	The EXPRESS	April 8, 1999
The egg that goes to work on your health	Daily Mail	April 7, 1999
New enriched egg could bring health benefits	Farmers Guardian	April 9, 1999

However, more research should be done in this fascinating area to convince costumes to go for designer eggs. Further development of various types of designer eggs could be an important contribution to functional food development with a consequent improvement of the human diet.

'Let food be your medicine and medicine be your food' (Hippocrates).

6.10 Conclusions

Se-eggs and Se-chicken meat are perfectly suited for the category of functional foods. A single egg or 100 g chicken meat can deliver 50% of the RDA in Se and since most of European countries are Se-deficient (Surai, 2006), this could have additional benefits, beyond those provided by normal eggs and meat. Eggs and meat form an essential part of many different foods and dishes and could enhance their quality beyond the nutritive value. Se-enriched eggs and meat could also have a substantial effect on gastro-intestinal functions (one of the purposes of functional foods) providing Se for the antioxidant enzyme GI-GSH-Px, which is responsible for the prevention of oxidised lipid absorption and may also protect against heart disease and cancer. Furthermore, Se delivered in eggs and meat could have a beneficial effect on the antioxidant/prooxidant balance in the intestine (Surai, 2006). Regarding diversity in enriched poultry products, it is interesting to note that Se-enriched quail eggs are already commonly seen on supermarket shelves in Ukraine and Belarus (Figure 6.7). Se-enriched pork and beef as well as Se-milk production is a next step in widespread functional food production.

Decreased Se levels in feeds and foods, in many cases, reflect the consequences of our agricultural practises. For example, usage of inorganic fertilisers rich in sulphur

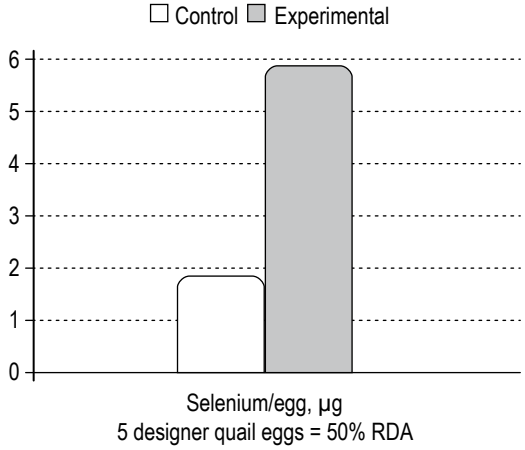

Figure 6.7. Quail egg enrichment with selenium (adapted from Karadas *et al.*, 2004).

interferes with Se assimilation from soil, and soil acidification also decreases Se availability. Therefore, eggs produced by free-range poultry fed on natural feed sources grown on well-balanced soils 100-200 years ago would have contained much higher Se concentrations than we currently see in eggs from many European and Asian countries. Recent observations (Pappas *et al.*, 2006) indicated that the Se levels in eggs from wild birds were substantially higher than in commercial hatching eggs. Se-enrichment of eggs, meat and milk may be viewed as merely production of naturally-designed food ingredients. Indeed, production and commercialisation of such organic Se sources as selenised yeast have already opened a new era in Se supplementation of animals and has given a real chance for producers to differentiate and add value to their poultry products and to meet the increasingly diverse requirements of consumers.

Se-eggs in many countries have successfully made their way from niche markets to main stream food sales. Indeed, it is possible to provide consumers with a range of animal-derived products that have been nutritionally improved in such a way that they can deliver substantial amounts of health-promoting nutrients to improve the general diet and help to maintain health. Therefore, without changing eating habits and traditions of various populations, it is possible to solve problems related to deficiency of various nutrients, in particular selenium. The consumer will go to the same supermarket to buy the same animal-derived products (eggs, milk and meat), cook and consume them as usual. The only difference will be in the amount of specific nutrients delivered with such products. Indeed, Se-enriched eggs and chicken meat could help general population in many countries worldwide, especially in Europe, to meet Se requirement and to keep healthy diet.

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Chapter 7
Selenium and immunity

If you give a man a fish, you feed him for a day,

If you teach him to fish, you feed him for lifetime

7.1 Introduction

Animal/poultry defence against various diseases depends on the efficacy of the immune system responsible for elimination of foreign substances (e.g. parasites, bacteria, moulds, yeast, fungi, viruses and various macromolecules) or the creation of specific inhospitable conditions within the host for a wide range of pathogens. This protective capacity is based on the effective immune system which is considered to be an important determinant of animal health and wellbeing. In that sense, a remarkable ability of components of the immune system to distinguish between self and non-self is a great achievement of animal evolution.

Commercial poultry production is based on balanced feed, providing requirements in major nutrients and optimised environmental conditions. However, it is very difficult to avoid various stresses that are responsible for immunosuppression and increased susceptibility to various diseases, and as a result decreased productive and reproductive performance of poultry. For example, mycotoxins are among major immunosuppressive agents in poultry diet. In such situations immunomodulating properties of certain macro- and micronutrients are of great importance for the poultry industry. In fact, almost all nutrients in the diet play a crucial role in maintaining an 'optimal' immune response, and both insufficient and excessive intake can have negative consequences on the immune status and susceptibility to a variety of pathogens. Indeed, immunocompetence can be presented as a 'violin music'. One can have a best violoncellist in the world with a Stradivari violin in the hands, however, before the violin is properly tuned there is no music, just noise. That is exactly what is happen with an immunocompetence when the immune system is overreacted. This is associated with allergy and other immune conditions. Furthermore, to maintain such an increased immune reactivity important nutrients are used. On the other hand, an underreacting immune system is also a problem, since it is not able to adequately protect the body from invaders, including microbes, viruses, etc. Only a well-tuned immune system can do a great job of the optimal protection without big losses to productive and reproductive performance of poultry. During the last 20 years, data have accumulated indicating that selenium is among the major immunomodulating agents. Also, the amount of selenium needed for immunomodulation is usually higher than that for animal growth and development (Surai, 2006). In fact, selenium has been shown to have immunomodulatory effects in a variety of species when administered in

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quantities in excess of the established dietary requirements. Therefore, selenium as an essential component of selenocysteine-containing proteins is involved in most aspects of cell biochemistry. The function and immune cell activity is also Se-dependent and Se deficiency attenuates the host immune response, thereby increasing the risk of bacterial and viral infections.

7.2 Immune system and its evaluation

There are two major types of immune function in poultry: natural and acquired immunity (Figure 7.1). Natural immunity, called the innate immune system, includes physical barriers (e.g. skin, mucus coat of the gastrointestinal tract), specific molecules (e.g. agglutinins, precipitins, defensins, acute phase proteins, lysozyme, etc.), innate immune cells, including dendritic cells (DC), macrophages, heterophils, mast cells, natural killer (NK) cells, and natural killer T (NKT) cells, represent the first line of defence against pathogens and foreign agents (Table 7.1).

In the case of pathogen invasion, pathogen-associated molecular patterns (PAMP) present on the cells are recognised by the cells of the innate immune system through Toll-like receptors (TLR) and pattern recognition receptors (PRRs; Korver, 2012). Phagocytes, such as heterophils (the avian equivalent of the mammalian neutrophil), dendritic cells and macrophages are known to kill pathogens through the release of

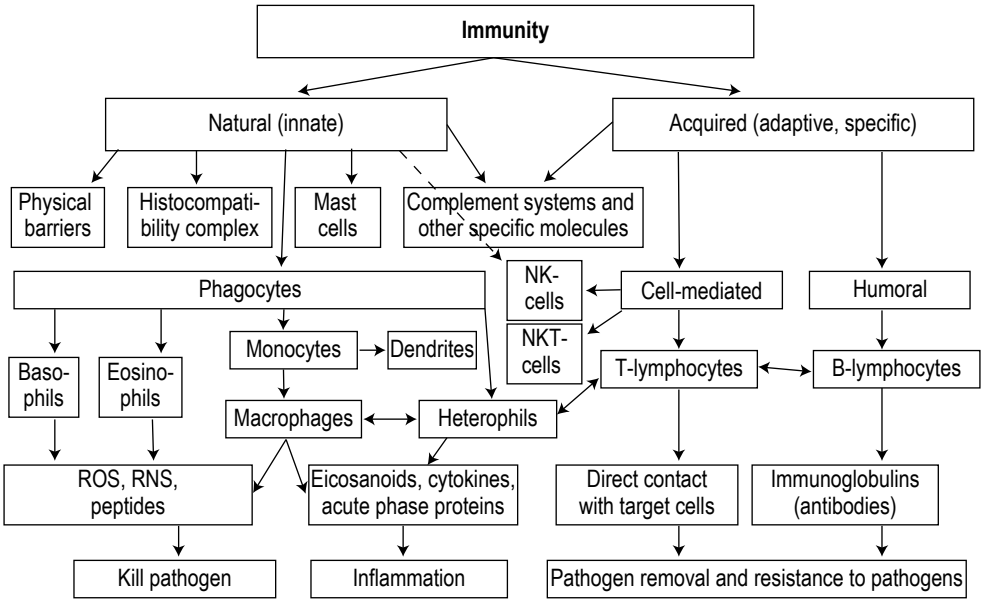

Figure 7.1. General scheme of the immune system (adapted from Surai, 2002, 2006). ROS = reactive oxygen species; RNS = reactive nitrogen species; NK cells = natural killer cells; NKT cells = natural killer T cells.

Table 7.1. Key elements of the immune system (adapted from Surai, 2006).

Element	Significance
Cells	
Monocytes, macrophages	phagocytosis, synthesis of interleukin (IL)-1, IL-6, IL-8 and other substances
Neutrophils	phagocytosis of bacteria, viruses and toxins
Eosinophils	destruction of parasites
Basophils	initiation of inflammatory processes
Dendritic cells	antigen processing, lymphocyte activation, cytokine secretion
Mast cells	release of inflammatory mediators
B cells (B lymphocytes)	production of plasma cells (immunoglobulins), antigen-specific; 10% of total lymphocytes
T helper cells (helper T lymphocytes)	antigen-specific, produce cytokines: IL-2, IL-3, IL-4, IL-5, IL-9 and IL-10; 55% of total lymphocytes
Cytotoxic T cells (T lymphocytes)	destruction of tumour cells and virus-infected cells; antigen-specific, 25% of total lymphocytes
Suppressor T cells (T lymphocytes)	inhibition of immune reactions (development of autoimmune diseases)
Natural killer cells	destruction of tumour cells and virus-infected cells, 10% of total lymphocytes
Macromolecules	
Immunoglobulins	binding of foreign cells and proteins; promotion of their ingestion by phagocytes
Interferons (IFN-α; IFN-β; IFN-γ)	activation of macrophages (IFN-γ); inhibition of viral replication
Complement system – a set of over 20 soluble glycoproteins	Destruction of foreign cells
Interleukins	regulation of specific types of leukocytes
Leukotrienes	promotion of inflammatory process
Lysozymes	dissolution of bacterial membranes
Collectines – a group of carbohydrate-binding proteins	act as opsonins in nonadaptive immune response to pathogen
Acute phase proteins – a group of plasma proteins produced in the liver in response to microbial stimulus	maximise activation of the complement system

toxic chemicals, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), and through degranulation. The phagocytes also process and present antigen to cells of the acquired immune system. Furthermore, a complement system activation is also involved in a pathogen destruction and elimination, as well as the release of histamine and heparin from mast cells helps dealing with invaders (Korver, 2012).

In comparison to mammals, the chicken has a different repertoire of immune genes, TLRs, chemokines and chemokine receptors. In fact, the chicken genome encodes at least two chicken-specific TLRs, namely TLR15 and TLR21 (Wu and Kaiser, 2011). The main difference is the absence in chicken of lymph nodes, the primary site of antigen presentation in mammals.

Macrophages perform a range of functions, including phagocytosis of foreign particles, destruction of bacterial and tumour cells, and secretion of prostaglandins and cytokines (Table 7.2), thereby regulating the activity of lymphocytes and other macrophages (Qureshi, 1998).

Macrophages express major histocompatibility complex (MHC) Class I, Class II and costimulatory molecules. Chicken macrophages play a central role in body defence against microbial infections and they express PRR including the extensively studied TLR (Wu and Kaiser, 2011). The immune system utilises at least fifty cytokines to deliver signals in the immune microenvironment (Delgoffe *et al.*, 2011). In fact, phagocytosis is the major mechanism by which microbes are removed from the body and is especially important for defence against extracellular microbes. As a result of stimulation (for example by microbes) monocytes differentiate into macrophages that are more powerful in mediating host defence (Klasing, 1998a).

Macrophages are equipped with a range of receptor systems including the scavenger receptors, complement receptors, Fc receptors, C-type lectins and mannose receptors mediating opsonic and non-opsonic recognition (Kaspers *et al.*, 2008). The phagocytic process includes several stages (Lydyard *et al.*, 2000):

- movement of phagocyte towards the microbe using chemotactic signals;
- physical contact of the micro-organisms with macrophage and attachment to the phagocyte surface with a nonspecific or receptor-mediated binding;
- endocytosis (engulfment) of microbe resulting in a phagosome by invagination of surface membrane;
- fusion of the phagosome with a lysosome;
- killing of microbes by bombarding them with oxidants (superoxide and hydroxyl radicals, hydrogen peroxide, nitric oxide, hypochlorous acid, etc.).

Similar to mammalian neutrophils, avian heterophils are highly phagocytic and capable of killing a variety of microorganisms after phagocytosis. In fact, avian heterophils are the first cell type recruited to the site of an infection and play an important role in innate immunity and the subsequent inflammatory response (Wu and Kaiser, 2011). It is interesting to note that avian heterophils constitutively express most of the TLRs found in the chicken, being principal players in the first line of immunological host defence against various infections (Kogut *et al.*, 2005). It has been shown that avian heterophils kill bacteria through two major mechanisms, including oxidative burst and degranulation. Microbicidal peptides released by degranulation include cathelicidins and defensins (Van Dijk *et al.*, 2009; Wu and Kaiser, 2011). Similar to mammalian neutrophils, avian heterophils use extracellular traps for invading pathogens (Chuammitri *et al.*, 2009). However, expression of MHC Class

Table 7.2. Macromolecules produced in immune cells (adapted from Surai, 2006).[1]

Name	Site of production	Principal effects
Il-1	MPH, epithelial cells	stimulation of T- and B-cell proliferation and production of inflammation-modifying proteins in the liver; induction of acute phase proteins; increase of effector cell access; tissue destruction
IL-2	T-helper cells, mast cells	proliferation of T- and NK cells, increase in NK cell activity; activation of production of cytokines
IL-3	T-helper cells	induction of proliferation and differentiation of Stem cells in bone marrow
IL-4	T-helper cells, mast cells	stimulation of T- and B-cell proliferation and IgG and IgE synthesis in plasma cells; increase in macrophage cytotoxicity; induction of Th2 and inhibition of Th1 responses
IL-5	T-helper cells, mast cells	stimulation of B-cell proliferation and activation and IgA synthesis In plasma cells stimulation of eosinophil production and accumulation
IL-6	MPH, T-helper cells, fibroblasts, endothelial cells; mast cells	induction of differentiation of B-cells and production of inflammation-modifying proteins in the liver; lymphocyte activation; fever
IL-7	thymic epithelial cells, bone marrow cells	stimulation of pre-T and pre-B-cell proliferation
IL-8 *	monocytes, MPH, fibroblasts, keratinocytes	promotion of neutrophil activation, adhesion to endothelial cells and migration to inflamed site
IL-9	T-helper cells	stimulation of mast-cell proliferation
IL-10	T-helper cells, MPH	inhibition of production of INF-γ and some ILs; B-cell activation
IL-11	mesenchymal cells	stimulates megakaryocyte production
IL-12	B cells, MPH	stimulation of production of T-helper cells type 1 and INF-γ synthesis by T cells and NK cells
INF-γ	T cells, NK cells	MPH activation; induction of Th1 and inhibition of Th2 responses
TNF-α	MPH, T cells	activation of vascular endothelium; fever; shock; mobilisation of metabolites
PAF	MPH	promotes neutrophil migration and eosinophil mobilisation
Fibroblast growth factor	MPH	induces division of connective tissue cells at sites of injury
Complement	MPH	lysis of foreign cells
α-I-protease inhibitor	MPH	inhibits proteases and breakdown of healthy tissues
α-2-macroglobin	MPH	inhibits proteases and breakdown of healthy tissues
Leukotrienes	MPH	promote leukocyte accumulation at inflammatory foci
Granulocyte colony-stimulating factor	MPH	increases the production of granulocytes (neutrophils) in the bone marrow
Granulocyte-macrophage colony-stimulating factor	MPH	increases granulocyte and monocyte production

[1] MPH = macrophages; NK = natural killer; IL = interleukin; INF = interferon; TNF = tumour necrosis factor; PAF = platelet-activating factor; Ig = immunoglobulin;* = chemokine.

I or II molecules in heterophils have not been shown yet (Wu and Kaiser, 2011). Inflammatory stimuli, such as lipopolysaccharides (LPS), or various infectious agents cause a dramatic influx of heterophils and the activation of heterophils by pathogens or cytokines induces the expression of various pro-inflammatory cytokines, such as IL-1, IL-6 and IL-8 (Juul-Madsen *et al.*, 2008). Therefore, the first line of defence against various pathogens, is the innate immune system, which is primed even in the absence of infection. It is characterised by a range of various molecules and cell-surface receptors – known as PRRs – that recognise generic molecular structures associated with different groups of pathogens (Maizels, 2009). These receptors perform many different functions, including mobilisation of macrophages and granulocytes, unleashing antimicrobial proteins and reactive metabolites. They are also involved in mobilisation of dendritic cells, which activate the lymphocytes of the adaptive immune system, inducing proliferation of T cells and antibody-producing B cells with variable receptors that specifically recognise the parasite (Maizels, 2009). It should be mentioned that evidence has been accumulated to show that innate immune cells can show memory-like behaviour. One of the key mechanisms that mediates these effects is epigenetic reprogramming. For example, Yoshida *et al.* (2015) showed that the stress response transcription factor ATF7 mediates LPS-induced epigenetic changes in macrophages and these changes lead to enhanced protection against pathogens.

Macrophage activation and phagocytosis of foreign particles are regularly accompanied by a so-called 'respiratory burst', an increase in the production of ROS, exerted by the enzyme complex NADPH oxidase (Figure 7.2).

Therefore, macrophages as well as other phagocyte leukocytes (e.g. neutrophils, monocytes and eosinophils) can synthesise toxic oxygen metabolites such as superoxide anion (O_2^-), hydroxyl radical (OH•), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), nitric oxide (NO), peroxinitrite ($ONOO^-$) hypochlorous acid (HOCl) and chloramines during the respiratory burst (Zhao *et al.*, 1998). For example, a bacterium coming into contact with the plasma membrane is enclosed in a plasma membrane vesicle containing NADPH oxidase, and exposed to an intensive flow of superoxide radical (Gille and Sigler, 1995). Superoxide radical can disproportionate to H_2O_2 which penetrates into the bacterium with a production of hydroxyl radical, which is ultimately a deadly weapon able to damage any biological molecules. In general, the production of ROS and reactive nitrogen species is a characteristic for both mammalian and avian macrophages (Qureshi *et al.*, 1998). It is interesting that, people whose phagocytes possess no functional NADPH oxidase, are shown to suffer from chronic infections of the skin, lung, liver and bones leading to a premature death (Halliwell and Gutteridge, 1999).

In general, ROS, RNS and eicosanoids (e.g. leukotrienes and prostaglandins) have received substantial attention as major metabolites produced by macrophages (Dietert and Golemboski, 1998). Macrophages bind, internalise, and degrade foreign antigens (e.g. bacteria) quite quickly by using ROS as a powerful weapon. It takes only 15 min for chicken macrophages to kill more than 80% of the internalised *Salmonella* (Qureshi *et al.*, 1998). Thus, natural immunity works rapidly and gives rise to an

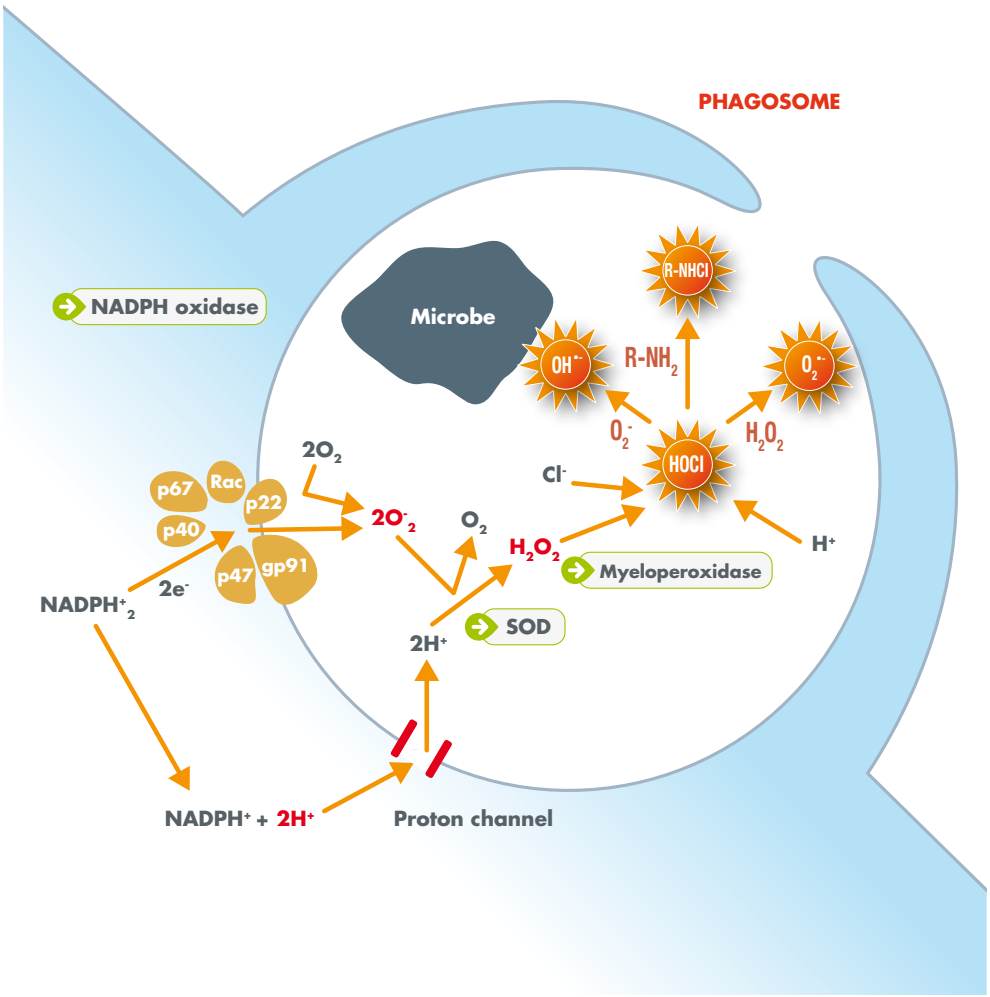

Figure 7.2. Redox balance, the key to macrophage function. SOD = superoxide dismutase.

acute inflammatory response. Macrophages contain various substances (including enzymes producing free radicals and small peptides with an antibiotic activity) involved in microbial killing. They also contain receptors for chemoattractive factors released by microbes. In addition to ROS, RNS and eicosanoids mentioned above, macrophages also synthesise and secrete a great number of communicational molecules, such as cytokines, including the pro-inflammatory cytokines interleukin (IL)-1, IL-6 and TNF-α. They also produce cytokine inhibitors, endocrine hormones, neurotransmitters (Klasing, 1998a) – which regulate specific immunity (initiating and directing the immune and inflammatory responses) – and many other related physiological responses. Therefore, these cells are important amplifiers of the immune response, both by cytokine production and by presenting parasite derived peptides to T cells.

The purpose of immune cell products is to destroy invading organisms. However, excessive or inappropriate production of these substances is associated with mortality and morbidity after infection and trauma, and with inflammatory diseases. Oxidants enhance IL-1, IL-8, and TNF production in response to inflammatory stimuli by activating the nuclear transcription factor NF-κB. Therefore, they also can cause a series of harmful effects, including killing of host cells, injuring cells and tissue directly via oxidative degradation of essential cellular components and damaging cells indirectly by altering the protease/antiprotease balance that normally exists within the tissue interstitium (Conner and Grisham, 1996). They also can cause cell mutations and sister chromatid exchanges (Weitzman and Stossel, 1981).

Therefore, overproduction of ROS or impaired antioxidant defence can result in oxidative damage to host macromolecules (McBride *et al.*, 1991) and extensive pathology and disease (Dietert and Golemboski, 1998). For example, in the case of a disease challenge, the overproduction of ROS may occur and they can leak from fagolysosomes into the surrounding cytoplasm and intracellular space. A number of antioxidant enzymes is expressed at the same time to protect the cells from the cytotoxic effects of ROS directed against engulfed microorganisms. However, low efficiency of these antioxidant mechanisms, for example due to selenium deficiency, could damage the cell's microbicidal and metabolic functions (Larsen, 1993). Indeed, macrophage function is disturbed, if there is a lack in antioxidant enzyme activity (Ebert-Dumig *et al.*, 1999). It was shown that TrxR activity as well as GSH-Px activity in these cells can be upregulated by the addition of selenium *in vitro* and *ex vivo*. Recent results revealed that TR1 is both a regulator and a regulated target in the macrophage gene expression network, and suggest a link between selenium metabolism and immune signalling (Carlson *et al.*, 2011).

NK cells were originally described as a population of granular lymphocytes with the ability to lyse tumour and virally-infected target cells. They differ from classical lymphocytes being larger in size, containing more cytoplasm and having electron dense granules. The mechanism of killing is mediated through release of its granule contents (perforins and granzymes) onto the surface of the infected cell (Lydyard *et al.*, 2000). In human NK cells comprise about 10-15% of all circulating lymphocytes. They produce cytokines and chemokines and can lyse target cells without prior sensitisation being crucial components of the innate immune system. They also exert cell cytotoxicity by recognising and inducing lysis of antibody-coated target cells and can be activated non-specifically by several cytokines via various receptors (Middleton *et al.*, 2002). The NK cells were shown to be extremely important in mucosal immunity (Rogers *et al.*, 2008). NK cells recognise and kill infected or transformed cells. First, activated NK cells make a tight contact with the target cell. Next, they polarise lytic granules toward the immunological synapse and release cytolytic proteins toward the target cell (Claus *et al.*, 2016). Indeed, NK cells are important in innate immunity. They were first described as guardians for the detection and clearance of transformed or virus-infected cells and recently they were also shown to be able to recognise and respond to bacteria-infected cells (Adib-Conquy *et al.*, 2014). Indeed, NK cells possess

receptors allowing them to sense and respond to viral and bacterial patterns, including TLRs. Upon TLR activation, NK cells are an important source of interferon (IFN)-γ and granulocyte macrophage colony-stimulating factor, cytokines necessary to fight infection (Adib-Conquy *et al.*, 2014). In fact, the consequences of TLR activation are diverse and include activation of cytokine, IFN and chemokine production, cell maturation, induction of degranulation and direct production of AMP, etc. (Juul-Madsen *et al.*, 2008).

TLRs are a family of conserved pattern recognition receptors that recognise specific microbial-associated molecular patterns and helping the cell distinguish between self and non-self materials (Coffey and Werling, 2011). In fact, TLRs provide a link between innate and adaptive immunity. Signalling by TLRs involves five adaptor proteins, known as MyD88, MAL, TRIF, TRAM and SARM. Recently additional functions for MyD88, apart from NF-B activation, have been revealed including activation of the transcription factors IRF1, IRF5 and IRF7, as well as a role outside the TLRs in IFN-γ signalling insights (O'Neill and Bowie, 2007). Furthermore, MAL and TRAM are shown to be bridging adaptors, with MAL recruiting MyD88 to TLR2 and TLR4, and TRAM recruiting TRIF to TLR4 to allow for IRF3 activation. Finally, the fifth adaptor, SARM, was shown to negatively regulate TRIF (O'Neill and Bowie, 2007). The presence of TLR ligands as adjuvants in conjunction with a vaccine was shown to increase the efficacy and response to the immunisation with a particular antigen (Coffey and Werling, 2011). Indeed, similar to innate immune cells, B cells express TLRs and B cell-intrinsic TLRs appear to cooperate with adaptive Ig receptors to achieve rapid humoral immunity and to preserve long-term humoral memory (Cerutti *et al.*, 2011).

Once a pathogen enters to the host, the initial non-specific response is an inflammatory reaction. In fact, during an inflammatory response, the innate immune cells release signals responsible for recruitment of distant immune cells to the site of infection, and for changing the metabolism of the bird. Indeed, an inhospitable environment for the invading pathogen is created in the body by increasing metabolic rate (fever), reducing feed intake, and initiating the preferential breakdown of skeletal muscle to support gluconeogenesis and synthesis of acute phase proteins in the liver (Korver, 2012). Therefore, inflammatory response results in a series of behavioural, immunologic, vascular and metabolic responses (Korver and Klasing, 2001). As a result, lethargy and anorexia are observed, growth rate could be slowed, muscle protein degradation increased with possible increased morbidity and mortality and decreased productivity. At the same time acute phase protein synthesis is increased. These proteins are involved in various aspects of host protection and help homeostasis restoration. In fact, in poultry, a number of acute phase proteins have been identified, including metallothionein, ovotransferrin, α1-acid glycoprotein, ceruloplasmin, fibronectin, serum amyloid A, mannan-binding protein and C-reactive protein, etc. (Korver, 2012). In general, the price for immunological defence against pathogens could be quite high.

Acquired or specific immunity includes humoral immunity and cell-mediated immunity (Figure 7.3). There are two major types of lymphocytes, B cells and T cells, of which humoral immunity is mediated by antibodies that are released by B-lymphocytes into the bloodstream. This immunity is based on the production of immunoglobulins (Table 7.3). They are responsible for specific recognition and elimination of various antigens: they bind and remove from the host invading organisms/substances.

Cell-mediated immunity is based on specific antigen recognition by thymus-derived T-lymphocytes. Due to this immunity, cells infected with a foreign agent, for example a virus, are destroyed via a direct contact between an activated T-cell and target (infected) cell (Qureshi *et al.*, 1998). Cell-mediated immunity is responsible for delayed-type hypersensitivity reactions, foreign graft rejections, resistance to many pathogenic microorganisms and tumour immunosurveillance (Wu and Maydani, 1998). Therefore, adaptive immunity is based on a tightly regulated interplay between antigen-presenting cells and T and B lymphocytes. They facilitate pathogen-specific immunologic effector pathways, formation of immunologic memory, and regulation of host immune homeostasis.

In birds, both T and B cell precursors originate in the bone marrow. Actual development of T cells takes place in the thymus and B cells develop in the bursa of Fabricius (Lahtala, 1998). Interactions between T and B-cells, as well as antigen presenting cells, are responsible for the development of specific immunity. These specific immunity

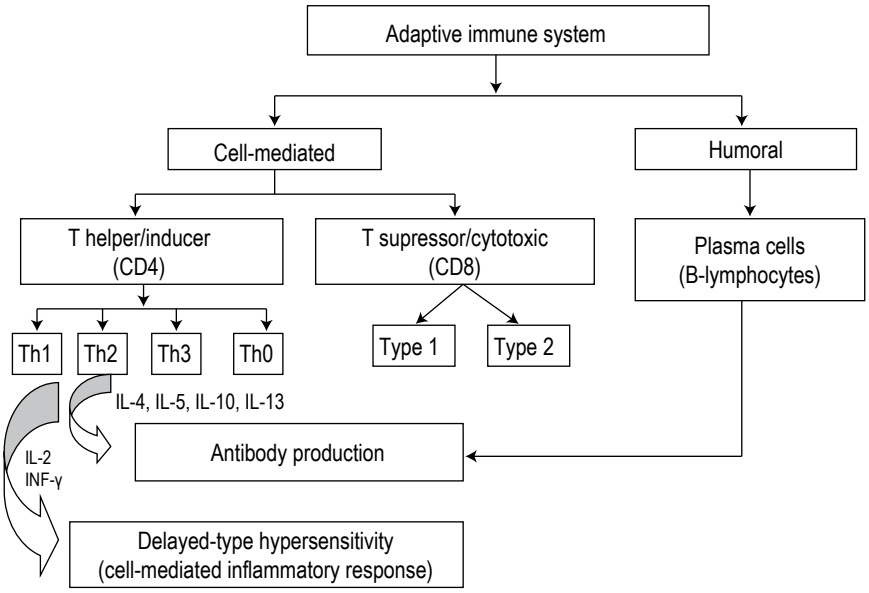

Figure 7.3. General scheme of adaptive immune system (adapted from Surai, 2006). IL = interleukin, INF = inferferon.

Table 7.3. Main immunoglobulin classes (adapted from Surai, 2006).

Immunoglobulin	Characteristics
IgG1, IgG2, IgG3, IgG4	largest quantity; provide the bulk of immunity to the most blood borne infectious agents; the only antibody class to cross the placenta to provide humoral immunity to the infant; vaccination asks the immune system to produce IgG specific for a particular antigen
IgA	a first line of defence against microbes entering through mucosal surfaces directly communicating with the environment; synthesised by plasma cells; prevents colonisation of mucosal surfaces by pathogens
IgM	the first antibody produced in an immune response in large quantity
IgD	present in humans, not documented in animals; functions as an antigen receptor on B cells
IgE	involved in allergy development and in immediate hypersensitivity syndromes such as hay fever and asthma

defence mechanisms are induced or stimulated by exposure to foreign substances, are specific for distinct macromolecules and increase in magnitude with each successive exposure to a particular macromolecule (Miles and Calder, 1998).

In comparison to natural immunity, specific immunity takes longer to develop, but is highly specific for antigens and has memory (Table 7.4). These two parts of the immune system work together through direct cell contact and through interactions involving such chemical mediators as cytokines and chemokines (Figure 7.4). Therefore, the chicken immune system requires the co-operation of macrophages, bursa-derived B-lymphocytes and thymus-derived T-lymphocytes with various other types of cells. The immune response involves cellular proliferation (T-lymphocytes), enhanced protein synthesis (including immunoglobulin synthesis by B-lymphocytes and acute phase protein synthesis by liver) and inflammatory mediator production. Physiological changes resulting from stimulation of the immune system include fever, anorexia and loss of tissue components (Grimble, 1997).

The two arms of the immune system (innate and acquired) are complementary and they operate in an integrated fashion to provide an effective protection of the host. As mentioned above, on the one hand, antigen is recognised and presented to the cells of the acquired immune system by innate immune cells. On the other hand, adaptive immune system cells (T helpers) regulate the bactericidal activity of phagocytes (e.g. macrophages and heterophils). Indeed, the innate immune system provides a rapid response to novel pathogens, but such a protective action can be metabolically and physiologically quite costly to the host. The acquired immune system can respond in a much more pathogen-specific fashion with minimal impact on the host, but depends on the interactions with the innate immune system (Korver, 2012).

Table 7.4. Key features of innate and adaptive immunity (adapted from Surai, 2006).[1]

	Innate	Adaptive
Appearance in evolution	primitive organisms	vertebrates
Induction time	fast (hours to days)	slow (days to decades)
Recognises	common 'pathogen-associated microbial patterns'	unique epitopes on each pathogen/ antigen
Cellular components	phagocytes (macrophages and neutrophils); NK cells; mast cells; dendritic cells	T and B cells
Generation of specificity	encoded in germline; has some specificity, no memory	somatic rearrangement; highly specific and has memory
Effector mechanisms	complement (alternative pathway); cytokines; chemokines; cell-mediated cytotoxicity	antibodies; cytotoxic T cells; classical complement activation; antibody-dependent cell-mediated cytotoxicity; cytokines; chemokines
Soluble mediators	macrophage-derived cytokines	lymphocyte-derived cytokines
Characteristic transcription factors	NF-κB (+JNK/AP1)	Jak/STAT, NF-κB, etc.
Physiological barriers	skin	cutaneous and mucosal immune systems
	mucosal membranes lysosyme stomach acid commensal bacteria	antibodies in mucosal secretions

[1] NF-κB = nuclear factor kappa B; NK = natural killer;

When analysing data related to immunomodulating properties of various nutrients it is necessary to pay special attention to methods used to assess immunological functions. For example, *in vivo* methods of immune function assessment are based on two main approaches: antibody response to vaccine or a delayed-type hypersensitivity (DTH) reaction. In the first method, immunisation with appropriate antigens (viral or bacterial) can elicit serum antibodies. So-called hemagglutination assay measures serum antibody concentration (titer) against antigens. Sheep red blood cells (SRBC) are often used as antigens.

This assay provides information about humoral immunity (B-cell responsiveness) and its association with cell-mediated immunity (T cell cooperation). The second (DTH) method is used to assess cell-mediated immune function.

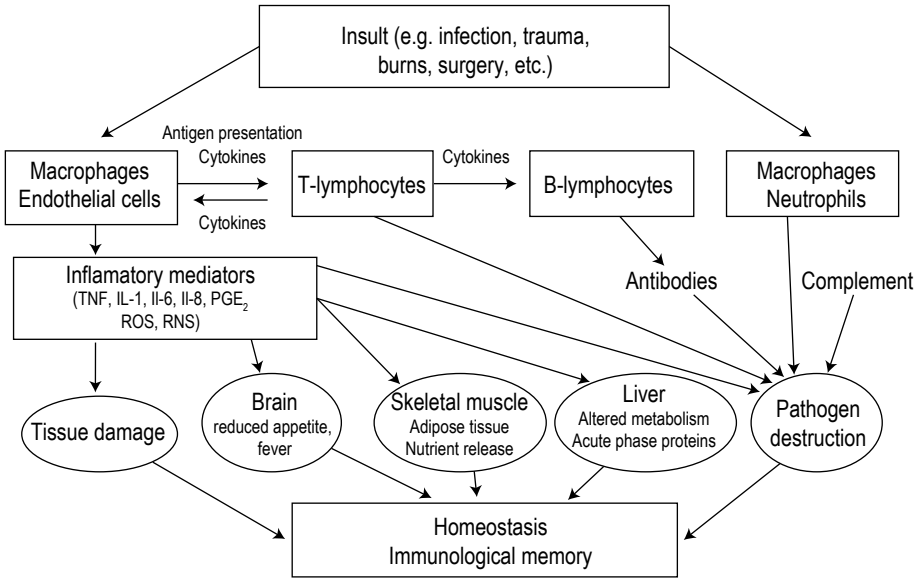

Figure 7.4. The natural and adaptive immunity interactions (adapted from Surai, 2006).

In vitro indices of immune function include (Wu and Meydani, 1998):

- *Lymphocyte proliferation assay*. Lymphocyte proliferation assay provides information about cell-mediated immune response and includes measuring the number of cells in culture with or without addition of stimulatory agent (mitogen). In this assay, isolated lymphocytes are incubated with mitogens which activate division of either T- or B-lymphocytes. Decreased proliferation may indicate impaired cell-mediated immunity. Various mitogens are used in such assays, but most often they include (Hayek *et al.*, 1996):
  - concanavalin A (ConA, a type of protein found in the jack bean, T-cell mitogen);
  - phytohemagglutin (PHA, a protein obtained from plant seeds, T-cell mitogen);
  - lipopolysaccharide (LPS, obtained from the membrane of Gram-negative bacteria, B-cell mitogen); and
  - pokeweed mitogen (PWM, T- and B cell mitogen).
- *Measuring cytokine production*. T cells produce a range of protein mediators called cytokines which regulate cell activation, growth, differentiation, inflammation and immunity.
- *Cytotoxicity assay.* This assay assesses activity of cytotoxic T lymphocytes (a group of T cells that kill other cells by recognising their cell-surface antigens) and NK cells (a group of non-T and non-B lymphocytes that kill virus-infected and tumour cells).
- *Flow cytometric analysis.* The assay deals with identifying the cells with different surface markers. The results can be used for understanding the cellular basis of immune response.
- *Plaque-forming cell (PFC) test*, which shows the number of antibody-producing cells.

Effect and role of animal immune system in modern animal production systems is difficult to overestimate. Banning feed grade antibiotics in Europe has made immune system competence the major factor determining efficiency of poultry production. Molecular immunology is developing very quickly and mechanisms of immunocompetence have received substantial attention (McCorkle, 1998; Saif and Swayne, 1998) and nutritional modulation of resistance to infectious diseases (Klasing, 1998b) is a frontline for future research.

Among the different nutrients playing an important role in modulating immune response (Klasing, 1998b) are natural antioxidants, including Se (Surai, 2006), which have been shown to be very promising (Figure 7.5). However, antioxidant roles in animal and human immune system modulation received only limited attention. Mechanisms of nutritional modulation of resistance to infectious diseases were divided by Klasing (1998b) into seven categories. However, selenium was not mentioned there.

It is well known that several indicators of immune responsiveness are depressed when animals are selenium and/or vitamin E deficient. In particular, decreases in both cellular and humoral immune function in man, laboratory and farm animals and chickens have been observed (Surai, 2006). Since these nutrients serve as antioxidants, membrane integrity may be affected by a deficiency. However, cellular integrity is very important for receiving, and responding to the messages needed to coordinate an immune response (Latshaw, 1991). Therefore, the antioxidant status of the host is a critical consideration in the optimal functioning of the immune system. Furthermore, modulating immune responses of birds by nutritional means targets in many cases specific immunity (Korver and Klasing, 2001).

7.3 Phagocyte functions

Phagocytosis is the process by which leukocytes and other cells ingest particulate ligands whose size exceeds about 1 μm. By ingesting microbial pathogens, phagocytes accomplish two important immune functions: initiate a microbial death pathway and build a bridge (send specific signals) between the natural and adaptive immune responses (Greenberg and Grinstein, 2002). As mentioned above, phagocytes play an important role in natural immunity. Among the 6 major specific rationales for modulating macrophage function in poultry analysed by Klasing (1998a), mitigation of immunosuppression arising from infectious diseases, dietary toxins or stress could be affected by dietary antioxidants. In fact, the availability of certain substrates and enzymatic cofactors can greatly influence the capacity for metabolite production by macrophages. Therefore, Se deficiency compromises the microbicidal activity of phagocytes (Serfass and Ganther, 1975). In particular, dietary deficiencies of Se and/or vitamin E have been shown to impair neutrophil and macrophage activities in chickens, including a decrease in peritoneal macrophages and decreased phagocytosis of red blood cells (Dietert *et al.*, 1990). The intracellular killing of yeast and bacteria by neutrophils and macrophages from chicken with selenium deficiency is reduced

Figure 7.5. Selenium deficiency impacts immunity.

(Larsen, 1993). Similar effects were observed in rat, goat, cattle and fish (for review see Larsen, 1993; Surai, 2006).

Thus, selenoproteins appear to be important in protecting those aspects of phagocytic function that are sensitive to the destructive properties of exogenous H_2O_2. In fact, Se-deficient granulocytes did not metabolise H_2O_2 as well as replete granulocytes, and H_2O_2 caused damage to the O_2-generating system. For example, prolonged incubation with stimulants and prior incubation with an H_2O_2-generating system caused loss of activity of the membrane-bound, NADPH-dependent, O^-_2-generating system in Se-deficient granulocytes with low GSH-Px activity (Baker and Cohen, 1984). It seems likely that macrophage function is insufficient in the case of altering the generation of a respiratory burst (e.g. hereditary chronic granulomatous disease) or when there is a lack in antioxidant enzyme activity. Thioredoxin has been identified as a lymphocyte growth factor and might therefore be involved in the crosstalk between macrophages and lymphocytes (Ebert-Dumig *et al.*, 1999).

Regulatory effects of Se and vitamin E on phagocyte functions have been shown in experiments with farm and laboratory animals as well as in various *in vitro* systems. For example, both vitamin E and selenium enhanced the chemotactic and random migration of polymorphonuclear leukocytes (PMN) and increased the production of superoxide following stimulation with phorbol myristate acetate (Ndiweni and Finch, 1996). *In vitro* Se supplementation of the J774.1 macrophage cell line enhanced phagocytosis, degranulation by the release of beta-glucuronidase after N-formyl-methionyl-leucyl-phenylalanine or cytochalasin B, and the production of superoxide anion after phorbol myristate acetate stimulation of these cells (Safir *et al.*, 2003). Selenium supplementation also substantially increased the release of TNF, IL-1 and IL-6 after lipopolysaccharide stimulation compared to Se-deficient cells.

The *in vitro* effects of an inorganic selenium salt on phagocytic functions of human neutrophilic granulocytes from donors with a low activity of GSH-Px have been investigated (Urban and Jarstrand, 1986). The phagocytic and bactericidal activities were significantly increased in granulocytes exposed to Se in physiological concentrations (100 and 200 ng Se/ml). However, at 2,000 ng Se/ml these activities were found to be equal to or lower than control levels, probably reflecting Se toxicity. It is necessary to bear in mind that the effect of supplemental dietary Se on phagocyte function depends on many factors, including background Se level, dose and form of Se used, age and sex of animals tested, etc. For example, ingestion of 400 μg/day of sodium selenite (182.8 μg pure Se) by volunteers resulted in a significant increase in plasma Se levels (Greenman *et al.*, 1988). However, the phagocytic function of PMNs measured by ingestion of Oil Red O paraffin droplets and chemiluminescence tests was not significantly affected. Therefore, it was concluded that inorganic selenium was not an efficient stimulating agent of phagocytosis in humans.

High Se doses could be detrimental for immune cells. For example, data reported by Shilo and Tirosh (2003) showed that exposing Jurkat T cells or J774.2 macrophages to >5 μM sodium selenite induced cell death. They suggested that selenite induces

changes in the balance between mitochondrial superoxide and hydrogen peroxide production, which can facilitate cell death in immune system cells. The authors considered these changes as a mechanism by which Se down-regulates the immune system's inflammatory response and protects against overproduction of peroxides. It seems likely that the level of NO and heat shock protein (HSP) expression levels in neutrophils can be influenced by Se status. In particular, it was shown that Se deficiency increased the mRNA levels of HSP and inducible NO synthase (iNOS) and induced a higher level of NO in chicken neutrophils (Chen *et al.*, 2014b). Selenoproteins are important for the balanced biosynthesis of pro- and anti-inflammatory oxylipids during inflammation. Indeed, decreased selenoprotein activity resulted in the accumulation of ROS, increased cyclooxygenase and lipoxygenase expression and decreased oxylipids with known anti-inflammatory properties, such as arachidonic acid-derived lipoxin A_4 (LXA_4) and linoleic acid-derived 9-oxo-octadecadienoic acid (9-oxoODE). Furthermore, treating macrophages with LXA_4 or 9-oxoODE diminished the oxidant-induced macrophage inflammatory response, as indicated by decreased production of TNF-α (Mattmiller *et al.*, 2014). Selenoproteins in macrophages enhance 15-PGDH-dependent oxidation of PGE2 to alleviate inflammation (Kaushal *et al.*, 2014). Interestingly, Se accelerates the differentiation and maturation of chicken dendritic cells (DCs) and selenoproteins are involved in regulation of the differentiation and maturation of DCs and are closely correlated to surface markers of chicken DCs (Sun *et al.,* 2017).

Since various mycotoxins are shown to reduce macrophage viability and effector functions (Bondy and Pestka, 2000; Surai and Dvorska, 2005) a protective immunomodulating effect of antioxidants, including Se, could be especially important in commercial conditions associated with mycotoxicoses (Hegazy and Adachi, 2000). Furthermore, optimised management of macrophage metabolite production by nutritional means is a crucial factor for bacterial resistance of poultry (Dietert and Golemboski, 1998).

7.4 Antibody production

Research data, accumulated over the last 50 years, clearly indicate that antioxidant deficiency is associated with an impaired immune system in human, and laboratory and farm animals. In fact, the effects of Se on immunity have been studied since 1972 when Berenshstein showed that Se enhanced humoral immunity, followed by a series of publications by Spallholz who tested immunomodulating properties of Se in mice. His results showed that adding 2.8 mg/kg Se to the diet stimulated antibody synthesis to SRBC while 1.25 mg/kg Se increased number of plaque-forming and concentration of IgM (Spallholz *et al.*, 1973b). It is interesting that the concentration of IgG was also affected by Se supplementation in mice at 1-3 mg/kg (Spallholz *et al.*, 1973a). Sodium selenite administered to mice i.p. (5 μg Se) enhanced the primary immune response to the sheep red blood cell antigen (Spallholz *et al.*, 1975). Enhancement of the primary immune response was greatest when Se was administered prior to or simultaneously with the sheep red blood cell antigen.

For the next stage of research, related to immunomodulating properties of Se, a chicken model was widely used. For example, when two-week-old chickens were maintained on Se- or Se-vitamin E deficient diets from hatching, they had reduced antibody titres to SRBC (Marsh *et al.*, 1981). In the same experiment three-week old chicks had decreased antibody production only in combined Se-vitamin E deficiency. Therefore, it was suggested that Se and vitamin E may be important components of immune function, and their effects depend on antigen concentration, sex, and age (Larsen, 1993).

In contrast to selenium and/or vitamin E deficiency, antioxidant supplementation of the diet has been shown to have an immunomodulating effect and the level of vitamin E or Se required for optimal immune function may be higher than that required for growth and other physiological functions. The simplest tests with antibody production against SRBC clearly showed a beneficial effect of vitamin E and selenium.

Newcastle disease challenge is often used to assess immunostimulating properties of various antioxidants. For example, the immune response of chicks vaccinated with live Newcastle disease vaccines was significantly improved by Se and vitamin E. Diets were supplemented 14 days before vaccination at a ratio of 0.25 and 300 mg/kg, respectively, and improvement in hemaglutination inhibition (HI) antibody titres and protection rate against challenge with velogenic Newcastle disease virus was observed (Bassiouni *et al.*, 1990). When Se was added to the feed of White Leghorn chickens (between 0.1 and 0.8 mg/kg) prior to challenge with either *Escherichia coli* or sheep erythrocyte antigen, an antibody titre against sheep erythrocytes increased by 77% (Larsen *et al.*, 1997). Following chilling, antibody titre response was substantially reduced and the titre reduction was prevented by dietary additions of Se between 0.1 and 1.2 mg/kg (Larsen *et al.*, 1997). Furthermore, significantly higher antibody titres at 10 days post-immunisation for Newcastle disease virus were attributed to 0.06 mg/kg and 150 IU/kg Se and vitamin E, respectively (Swain *et al.*, 2000). The antibody immune response in White Leghorn chicks was also significantly improved for the Se enrichment diet in the *Salmonella* or *Salmonella* + aflatoxin inoculated groups (Hegazy and Adachi, 2000). Selenium can also be used with drinking water. For example, Deng *et al.* (1999) studied effects of sodium selenite solution on the humoral immunity and erythrocyte immunity function against Newcastle disease in chicks. Their results showed that the HI titre of the control group was much lower than that of the 4 Se-supplemented groups. At the same time, the erythrocyte immune function of the control group was lower than that of the tested groups. Similarly, when chicken were vaccinated with attenuated strain C30-86 of Newcastle disease virus via drinking water at 3 and 21 days of age supplementation with Se through water increased HI antibody titre by stimulating antibody production (Yang *et al.*, 2000). Furthermore, Se supplementation helps increasing post vaccination humoral immune response against infectious bursal disease (IBD) in broiler chicks (Arshad *et al.*, 2005). It was shown that organic Se had more pronounced effect on humoral immunity of the growing chickens in comparison to sodium selenite. In particular, it was effective in improving antibody production against Newcastle disease (Kovalenko *et al.*, 2008). In fact, at day

39 IgG concentration in chicken plasma increased from 7.42 to 9.66 mg/ml ($P<0.05$) and IgM from 0.80 to 1.06 mg/ml ($P<0.05$) due to organic Se supplementation.

Immunomodulating properties of antioxidants were shown with a range of animal species, including various farm and laboratory animals. Therefore, selenium and/or vitamin E deficiencies are associated with a compromised humoral immune response not only in chicken but also in mouse, horse, pig, cattle, calves, lambs and sheep (Larsen, 1993) and inclusion of increased doses of these nutrients into the diet is proven to improve the immune response (Surai, 2006).

7.5 Lymphocyte functions

It has been suggested that Se and vitamin E deficiencies affected T lymphocytes to a greater extent than B lymphocytes (Larsen, 1993). This was explained to be a result of higher level of polyunsaturated fatty acids (PUFAs) in T lymphocytes and higher membrane fluidity. In fact, vitamin E and Se deficiencies may affect both the maturation of specific lymphocyte subpopulations and the functional and proliferative capabilities of the peripheral lymphocytes. A combined Se- and vitamin E deficiency in chicks showed a more severe depression of T-cell response than just Se-deficient chicks. For example, in an experiment of Chang *et al.* (1994) the dietary deficiencies in vitamin E and selenium (basal diet without vitamin E and Se supplementation starting from hatching) resulted in a significant inhibition of T-lymphocyte proliferation. In particular, a decreased proportion of peripheral T cells and more specifically a decreased number of $CD4^+$ peripheral blood leukocytes were observed. It is interesting to note that so-called regulatory $CD4^+$ T cells are capable of controlling the activity of other lymphocytes and they protect the integrity of tissues and organs *in vivo* and also play a major role in the systemic homeostatic mechanisms that control total lymphocyte numbers (Annacker *et al.*, 2001). The proliferative response to both ConA and PHA was impaired by vitamin E and Se dietary deficiencies. However, the proliferative response could be fully reconstituted after vitamin E and Se supplementation for periods longer than one week.

Selenium deficiency in growing chickens was associated with impaired bursal growth and Se-vitamin E deficiency caused inhibition in thymus growth (Marsh *et al.*, 1986). In this experiment reduced numbers of lymphocytes were seen in both the thymus and bursa in combined Se-vitamin E deficiency. On the other hand, a significant increase in relative weight of the bursa of Fabricius was observed in broiler chicks at a supplementation level of 0.10 mg/kg selenium and 150 IU/kg vitamin E (Swain *et al.*, 2000). It is interesting to note that bursectomy in chickens caused a small but significant fall in spleen Se concentration, but not in that of other tissues (Abdel-Ati *et al.*, 1984). It has been shown that Se deficiency alone or in combination with vitamin E is associated with depressed splenocyte ability to proliferate in culture (Marsh *et al.*, 1987). Such a depression was not due to reduced lymphocyte viability in culture. Marsh *et al.* (1986) suggested that the primary lymphoid organs are major targets of Se and vitamin E dietary deficiencies and provided a possible mechanism

by which immune function may be impaired. In their experiment with chickens specific deficiencies of Se or vitamin E significantly impaired bursal growth. Thymic growth was impaired only by the combined vitamin E-selenium deficient diet. Severe histopathological changes in the bursa resulted from the combined deficiency and these were detectable by 10-14 days after hatching (Marsh *et al.*, 1986). These changes were characterised by a gradual degeneration of the epithelium and an accompanying depletion of lymphocytes. On the other hand, chicks receiving Se (1 mg/kg) and vitamin E (300 IU/kg) had significantly higher cellular immune responses in terms of percent leukocyte migration inhibition (Swain *et al.*, 2000).

In a series of recent publications a relationship between Se deficiency and immune organ growth, development and structure has been described in details. In particular, it has been shown that chickens fed deficient in Se diets exhibited oxidative stress associated with lesions in immune organs, decreased serum IL-1β, IL-2 content, and serum TNF content and impaired the immune function of chickens (Zhang *et al.*, 2012). In fact, Se concentrations in thymus, bursa and spleen of the low-Se (0.032 mg/kg Se) chicken group were significantly lower than in the control group. Furthermore, low-Se diet compromised antioxidant defences indicative by a decrease in the activities of total antioxidative capacity, superoxide dismutase (SOD), GSH-Px, and an increase in xanthine oxidase activity and malondialdehyde (MDA) content in the immune organs. In addition, pathological lesions and DNA damage of immune tissues were observed in low-Se group, while the serum IL-1β and IL-2 contents decreased, and TNF content increased (Zhang *et al.*, 2012). Similarly, it was shown that low Se diet intake (0.0342 mg/kg Se) caused oxidative stress, evidenced by decrease in GSH-Px and catalase activities and increase in MDA contents, associated with increased apoptosis, arrested cell cycle, and injured structure and immune function of the spleen in chickens (Peng *et al.*, 2012). Indeed, the weight and relative weight of the spleen were significantly decreased in the low-Se group when compared to the control group (0.2 mg/kg Se) and splenic lesions were characterised by lymphocyte depletion and congestion of red pulp. In addition, the percentage of apoptotic splenocytes was greatly increased in the low-Se group when compared to the control group (Peng *et al.*, 2012). It seems likely that low-Se diet inhibits the development of the thymus by arresting the cell cycle and decreasing IL-2 content. For example, low-Se diet caused a decrease in S-phase cells in the thymus and mitochondrial injury with increased apoptotic cells was observed. Furthermore, low-Se diet decreased the serum IL-2 content and mitogenesis of peripheral blood lymphocytes to concanavalin A in comparison with those of control group (Peng *et al.*, 2011e) and decreased T cell subsets (Peng *et al.*, 2011b) and restrained the development of the bursa of Fabricius and thymus by cell cycle arrest, apoptosis and decreasing the IL-2 content (Peng *et al.*, 2011d).

It seems likely that the oxidative stress and NO overproduction play a causative role in the apoptosis of immune tissues induced by Se deficiency. For example, free radical production, NO and iNOS activity in blood and the immune organs (bursa, thymus and spleen) of the chicken in low-Se group (0.032 mg/kg Se) were significantly increased compared to the control group. In addition, the expression of Fas and caspase-3 mRNA increased and apoptosis was observed in chicken immune

organs in the low-Se group. The degree and number of apoptotic cells rose in a time-dependent manner (Zhang *et al.*, 2013). In fact, selenoproteins, including SelW, Sel15 and SelT play important roles in the protection of immune organs of birds from inflammatory injury by the regulations of inflammation-related genes. In fact, SelW is widely expressed in immune organs of birds and Se-supplementation of the feed increases SelW expression in the thymus and the bursa of Fabricius (Yu *et al.*, 2011). Recently, it has been shown that Se-deficient diets significantly decreased the mRNA expression of SelW, and induced a significantly up-regulation of pro-inflammatory signalling: cyclo-oxygenase 2 (COX-2), iNOS, NF-κB, prostaglandin E synthase (PTGEs) and TNF-α mRNA levels in chicken immune organs, including spleen, thymus and bursa of Fabricius (Yu *et al.*, 2015). Furthermore, chicken Sep15 preserves the typical characteristic of a selenoprotein and may play some role in the redox regulation. Chicken Sep15 was shown to decrease by Se deficiency in immune organs (Sun *et al.*, 2014). Furthermore, Sep15 deficiency induced the occurrence of higher oxidative stress and enhanced the sensitivity of cells to H_2O_2. Se deficiency in chickens also induced lower levels of SelT and induced oxidative stress in immune organs, including reduced catalase (CAT) activity, and increased levels of H_2O_2 and hydroxyl radical (-OH; You *et al.*, 2014). In addition, the histopathological analysis showed that immune tissues were injured in the low-Se groups. Furthermore, *in vitro*, H_2O_2 induced a significantly up-regulation of the mRNA levels of inflammation-related genes (iNOS, COX-2, NF-κB, PTGEs, and TNF-α) in cultured splenic lymphocyte. However, when lymphocytes were pretreated with Se before treatment with H_2O_2, the expression of inflammation-related genes were significantly decreased. On the other hand, silencing of SelW significantly up-regulated the inflammation-related genes in cultured splenic lymphocytes. The results suggested that the expression levels of inflammatory factors and SelW can be influenced by Se in birds (Yu *et al.*, 2015). It is well established that Se deficiency causes defects in the chicken bursa of Fabricius and Se-related immunosuppression is usually accompanied by a downregulation of mRNA expression levels of selenoproteins and an upregulation of the HSP mRNA expression levels. It was shown that in chicken bursa of Fabricius mRNA expression levels of selenoproteins, such as TrxR1, TrxR2, TrxR3, Dio1, Dio2, Dio3, GSH-Px1, GSH-Px2, GSH-Px3 GSH-Px4, SelP1, SelO, Sel15, SelX1, SelS, SelI, SelU, SelH, and SPS2, were significantly decreased due to Se deficiency (0.033 mg/kg for 15-55 days; Khoso *et al.*, 2015). Furthermore, the mRNA and protein expression levels of HSPs (HSP27, HSP40, HSP60, HSP70, and HSP90) were also significantly increased in comparison to Se adequate chickens (0.15 mg/kg). The expression levels of IL-2, IL-6, IL-8, IL-10, IL-17, IL-1β, IFN-α, IFN-β, and IFN-γ were significantly decreased and TNF-α was significantly increased due to Se deficiency compared with the control group (Khoso *et al.*, 2015).

Recently it has been shown that in chicken erythrocytes the expression of 24 selenoproteins and 7 cytokines (IL-2, IL-4, IL-8, IL-10, IL-12β, transforming growth factor (TGF)-β4, and IFN-γ) decreased, and the expression of 3 cytokines (IL-1γ, IL-6 and IL-7) increased due to Se-deficiency (Luan *et al.*, 2016). The increase of pro-inflammatory cytokines (IL-1γ, IL-6, and IL-7) suggested that the immune system of chickens was damaged by the Se deficiency. There was a close correlation

between selenoprotein and cytokine expression levels and a Se diet revealed an important relationship between Se and the chicken immune system (Luan *et al.*, 2016). Se deficiency in chickens caused defects in immune organ morphology and function, as evidenced by abnormal histological structures: red pulp broadening and lymphocytes in the cortex and medulla of the thymic lobule decreased distinctly and distributed loosely (Yang *et al.*, 2016a). In Se deficiency in broiler chicks with signs of exudative diathesis, the antioxidant function declined remarkably, and most of the HSP expression levels increased significantly in the spleen, thymus, and bursa of Fabricius (Yang *et al.*, 2016a). At the same time mRNA levels of HSP27, HSP40, and HSP70 decreased in thymus tissues of the Se-deficient chickens. It was shown that Se deficiency decreased the mRNA levels of 23 selenoproteins in the thymus, spleen, and bursa of the Fabricius tissues of broiler chickens (Yang *et al.*, 2016b). Furthermore, among 23 selenoproteins, the mRNA levels of Dio1 in the thymus, Txnrd2 in the spleen, and Txnrd3 in the bursa of Fabricius decreased significantly (90.9, 83.3 and 96.8%, respectively). Indeed, Se deficiency mainly influenced the expression of antioxidative selenoproteins, especially, GSH-Px, thioredoxin reductases and iodothyronine deiodinases in chicken immune organs (Yang *et al.*, 2016b).

Inclusion of selenium in the chicken diet (0.3-0.6 mg/kg) for 2 months significantly increased the response of peripheral blood lymphocytes to phytohemagglutinin and NK cell activities, accelerating the development of cell-mediated immunity in chickens (Huang and Chen, 1999). In general, splenic NK cell activity is enhanced in selenium-supplemented, healthy animals (Dhur *et al.*, 1990). The results of Colnago *et al.* (1984b) indicate that dietary Se supplementation modifies the number of peripheral blood leukocytes in chickens infected with coccidia. In particular selenium significantly increased blood leukocyte number 8 h after challenge in one experiment and produced numerically higher leukocyte number in three other experiments. Immunisation of chickens against coccidiosis can be enhanced by Se supplementation (Colnago *et al.*, 1984a).

It seems likely that Se alters the kinetics of expression of high affinity (p55/p75) IL-2 receptors (IL-2R). For example, dietary (2 mg/kg for 8 weeks) or *in vitro* (1×10^{-7} M) supplementation with Se results in a significant upregulation of the expression of both the p55 and p70/75 IL-2 binding sites on the surface of ConA-stimulated lymphocytes from mice (Roy *et al.*, 1993). This resulted in the formation of significantly higher numbers of high affinity IL-2R/cell with the preservation of the normal ratio of high affinity to total IL-2 binding sites/cell. The authors suggested that Se could accelerate the clonal expansion of activated lymphocytes.

As shown above, Se is proven to have an important function in maintaining proliferative capacity of T- and B-cells (Surai, 2006). It has been suggested that Se may modulate the expression of IL-2R on the cell surface, which could lead to the altered ability of lymphocytes from Se-deficient animals to respond to mitogen and antigens (Larsen, 1993). Again this phenomenon is a characteristic of various farmed and laboratory animals (Surai, 2006).

7.6 *In vitro* effects of selenium on immune cells

Natural antioxidants are essential nutritional factors that affect the development and expression of cell-mediated immune responses. Various *in vitro* models were used to assess effects of Se on lymphocyte functions with rodent and human lymphocytes been used most frequently. It has been shown that dietary (2 mg/kg for 8 weeks) or in *in vitro* (1×10^{-7} M) supplementation with Se (as sodium selenite) results in a significant enhancement of the proliferative responses of spleen lymphocytes from C57B1/6J in response to mitogen stimulation. In contrast, Se deficiency (0.02 mg/kg for 8 weeks) had an opposite effect. The alterations in the ability of cells to proliferate were apparently related to the ability of Se to alter the kinetics of expression of high-affinity IL-2 receptors on the surface of activated lymphocytes. This was associated with enhanced or delayed clonal expansion of the cells. The changes in tumour cytotoxicity were paralleled by changes in the amounts of lymphotoxin produced by the activated cells. The results also suggested that Se exerts its effect 8-24 h after stimulation, and that it most likely affects processes in the cytoplasmic and/or nuclear compartments of activated lymphocytes (Kiremidjian-Schumacher *et al.*, 1992).

The impairment in lymphocyte proliferative response to antigens in Se and/or vitamin E deficiency could be a result of lipid peroxidation and damages to membrane structures and more importantly to membrane receptors (Larsen, 1993). As a result cell-to-cell communication could be compromised. In this case a protective effect of antioxidants could be a crucial factor in immune system competence. For example, it was found that lipid peroxidation in lymphocytes before ConA stimulation was lower than that after stimulation and that SOD promoted lymphocyte proliferation dose dependently. The addition of Na_2SeO_3 to lymphocytes culture or supplementation in drinking water to mice decreased the lipid peroxidation in lymphocytes stimulated by ConA. In the presence of Se, there was an inverse correlation between the levels of LPO in lymphocyte and the stimulated proliferation (Sun *et al.*, 1995). *In vitro* vitamin E and selenium supplementation was able to enhance significantly the depressed PMN-mediated chemotaxis and phagocytosis or monocyte chemoattractant protein-1 (MCP-1) production in elderly subjects (Ventura *et al.*, 1994).

Thus, selenium appears to be capable of selectively regulating the generation of functional lymphocyte subsets *in vitro*. For example, Se at physiologic concentrations can inhibit human lymphocyte proliferation in response to irradiated tumour cells in mixed lymphocyte/tumour cell cultures (MLTC; Petrie *et al.*, 1989). Furthermore, the various lymphocyte functional activities generated in these cultures exhibited different levels of sensitivity to the effects of selenium. In particular, the generation of suppressor-cell activity in MLTC was strongly inhibited by the presence of physiologic levels of Se, while the development of cytotoxic T-lymphocyte activity in identical cultures was not affected by Se. Production of interleukin-2 in these cultures showed an intermediate sensitivity to the effects of Se (Petrie *et al.*, 1989).

The effect of Se on NK and lymphokine-activated killer (LAK) cell activities and proliferative responses of human lymphocytes was studied *in vitro* (Nair and Schwartz,

1990). Direct addition of Se at 1.0 μg/ml to the mixture of target and effector cells significantly suppressed the NK activity of normal lymphocytes. Similarly, when lymphocytes were preincubated with Se at concentrations as low as 0.2 μg/ml for a period of 48 h, a significant inhibitory effect on NK activity was observed. Lymphocyte proliferative responses to T cell mitogens, such as phytohemagglutin (PHA) and ConA, were also significantly suppressed by direct addition of Se at 0.5-1.0 μg/ml. The inhibitory effect of Se was not due to non-specific toxicity of effector cells as demonstrated by viability nor was the effect directed against target cells. Therefore, although Se is an essential micronutrient for various immune mechanisms, an excess of Se may have a deleterious effect on certain immunological functions.

As mentioned above there are principal differences in metabolism of organic and inorganic selenium. This could be a reason of differences in stimulating activity of different forms of selenium on immune system. For example, sodium selenite and SeMet were tested in parallel, and their capability to inhibit or to increase the antibody production by human lymphocytes *in vitro* was investigated (Borella *et al.*, 1996). Low doses of Se (0.5-2.0 μM) added as sodium selenite or SeMet did not alter the secretion of antibodies. When Se was added at higher levels, instead, an inhibitory effect was found using selenite, whereas a progressive increase in immunoglobulin production was observed after exposure to SeMet. Therefore, there is an advantage in using organic selenium for immune system stimulation in comparison to selenite.

In general, it is well known that selenium, especially in the form of selenite, is clastogenic for cultured lymphocytes. For example, human lymphocyte cultures were treated with increasing concentrations (from 8.0×10^{-8} to 8.0×10^{-5} M) of sodium selenite and SeMet 24 h after stimulation with PHA and were assessed for chromosomal aberrations at 48 h (Khalil, 1989). The yield of abnormal metaphases was dependent on the dose and the form of selenium used. At 8.0×10^{-5} M the proportion of aberrant cells reached 53.5 and 43.0% for selenite and SeMet, respectively. The selenium-induced chromosomal aberrations were primarily of the chromatid type and included breaks and fragments. *In vitro* exposure of human peripheral blood lymphocytes to high concentration of two inorganic salts of selenium-sodium selenite (2.9×10^{-5} M) and sodium selenate (2.65×10^{-5} M) was found to be lethal. Lower concentrations of sodium selenite (from 2.32×10^{-7} to 2.9×10^{-6} M) and sodium selenate (from 1.06×10^{-6} to 5.3×10^{-6} M) induced chromosomal aberrations and reduced cell division in proportions directly related to the dose (Biswas *et al.*, 2000). Clearly, sodium selenite was considerably more clastogenic than sodium selenate.

It seems likely that only low levels of selenium are essential for T-cell mitogenesis even in selenium-insufficient splenic cells. For example, T-cell proliferation of the selenium-insufficient splenic cells induced by ConA was increased by addition of Na_2SeO_3, Na_2SeO_4, Na_2Se, seleno-DL-cystine, seleno-L-methionine and selenocystamine (Ueno *et al.*, 2008). Their promoting action was observed at levels lower than 0.1 μmol/l and was completely suppressed at the highest concentration (1 μmol/l). However, recovery of cGSH-Px activity in the selenium-insufficient cells by supplementary Na_2SeO_3 was only partial, while TrxR activity was readily recovered from selenium deficiency.

The stimulation of T-cell mitogenic response by the physiological levels of selenite is predominantly caused by increased TrxR activity, which may lead to reduction of Trx-1 dependent intracellular expression level and promotion of DNA binding of NF-κB (Ueno *et al.*, 2007).

Se supplementation increases the production of 15d-PGJ2 as an adaptive response to protect cells against oxidative stress-induced pro-inflammatory gene expression (Vunta *et al.*, 2007). It seems likely that Se supplementation of macrophages negatively regulates the LPS-dependent production of iNOS, a proinflammatory gene. Se plays an important role as an anti-inflammatory agent by tightly regulating the expression of pro-inflammatory genes in immune cells (Vunta *et al.*, 2008). It seems likely that Se down-regulates iNOS gene expression and NO production in the LPS-stimulated macrophages through inhibition of the NF-κB activation pathway (Yun *et al.*, 2007). Indeed, selenium is important in the shunting of arachidonic acid metabolism toward the production of PGD(2) metabolites. For example, treatment of selenium-deficient macrophages with rosiglitazone, a peroxisome proliferator-activated receptor γ ligand, up-regulated hematopoietic-PGD(2) synthase, while the expression of NF-κB-dependent thromboxane synthase and microsomal PGE(2) synthase was down-regulated by selenium (Gandhi *et al.*, 2011). Therefore, the anti-inflammatory activity of Se using LPS and IL-4-treated macrophages from mice fed Se-deficient and Se-adequate diets were investigated (Nelson *et al.*, 2011). Supplementation with Se (100 nmol/l) of IL-4-treated macrophages significantly increased the expression of alternatively activated macrophage (M2) markers, Arg-I, Fizz1, and Mrc-1. At the same time, expression of classically activated macrophage (M1) markers, TNF-α, and IL-1β, was significantly decreased in LPS-treated macrophages that were cultured in Se and IL-4. Furthermore, macrophages treated with inhibitors of PPARγ and STAT6, pivotal transcription factors that mediate the activity of Se and IL-4, respectively, showed complete ablation of Se-dependent expression of M2 markers (Nelson *et al.*, 2011). It is interesting to note that subjects with a decreased serum Se concentration may be exposed to a greater chronic oxidative stress due to neutrophil ROS production (Lee *et al.*, 2011).

Recently, the effects of Se on T-cell proliferation and IL-2 production were studied in primary porcine splenocytes (Ren *et al.*, 2012). It seems likely that Se can improve the redox status of splenocytes and enhance mitogen-induced T-cell activation. For example, splenocytes were treated *in vitro* with different mitogens in the presence of 0.5-4 μmol/l sodium selenite. It was shown that Se significantly promoted T-cell receptor (TCR) or ConA-induced T-cell proliferation and IL-2 production but failed to regulate T-cell response to PHA. In addition, Se significantly increased the levels of cytosolic GSH-Px1 and TrxR1 mRNA, the activity of GSH-Px1 and the concentration of reduced glutathione (GSH) in the unstimulated, or activated splenocytes. In the same experiment, N-acetylcysteine, a pharmacological antioxidant, increased T-cell proliferation and IL-2 production by TCR and ConA stimulated splenocytes confirming that T-cell proliferation is sensitive to the redox status (Ren *et al.*, 2012). A new role for selenoproteins in the epigenetic modulation of proinflammatory genes has been suggested (Narayan *et al.*, 2015). In particular the authors showed that selenium

supplementation decreased acetylation of histone H4 at K12 and K16 in COX-2 and TNF-α promoters, and of the p65 subunit of the redox sensitive transcription factor NF-κB in primary and immortalised macrophages. It seems likely that the ability of selenoproteins to skew the metabolism of arachidonic acid contributes, in part, to their ability to inhibit histone acetylation (Narayan *et al.*, 2015).

7.7 Disease resistance

The final goal of the improvement of the immune system is to increase resistance to various diseases. Indeed, this option was extensively studied with chickens during 1980-1990. For example, a combination of vitamin E with Se resulted in reduced mortality and increased body weight gain in chickens infected with *Eimeria tenella* (Colnago *et al.*, 1984). The same authors showed that dietary supplementation with selenium or vitamin E reduced mortality and increased body weight gain of nonimmunised chickens infected with *E. tenella* in three of four experiments. When chicks were inoculated with virulent Marek's disease (MD) virus at 10 days of age, selenium (0.6 mg/kg) decreased the morbidity and mortality from MD. In particular, Se increased the ability of cells to remove ROS and lipid peroxides, and decreased the degree of tissue damage caused by ROS (Huang and Chen, 1996). In another experiment, one-day old chicks were fed on a basal diet containing Se at 0.086 mg/kg (group I) or a basal diet supplemented with Se at 0.3 mg/kg (group II) or 0.6 mg/kg (group III). The chickens were infected with infectious bursal disease virus at day 39. Ten days later the mortality rates in groups I, II and III were 33.3, 12.4 and 10.6%, respectively, and the infection induced inhibition of T lymphocyte transformation was less in the Se supplemented birds (Bu *et al.*, 1996). When Se was added to the feed of White Leghorn chickens prior to challenge with either *E. coli* or sheep erythrocyte antigen, the incidence of death or lesions was reduced from 86 to 21% at the optimal dose of Se (0.4 mg/kg feed; Larsen *et al.*, 1997). Lower Se values were measured in chickens infected with *Ascaridia galli* compared to controls, and this was related to a lower degree of Se absorption and the regeneration of the intestinal mucosa in infected birds (Damyanova *et al.*, 1995). Immunostimulating and disease-preventing effects of natural antioxidants are not restricted to avian species, but were also obvious with other farm animals (Teige *et al.*, 1982).

Therefore, the results presented above clearly showed that various components of the immune system as well as general animal health were improved when Se and/or vitamin E were added to deficient diets or supplemented at levels far above those required for growth. The benefit of Se supplementation would be greatest in situations when animals were infected with a particular pathogen. In these cases, clinical signs of infection could be reduced. Furthermore, for optimising the animal's resistance to disease, Se requirement is higher than that for adequate growth, feed efficiency, egg production or even reproduction (Nockels, 1988). The optimal doses of Se for maximum disease protection depends on many factors and need further elucidation (Tengerdy, 1990).

It is well known that Se and other antioxidants are able to protect cells from free radical damage, reduce the detrimental effects of certain eicosanoids, and enhance humoral and cellular immune responses in disease (Nockels, 1988). Improved immune system could ultimately lead to higher resistance of animals to various diseases. It is important to remember that during disease challenge nutrient assimilation from the diet could be further compromised as a result of absorption impairment or decreased feed consumption. This could lead to decreased efficiency of antioxidant system leading to immunocompetence declining. For example, Hao *et al.* (1999) studied the activities of Se-GSH-Px and lipid peroxidation in central and peripheral immune organs and main visceral organs of broilers experimentally infected at one day of age with virulent Marek's disease virus (vMDV). They showed that in infected birds the Se-GSH-Px activity was significantly decreased and lipid peroxidation was enhanced. Therefore, preventing these changes in antioxidant defence system is believed to be an effective means to maintain immune system efficiency and this could be associated with better survival infected chicks.

7.8 Immunoprotective effects of Se in stress conditions

Since more than half of known selenoproteins are involved in cell signalling, maintenance of redox balance of the cell and antioxidant defences, protective role of Se on immunity would be most pronounced in various stress conditions. This includes immunosuppressive action of mycotoxins, oxidative stress-related changes in heat stress conditions, heavy metal contamination, and usage of various drugs, including antimicrobials, gut challenge with pathogens or vaccinations.

7.8.1 Mycotoxins

Our previous research established that oxidative stress is the major mechanism of mycotoxin toxicity and their immunosuppressive action (Surai, 2002, 2006; Surai and Dvorska, 2005). Among various mycotoxins, aflatoxins are considered to be most toxic and immunosuppressive. It was shown that dietary Se, through a mechanism of apoptosis regulation, may ameliorate AFB_1-induced lesions of thymus and accordingly improve the impaired cellular immune function. In fact, in chickens fed AFB_1-contaminated diet with 0.2-0.6 mg/kg Se the percentage of apoptotic thymocytes and the expression of caspase-3 and Bax were decreased, while the expression of Bcl-2 was increased compared to AFB_1 group (Chen *et al.*, 2013b). In addition, 0.6 and 0.8 mg/kg Se could also restore the decreased percentages of peripheral blood T-cell subsets and the contents of serum IL-2 and IFN-γ induced by 0.3 mg/kg AFB_1 in the diets, improving cellular immune function in chickens (Chen *et al.*, 2013a). Indeed, dietary Se (0.2-0.6 mg/kg) increased the relative weight of bursa of Fabricius and contents of serum immunoglobulin, and ameliorated histopathological lesions caused by AFB_1 (Chen *et al.*, 2014c). The percentage of apoptotic bursal cells in Se-supplemented groups were lower than those in the AFB_1 group. Moreover, compared to the AFB_1 group, the mRNA expression of Bax and caspase-3 in the Se-supplemented groups decreased, while the expression of Bcl-2 increased. Therefore, dietary Se can protect

chicken from AFB_1-induced impairment of humoral immune function by reducing bursal histopathological lesions and the percentage of apoptotic bursal cells (Chen *et al.*, 2014c). In fact, 0.3 mg/kg AFB_1 could reduce the humoral immune function of the ileum mucosa of chickens, but 0.4 mg/kg supplemented dietary selenium could protect the mucosal humoral immune function from AFB_1-induced impairment. In particular, compared with those in the AFB_1 group, the numbers of IgA(+) cells, as well as the IgA, IgG, and IgM contents were increased in the AFB_1 + Se group, and these data had no difference between the AFB_1 + Se and the control group (He *et al.*, 2014b). Similarly, compared with those in the control group, the percentage of CD3+, CD3+CD4+, CD3+CD8+ intraepithelial lymphocytes (IELs) and lamina propria lymphocytes, the CD4+/CD8+ ratio of IELs, and the mRNA contents of IL-2, IL-6, and TNF-α were decreased in AFB_1 group (He *et al.*, 2014a). However, compared to those in AFB_1 group, these parameters of the AFB_1+Se group (0.4 mg/kg) were increased to be close to those in the control group showing immune-protective effects in mycotoxicosis. Furthermore, dietary Se (0.2-0.6 mg/kg) could also improve the cellular immune function impaired by AFB_1 through increasing the relative weight of spleen and percentages of splenic T cell subsets, and alleviating histopathological spleen damage (Chen *et al.*, 2014a). Mitochondrial swelling assays showed that opening of the mitochondrial permeability transition pores was increased in ducklings that had received AFB_1 for 14 and 21 days and Se significantly attenuated these adverse effects of AFB_1 (Shi *et al.*, 2015).

It seems likely that selenium could assert an important effect against the immunotoxic effects of T-2 toxin against T lymphocytes. For example, after a sublethal dose of T-2 toxin alone, the number of CD8(+) T-lymphocytes was significantly decreased at 12 h and normalised at 48 h, while level of CD3(+) and CD4(+) T-lymphocytes were significantly increased at 24 h and returned to normal after 48 h. When Se was injected into the mice 24 h before or concurrent with the T-2 toxin, the effects on CD8(+) cells were mitigated. It is interesting to note that only when Se was given with the toxin could the effects on the CD3(+) and CD4(+) cells be altered (Salimian *et al.*, 2014). Furthermore, in mice after injection of a sublethal dose of T-2 toxin, the number of B cells (CD19+) significantly decreased at 12 h and became normal at 72 h. When selenium was injected both 24 h before and simultaneously with T-2 toxin, it was able to inhibit B lymphocyte (CD19+) reduction (Ahmadi *et al.*, 2015). In piglet splenic lymphocytes, Se can alleviate deoxynivalenol (DON)-induced damage to antioxidant enzymes by improving glutathione peroxidase activity (Wang *et al.*, 2016b). It seems likely that Se could have a protective effect during DON contamination of the poultry feed. For example, chicken blood granulocyte phagocytic activity was not reduced by dietary DON treatment but numbers of heterophils were increased. In the DON plus Se yeast group phagocytic activity was the same as in the control group. The Se-yeast and DON plus Se-yeast groups had increased numbers of CD3(+), CD4(+), and CD8(+) T-cells, as well as IgM(+) B-cells in their blood compared to both control and DON-groups (Levkut *et al.*, 2009). Furthermore, Se-yeast supplemented to the DON contaminated diet prevented suppression of blood phagocytic activity of broilers (Placha *et al.*, 2009).

7.8.2 Heat stress

Scientific evidence is accumulating to show compromised immunocompetence under heat-stress (HS) condition in avian species. This is associated with altered the heterophil to lymphocyte (H/L) ratio, reduced antibody production and decreased phagocytic potential (Habibian *et al.*, 2015). Recent studies have shown that Se improved the immune functions in HS treated broilers (Liao *et al.*, 2012). In particular, it was shown that HS severely reduced growth performance and immunocompetence of broilers, whereas organic selenium supplementation (0.2-0.4 mg/kg) was able to alleviate the deleterious effects of heat stress. (Niu *et al.*, 2009). In fact, Se supplementation was associated with improved immunity indicated by increased numbers of abdominal exudate cells (AEC), percentage of macrophages in AEC, phagocytic macrophages, and internalised opsonised and unopsonised SRBC. Furthermore, both primary and secondary antibody responses were characterised by increasing titres of antibodies to SRBC by dietary Se when birds were exposed to HS (Niu *et al.*, 2009). Similarly, the production of antibodies against SRBC and IBD was significantly increased due to organic selenium supplementation (0.3 or 0.5 mg/kg) in heat-stressed broiler chicks (Da Silva *et al.*, 2010). The liver and lymphoid organ weights, as well as IgM and IgG, and antibody titers for primary and secondary antibody responses to SRBC were reduced significantly under HS, whereas Se addition showed a significant protective effect (Habibian *et al.*, 2014). It seems likely that under HS conditions Se supplementation can increase antioxidant status and cell-mediated immunity. For example, an experiment was conducted to determine the effect of supplementing various concentrations (0, 0.1, 0.2, 0.3, or 0.4 mg/kg diet) of Se on growth performance, carcass traits, oxidative stress, and immune responses in commercial broiler chickens reared in open-sided poultry house under tropical climatic conditions. It was shown that lipid peroxidation in plasma decreased, while activities of GSH-Px and glutathione reductase in plasma increased linearly with Se concentration in diet. Furthermore, the cell-mediated immunity (lymphocyte proliferation ratio) increased linearly with increasing dietary Se concentration (Rao *et al.*, 2013). Similarly, heat-stressed chicks fed Se-supplemented diets had higher average daily feed intake, Se concentrations in liver and breast muscle, liver GSH-Px activity, serum antibody titers against H5N1(Re-4 strain), H5N1(Re-5 strain) and lower mortality compared with the control. It is interesting to note that 0.30 mg/kg Se was more effective than 0.15 mg/kg Se (Liao *et al.*, 2012). More recent results indicate that HS (the chickens were exposed to 8 h of 23.9 °C, 4 h of 23.9 to 37 °C, 8 h of 37 °C, and 4 h of 37 to 23.9 °C) induced higher levels of TNF-α, IL-4, HSP27, HSP70, and MDA levels, but lower level of IFN-γ, IL-2, GSH-Px and SOD in spleen and the responses were ameliorated by the Se treatment (0.3 mg/kg). Indeed, selenium supplementation decreased the elevated levels of TNF-α and IL-4 and increased the diminished levels of TNF-γ and IL-2 induced by heat stress (Xu *et al.*, 2014). The immune dysfunction induced by heat shock was alleviated by treatment with Se (0.3 mg/kg) and polysaccharide (PAMK) in different immune organs. Indeed, Se specifically regulated the TNF-α and IFN-γ pathways in immune organs (Xu and Tian, 2015).

7.8.3 Antimicrobials

Enrofloxacin (EFX) is an important antimicrobial used in veterinary practice, but it is known to exert immune suppression and oxidative stress. In particular, it was shown that the activity of cellular, humoral immune response and enzymatic and non-enzymatic antioxidants, has significantly been decreased as a result of EFX treatment of broiler chickens. In such conditions nano-Se supplementation (0.6 mg/kg feed) greatly restores these values towards the control (Shirsat *et al.*, 2016).

7.8.4 Heavy metals

Cadmium (Cd) is a toxic heavy metal of increasing environmental concern due to its wide variety of adverse effects. In fact, Cd consumption causes oxidative stress and has immunosuppressive action (Pappas *et al.*, 2011). *In vitro*, a statistically significant increase in the mRNA expression of HSPs in chicken splenic lymphocytes was observed due to Se treatment. Furthermore, treatment of chicken splenic lymphocytes with Se in combination with Cd enhanced the mRNA expression of HSPs, which were reduced by Cd treatment alone (Chen *et al.*, 2012b). It was shown that cadmium induced nitric oxide-mediated apoptosis of immune organs, and selenium played a protective effect against cadmium-induced apoptosis in the immune organs of chickens. Indeed, Se supplementation (10 mg/kg) during dietary cadmium (150 mg/kg $CdCl_2$) reduced the production of nitric oxide, the mRNA level and activity of inducible nitric oxide synthase, ultrastructural damage, and apoptosis in the immune organs of chicken (Liu *et al.*, 2014a). A high level of MDA and ROS production were observed in chicken splenic lymphocytes of the Cd treatment group; furthermore, the activities of catalase (CAT), GSH-Px, SOD, and the mitochondrial inner transmembrane potential ($\Delta\Psi m$) were significantly lower in the Cd treatment group than that of the control. Se supplementation significantly improved the activities of the antioxidant enzymes and reduced MDA and ROS levels compared to Cd treatment group, although levels were not restored to that of the control group (Liu *et al.*, 2014b). The protective effects of Se against Cd toxicity in chicken splenic lymphocytes were confirmed by the increase in select cytokines (NF-κB, iNOS, COX-2, TNF-α, and PGE2), NO content and iNOS activity (Liu *et al.*, 2015b). Indeed, compared with the control group and the Se-alone-treated group, the mRNA expression levels of IL-2, IL-4, IL-10, IL-17, and IFN-γ in chicken splenic lymphocytes decreased significantly in the Cd-treated group. In such conditions, Se showed a significant protective effect by slowing down a decrease in cytokine production due to Cd contamination (Xu *et al.*, 2015a). Selenium significantly increased the expression of selenoproteins K, N, S and T, which were reduced by cadmium in chicken splenic lymphocytes (Zhao *et al.*, 2014). Therefore, Se partly attenuates immune toxicity induced by Cd in chicken splenic lymphocytes.

It was also shown that Pb poisoning induced mRNA expression of HSPs and inflammatory cytokines in the peripheral blood lymphocytes of chickens, while Se alleviated the Pb-induced increase in HSP and inflammatory cytokine (Sun *et al.*, 2016). In beluga whales lymphocyte proliferative responses were reduced following exposure to 1 μM of $HgCl_2$ and 0.33 μM of MeHgCl. Concurrent exposure of Se

provided a degree of protection against the highest concentrations of inorganic Hg or organic Hg for T-lymphocytes (Frouin *et al.*, 2012). It was also shown that non-adherent splenic cells treated *in vitro* with an extract of *Pteridium aquilinum* showed diminished NK cell activity that was not only prevented by selenium co-treatment, but also fully reversed by selenium post-treatment (Latorre *et al.*, 2011).

7.8.5 Gut challenge

Necrotic enteritis (NE) is considered to be an important factor in poultry production and its negative consequences for chicken growth, development and health are well known (Moran, 2014). Recent results have shown that dietary supplementation of newly hatched broiler chicks with 0.25 Se mg/kg from hatch significantly reduced NE-induced gut lesions compared to infected birds given a non-supplemented diet (Xu *et al.*, 2015b). The levels of serum antibody against the NetB toxin in the chicks fed with 0.25 and 0.50 mg/kg Se were significantly higher than the non-supplemented control group. The transcripts for IL-1β, IL-6, IL-8, iNOS, LITAF (lipopolysaccharide-induced tumour necrosis factor-α factor), and GSH-Px7, as well as avian beta-defensin (AvBD)6, AvBD8, and AvBD13 were increased in the intestine and spleen of Se-supplemented groups. The authors concluded that dietary supplementation with optimum levels of Se exerted beneficial effects on host immune response to NE and reduced the negative consequence of NE-induced immunopathology (Xu *et al.*, 2015b).

7.8.6 Selenium deficiency and viruses

It is generally accepted that the nutritional status of the host is associated with both severity and susceptibility to infectious disease. In particular, oxidative stress via RNA virus infections can contribute to several aspects of viral disease pathogenesis, including apoptosis, loss of immune function, viral replication, inflammatory response and loss of body weight (Reshi *et al.*, 2014). Indeed, the excess production of superoxide in the influenza A virus infection is detrimental and the downregulation of the superoxide markedly alleviates lung injuries caused by the influenza virus and viral replication, irrespective of the infected viral strain (Suliman *et al.*, 2001). Influenza infection also leads to the thymus-specific elevation of the mitochondrial superoxide, which interferes with the normal functioning of T-cell lymphocytes in influenza A virus infections (Allen and Balin, 1989). Furthermore, inadequate nutrition impairs the functioning of the immune system, thus resulting in increased susceptibility to infection. It has been suggested (Beck and Levander, 2000) that the nutritional status of the host not only affects the immune response, but also substantially can affect the viral pathogen. In particular, in a mouse model, a benign strain of coxsackievirus B3 became virulent and caused myocarditis in Se and vitamin E-deficient mice (Beck and Levander, 2000). The change in pathogenicity was a result of mutations in the viral genome, which changed an avirulent virus into a virulent one. It is interesting that six nucleotide changes were found in the virus that replicated in the deficient mice, and once these mutations occurred, even mice with normal nutrition became susceptible to disease (Beck, 1999). Therefore, mice deficient in Se were more susceptible to

infection with coxsackievirus, as well as with influenza virus. It was also suggested that the immune system was altered in the Se-deficient animals, as was the viral pathogen itself. Mice fed a diet deficient in Se suffer much more severe lung pathology after infection with the influenza virus than Se-adequate controls (Beck *et al.*, 2001).

Sequencing of viral isolates recovered from Se-deficient mice demonstrated mutations in the viral genome of both coxsackievirus and influenza virus (Beck, 2001). These changes in the viral genome are associated with the increased pathogenesis of the virus. The effects of 10 week of Se supplementation (5 mg/kg) in drinking water on immune responses and resistance to a myocarditic Coxsackie virus B3 (CVB3) infection were studied in female Balb/c mice (Ilback *et al.*, 1998). Se supplementation reduced CVB3-induced mortality: at day 14 post-inoculation, survival was 58% in the Se-treated group compared to 25% in the untreated group. Anti-viral protective effects of Se has also been shown in the recall responses to polio virus vaccination of healthy volunteers with marginal selenium status (Broome *et al.*, 2004).

Experimental mice that were kept on a Se-replete or Se-deficient diet for 28 days, infected intraperitoneally with CVB3 while maintaining their previous diets, were examined for next 90 days for several parameters indicative of the infection or disease (Jun *et al.*, 2011). It was shown that the mice on the Se-deficient diet exhibited a higher mortality, lower serum GSH-Px activity, evident histopathological changes indicative of myocarditis, and a higher level of viral RNA in the heart. Therefore, Se-deficiency creates a chronic myocarditis-prone condition by fostering the active virus replication. In general, in some RNA viruses, including coxsackievirus B3 (CVB3/0) (cause of Keshan disease), human immunodeficiency virus (HIV), influenza A virus, SARS coronavirus, and Ebola virus, Se deficiency may cause accumulation of mutations in their genome, leading to changes in the virulence-associated genetic structures (Harthill, 2011). In such conditions antioxidant protection is a key for overcoming virus-related changes in the cell/body. For example, sodium selenite was shown to suppresses hepatitis B virus protein expression, transcription, and genome replication in hepatoma cell models in a dose- and time-dependent manner (Cheng *et al.*, 2016). Se-supplementation in healthy human adults with marginal Se status resulted in both beneficial and detrimental effects on cellular immunity to flu that was affected by the form of Se, supplemental dose and delivery matrix (Ivory *et al.*, 2017). It seems likely that Se can attenuate the porcine circovirus type 2 (PCV2) infection in animals through altering the systemic inflammation and maintaining the normal organ morphology. For example, dietary Se supplementation had little effect on the PCV2 virus load in the liver, spleen and lung. However, mice in the Se-supplemented group showed a significant decrease in microscopic lesion scores in the lung and spleen compared to those in the control group (Liu *et al.*, 2015a). In another study, PCV2 replication was inhibited by SeMet at a high concentration (6 μM) and the increase in PCV2 replication by oxidative stress was blocked by SeMet at physiological concentrations (2 or 4 μM). Furthermore, PCV2 infection caused a decrease in GSH-Px1 activity. It is interesting to note that SeMet did not significantly block the promotion of PCV2 replication in GSH-Px-knockdown cells. Indeed, GSH-

Px plays a key role in blocking the promotion of PCV2 replication (Chen *et al.*, 2012a). Se supplementation can be considered as a widely available adjuvant therapy in viral and bacterial infections to support therapy and/or to improve immunocompetence of farm animals and poultry (Steinbrenner *et al.*, 2015). A general scheme of Se-virus interaction is shown in Figure 7.6.

7.8.7 Se overdose and immunity

Selenium is toxic at high doses so it is necessary to carefully choose the correct dose of this trace element for an experiment. Therefore, over-supplementation with Se, in the presence or absence of vitamin E and in amounts insufficient to cause clinical toxicity, may impair vaccination responses. Se supplementation with sodium selenite should be carefully monitored, since Se excess (more than 5 mg/kg) could cause profound immunologic inhibition (Peng *et al.*, 2009). Therefore, excessive Se (more than 5 mg/kg as sodium selenite) intake could impair cellular immunity in broilers, causing lesions of thymus and a decrease of T-cell subsets (Peng *et al.*, 2011a), causing growth retardation of spleen by cell cycle blockage in young chickens (Peng *et al.*, 2011c).

The effects of supranutritional dietary Se (3 mg/kg) on selenoprotein expression in three immune organs of chickens were studied. The results showed that high dietary selenite depressed growth performance of chicken and down-regulated 9 and 3 selenoprotein genes in thymus and spleen, respectively, while only Sepp1 was up-regulated in the bursa of Fabricius. Also 3, 3 and 7 inflammation-related genes were up-regulated in these three organs, respectively. Supranutritional Se elevated activities of SOD, total antioxidant capacity and GSH-Px, mainly in early stages (Tang *et al.*, 2017). Similarly, excessive Se supplementation (5-15 mg/kg) decreased the activities of GSH-Px and SOD, but increased levels of MDA in chicken spleen in a dose- and time-dependent manner. In addition, the endoplasmic reticulum stress genes GRP78 and ATF6 were highly expressed, and the apoptosis genes caspase 3 and 12 were increased, but Bcl 2 was decreased by Se treatment (Wang *et al.*, 2016a).

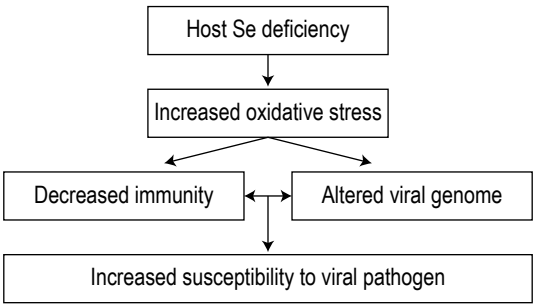

Figure 7.6. Effect of Se on viral diseases (adapted from Surai, 2006).

7.9 Molecular mechanisms of immunomodulating properties of selenium

To elucidate the role of Se-containing proteins in the immune function, the expression of this protein class in T-cells or macrophages of mice was knocked-out (Carlson *et al.*, 2010). The results of deficiency in Se-proteins can be summarised as follows:

- Reduced pools of mature and functional T-cells in lymphoid tissues and an impairment in T-cell-dependent antibody responses.
- An inability of T-cells to suppress reactive oxygen species production, which in turn affected their ability to proliferate in response to T-cell receptor stimulation.
- An altered regulation in extracellular matrix-related gene expression and a diminished migration of macrophages in a protein gel matrix.

It is believed that several mechanisms are involved in Se-dependent modulation of the immune system (Figure 7.7; Surai, 2002, 2006; Wu and Meydani, 1998):

- *Protection of cell membranes and receptors*. It is well recognised that sophisticated antioxidant defences directly and indirectly protect the host against the damaging effects of cytokines and oxidants. In particular, indirect protection is afforded by antioxidants, which reduce activation of NF-κB, thereby preventing up-

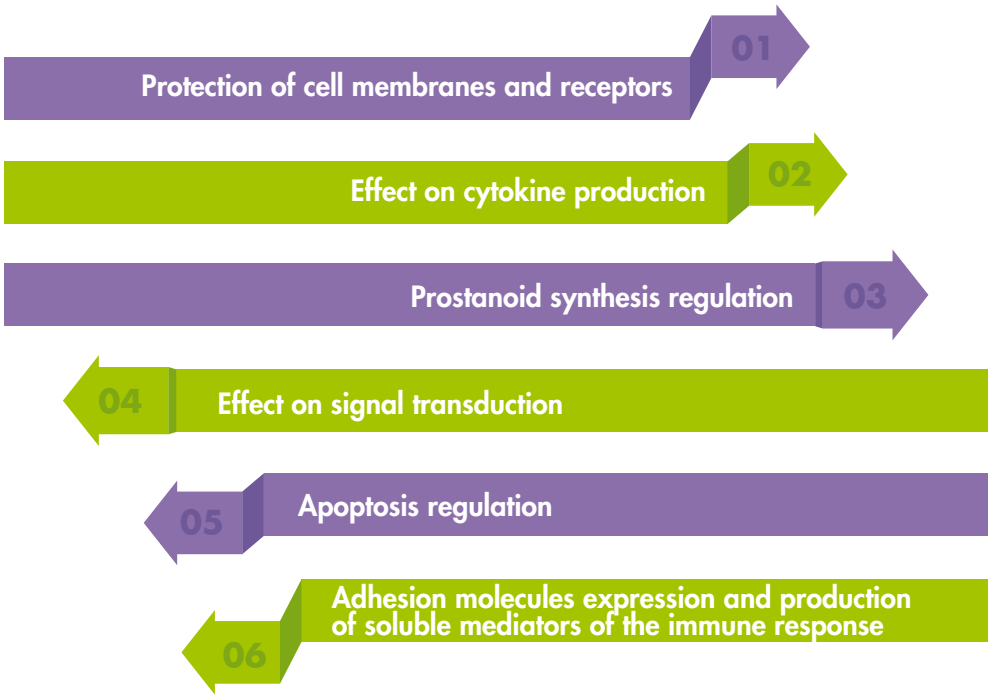

Figure 7.7. Immunomodulating properties of selenium.

regulation of cytokine production by oxidants. On the other hand, cytokines increase both oxidant production and antioxidant defences, thus minimising the damage to the host. Antioxidants prevent oxidative stress-induced damage to immune cells. It is necessary to take into account that cellular integrity is very important for receiving, and responding to the messages needed to coordinate an immune response. As mentioned above, phagocytosis is the major mechanism for removal of microbes from the body. The immune system generates ROS as part of its defence function and these ROS are an important weapon to kill pathogens. However, chronic overproduction of ROS can cause damage to immune cells and compromise their function (Wu and Meydani, 1998). In fact, immune cells are rich in PUFAs which are very susceptible to free radical attack. It is well recognised that many immunological functions are membrane-dependent. These are antigen recognition, receptor expression, secretion of antibodies and cytokines, lymphocyte transformation, and contact cell lysis (Wu and Meydani, 1998). In particular, the receptors are important for antigen recognition and the secretion of various chemical mediators, such as interferon, tumour necrosis factor, prostaglandins and interleukins. Therefore, lipid peroxidation can change membrane structure and properties (e.g. fluidity, permeability, flexibility, etc.) which would affect immune cell functions. In contrast, antioxidants are able to prevent those damaging effects of ROS and maintain immune function. For example, H_2O_2 depressed lymphocyte proliferation (Metzger *et al.*, 1980), while vitamin E decreased H_2O_2 formation by PMN (Baehner *et al.*, 1977). It was found that H_2O_2 deeply injured lymphocytes immunocompetence and administration of Se counteracts this damage (Sun *et al.*, 1995). It is well known that macrophage activation and phagocytosis of foreign particles are regularly accompanied by a so-called 'respiratory burst', an increase in the production of ROS, exerted by the enzyme complex NADPH oxidase. A number of selenoproteins is at the same time expressed to protect the cells from the cytotoxic effects of ROS directed against engulfed microorganisms. Selenoproteins participating in antioxidant defences (GSH-Px, thioredoxin reductases, methionine sulfoxide reductase B, etc.) are able to protect neutrophils from oxygen-derived radicals that are produced to kill ingested foreign organisms (Ebert-Dumig *et al.*, 1999). Therefore, as a constituent of selenoproteins, Se is needed for the proper functioning of neutrophils, macrophages, NK cells, T lymphocytes and some other immune mechanisms (Ferencik and Ebringer, 2003; Surai, 2006).

- *Effect on immunomodulator production.* There is a range of regulatory molecules produced by immune cells. For example, IL-2, a lymphocyte growth factor, is recognised as an important immunomodulatory molecule. Oxidative stress suppresses IL-2 production and antioxidants can help to overcome this suppression. Therefore, Se up-regulates the expression of the T-cell high affinity IL-2 receptor and provides a vehicle for enhanced T-cell responses. Binding of IL-2 by the IL-2 receptor induces proliferation of T-lymphocytes. For example, it has been suggested that Se can increase the inducibility of IL-2 receptor whereas vitamin E may counteract the down-regulatory effect of cAMP on IL-2 activity (McCarthy, 1997). The mRNA for IFN-γ was much less abundant in Se-deficient vs Se-adequate mice and mRNA for IL-2 was also lower in the Se-deficient mice (Beck *et al.*, 2001).

- *Prostanoid synthesis regulation*. Antioxidants alter the production of immunomodulatory molecules, such as prostaglandins and leukotrienes, thereby altering the ratio between immunosuppressive and immunostimulating eicosanoids. The relationship between antioxidants and inflammatory reactions is shown in Figure 7.8. From the figure it is clear that poor antioxidant defence is associated with enhanced inflammation, overproduction PGE_2 resulting in suppression of lymphocyte activity (Grimble, 1997). For example, at low concentrations, PGE_2 is essential for cellular immunity; however, increased PGE_2 concentration is associated with a suppression of cellular and humoral immunity, including antibody formation, DTH, lymphocyte proliferation, and cytokine production. Lymphocytes from Se-deficient rats stimulated by an ionophore produced significantly less prostaglandins in comparison to Se-supplemented animals and characterised by a decreased activation of phospholipase D (Cao *et al.*, 2002). It was postulated that dietary Se status, which in turn determines tissue Se concentration, plays an important role in the regulation of arachidonate metabolism that is affecting the 5-lipoxygenase pathway. This may be one of the biochemical mechanisms underlying the inhibition of lymphocyte proliferation and the decrease in resistance to infectious diseases observed in Se-deficient animals (Cao *et al.*, 1992). 5-lipoxigenase (5-LO), a key enzyme in the biosynthesis of proinflammatory leukotrienes, is regulated by the cellular redox status and required hydroperoxide for activation. Therefore, cellular concentrations of hydroperoxides might be an important factor regulating leukotriene synthesis (Werz *et al.*, 2000). In fact, granulocyte-derived ROS can activate B-lymphocyte

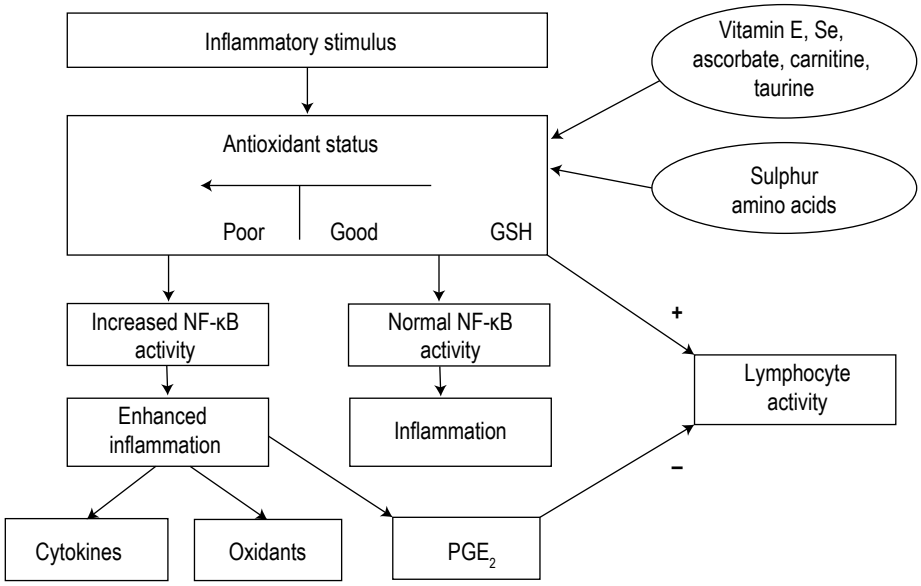

Figure 7.8. Antioxidants and inflammation (adapted from Surai, 2006).

5-LO. Since leukotrienes are involved in regulation of cell proliferation, activation and maturation, they regulate immune responses.

- *Effect on signal transduction*. Natural antioxidants, such as Se and vitamin E, may protect against oxidant-mediated inflammation and tissue damage by virtue of their ability to scavenge free radicals and inhibit the activation of NF-κB (and possibly other oxidant-sensitive transcription factors). *In vitro* Se supplementation at low levels increased the production of interferon by human peripheral lymphocytes, while high Se doses were detrimental for interferon production (Watson *et al.*, 1986). When 13 synthetic seleno-organic compounds, were studied, seven of them were found to be inducers of IFN-γ and/or TNF-α in human peripheral blood leukocyte cultures (Piasecki *et al.*, 1992). In fact, NF-κB is required for maximal transcription of many inflammatory cytokines and adhesion molecules (Hughes, 1999). Furthermore, Se at physiological levels mediates inhibition of the activation of the transcription factor NF-κB which regulates genes that encode inflammatory cytokines (Maehira *et al.*, 2003). Thus, maintaining an adequate antioxidant status may provide a useful approach in attenuating the cellular injury and dysfunction observed in some inflammatory disorders. (Conner and Grisham, 1996). In fact, there is an inverse relationship between cellular Se status and the inducible form of nitric oxide synthase expression in LPS-stimulated cells (Prabhu *et al.*, 2002). Following LPS stimulation, the nuclear localisation of NF-κB was significantly increased in Se-deficient macrophages, thereby leading to increased expression of pro-inflammatory enzyme cyclooxygenase-2 (Zamamiri-Davis *et al.*, 2002). It is necessary to underline that non-toxic concentrations of reactive metabolites of oxygen and nitrogen play an important role in regulating the expression of genes involved in the inflammatory response and in modulating apoptosis (Jourd'heuil *et al.*, 1997). At the same time, an immune response requires extensive communication between a wide range of cell types (Klasing, 1998b) and special cell receptors are of great importance in this communication. Therefore, the protective effect of antioxidants in the prevention of membrane and receptor damages due to peroxidation could provide an important way of enhancing the immune system.
- *Apoptosis regulation*. Antioxidants are considered to prevent apoptosis caused by oxidative stress. This could have a great effect on immune cell apoptosis preventing immunosuppression. It has been shown that *in vitro* selenium deficiency in a subset of hepatocellular carcinoma-derived cell lines causes oxidative stress and cytochrome c release with subsequent cell death by apoptosis (Irmak *et al.*, 2003). New techniques are now available, which can be used to re-evaluate old data on Se-deficiency. For example, Se and vitamin E deficiency in chickens significantly increased caspase-like activity suggesting that cell death associated with exudative diathesis can follow the apoptotic pathway (Nunes *et al.*, 2003). Immunomodulating properties of Se could be mediated via prevention of apoptosis of immune cells in the case of mycotoxicoses. Indeed, many mycotoxins are immunosupressive and they can cause apoptosis (Surai, 2002, 2006), therefore Se supplementation of mycotoxin-contaminated feed could potentially have a beneficial effect. Since more than 25% of the world's grain production is contaminated with mycotoxins, sub-clinical mycotoxicoses seem to be a real problem (Da Silva *et al.*, 2018).

- *Adhesion molecules expression and production of soluble mediators of the immune response*. It has been shown that Se is able to affect the expression of adhesion molecules that are crucial in the inflammatory process (Jahnova *et al.*, 2002). In fact, the inhibitory effect of Se on adhesion molecule expression has been studied in cultured endothelial cells after interferon-gamma stimulation (Horvathova *et al.*, 1999). The data suggest that Se affects the expression of P-selectin, ICAM-1, VCAM-1, and ELAM-1 in a dose-dependent manner.

7.10 Immunocommunication, free radicals and selenium

As mentioned above, the immune system of the animal is based on natural and adaptive immunity. Natural immunity is dependent on the efficient function of phagocytic cells, namely neutrophils/heterophils and macrophages. These cells are equipped with an array of microbicidal weapons, such as proteases, enzymes that hydrolyse proteins disrupting membranes. This weaponry is stored in granules in the cytoplasm. Furthermore, these cells have a powerful system for generating large amounts of ROS that are used as an effective chemical weapon to kill pathogens. However, on escape from the phagosome, the same free radicals become dangerous and can damage biological molecules, which compromises phagocyte function and reduces adaptive immunity. Phagocytes also produce communication molecules (eicosanoids, cytokines, etc.) that are used for effective communications between the various immune cells.

Invading pathogens are controlled by the natural and adaptive branches of the immune system. It seems likely that in chickens the establishment of the adaptive immunity is not sufficiently quick to eradicate microorganisms and natural immunity is responsible for recognition of invading pathogens by specific receptors. Binding of pathogen to those receptors induces the production of ROS and RNS, pro-inflammatory cytokines, as well as communicating molecules which are responsible for sending regulatory signals to the adaptive immunity (Werling and Jungi, 2003).

As mentioned above, adaptive immunity is based on the activity of B- and T-lymphocytes, which produce antibodies to specific non-self substances (B-lymphocytes) or directly attach to them (T-lymphocyte) and remove them from the cell. In general, in chicken an overall pool includes about 100 million T cells (Juul-Madsen *et al.*, 2008). Specific adaptive immune responses rely on the major histocompatibility complex's restricted recognition of peptide antigens derived from pathogens to activate a variety of effector T cells (helper and cytotoxic cells) that interact with B cells that produce effector proteins called antibodies (Castle, 2000). The adaptive immunity is characterised by a high plasticity to recognise up to 10^{11} distinct structures and is tightly regulated to turn on or off a response aiming at eradication of pathogens but not the destruction of self. The healthy animal or human resistance to infection relies on a balance between natural and adaptive immunity. Regulation of the immune system is extremely complex. We are only starting to understand how the immune system co-ordinates the body's response to a disease or invading

pathogen. It seems likely that communication between immune cells is a crucial factor of immunocompetence (Surai, 2006). Interaction between the different immune cell types that make up the components of host defence is carried out by the relative mix of cytokines, hormone-like proteins, as well as other communicating molecules (Castle, 2000). The innate response, including its inflammatory component, reacts initially to the stimulus, acting directly to eliminate it by the activities of the complement system or phagocytosis.

The complement system, a chief component of the innate immunity, was identified more than 100 years ago as a heat-labile factor in blood serum that kills bacteria. Complement is a complex, self-regulatory system of approximately 35 proteins that are usually designated as either complement components (C) or complement factors which are designed to attack and kill extracellular bacteria in body fluids (Vorup-Jensen and Boesen, 2011). Therefore, microbial pathogens are attacked and eliminated by pore-forming proteins specially adapted for their targets. These specific proteins are able to inflict physical damage, thereby damaging permeability barriers and structural protection (McCormack *et al.*, 2013). Penetration is followed by additional effectors to complete target destruction. In fact, the complement system is highly inducible with the liberation of both antimicrobial elements (e.g. the C9 membrane attack complex (MAC)) and elements that attract and modulate the cellular immune system (Juul-Madsen *et al.*, 2008). Indeed, the combination of physical and chemical attack is highly efficient allowing animal/poultry survival despite continuous attacks by various microbes, being huge in number and possessing the ability to rapidly replicate and mutate (McCormack *et al.*, 2013).

Cytokines produced by monocytes and macrophages regulate this response and also act on the liver, skeletal muscle, adipose and brain changing their metabolism and stimulating various responses. The cytokines also interact with T-lymphocytes (Calder, 2001).

If we imagine that the immune system is an army fighting against invaders (microorganisms, viruses, etc.) then we would expect them to have something like mobile phones to receive and send signals to each other. Remarkably enough, major immune cells (macrophages, neutrophils/heterophils, T- and B-lymphocytes) have on their surface something like 'mobile phones' called receptors. Those receptors are extremely sensitive to communicating molecules, but they are also sensitive to free radicals and can be easily damaged. In such a situation without proper communication all those huge armies of immune cells would become useless. They also can start fighting each other, as well as eventually destroying immunocompetence and causing autoimmune reactions. If we imagine that immune cells are soldiers using chemical weapon to kill the enemy, then special gear protecting them from their own weapon would be a crucial for effective battle. In the case of immune cells, such gear is represented by natural antioxidants with Se-GSH-Px, thioredoxin reductase and other selenoproteins being major defences. Indeed, if not properly protected, macrophage functions could be compromised, including initial overproduction of free radicals with consecutive damages to specific enzymatic systems resulting in

decreasing efficiency of oxidative burst and apoptosis. Based on the presented model it is clear that antioxidant defence is a crucial factor of immuno defence in the body and Se plays a central role in this defence.

There is a great body of evidence confirming this idea. For example, Roy *et al.* (1993) indicated that supplementation of C57BL/6 mice with Se resulted in increased expression of IL-2R on ConA-stimulated lymphocytes resulting in a greater number of high-affinity IL-2R per cell and enhanced proliferation and differentiation of lymphocytes. A similar effect of Se was seen on IL-2R in response to allergens: increases in CD25 were found on T helper (Th) cells with increases in dietary Se (Hoffmann *et al.*, 2007). A clear correlation between Se supplementation and lymphocyte proliferation, which was preceded by enhanced expression of high-affinity IL-2R was shown in human study (Roy *et al.*, 1994). It should be mentioned that Th cells are involved in providing effective immune responses to a wide variety of antigens and they depend on IL-2R signalling for activation, proliferation, and differentiation (Zhu *et al.*, 2006). The peripheral blood mononuclear cell (PBMC) membrane fluidity, IL-2 production and IL-2R expression in patients with chronic hepatitis were significantly lower than those in healthy blood donators, while MDA concentration was significantly increased. Both *in vitro* and *in vivo* administration of selenium could reverse the above parameters (He *et al.*, 2004). It was also shown that Se suppresses the activation of transcription factor NF-κB and IRF3 induced by TLR3 or TLR4 agonists confirming an ability of Se to modulate signalling pathways of TLRs leading to decreased inflammatory gene expression (Youn *et al.*, 2008). Furthermore, consumption of the low-Se yeast by healthy free living men increased counts of NK cells and T lymphocytes expressing both subunits of the high affinity interleukin-2 receptor (IL2R; Hawkes *et al.*, 2009). The receptor integrity and expression were not studied in the aforementioned study, but it could well be that they were damaged. Clearly, selenoproteins mediate T cell immunity through an antioxidant mechanism. It seems likely that dietary Se levels could modulate free thiol levels, receptor expression/integrity and specific signalling events during CD4(+) T cell activation, which influence their proliferation and differentiation. For example, mice fed low (0.08 mg/kg), medium (0.25 mg/kg), or high (1.0 mg/kg) Se diets for 8 weeks were challenged with a peptide/adjuvant (Hoffmann *et al.*, 2010). It was shown that antigen-specific CD4(+) T cell responses were increased in the high Se group compared to the low and medium Se groups. T cell receptor signalling in *ex vivo* CD4(+) T cells increased with increasing dietary Se, in terms of calcium mobilisation, oxidative burst, translocation of nuclear factor of activated T cells, and proliferation. Furthermore, the high Se diet increased expression of IL-2 and the high affinity chain of the IL-2 receptor compared to the low and medium Se diets. In the low Se diet group there was a decrease in free thiols compared to the medium and high Se diets. Addition of exogenous free thiols eliminated differences in CD4(+) T cell activation among the dietary groups (Hoffmann *et al.*, 2010). Similarly, selenoprotein deficiency in T cells led to an inability of these cells to suppress ROS production, which in turn affected their ability to proliferate in response to T cell receptor stimulation (Carlson *et al.*, 2010; Shrimali *et al.*, 2008). Recently, it has been confirmed that Se can influence immune response by changing the expression of cytokines and their

receptors or by making immune cells more resistant to oxidative stress (Naziroglu *et al.*, 2012). Furthermore, supranutritional Se-yeast supplementation to sheep increased whole blood neutrophil expression of genes involved in innate immunity, including L-selectin, IL-8 receptor and TLR4 (Hugejiletu *et al.*, 2013). It was shown that spleen oxidative stress induced by a high-fat diet in mice induced a decreased expression of genes associated with antioxidant defence, as well as Fc receptor. Antioxidant (lipoic acid) supplementation attenuated the aforementioned changes (Cui *et al.*, 2012). It is interesting that receptor-mediated Ca(2+) flux was decreased in T cells, neutrophils, and macrophages from selenoprotein K-deficient (Sel K(-/-)) mice compared to the controls. Furthermore, Ca(2+)-dependent functions, including T cell proliferation, T cell and neutrophil migration, and Fcγ receptor-mediated oxidative burst in macrophages, were decreased in cells from Sel K(-/-) mice compared to that in cells from the controls (Verma *et al.*, 2011). Recently, it has been shown that organic selenium supplementation in sheep was associated with the enrichment of B and T cell receptors signalling pathways (Elgendy *et al.*, 2016). Furthermore, effective activation of macrophages through phagocytic Fcγ receptors (FcγR) has been shown to require selenoprotein K (SelK; Norton *et al.*, 2017). In fact, SelK deficiency through genetic deletion or low selenium in culture media leads to low expression of the IP3 receptor (an important element for the activation and function of immune cells) due to a defect in the palmitoylation receptor (Fredericks *et al.*, 2014).

Recently, a lot has been learned about innate receptors, such as Toll-like receptors, that activate antigen-presenting cells in response to microbial products and initiate an immune response. It has been shown that Se status was associated with IL-2R levels. It is well established that the immune response is regulated by very complex interactions between immune cells via the cytokine system as well as via other signalling molecules. Indeed, there are many various cytokines responsible for immune cell communication. The most difficult question remains to be answered: 'how do cells interpret these signals that modulate the immune response?'. A key aspect of the answer lies at the receptor and the multiple modifications and regulatory proteins that collectively shape a cytokine response (Delgoffe *et al.*, 2011). For example, for an optimal and appropriate immune response, T cells require activation through TCR, which recognises a specific antigen presented in the context of MHC (Williams and Kwon, 2004). This recognition also confers the ability of T cell responses to distinguish between 'self' and 'non-self'. Engagement of the TCR, throughout the lifetime of the T cell, controls the survival, proliferation, and/or differentiation of T cells. Thus, signalling through the TCR has important consequences for a proper immune response and the effectiveness of that response. In fact, receptor expression can be dynamically modulated by the cell, based on cell type, stimulation, and cytokine activity. Some receptors are constitutively expressed, while others require additional signals in order to be upregulated and properly expressed. In addition, a lot of cytokine receptors are formed from multiple chains that have distinct modes of regulation. Furthermore, cells can temporally modulate the expression of certain receptors thereby altering the cytokines to which a particular cell is responsive (Delgoffe *et al.*, 2011). As a result of cytokine stimulation, cells can become activated and differentiate. Most of the physiologic outcomes observed in the immune system are mediated by cytokines. It seems likely that T-cell

activation requires two distinct signals: the first signal (recognition) relies on the interaction between the TCR and a MHC-peptide complex, while the second signal is provided by the cross-linking between co-stimulatory molecules on activated T cells. The loss of one or more of such regulatory and/or co-stimulatory molecules can be detrimental for immunocompetence (Pizzi *et al.*, 2016).

The immune response to pathogens is characterised by a biphasic response: the initial non-specific innate immune response and the subsequent pathogen-specific adaptive immune response. It is well established that the innate immune response is mediated mainly by macrophages, dendritic cells and neutrophils, and occurs rapidly after these cells encounter a pathogen, while the adaptive immune response is controlled by T and B cells, which takes place several days after pathogen invasion (Kingeter and Lin, 2012). It is necessary to underline that the innate immune response is crucial for the development of the adaptive immune response. First, phagocytic cells, such as dendritic cells and/or macrophages, engulf extracellular pathogens or infected host cells, and present PAMPs to T cells. In this way, the innate immune system indicates the presence of a pathogen to the cells of the adaptive immune system and the appropriate pathogen-specific adaptive lymphocytes are activated (Kingeter and Lin, 2012). As a result, signal transduction pathways are triggered resulting in the activation of various transcription factors, such NF-κB responsible for the production of pro-inflammatory cytokines and chemokines, which are involved in further regulation of the immune response. Indeed, immune cell receptor expression and integrity are major determinants of bird immunocompetence. In fact, we can consider immunocompetence as an effective communication between all major cells of the immune system. It should be mentioned that both the innate and adaptive immunity are closely related and there is a complex interplay between innate and adaptive responses. In fact, the innate immune system can be considered as an effector arm of the adaptive response. For example, interactions between T cells and macrophages are of great importance. T cell responses are associated with the production of IFN-γ, a cytokine possessing the ability to activate macrophages, increasing their capacity to phagocytose and kill invading microorganisms. Similarly, macrophages express Fc receptors that bind immunoglobulin (produced by B cells) and enhance recognition and engulfment of foreign pathogen-derived material. There is also a close interactions between T cells and goblet cells resulting in increased secretion of defensins and altered mucus composition (Juul-Madsen *et al.*, 2008). Indeed, initial encounter of pathogens with the innate system leads not only to the destruction of the pathogens but also initiates a cascade of important immunological events, including recruitment of various immune components, as well as induction and modulation of the adaptive immune system. In such interactions immune receptors are of great importance and their protection in stress conditions is a key for effective immunocompetence. For example, in the cellular innate immune system up to 100 different receptors are expressed at a relatively high frequency (Juul-Madsen *et al.*, 2008) and each receptor is usually expressed on millions of innate and adaptive immune cells. Based on common structural features, cytokine receptors can be grouped into several families including the class I (haematopoietin) cytokine receptor family, the class II (IFN/IL-10) cytokine receptor family, the TNFRSF, the IL-1 receptor family, the TGF-β receptor family and

the chemokine receptors (Kaiser and Staheli, 2008). Furthermore, multiple innate immune receptors, including TLR2, TLR4, TLR5, TLR9, NOD1/2, and NLRP3, have been implicated in the recognition of metabolic stress and initiation of inflammatory responses in various tissues (Jin *et al.*, 2013). Indeed, Se increases lymphocyte proliferation, expression of the high-affinity IL-2R, cytolytic T lymphocyte efficacy/activity, and NK-cell function, enhancing resistance to infections through modulation of interleukin production and subsequently the Thl/Th2 response. In fact, Se can upregulate the expression or protect from damages by ROS of receptors for IL-2 and other cytokines on the surface of activated lymphocytes and NK cells. This event favours the interaction of this cytokine with its respective receptors (Puertollano *et al.*, 2011; Surai, 2002, 2006).

It is proven that Se- and vitamin E deficiencies are associated with compromised functions of natural and adaptive immunity. In particular phagocytic functions, lymphocyte proliferation and antibody production are compromised (Surai, 2002, 2006). On the other hand, Se supplementation is shown to improve immunocompetence and increase resistance to various diseases. This is true for variety of farm and companion animals, including poultry, cows, sheep, horses, pigs, fish, cats and dogs. A summary of the effects of a compromised antioxidant system on the immune system is shown in Figure 7.9.

The importance of a delicate turning of the immune system is reflected by various observations showing that over-reacting of the immune system has detrimental

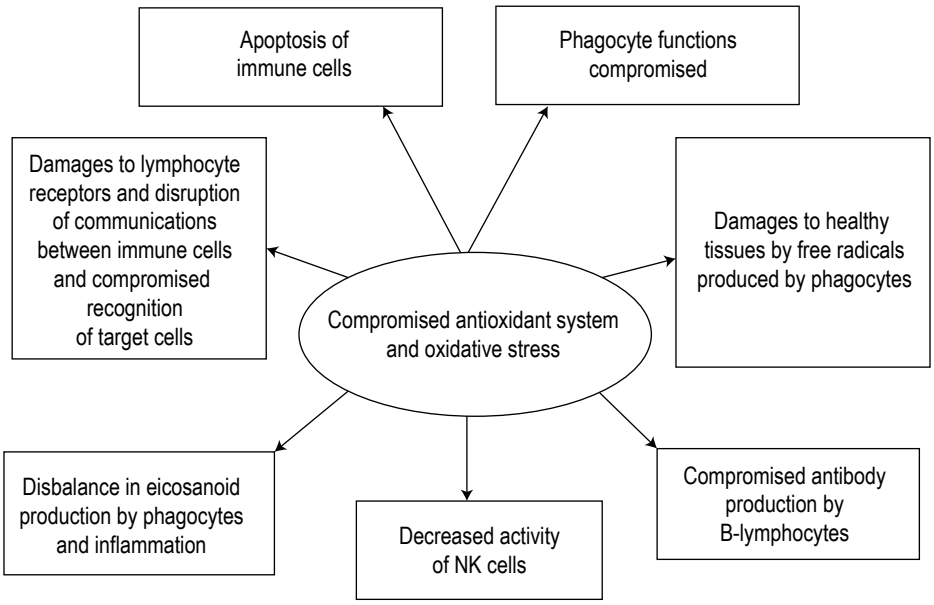

Figure 7.9. Oxidative stress and the immune system (adapted from Surai, 2002, 2006).

consequences, like immunosuppression. For example, in some individuals, the immune system recognises host antigens as 'non-self', attacking them and producing tissue damage leading to chronic inflammatory or autoimmune diseases. The immune system can also become sensitised to usually benign antigens from the environment causing allergies (Calder, 2001). It seems likely, that in these immune system impairments miscommunication between immune cells plays a crucial role.

The chicken immune system is functionally immature at hatch. Postnatal development of the immune system is associated with the accumulation of polyunsaturated fatty acids and a desperate need for antioxidant protection. Therefore, expression of selenoproteins in immune cells during early development would be a crucial factor in the regulation of the development of immunocompetence. However, selenium reserves in the newly hatched chicks are very limited when inorganic selenium is used in the maternal diet. In great contrast, in a number of studies, organic selenium was shown to be able to significantly increase Se concentration in eggs. This selenium is absolutely essential for the formation of effective antioxidant defences resulting in effective immune system maturation and immunocompetence building.

7.11 Conclusions

Selenium affects all components of the immune system, including the development and expression of nonspecific, humoral, and cell-mediated responses (Figure 7.10). In general, a deficiency in Se appears to result in immunosuppression, whereas supplementation with low doses of Se appears to result in augmentation and/or restoration of immunologic functions. On the one hand, a deficiency of Se has been shown (Kiremidjian-Schumacher and Stotzky, 1987) to inhibit:

- resistance to microbial and viral infections;
- neutrophil function;
- antibody production;
- proliferation of T and B lymphocytes in response to mitogens; and
- cytodestruction by T lymphocytes and NK cells.

On the other hand, Se supplementation has been shown (Kiremidjian-Schumacher and Stotzky, 1987) to stimulate:

- the function of neutrophils;
- the production of antibodies;
- the proliferation of T and B lymphocytes in response to mitogens;
- the production of lymphokines;
- NK cell-mediated cytodestruction;
- delayed-type hypersensitivity reactions;
- allograft rejection; and
- the ability of a host to reject transplanted malignant tumours.

Figure 7.10. Effects of selenium on immunity.

When considering immuno-facilitating properties of selenium it is necessary to take several points into account:

- Individual antioxidants in the body interact with each other (Se, vitamin E and C, carotenoids, etc.) and with prooxidants (iron, high level of PUFAs, mycotoxins), which themselves have immunostimulating or immunosuppressive effects. Therefore, in every experiment the results reflect a sum of all these interactions and if background dietary concentrations of those nutrients differ, results could be completely different. This could explain the inconsistency of some results published over the last 30 years. Furthermore, antioxidants can suppress respiratory burst, however, it is not clear at present if there is a limit to this suppression after which the phagocyte anti-microbial activity would be compromised.
- Immunomodulating effect of antioxidants is shown to be maximal at their supplementation usually above the requirement for maximal growth and maintenance of reproduction. It could well be that the Se and vitamin E doses that are adequate for maximal productivity in healthy, unchallenged birds are not optimal for immunocompetence and disease resistance.
- There is only limited information on the effect of antioxidants on intestinal immune structures (Fisinin and Surai, 2013), which could be crucial barriers to external pathogens (Qureshi *et al.*, 1998). Free radicals can be damaging to intestinal structures (Hoerr, 1998), whereas antioxidant functions of selenium are responsible for the prevention damages to intestinal lymphoid structures as well as damages to intestinal enterocyte membranes. This could especially be important in

relation to digestive immunosuppression caused by toxins/mycotoxins, nutritional deficiencies and infectious agents (Hoerr, 1998). Accumulation evidence indicates a non-immunological protective effect of selenium from various toxic agents including cadmium (Zasadowski *et al.*, 1997), monensin (Yarsan, 1998), salinomicin (Zarski *et al.*, 1995) and mercury (Maretta *et al.*, 1995) in chickens.

- Selenium source (organic vs inorganic) seems to be an important element of its immunomodulating properties. Organic selenium appears to be at an advantage because it is better assimilated from feed and better accumulated in tissues. Indeed, with the same dose of supplementation organic selenium can deliver more of the element to the target tissues and because of the toxicity of high selenium levels this could be a solution to avoid adverse effects of selenium overdose. For example, results presented by Johnson *et al.* (2000) indicated that splenic macrophages and lymphocytes are sensitive to Se intoxication and there is a disparity in the immune system of the toxicity of inorganic and organic forms of Se administered through drinking water; inorganic Se being more toxic. In fact, sodium selenite at 9 mg/kg in the drinking water for 14 days increased the production of proinflammatory cytokines, TNF-α and IL-1β, in LPS-stimulated splenic macrophages. However, mice exposed to Se as SeMet in the drinking water did not display any effects on the parameters examined at the dose range in the study. Furthermore, Se supplementation in organic form could have a beneficial effect during acute phase response (APR) in many infection diseases. APR is characterised by the synthesis of acute phase proteins, fever, accelerated whole-body protein turnover, high rates of hepatic gluconeogenesis (Klasing, 1998b) and decreased Se concentration in plasma (Sattar *et al.*, 1997). It is interesting that acute infections decrease serum Se levels regardless of the infective agent (Sammalkorpi *et al.*, 1988). Therefore, if the body has selenium reserves in the form of SeMet in muscles, during acute phase response, it would be liberated as a result of skeletal muscle protein catabolism by proteosome action and used for re-synthesis of new selenoproteins which could decrease oxidative stress.
- First two weeks post-hatch represent most important period of immune system development and maternal diet is shown to have a profound effect on this process (Fisinin and Surai, 2012; Klasing, 1998b; Surai and Sparks, 2001). In particular, the first week of chick life is a period of rapid expansion of the leukocyte population, seeding of lymphoid organs and other events ultimately leading to the production of unique clones of lymphocytes that will mediate immunity in postnatal development (Klasing, 1998b). In this respect effects of various combinations of natural antioxidants await investigation.
- Because of the complexity of regulation of the immune response and lack of understanding of molecular mechanisms involved in such a regulation, immunomodulation should be directed toward the correction of dysfunctional situation created by immune system immaturity, stress, immunosuppressive disease, or genetics (Klasing, 1998a). In this respect natural antioxidants, especially Se, could have a prominent role.
- As a result of antioxidant (selenium) deficiency, increased oxidative stress of a host can lead to increased virus mutation rate and change in a viral pathogen (Beck, 1999), resulting in emerging viral pathogens with new pathogenic properties.

Se deficiency was associated with a change in the viral genotype, converting the virus from a benign to a virulent strain (Beck, 1998). This possibility has not been fully exploited in animal/poultry production, but seems important to study more extensively.

- There is a need to study antioxidant composition and fatty acid profile of immunocompetent tissues depending on chicken age and nutritional supplementation of antioxidants.

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Chapter 8
Antioxidant-prooxidant balance in the gut

A good beginning makes a good ending

8.1 Introduction

A delicate balance between antioxidants and prooxidants in cells is an important determinant of various physiological processes and maintenance of this balance is the main aim of the so-called integrated antioxidant system of the animal body. This system was developed during evolution to provide an antioxidant defence and give a chance for animals to survive in an oxygenated atmosphere. Recent data suggest that the antioxidant-prooxidant balance starts in the intestine (Figure 8.1). Indeed, the redox status of the gut is involved in regulation of many important physiological processes.

8.2 The gastrointestinal tract as a major site of antioxidant action

It is well appreciated that some antioxidants, such as antioxidant enzymes, including glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), catalase, glutathione (GSH), thioredoxin (Trx), coenzyme Q, can be synthesised in the body. However, the diet is the major provider of nutrients possessing antioxidant properties directly (vitamin E, vitamin A, carotenoids, ascorbic acid, flavonoids, etc.) or essentials for the synthesis of antioxidant enzymes. For example, selenium (Se) is an essential part of a range of selenoproteins performing antioxidant functions (GSH-Px, thioredoxin reductase (TrxR), selenoproteins P and W, etc.); manganese (Mn) is an integral part of mitochondrial Mn-SOD; zinc (Zn) and copper (Cu) are integral parts of cytosolic Cu,Zn-SOD; iron (Fe) is an integral part of catalase.

When food is consumed and appears in the stomach and then in the small intestine, it contains a range of antioxidants but may also contain a range of potentially dangerous substances. Keeping a balance between antioxidants and prooxidants in the intestine is a very important task in which diet plays a crucial role. In general, the prooxidants which can be found in the gastrointestinal tract (GIT) are summarised in the next paragraphs.

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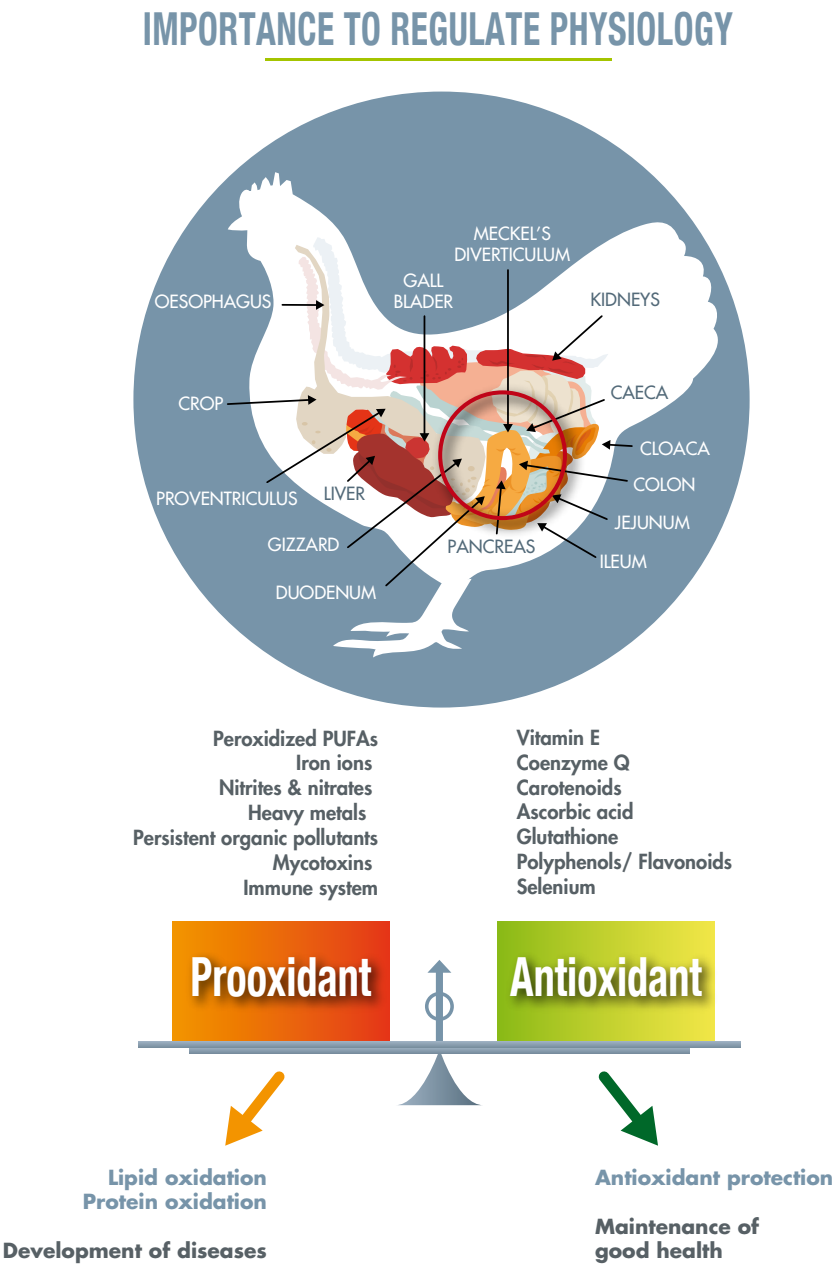

Figure 8.1. Antioxidant-prooxidant balance in the gut.

8.3 Prooxidants in the gastrointestinal tract

8.3.1 Peroxidised poly-unsaturated fatty acids

Lipid hydroperoxides (LOOH) derived from unsaturated fatty acids are important intermediates of peroxidative reactions induced by reactive oxygen species (ROS). In fact, lipid hydroperoxides are not stable and in the presence of transition metal ions can decompose producing new free radicals and cytotoxic aldehydes (Diplock, 1994). Oxidised lipids are partly absorbed in the digestive tract (Staprans *et al.*, 1999) and incorporated into membrane phospholipids altering their structure and properties (Hayam *et al.*, 1997; Staprans *et al.*, 1994). In animal models it has been shown that oxidised lipids in the diet can suppress growth (Calabotta and Shermer, 1985; Lin *et al.*, 1989), reduce vitamin E level in tissues increasing their susceptibility to lipid peroxidation (Sheehy *et al.*, 1994), increase tissue protein oxidation (Hayam *et al.*, 1997) and increase the number of aberrant crypts in the intestine (Yang *et al.*, 1998). The consumption of oxidised fats is associated with diarrhoea, liver enlargement, growth depression and histological changes in tissues of experimental animals (Andia and Street, 1975; Cutler and Schneider, 1973; Koshio *et al.*, 1994; Shibata *et al.*, 1992). Indeed, feeding weaning pigs diets containing 10% thermally oxidised lipids for 38 days appeared to impair oxidative status (Liu *et al.*, 2014) and negatively affected growth performance and liver triglyceride concentration of young pigs, which was associated with an upregulation of fatty acid catabolism pathways (Liu *et al.*, 2014). Furthermore, the oxidised fat in the pig diet up-regulated sterol regulatory element-binding protein and its target genes in liver and small intestine (Luci *et al.*, 2007). The gastrointestinal epithelium of swine and chickens respond to the oxidant stress imposed by oxidised fat by increased enterocyte turnover and the gut associated immune system was compromised (Dibner *et al.*, 1996). From data presented above it is clear that lipid peroxidation in the feed is an important source of toxic products and a potential source of free radicals in the animal digestive tract.

8.3.2 Iron ions

Iron is recognised as an essential nutrient; however, iron absorption from a diverse diet has shown to be about 15% (Department of Health, 1991). The main problem with iron nutrition is its reactivity and possible involvement in free radical generation. Iron ions are considered to catalyse the formation of the hydroxyl radical and accelerate the decomposition of lipid hydroperoxides (Davies and Slater, 1987) and to stimulate lipid peroxidation (Braughler *et al.*, 1986; Minotti and Aust, 1987). Iron dietary supplementation could represent an important source of potentially dangerous free iron in the digestive tract which could also be involved in lipid peroxidation in the intestine (Hollan and Johansen, 1993). For example, iron supplementation in relatively high doses resulted in abnormal iron accumulation and increased lipid peroxidation in rats (Knutson *et al.*, 2000). Iron supplementation amplified the inflammatory response and enhanced the subsequent mucosal damage in a rat model of colitis (Reifen *et al.*, 2000). In the conditions of dietary iron overload in rats, *in vivo* hydroxyl radical generation (ultimately responsible for iron-induced injury) was demonstrated

(Kadiiska *et al.*, 1995). Iron can also interact with other nutrients stimulating free radical production. For example, it has been shown that iron in combination with the secondary bile acids, lithocholic and deoxycholic acids and the vitamin K group can generate free radicals (Blakeborough *et al.*, 1989). Clearly, feed iron supplementation represents a possible source of catalytic iron in the GIT responsible for oxidative stress and lipid and protein oxidation.

8.3.3 Nitrites and nitrates

Nitrates is consumed in the diet, through feed and drinking water (Cammack *et al.*, 1999). Therefore, animals are subjected to significant nitrate and nitrite levels in feed and water, as well as those formed *in vivo*. Nitrites and nitrates formed from nitrogenous sources by microorganisms in saliva and the intestine are considered to be the major source of animal and human exposure under physiological conditions (Chow and Hong, 2002). It is generally accepted that nitrate is concentrated in the saliva and rapidly conversed to nitrite by facultative anaerobic bacteria. Benjamin *et al.* (1994) showed that nitrite is converted to NO under the highly acidic conditions (pH 3) which occur in the lumen of the stomach. It was observed that the generation and accumulation of NO from typical nitrite concentrations found in biological tissues increased 100-fold when pH fell from 7.4 to 5.5 (Zweier *et al.*, 1999). Therefore, nitrate and nitrite can generate NO• radicals by either direct disproportionation or reduction under the acidic and highly reduced conditions (Chow and Hong, 2002). More importantly, NO• can be a source of another reactive free radical, peroxynitrite ($ONOO^-$), which is 1000 times more oxidising than H_2O_2 and has a half-life in solution of about 1-2 seconds (Van Dyke, 1997). The amount of nitrites and nitrites in the diet vary substantially. However, in combination with other prooxidants in the digesta they can be involved in free radical formation and lipid peroxidation.

8.3.4 Heavy metals

Agricultural use of phosphate fertilisers and sewage sludge, and industrial use of cadmium have been identified as a major cause of widespread dispersion of heavy metals at trace levels into animal feed and human foodstuffs. It is well known that heavy metals can cause oxidative stress and stimulate lipid peroxidation (Surai, 2006). For example, cadmium increased lipid peroxidation in liver, kidney, and testes of rats and reduced metallothionein and total sulfhydryl in liver and kidney (Khandelwal *et al.*, 2002). In renal tubular epithelial cells of rats a significant decrease in activities of GSH, GSH-Px, SOD and an increased malondialdehyde (MDA) formation were observed as a result of the treatment with lead and cadmium (Wang *et al.*, 2002). It is believed that lead can alter certain membrane bound enzymes and may cause oxidative stress. For example, exposure of HepG2 cells to lead ions decreased cell viability and stimulated lipid peroxidation of cell membranes decreasing the fluidity in the polar surface of cell membranes (Chen *et al.*, 2002). Levels of lipid peroxidation products, such as MDA, conjugated diene and hydroperoxide were increased in liver, lung and kidney of lead-treated rats. Consumption of lead in drinking water by rats imposed oxidative stress, increasing lipid peroxidation in

peripheral blood mononuclear cells and liver (Ercal *et al.*, 2000). Administration of exogenous antioxidants in lead-treated animals significantly reduced the prooxidant effect of the toxicant (Upasani *et al.*, 2001).

Mercury is also a strong prooxidant and able to increase lipid peroxidation and decrease GSH content in liver of Swiss albino mice. In particular, mercury treatment enhanced lipid peroxidation in kidney, testis and epididymus of rats (Mahboob *et al.*, 2001). Heavy metal concentrations in major feed and food sources are quite low; however, in combination with other prooxidants they potentially can be involved in generation of free radicals and cause oxidative stress in the GIT.

8.3.5 Persistent organic pollutants

Persistent organic pollutants (POPs) comprise a class of chemicals that are among the most insidiously dangerous compounds and include many organochlorine pesticides (OCPs). Examples of persistent organic pollutants found in food and feed include dioxins, polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers, and some pesticide chemicals (Jensen *et al.*, 2001). Contaminating/accumulating levels of OCPs in extractable fats from a basal diet, eggs and seven tissues and excreta of laying hens that were kept in a general poultry farm of Japan were examined (Furusawa and Morita, 2000). Dieldrin and dichlorodiphenyltrichloroethane (DDT) were shown to contaminate the dietary fats at the level of 10-70 μg/kg. In 1989-1994, the US Food and Drug Administration (FDA) analysed 545 samples of mixed feed rations and found that only 88 samples (16.1%) did not contain detectable pesticide residues (Lovell *et al.*, 1996). Malathion, chlorpyrifos-methyl, diazinon, chlorpyrifos, and pirimiphos-methyl were the most commonly detected pesticides comprising 93.4% of all pesticide residues detected. Their median values in samples containing quantifiable levels ranged from 0.014 to 0.098 mg/kg.

Animal products like milk and meat are often found to be contaminated with residues of persistent pesticides and other toxic substances. The major entry source of these compounds to animal body is contaminated feed and fodder. The frequency of occurrence and contamination levels of OCP residues in different kinds of animal concentrate feed and straw samples collected from Bundelkhand region of India were determined (Nag and Raikwar, 2011). Out of 533 total samples, 301 (i.e. 56.5%) samples were positive, containing residues of different OCPs. In the case of the DDT complex, i.e. DDD, DDE and DDT, the concentration ranged between 0.016 and 0.118 mg/kg. It has been shown that organochlorines can cause oxidative stress. For example, the adverse effect of organochlorine pesticide methoxychlor on the male reproductive system was shown to be due to induction of oxidative stress in the testis (Latchoumycandane and Mathur, 2002; Latchoumycandane *et al.*, 2002). Similarly, the pesticide hexachlorocyclohexane compromised antioxidant defence and induced oxidative stress in the cerebral hemisphere of rats (Sahoo *et al.*, 2000). Even if the levels of the aforementioned toxicants are in the accepted limits, they are still able to participate in lipid peroxidation. Therefore, persistent organic pollutants represent an

important health hazard for animals and they also can be involved in promotion of lipid peroxidation in the gut.

8.3.6 Mycotoxins

At least 25% of world's grain production is contaminated with mycotoxins, which are a worldwide problem (Fink-Gremmels, 1999). Indeed, mycotoxins are the major feed- and food-derived stressors (Surai *et al.*, 2010). Recently, the occurrence of ochratoxin A (OTA) in complete poultry feeds (n=80) and poultry feed ingredients (n=286) from Pakistan has been evaluated (Sherazi *et al.*, 2015). Contamination frequency and mean OTA levels were 31% and 51 μg/kg in feed ingredients, and the corresponding values for complete feeds were 38% and 75 μg/kg. In an Argentinian study on mycotoxins in poultry feed, 44 out of 49 samples (90%) were contaminated with deoxynivalenol (DON; median 222 μg/kg) and OTA (median 5 μg/kg). In addition, 44 out of 49 samples were contaminated with aflatoxins (median 2.7 μg/kg), 42 samples (86%) with zearalenone (ZEA; median 50 μg/kg), and 38 samples (78%) with T-2 toxin (median 50 μg/kg; (Greco *et al.*, 2014). A total of 55 feed ingredients as well as 76 complete swine feeds were randomly collected from 15 swine farms located in the Beijing region of China from July to August 2011 and mycotoxins were analysed (Li *et al.*, 2014). It is important to note that DON and ZEA were the most prevalent mycotoxins found. DON was detected at percentages of 93, 92, 54, 100 and 97% with a mean level of 1.01, 0.44, 0.05, and 0.65 mg/kg in the samples of corn, wheat bran, soybean meal, and complete feeds, respectively. The detected percentages of ZEA were 100, 100, 54 and 100% with mean levels of 109.1, 14.9, 9.2 and 58.9 μg/kg in the same samples. In another study from China, of the 420 analysed feedstuff samples, the incidence of T-2 toxin, ZEA and fumonisin B_1 (FB_1) was 79.5, 85.2 and 96.1%, respectively, and detected concentrations ranged from 10-735, 35-1,478 and 20-6,568 μg/kg, respectively (Wang *et al.*, 2013). Indeed, during an 8-year period, 17,316 samples of feed and feed raw materials from all over the world were analysed for contamination with aflatoxins, OTA, ZEA, DON and fumonisins. Overall, 72% of the samples tested positive for at least one mycotoxin and 38% were found to be co-contaminated (Streit *et al.*, 2013). Therefore, the chances mycotoxins occurring in pig/poultry diet are very high.

It is necessary to mention that OTA alters both the barrier and absorption function of the intestinal epithelium, causing intestinal injuries, including inflammation and diarrhoea (Maresca *et al.*, 2001). Inhibition of protein synthesis and induction of apoptosis are the main mechanisms of DON toxicity in the intestinal cells (Maresca *et al.*, 2002). In general, the main mycotoxin contaminants of food – aflatoxin B_1, fumonisins, T-2 toxin, DON, ZEA and OTA – have been shown to compromise the antioxidant system and stimulate lipid peroxidation *in vivo* and *in vitro* (Surai, 2002; Surai and Dvorska, 2005; Surai *et al.*, 2010). Mycotoxins are considered to be unavoidable contaminants of most food and feed ingredients and they are potent prooxidants able to affect a range of genes (Surai *et al.*, 2008). Therefore, even in comparatively low concentrations (lower than the officially allowed limits) they still

represent an important source of free radical generation in the GIT (Awad *et al.*, 2013; Da Silva *et al.*, 2018; Grenier and Applegate, 2013; Maresca, 2013; Pestka, 2010; Smith *et al.*, 2012).

8.3.7 Immune system

The immune system is considered to be an important source of ROS in the human body (Surai, 2002) and intestinal immunity is not an exception (Fisinin and Surai, 2013b). Indeed, the intestinal tract is considered to represent the largest immune organ of the human body responding to the challenge of bacteria or food antigens by production of ROS (Halliwell *et al.*, 2000). Mucosal surfaces covered by a layer of epithelial cells represent the most critical interface between the organism and its environment, since the mucosal interstitia of the intestine is continuously exposed to large amounts of dietary and microbial antigens. Therefore, epithelial cells engage in cross talk with luminal bacteria and their products and produce mediators and signals that are key components of host innate and acquired mucosal immunity (Maaser and Kagnoff, 2002). The mucosal immune system is a first line of defence against foreign antigens, including microbial and dietary antigens. Under normal circumstances it employs tightly regulated dynamic mucosal intra- and internets consisting of inductive (e.g. Peyer's patch) and effector (e.g. intestinal lamina propria) tissues, and maintains an appropriate immunological homeostasis between the host and mucosal environments (Kiyono *et al.*, 2001). Hence, the mucosal immune system has evolved efficient mechanisms to distinguish potentially pathogenic from non-pathological antigens. For example, the mucosal immune compartment must be able to choose the appropriate effector function (e.g. tolerance vs clearance) necessary to deal with each encountered antigen whether it is innocuous or pathogenic in nature (Laroux *et al.*, 2001). However, abrogation of these mucosal defence mechanisms may alter the immunological homeostasis in the GIT and induce pathological changes, including chronic active inflammation, mucosal atrophy and tissue injuries (Nagura *et al.*, 2001). It is important to stress that under inflammatory conditions in the intestine the maintenance of the epithelial barrier could be broken.

It is necessary to underline that the nutrient requirement to maintain a highly active immune system in the digestive tract could be quite high. In fact, different sources of injury to the intestinal mucosa (nutritional, infectious or allergic) act via a common mechanism of cell-mediated immune damage and nutrient repletion is required for restoration of the immune function (Cunningham-Rundles, 2001). In particular, the immunomodulating properties of natural antioxidants (Surai, 2002) could be of great advantage for the intestinal immunity. Furthermore, the intestinal epithelium can modulate the level of immune activity in the mucosal immune system according to the environment of the intestinal lumen (Sanderson, 1999). Data presented above indicate that the intestinal immune system can generate free radicals in response to various antigens including microbes and some food allergens.

8.3.8 Combinations of prooxidants in the gut and their detrimental effects

The physicochemical environment of the gastrointestinal tract depends on many factors, with diet, bacterial metabolites and body secretion being major determinants (Sanderson, 1999). There is a delicate balance between the environment of the lumen and epithelial cell functionality, and dietary factors are responsible for gene expression in the intestine and its adaptation. In this regard, oxidative stress could cause changes in this balance affecting the absorption of nutrients. Even if each of those lipid peroxidation promoters is present at a very low concentration, their combination could be much more powerful. For example, as mentioned above, lipid hydroperoxides can produce peroxyl radicals in presence of iron or copper ions and once the chain reaction of lipid peroxidation has started many other food-derived PUFAs can be oxidised. It has been calculated that the pH and temperature, as well as presence of oxygen in stomach can be favourable for lipid peroxidation (Kanner and Lapidot, 2001).

Data provided above indicate that the average pig or poultry diet contains a range of various prooxidants. They include oxidised PUFAs, nitrites, nitrates, heavy metals, mycotoxins, persistent organic pollutants, etc. In many cases those contaminants are found in the feed in low or very low concentrations, however, their various combinations in the feed could be an important source of free radical production in the gut.

8.4 Antioxidant defences in the gastrointestinal tract

An effective antioxidant protection in the gastrointestinal tract is needed to maintain gut health and this protection is based on food derived antioxidants (Surai, 2006).

8.4.1 Vitamin E

Vitamin E is the main biological chain-breaking antioxidant, located in the biological membranes of various tissues. In food and feed ingredients, vitamin E can be found in the form of 4-tocopherols and 4-tocotrienols. It is possible that the gut is a special place for γ-tocopherol and tocotrienols to play their antioxidant role. Alpha-tocopherol is not stable and easily oxidised during food processing and therefore commercial vitamin E preparations contain the stable esterified form of vitamin E or a mixture of tocopherols. For farm animals and poultry premixes the main source of vitamin E and its level in feed ingredients is often not taken into account during feed formulation (Surai, 2002).

The main reason for vitamin E dietary supplementation for poultry and farm animals is to maintain their optimal health and high productive and reproductive performance. This includes positive effects on male and female reproduction, immunocompetence, effective growth and development, high quality of eggs and meat as well, as decreased negative consequences of various stresses (Fotina *et al.*, 2013; Surai, 1999b, 2002,

2006, 2014; Surai and Fisinin, 2012, 2014). Extensive research and wide commercial application for a number of years clearly showed the essentiality of vitamin E in animal/poultry nutrition. Recently, it has been shown that vitamin E recycling in the cell is key for its antioxidant activity. Ascorbic acid, selenium, vitamins B1 and B2 are important elements of vitamin E recycling. Therefore, when recycling is effective, even a low vitamin E concentration, for example in the embryonic brain, can prevent lipid peroxidation *in vivo* (Surai, 1999b, 2002). There is a range of anti-stress premixes with increased vitamin E content, but their efficacy is variable. After 90 years of extensive research in the field of vitamin E we greatly appreciate its unique role in biological systems, in maintaining growth, development and general health of humans and animals. High concentrations of vitamin E were present in the mucosa of the duodenum and jejunum, with a trend to lower levels in the ileum and ceca, and significantly less in the colon of the chicken (McLean *et al.*, 2005). The different isoforms of vitamin E were absorbed from the digesta by the mucosa without any major selectivity. However, the liver was greatly enriched with alpha-tocopherol over the other isoforms, indicating a high degree of discrimination by this tissue. Clearly, vitamin E can be considered as a main contributor to the antioxidant potential of the digesta (Surai, 2006; Surai and Fisinin, 2014).

It should be also mentioned that beyond a direct antioxidant activity, vitamin E is involved in modulation of enzyme transcription and/or activity by interacting with genes involved in oxidative stress, proliferation, inflammation and apoptosis (Mocchegiani *et al.*, 2014). These genes include SOD, NO synthase, cyclooxygenase-2, NAPDH oxidase, NF-κB, phospholipase A2, protein phosphatase 2A, 5-lipooxygenase, activator protein-1, cytochrome P450, BCL2-like 1 as well as a lot of other genes. However, at present, it is not clear if all aforementioned genes are affected by vitamin E in the gut.

8.4.2 Coenzyme Q

CoQ, known also as ubiquinone, was discovered in 1957. The name ubiquinone is related to its 'ubiquitous' presence in all cells and the name coenzyme Q reflects the chemical structure of the compound containing one quinone group and 10 isoprenyl units. Coenzyme Q_{10} (CoQ_{10}) exists both in an oxidised and a reduced form, called ubiquinone and ubiquinol, respectively (Overvad *et al.*, 1999). In general, dietary supplementation of CoQ does not affect the endogenous synthesis of CoQ in tissues. However, oxidative stress (physical exercise, thyroid hormone treatment, cold adaptation, vitamin A deficiency, etc.) is associated with increased CoQ synthesis reflecting a cellular adaptation (Ernster and Dallner, 1995). Therefore, CoQ synthesis is considered to be an adaptive mechanism in response to stress conditions when other antioxidants are depleted. For example, in vitamin E and Se deficient rats CoQ concentration is elevated and the CoQ-dependent reductase system activated (Navarro *et al.*, 1998).

Antioxidant properties of CoQ are directly related to the protection in the GIT. For example, in rats treated *per os* with sodium nitrite increases in thiobarbituric

acid reactive substances in the small intestinal mucosa and liver were observed. Pre-treatment of nitrite-poisoned rats with CoQ_{10} mitigated lipid peroxidation and increased the total antioxidant status in animal blood (Grudzinski and Frankiewicz-Jozko, 2001). The protective effect of administered CoQ_{10} against small intestinal damage caused by ischemia reperfusion was also shown (Matsusaka *et al.*, 1992). In rat intestine, administration of CoQ_{10} normalised a sharp gamma-irradiation-induced inhibition of transformation of phosphatidylcholine from phosphatidylethanolamine (Novoselova *et al.*, 1985). Compared to paired non-inflamed mucosa, concentration of CoQ_{10} was significantly decreased in inflamed mucosa (Buffinton and Doe, 1995). The decreased antioxidant defences may severely compromise the inflamed mucosa, rendering it more susceptible to oxidative tissue damage, hindering recovery of the mucosa and return of epithelial cell layer integrity. Therefore, antioxidant and other regulating functions of CoQ_{10} could be extremely important in the GIT.

8.4.3 Carotenoids

Carotenoids comprise a family of more than 750 compounds responsible for a variety of bright colours in nature (Maoka, 2009). It is well appreciated that carotenoids are important elements of the antioxidant system, possessing antioxidant activities and participating directly or indirectly (for example, by recycling vitamin E or regulating expression of various genes) in antioxidant defences. Recently, an important role of canthaxanthin with a special emphasis to carotenoid antioxidant activities in breeder nutrition has been described (Surai, 2012a,b). Biological functions of these natural pigments in relation to animals or humans are not well defined but their antioxidant properties seem to be of major importance. In mixtures with other antioxidants they could be much more effective than on their own, and the GIT could be a major place for these compounds to exert their activity. Furthermore, carotenoids can induce the expression of genes related to antioxidant defences. For example, recently it has been shown that lutein modulates the expression of a range of genes related to oxygen transporters and decreases DNA damage and oxidative stress in mice (Serpeloni *et al.*, 2014).

Carotenoid assimilation from the diet varies significantly depending on many conditions; however, it seems likely that a substantial proportion of ingested carotenoids can be found in all segments of the digestive tract. In particular, the chicken mucosa of the duodenum and jejunum contain the highest concentrations of carotenoids, with much lower levels in the ileum and colon (McLean *et al.*, 2005). Therefore, in combination with other dietary antioxidants carotenoids could promote antioxidant defence in the gut. Furthermore, carotenoid activities related to the promotion of cell differentiation, regulation of cell proliferation and intracellular communication via gap junctions, as well as regulation of the detoxifying enzymes and enhancement of immune system (Surai, 2002, 2012a,b) could also be of great importance in the gut.

8.4.4 Ascorbic acid

Vitamin C is referred to as L-ascorbic acid and its two-electron reduction product dehydro-L-ascorbic acid. Most animal species synthesise ascorbic acid (AA) from glucose, but human subjects are not able to synthesise it. Therefore, AA is an essential dietary component playing an important role in many physiological processes. It is a hydrophilic antioxidant functioning in an aqueous environment and possessing high free-radical-scavenging activity. It can participate in vitamin E recycling thus maintaining an efficient antioxidant defence (Surai, 2006). Due to its high reducing potential, in combination with iron ions AA can be a prooxidant. However, it is believed that in physiological conditions and in the GIT AA performs mainly its antioxidant function. In fact, AA inhibits chemical synthesis of nitrosamines (animal carcinogens) in the gastric contents and there are suggestions that intake of AA much higher than RDA may reduce the risk of diseases, such as heart disease and cancer (Hathcock, 1997). AA is synthesised in farm animals and poultry, but in stress conditions, additional dietary vitamin C supplementation or its provision with drinking water has shown to be helpful.

8.4.5 Glutathione

GSH is the most abundant non-protein thiol in mammalian cells, and considered to be an active antioxidant in biological systems providing cells with their reducing milieu. It is well known that GSH can be synthesised in the animal and human body. It is abundantly distributed in the mucosal cells of the GIT in man and its highest concentration is found in the duodenum (Loguercio and Di Pierro, 1999). Cellular GSH plays a key role in many biological processes: the synthesis of DNA and proteins, including cell growth and proliferation; regulation of programmed cell death; immune regulation; transport of amino acids; xenobiotic metabolism; and redox-sensitive signal transduction (Aquilano *et al.*, 2014; Hansen and Harris, 2015). Furthermore, the GSH thiolic group can react directly with H_2O_2, superoxide anions, hydroxyl radicals, alkoxyl radicals and hydroperoxides (Ribas *et al.*, 2014). Under stress conditions GSH prevents the loss of protein thiols and vitamin E and plays an important role as a key modulator of cell signalling (Griffiths *et al.*, 2014). In addition to body synthetic activity, feed also provides GSH. In general, it is difficult to overestimate a protective role of GSH in the gut (Aw, 1998, 2005; Circu and Aw, 2011; Jefferies *et al.*, 2003).

8.4.6 Polyphenols/flavonoids

Natural polyphenols comprise a big group of compounds with flavonoids, low molecular weight polyphenolic substances based on the flavan nucleus, being the most studied ones. They are widespread in nature, occurring in all plant families. The list of flavonoids substantially increased during the last decades accounting for over than 8,000 individual compounds (Pietta, 2000). It seems likely that the gut is the major place of antioxidant action of polyphenols (Surai, 2014). Indeed, reduction of

oxidative damage, modulation of colonic flora and variation in gene expression are involved in the modulation of intestinal function by polyphenols. In order to study the molecular effects of wine polyphenols at the gene level, microarray technology was used: rats were treated with 50 mg/kg wine polyphenols for 14 days, mixed in the diet. It was shown that two major regulatory pathways were down-regulated in the colon mucosa of polyphenol-treated rats: inflammatory response and steroid metabolism (Dolara *et al.*, 2005). Since flavonoids are usually consumed in concentrations much higher than other antioxidant compounds, their protective effect during digestion is of great importance. For example, it has been shown that flavonoids not only prevented the accumulation of peroxidised lipids, but also could switch prooxidant properties of heme-proteins to antioxidant ones (Kanner and Lapidot, 2001). Dietary polyphenols can also modulate *in vivo* oxidative damage in the gastrointestinal tract of rodents (Giovannelli *et al.*, 2000) supporting the hypothesis that dietary polyphenols might have both a protective and a therapeutic potential in oxidative damage-related pathologies. Indeed, the antioxidant-prooxidant balance (redox status) in various parts of the intestine would ultimately depend on the level of antioxidants and prooxidants provided with the diet and released by the cells themselves, as well as on the level of absorption of both antioxidants and prooxidants. In a model system mimicking stomach conditions it was shown that both lipid peroxidation and co-oxidation of vitamin E and beta-carotene were inhibited at pH 3.0 by red wine polyphenols (Gorelik *et al.*, 2005).

Redox signalling in gut inflammation is complex and poorly understood. However, it is generally accepted that homeostatic control of the intestinal epithelial redox environment is central for nutrient digestion and absorption, stem cell proliferation, apical enterocyte apoptosis, and immune response (Circu and Aw, 2012). Indeed, polyphenols may play a role on intestinal mucosa integrity, inflammation and permeability (Martínez *et al.*, 2013). For example, wine phenolics were able to prevent or delay the progression of intestinal diseases characterised by oxidative stress and inflammation, acting as both free radical scavengers and modulators of specific inflammation-related genes involved in cellular redox signalling (Biasi *et al.*, 2014). They exert their effects by modulating cell signalling pathways, mainly activated in response to oxidative and inflammatory stimuli, and Nrf2 and NF-κB are the principal downstream effectors (Biasi *et al.*, 2011). It is possible to suggest that there is a biological reason for some antioxidants not to be absorbed completely, thereby providing antioxidant protection in the lower parts of the intestine. Comparatively low bioavailability and antioxidant potential of various flavonoids could be beneficial for the human/animals providing antioxidant protection in various parts of the digestive tract, including the large intestine where levels of other antioxidants would be quite low.

8.4.7 Synthetic antioxidants

Antioxidants in feed may be of endogenous origin or may be added externally to preserve their lipid components from peroxidation. Synthetic antioxidants, such as ethoxyquin and its blends with other antioxidants, such as propyl gallate (Lu *et*

al., 2014a,b,c,d) are used to stabilise fat in poultry and pig diets and they play an important role in antioxidant defences of the gut.

8.5 Specific place for Se-dependent enzymes in antioxidant defence of the gastrointestinal tract

Food derived antioxidant enzymes would be inactivated during thermal food processing. However, the GIT contains internally-originated antioxidant enzymes SOD, GSH-Px and CAT and they represent an important mechanism of the enterocyte defence from oxidative damage. A specific gastrointestinal GSH-Px (GI-GSH-Px) has been described in 1993 (Chu *et al.*, 1993). GI-GSH-Px activity was present in both the villus and crypt regions of the rat mucosal epithelium and its activity nearly equalled that of classical GSH-Px throughout the small intestine and colorectal segments (Esworthy *et al.*, 1998). GI-GSH-Px could be considered to be a barrier against hydroperoxide resorption (Brigelius-Flohe, 1999; Brigelius-Flohé and Maiorino, 2013). Furthermore, in the gastrointestinal tract there are at least three more selenoproteins, including plasma GSH-Px, selenoprotein P and thioredoxin reductase (Mork *et al.*, 1998).

Glutathione and glutathione-dependent enzymes contribute significantly towards intestinal antioxidant defences. In fact, an important peroxide detoxification pathway in the intestine is based on the GSH redox system (LeGrand and Aw, 1998). In this system GSH-Px reduces peroxides at the expense of GSH oxidation. Oxidised glutathione is reduced back to the active form by glutathione reductase utilising reducing potential of NADPH which is produced in the pentose phosphate pathway.

Various studies in mouse models of colon cancer and selenoprotein gene deletion studies indicated that selenoproteins play a pivotal role in the maintenance of gut homeostasis (Irons *et al.*, 2006; Reeves and Hoffman, 2009). In particular, GSH-Px, selenoprotein S (SelS), and selenoprotein P (SePP1) have been extensively studied for their redox regulation, antioxidant and anti-inflammatory roles in preventing chronic intestinal inflammation (Reeves and Hoffman, 2009; Speckmann and Steinbrenner, 2014). Indeed, GSH-Px1 is shown to be expressed in all cell types of the gut, whereas GSH-Px2 is predominantly expressed in the epithelial cells, including the paneth cells, of the gastrointestinal mucosa (Brigelius-Flohe *et al.*, 2001; Esworthy *et al.*, 1998; Florian *et al.*, 2001) and GSH-Px4 is found to be expressed in epithelial cells and the lamina propria of the intestine (Speckmann *et al.*, 2011; Takahashi *et al.*, 1987). Furthermore, SelW is widely expressed in the gastrointestinal tract tissues of birds and the transcription of the SelW gene is very sensitive to dietary Se (Li *et al.*, 2011). In fact, the expression of the SelW mRNA in the gastrointestinal tract tissues of chickens was shown to correlate with the dietary Se concentrations (Gao *et al.*, 2012).

The changes in GSH-Px and other selenoprotein expression in the gut due to Se deficiency may be the major cause of injury in the intestinal tract. In fact, recent results indicated that Se deficiency induced oxidative damage in the intestinal tract of

chickens and that low levels of GSH-Px and high contents of NO may exert a major role in the injury of the intestinal tract induced by Se deficiency (Yu *et al.*, 2015). Indeed, Se deficiency induced higher inflammatory damage and MDA levels, which were accompanied by higher levels of iNOS and NO but lower levels of GSH and GSH-Px. It was shown that duodenum and jejunum were the primary organs targeted by Se deficiency, while the rectum has a certain tolerance for the lack of Se (Yu *et al.*, 2015). In fact, Se deficiency induced high expression levels of PTGE, COX-2, TNF-α, and NF-κB in the gastrointestinal tract tissues (Gao *et al.*, 2016). The effects were more pronounced in the duodenum and small intestine than those in other parts of the gut. Furthermore, Se deficiency induced the production of pro-inflammatory factors, thus aggravating inflammatory lesions in the gastrointestinal tract. In particular, Se deficiency increased the expression of NF-κB which promotes inflammation (Liu *et al.*, 2006). Therefore, one of the most important consequences of Se deficiency in chicken is an induction of inflammation in the gastrointestinal tract (Placha *et al.*, 2009). Furthermore, chickens fed with low-Se diet exhibited histological changes, lower H_2S production, and lower mRNA expression of H_2S-producing enzymes, as well as higher mRNA expression of intestinal inflammatory factors (TNF-α, NF-κB p50, COX-2, and PTGES) compared to controls (Wu *et al.*, 2016). Similarly, a decreased level of GSH-Px induced by Se deficiency has been reported to be the cause of damage to intestinal tissues. In fact, Se deficiency decreased GSH-Px activity in the colon of humans causing oxidative stress (Pawłowicz *et al.*, 1991). GSH-Px activity and mRNA level were significantly depressed in the ileum of Se-deficient mice (Esworthy *et al.*, 2005). Similarly, Se deficiency in rats reduced GSH-Px and other antioxidant enzymes in intestines and led to DNA damage and oxidative stress (Rao *et al.*, 2001) as well as increased lipid peroxidation and decreased GSH levels, thus exacerbating the intestinal mucosal injury in the intestine of rats (Bolkent *et al.*, 2007).

In mouse, supplementation with Se-enriched milk proteins and Se-yeast up-regulated the expression of gut antioxidant selenoproteins, enhancing the capacity for cell protection from oxidative damage (Hu *et al.*, 2010). It was also proven that dietary macro-fungal organic Se from Se-enriched *Agaricus bisporus* protected the gastrointestinal tract in rats from the effects of heat induced oxidative stress, by restoring epithelial ion transport and barrier functions, and elevating the expression of GSH-Px-1 and gastrointestinal specific GSH-Px-2, selenoenzymes relevant to mitigating oxidative stress (Maseko *et al.*, 2014, 2014a). Furthermore, it was shown that dietary supplementation of young broilers with Se (0.25-1 mg/kg) is beneficial to reduce the negative consequence of necrotic enteritis (NE) (Xu *et al.*, 2015). In particular, chickens fed with 0.50 mg/kg Se showed significantly increased body weights and antibody levels against NetB, and significantly reduced gut lesions compared with non-supplemented chickens reducing negative consequence of NE-induced immunopathology (Xu *et al.*, 2015a). Similarly, in pigs an increase of dietary Se and vitamin E mitigated the impacts of heat stress on intestinal barrier integrity, associated with a reduction in oxidative stress (Liu *et al.*, 2016).

The aforementioned data clearly indicate that Se as a part of various selenoproteins play an important role in protection of gut integrity by regulating antioxidant/prooxidant

balance and redox status of the gut. Furthermore, Se deficiency attenuated chicken duodenal mucosal immunity via activation of the NF-κB signalling pathway regulated by redox activity, which suggested that Se is a crucial host factor involved in regulating inflammation (Liu *et al.*, 2016a). Furthermore, anti-inflammatory properties of Se in the gut due to its regulatory effects on transcription factor NF-κB (Narayan *et al.*, 2015; Tyszka-Czochara *et al.*, 2016; Wrobel *et al.*, 2015) are of great importance. Indeed, recent reports demonstrated the ability of selenium to inhibit the acetylation of non-histone and histone proteins by histone acetyltransferase p300 and there was an epigenetic modulation of the expression of proinflammatory genes, including NF-κB member p65, in macrophages (Narayan *et al.*, 2015). On the other hand, direct effect of selenium on the composition of the gut microbiota in mice (Kasaikina *et al.*, 2011) deserve more attention and further investigation. Furthermore, prooxidant properties of selenite could have a detrimental effect on the gut. Indeed, the duodenal and intestinal mucosa of the chicken ileum was negatively affected by inorganic Se at 0.3 mg/kg supplementation (Attia *et al.*, 2010). In fact, in the duodenum of chickens fed on 0.3 mg/kg selenite vacuolar and hydropic degeneration of the epithelial cells lining the intestinal crypts were seen, while in the ileum of chickens from the same group an excess of mononuclear cell infiltration and aggregation in between degenerated and necrotic intestinal glands was noticed (Attia *et al.*, 2010).

8.6 Role of vitagenes in the gut defence

To adapt to environmental changes and survive different types of injuries, eukaryotic cells have evolved networks of different responses which detect and control diverse forms of stress. Recently, a vitagene concept has been developed. The term 'vitagene' was introduced by Rattan (1998) who considered them as a range of genes to be involved in regulation of various protective mechanisms in stress conditions. Later, the vitagene concept has been further developed by Calabrese and colleagues (Calabrese *et al.*, 2004, 2007, 2014; Cornelius *et al.*, 2013a,b,c) with major emphasis to prosurvival mechanisms controlled by the vitagene network. Furthermore, possible roles of vitagenes in the protection of chickens against various stresses have been reviewed recently (Fisinin and Surai, 2011a,b; Surai and Fisinin, 2012, 2016a,b,c,d; Velichko *et al.*, 2013). In accordance with Calabrese *et al.* (2007, 2014) the vitagene family includes genes that are strictly involved in preserving cellular homeostasis during stress conditions. In fact, the vitagene family includes heat shock proteins (HSPs), such as haeme oxygenase-1 (HSP32, HO-1), HSP60 and HSP70, the thioredoxins (Trx)/thioredoxins reductase (TrxR) system and sirtuins (Figure 8.2). It seems reasonable to extend the list of potential candidates of the vitagene family. In particular, SOD, a major inducible enzyme of the first level of antioxidant defence, meets the selecting criteria to be included into the vitagene family. The products of the mentioned genes are responsible for the detection and control of diverse forms of stress and cell injuries. The cooperative mechanisms of the vitagene network are reviewed in recently published comprehensive reviews (Calabrese *et al.*, 2014; Cornelius *et al.*, 2013a,b; Trovato Salinaro *et al.*, 2014) with a major conclusion indicating an essential

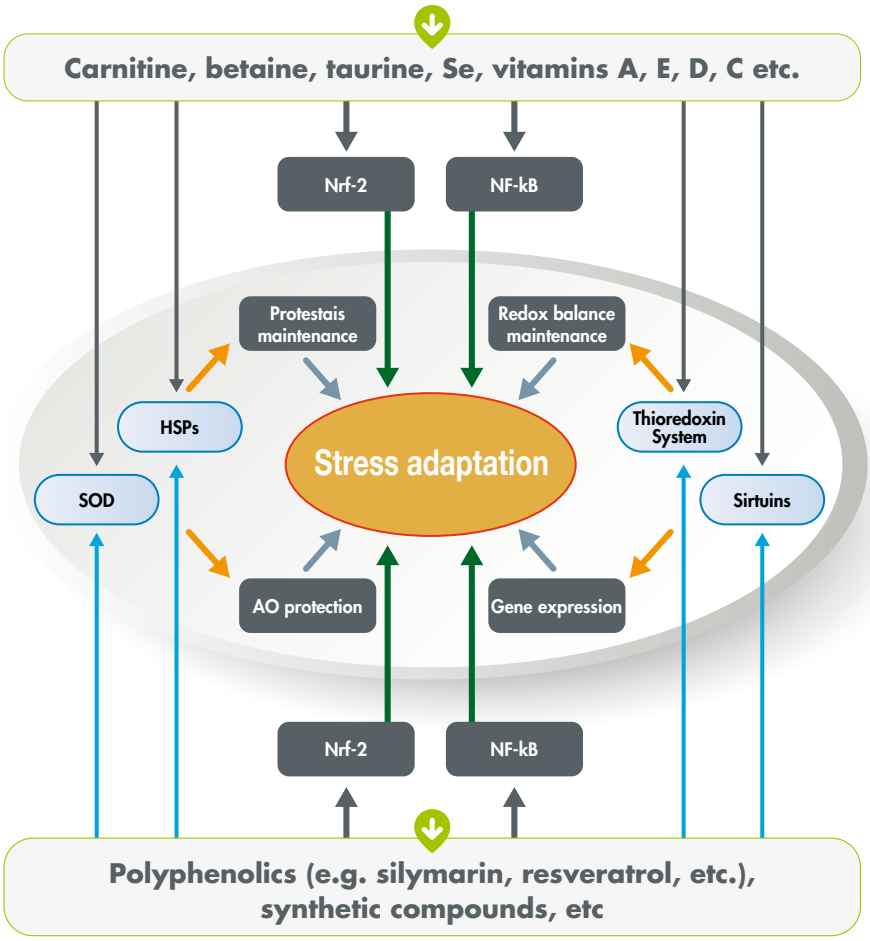

Figure 8.2. Vitagene – a gut defense.

regulatory role of the vitagene network in cellular and whole organism adaptation to various stresses.

Recently, new mechanisms of such adaptive defences of the gastrointestinal mucosa at the intracellular level have been characterised. One of these responses, known as the heat shock response is considered to be a universal fundamental mechanism necessary for cell survival under a variety of unfavourable conditions (Santoro, 2000). As mentioned above, intestinal cells are challenged with a great variety of potentially toxic compounds and their protection is a vital part of the strategy to maintain human health. In mammalian cells, the induction of the heat shock response requires the activation and translocation to the nucleus of one or more heat shock transcription factors, which control the expression of a specific set of genes encoding

cytoprotective heat shock proteins (Santoro, 2000). Indeed, HSPs have a broad range of functions related to their major role in cellular homeostasis and protect cells against apoptosis and cell death. The physiological expression of cytoprotective HSP27 in the gut was investigated in eighteen 7-wk-old pigs (Liu *et al.*, 2012). Indeed, HSP27 was expressed in all the samples from ileum and colon and the expression was most intensive in the apical intestinal epithelium in close contact with luminal contents and lighter in crypt cells. Furthermore, the ileal Peyer's patches showed a strong expression of HSP27, which was highly correlated with HSP27 expression in the ileal epithelial cells. The expression of HSP27, heat shock cognate 70 (HSC 70), HSP70 and HSP90 along the GIT of young pigs and the effect of weaning on this expression were studied (David *et al.*, 2002). Pigs were weaned at 28 or 21 d and slaughtered at various times post-weaning. All HSPs were expressed in the GIT segments studied before and after weaning. However, there was a site specificity in HSP expression in the gut. For example, the expression of HSP27 and HSP70 was increased in the stomach and duodenum between 6 and 12 h post-weaning and between 24 and 48 h in the mid-jejunum, ileum and colon. At the same time, their expressions were transiently decreased in the ileum. Expression of HSP90 increased in the stomach and jejunum but decreased in the duodenum, ileum and colon. Similar results were obtained at both ages of weaning. Indeed, in normal porcine GI tract HSP expression is gut region- and cell type-specific in response to dietary components, microbes, and microbial metabolites to which the mucosa surface is exposed (Liu *et al.*, 2014b).

Therefore, HSPs function as molecular chaperones in regulating cellular homeostasis and promoting survival. However, if the stress is too high, a signal that leads to programmed cell death, apoptosis, is activated, thereby providing a finely tuned balance between survival and death (Kopecek *et al.*, 2001). In addition to extracellular stimuli, several non-stressful conditions induce HSPs during normal cellular growth and development. In particular, the HSP family is activated under oxidative stress and provides an important protection against protein denaturation and modifications by capping and refolding, or drives damaged proteins into appropriate proteolytic pathways (Yenari, 2002). In fact, HSPs have been assigned to multiple subcellular sites and implicated in multiple functions ranging from stress response, intracellular trafficking, antigen processing, control of cell proliferation, differentiation, and tumorigenesis (Wadhwa *et al.*, 2002). Therefore, in response to environmental or physiological stress cells increase synthesis of HSP (Tsukimi and Okabe, 2001). It has been suggested that the conserved heat shock protein HSP33 functions as a potent molecular chaperone with a highly sophisticated regulation. In fact, at the transcriptional level, the HSP33 gene is under heat shock control; at the posttranslational level, the HSP33 protein is under oxidative stress control (Graf and Jakob, 2002). Therefore, redox-regulated chaperone activity of HSP33 specifically protects proteins and cells from the detrimental effects of reactive oxygen species.

ROS-mediated damage has been implicated in the pathophysiology of the gastrointestinal mucosa and HSPs are suggested to play an important role in cytoprotection against oxidative stress-induced injury (Prabhu and Balasubramanian, 2002). For example, the mammalian intestinal epithelial cells respond to heat stress

by producing heat shock proteins that provide protection in stress conditions, which would otherwise lead to cell damage or death. The protective effects of HSPs are seen in heat stress, infection, and inflammation (Malago *et al.*, 2002). Similarly, glucocorticoid protection of rat intestinal cells against oxidant-induced stress was mediated by HSP72 (Urayama *et al.*, 1998).

The molecular mechanisms of heat shock response-induced cytoprotection are beyond this review. However, they involve inhibition of proinflammatory cytokine production and induction of cellular proliferation for restitution of the damaged epithelium (Malago *et al.*, 2002). It is interesting to note that HSP72, the stress-inducible form of HSP70, was detected in samples from rat distal colon, proximal colon, and terminal ileum, but was not found in proximal small bowel or other organs (liver, kidney, spleen, heart, and brain) of unstressed animals (Beck *et al.*, 1995). HSPs play an important role in gastric mucosal defence under conditions of stress. For example, exposure of rats to restraint and water-immersion stress caused rapid HSP70 mRNA expression and HSP70 accumulation in gastric mucosa and the extent of HSP70 induction inversely correlated to the severity of mucosal damage (Rokutan, 1999). Therefore, HSP70 is involved in repair of partially damaged proteins and substantially contributes to protection of the gastrointestinal mucosa against various necrotising factors (Tsukimi and Okabe, 2001).

It is known that HO-1, known as HSP32, can be induced by various stresses. Indeed, HO-1 induction and the maintenance of its appropriate activity is critical in protecting the intestinal epithelial cells from oxidative injury (Fujii *et al.*, 2003). It is interesting that in the aforementioned experiment HO-1 was markedly induced following LPS treatment in the mucosal epithelial cells in the upper intestine (duodenum and jejunum) but not in the lower intestine (ileum and colon). It seems likely, that there is a delicate interaction between HSPs and other antioxidant defence mechanisms to maintain mucosal integrity and repair of acute mucosal damage.

Thioredoxin and TrxR are also considered to be important members of vitagene family. Their role in the gut is not clear at present, but they participate in maintenance of the redox balance in the gut. The presence of a full complement of Trx/TrxR proteins in stomach, duodenum, jejunum, ileum and colon suggests their function in antioxidant defence and redox regulation in the intestinal tract (Godoy *et al.*, 2011). It is important to note that Trx expression is particularly high in the small intestinal mucosa and colon (Gasdaska *et al.*, 1996), while TrxR expression in small intestine is substantially higher than in colon. All three thioredoxin reductases are expressed in the intestine at least at the mRNA level, which is relatively unaffected by marginal selenium deficiency and in the case of TrxR2 and TrxR3 rather increased when selenium becomes limited (Kipp *et al.*, 2009). Furthermore, the selenoproteins GSH-Px2, TrxR2 and TrxR3 in the gut are regulated by the Wnt pathway (Kipp *et al.*, 2012). It has been also shown that unstimulated lamina propria T lymphocytes exhibited high expression of Trx, which is involved in the regulation of intracellular redox homeostasis in these cells (Sido *et al.*, 2005). The authors suggested that Trx may play a key role in the specialised intestinal microenvironment in amplifying immediate

immune responses. In fact, membrane-bound Trx converts human β-defensin 1 to a potent antimicrobial peptide *in vivo* (Jaeger *et al.*, 2013). Takaishi *et al.* (2003) identified rat Trx as a growth-promoting factor for intestinal epithelial cells, while Higashikubo *et al.* (1999) showed that cellular oxidative shock caused an increase in the activity of thioredoxin, which is involved in the defence mechanism against oxidative stress. In particular, H_2O_2 was cytotoxic to the small intestine epithelial cell line, IEC-6, and the glutathione S-transferase and thioredoxin reductase activities and SH content decreased dose-dependently with H_2O_2, while thioredoxin activity increased at low H_2O_2 concentrations.

8.7 Critical periods of the gut development

8.7.1 Chick placement

Chick viability is an important factor in determining profitability and, from fertilisation to placement at the broiler farm, factors such as egg quality, egg storage conditions, incubation conditions and post-hatch environment will all affect chick quality (Decuypere *et al.*, 2011). It is well appreciated that time between chick hatch and placement is stressful due to dehydration and yolk sac reserve depletion. Indeed, if we put together hatching time inside the hatcher, time of chick processing and transportation, and finally, placement at the farm, it could take up to 36-48 h before a newly hatched chick has access to feed and water and during this time body weight decreases quickly (Noy and Sklan, 1999). It has been shown that in the hatching chick, the small intestine matures in a manner similar to neonatal mammals, with specific ontogenetic timetables in the different small intestinal segments; however, the most dramatic changes occur within the first 24 h post-hatch (Geyra *et al.*, 2001). There is an inverse relationship between duration of post-hatching holding time and subsequent chick performance (Hager and Beane, 1983; Pinchasov and Noy, 1993). Therefore, immediate access to feed and water can increase body weight of the growing chick at 3 weeks of age (Sklan *et al.*, 2000) or at market age of broilers (Vieira and Moran, 1999). It should be mentioned that there is the hatch window (24-36 hours) or the spread between late and early hatchers which depends on the homogeneity/heterogeneity of the incubating eggs including egg size and breeder age. A spread in the hatching period will increase the numbers of chicks sitting extra hours in stressful conditions of the hatcher without food or water. In fact, hatching process places a stress upon the emerging chick and natural antioxidants (vitamin E and carotenoids) have evolved to reach the maximum concentration in the liver to protect the unsaturated lipid in the tissues and so limit lipid peroxidation (Surai *et al.*, 1996, 1998). Furthermore, any delay in accessing food (Bigot *et al.*, 2003; Noy *et al.*, 2001) and/or water intake after hatching as well as hatchery treatments such as vaccination, sexing and transport to the farm can result in additional stress (Geyra *et al.*, 2001a). Indeed, extended time in the hatcher (36 h) was associated with decreasing vitamin E and coenzyme Q concentrations in chicken tissues (Karadas *et al.*, 2011). Given the relatively high temperature and humidity in the hatcher, it is easy to make the argument that the chick may be under chronic oxidative stress during this holding time. Therefore, antioxidant

protection at hatching time is considered to be an important determinant of chick viability during first post-hatch days (Surai, 2000, 2002; Surai *et al.*, 1998a, 1999). During chick embryo development there is an antioxidant/prooxidant balance in the tissues which supports normal embryonic development and post-hatch chick viability. It has been suggested that an accumulation of the natural antioxidants like vitamins A, E and carotenoids as well as an increase in GSH-Px activity in the embryonic liver may have an adaptive significance, evolving to protect unsaturated lipids against peroxidation during the stress imposed by hatching (Figure 8.3; Surai, 2002).

The antioxidant system of the chicken embryo and newly hatched chick has been studied extensively (Surai, 2002). It was shown that it includes fat-soluble antioxidants (vitamin E and carotenoids) originating from the maternal diet (Surai *et al.*, 1996), as well as water-soluble antioxidants (ascorbic acid, glutathione, and uric acid) and antioxidant enzymes (superoxide dismutase, glutathione peroxidase and catalase) which are synthesised during embryonic development (Surai, 1999a; Surai *et al.*, 1999a). Previously, a dramatic decrease in vitamin E concentrations in the chicken liver for the first 10 days of post-hatch development has been shown for chickens, turkey, duck and goose (Surai *et al.*, 1998a). An increase in dietary vitamin E supplementation slowed down this process but did not change the trend (Surai,

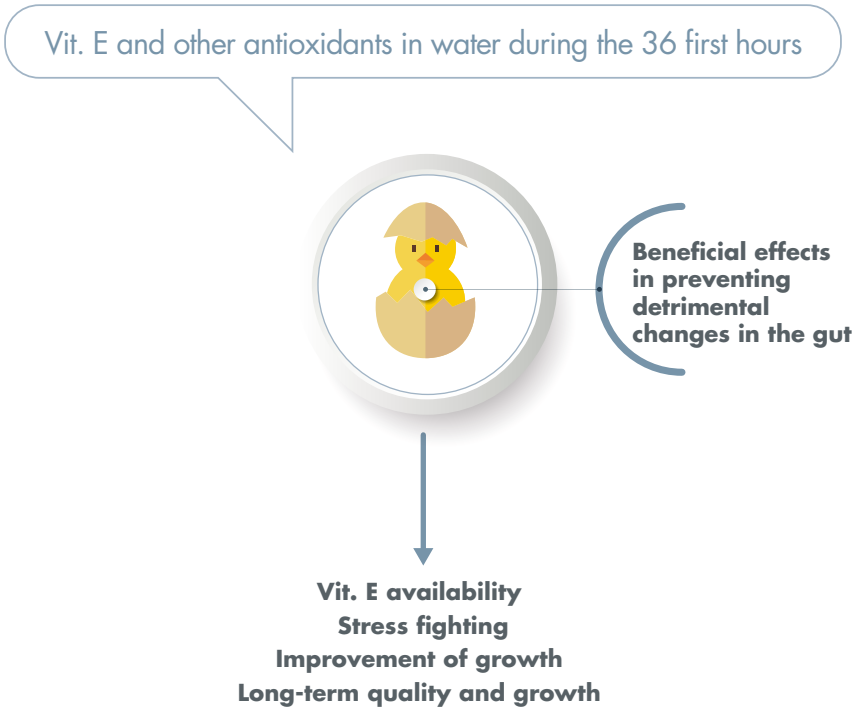

Figure 8.3. Chick placement – importance to regulate physiology.

2002; Surai *et al.*, 1999). Therefore, one of the most impressive features of vitamin E metabolism in avian embryonic tissues is an abrupt decrease in the concentration of this vitamin over the first two weeks of postnatal development. As indicated previously, the liver accumulates vitamin E during embryonic development to supply chickens with this vitamin in the first days of life after hatch (Surai *et al.*, 1996). This reserve of vitamin E is used by chickens during the first 2 weeks post-hatch. During this period vitamin E concentration in the liver decreased by 10 times in chickens, goslings and ducklings (Surai, 2002) and more than 50 times in turkeys (Soto-Salanova *et al.*, 1993). Marusich *et al.* (1975) suggested that the low levels of vitamin E in the liver of turkey poults resulted from the inefficient intestinal absorption of vitamin E, which can be explained as a result of low pancreatic lipase activity (Sell *et al.*, 1991) and restricted bile production (Freeman, 1976) as well as of greater (compared to chicken) production and excretion of tocopheryl glucuronides (Sklan *et al.*, 1982). In general, the capacity for fat and probably fat-soluble vitamin E absorption is incompletely developed in the newly-hatched chick, but it matures rapidly in the first week of life (Freeman, 1976; Noy and Sklan, 1998). On the other hand, during this period of development, tissues with incompletely developed antioxidant regulation require an effective protection against lipid peroxidation, which is afforded mainly by vitamin E. These data also confirmed the biological importance of a very high vitamin E concentration in the embryonic liver at hatching time.

Postnatal nutritional exposures are considered to be critical for the developmental maturation of many organ systems and optimal physiological functions. There is a growing body of evidence indicating that environmental exposure, including nutritional exposure, during these critical and sensitive periods of life can cause permanent changes in many physiological processes, which is known as 'programming' (Amarasekera *et al.*, 2013). In this regard, research data are accumulating to support the hypothesis that the vitamin E status of turkey poults and probably chickens may be inadequate during the first 3 weeks after hatching (Sell, 1996). A variety of approaches aimed at improving the vitamin E status of turkey poults have, in fact, been investigated; these have included dietary supplementation of the poults with high levels of α-tocopherol (Applegate and Sell, 1996; Surai, 2002), bile salts (Soto-Salanova *et al.*, 1993) and fat (Soto-Salanova and Sell, 1995), as well as vitamin E injection (Soto-Salanova and Sell, 1996) and alterations in provision of n-6 and n-3 polyunsaturated fatty acids (Applegate and Sell, 1996). When d-α-tocopherol was added in the drinking water, there was a temporary increase of α-tocopherol in tissues and a decreased susceptibility of red blood cells to haemolysis (Soto-Salanova, 1998). Moreover, day-old chickens were treated with 3.25 mg vitamin E/bird/day *per os*, via the drinking water, for two weeks. The vitamin E content of both the liver and the blood plasma was significantly higher in the treated chickens than in the untreated controls (Mezes, 1994). It seems likely, that provision of vitamin E and other fat-soluble vitamins (A and D3) with water at time of chicken placement can solve the problem of their low availability for newly hatched chicks. Such a supplementation helps chickens overcome stress of placement and has a positive effect on chicken growth and development. The new concept of fighting stresses was based on an idea that supplying birds with various antioxidants via the drinking water could

help them deal with stress conditions more effectively. Indeed, it was proven that inclusion of vitagene-regulating compounds (vitamin E, vitamin A, in combination with carnitine, betaine and other compounds) in water could be effective in fighting various stresses (Fisinin and Surai, 2011a,b, 2012g; Surai and Fisinin, 2012). This helps at chick placement, when the antioxidant system is crucial for the digestive and immune system development (Fisinin and Surai, 2012g). In particular, it was proven that inclusion of an anti-stress composition (PerforMax) at time of chicken placement into the drinking water at the trial improved chicken growth and feed conversion ratio (FCR) (Fotina *et al.*, 2011, 2014). Using the same anti-stress composition in commercial conditions improved FCR during a 39-day broiler growth trial. At the end of the trial, the improvement in FCR due to the anti-stress composition during the first three days post-hatch as well as before and after vaccination was highly significant (Velichko and Surai, 2014; Velichko *et al.*, 2013). Furthermore, using the same anti-stress composition (PerforMax/Magic Antistress Mix) for broilers grown for 35 days, at time of placement, as well as before and after vaccinations, significantly improved FCR (1.5 vs 1.56) and vaccination efficiency (Grigorieva *et al.*, 2017). In addition, it was shown that the anti-stress composition had an immune-modulating effect in broilers (Fotina *et al.*, 2011), growing ducklings (Surai *et al.*, 2012) and could be successfully used to prevent immunosuppression (Fisinin and Surai, 2013a,b). Improvement of the antioxidant system by supplying the antioxidant composition via the drinking water could help deal with various mycotoxins in feed, including DON (Fisinin and Surai, 2012a,b), OTA (Fisinin and Surai, 2012c,d), and T-2 toxin (Fisinin and Surai, 2012e,f). Furthermore, such a technology could also help fight heat stress (Surai and Fotina, 2013a,b).

The importance and efficacy of the anti-stress composition for rearing birds and adult egg type parent stock (Hy-Line) at one of the biggest egg producing farms in Russia (Borovskaya poultry farm, Tumen region) have recently been reviewed (Shatskich *et al.*, 2015). In particular it was shown that usage of the anti-stress composition containing vitagene-activating nutrients (vitamins, minerals, carnitine, betaine, etc.; PerforMax/Magic Antistress Mix) with drinking water at specific periods of increased stress can improve breeder's performance. In particular, there was an increase of 2% in the egg peak production and the peak plateau lasted about 50 days longer than that in the control birds. It is interesting to note that hen-housed egg production in the control group (260.8 eggs) was higher than the target for the line (253.4 eggs) and in the experimental group it was increased by 6 eggs. Furthermore, improved egg production was associated with increased weight of the oviduct in the experimental layers. It is also important to mention that FCR (feed per 10 eggs) also improved by usage of the anti-stress composition and was better than the target for the line. Notably, shell strength at age 26, 36 and 56 weeks was improved in the experimental group by 2.8, 5.6 and 5.6%, respectively. The most interesting finding was related to a significant increase of the carotenoid level in the egg yolk of experimental birds. Since carotenoids were not supplied with the anti-stress composition, this increase could be due to improved absorption of nutrients resulting from anti-stress composition use. This can also explain improved FCR in the experimental birds. The vitamin A level in the egg yolk from the experimental layers was also increased, probably

reflecting its transfer from the anti-stress composition. In particular, use of the anti-stress composition was associated with improved fertility at 16, 40, 48 and 56 weeks by 2.5, 2.7, 2.8 and 3.7%, respectively. In the same experimental group the hatch rate of viable chicks improved at 26, 32, 40, 48 and 56 weeks by 3.6, 2.1, 3.4, 4.9 and 4.3%, respectively (Latipova *et al.*, 2016; Shatskih *et al.*, 2015). Similarly, effects of an anti-stress composition (PerforMax/Magic Antistress Mix) on the rearing birds were studied (Shatskikh *et al.*, 2016). Similarly, the use of the anti-stress composition positively affected testes development of 15-, 26- and 56-week old cockerels. The liver of experimental birds was characterised by a significant increase in vitamin A content at various ages. There was an increase in Ca content of the bones of female birds at 15 weeks indicating better Ca reserves for future egg production. The results of balance experiments indicated that females and males of the experimental group were characterised by improved use of nitrogen, calcium and phosphorus from the diet. Indeed, the use of the anti-stress composition with water during periods of chicken stress positively affected the experimental birds (Shatskikh *et al.*, 2016). Furthermore, the effects of supplying an anti-stress composition (PerforMax/Magic Antistress Mix) at 100 g/100 l drinking water to Hy Line breeders on their progeny with specific emphasis to chick uniformity as an important determinant of rearing birds quality were studied (Shatskikh *et al.*, 2016). In total, there were 1,938 layers and 176 cockerels in control and experimental groups and the experiment lasted from day 106 until day 448. The obtained data indicate that supply of the anti-stress composition to breeders was associated with a significant improvement of the uniformity (at day 28) in progeny chicks obtained from breeders of various ages: 26 weeks (81.3 vs 67.3%), 32 weeks (85.5 vs 76.8%), 40 weeks (83.2 vs 68.8%), 48 weeks (75.5 vs 68%) and 56 weeks (73.7 vs 62%). It is interesting to note that there were no differences in weight of day old chicks between groups independent on breeder's age. Therefore, it seems likely that changes in egg composition by supplying breeders with important nutrients, including methyl donors (betaine, methionine, vitamin B12, etc.), could have epigenetic effects on the progeny chicks (Shatskikh *et al.*, 2016; Surai and Fisinin, 2016d).

Our previous investigations indicate that low quality neonatal nutrition resulted in long-term impairment in the capacity to assimilate dietary antioxidants. In fact, birds that have experienced relatively low-quality diets during their early growth had half the levels of lipophilic antioxidants (vitamins A, E and carotenoids) as adults than birds reared on the standard-quality diet (Blount *et al.*, 2003). This difference appeared despite that the birds had been on the same diet since 15 days of age – in fact, 85% of their lives. These results suggest that the quality of the rearing diet permanently affect the capacity of birds to assimilate lipophilic antioxidants from the diet. Effect of early nutrition in mammals is well recognised. In particular, retrospective studies investigating the effect of famine or season during pregnancy indicate that variation in early environmental exposure *in utero* leads to differences in DNA methylation of offspring (Dominguez-Salas *et al.*, 2012). This potentially may affect gene expression in the offspring via epigenetic mechanisms (Amarasekera *et al.*, 2013). Our recent results demonstrated substantial effects of diet on the development of behavioural traits, and that these effects differ both between the sexes and over different developmental periods (Noguera *et al.*, 2015).

It seems likely that early programming associated with epigenetic mechanisms plays a key role in chicken growth and development, and at time of chicken placement additional supplementation of fat-soluble vitamins, which are poorly assimilated from the diet, with water could be considered as an important solution for poultry industry.

8.7.2 Gut redox balance and microbiota

As was shown earlier in this chapter, under physiological conditions, the gut cell can tolerate a certain level of ROS due to its antioxidant capacity, which is critical for intestinal homeostasis (Tian *et al.*, 2017). However, under stress conditions an excessive ROS production can break an existing antioxidant-prooxidant balance and enhance membrane permeability, alter the inflammatory response, and caused lipid and protein modifications, DNA damage and apoptosis (Tian *et al.*, 2017).

The avian gastrointestinal tract harbours trillions of commensal microorganisms, collectively known as the microbiota (Bhat and Kapila, 2017). For example, the chicken intestinal tract is composed of the duodenum, jejunum, ileum, caecum, and colon, and there are significant differences in microbiota concentration and composition between the aforementioned gut sections (Xiao *et al.*, 2017). Interestingly, caecum is characterised by the most complex microbial community dominated by the phyla *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria* (Sergeant *et al.*, 2014). On the other hand, at the genus level, the major microbial genera across all gut sections were shown to be *Lactobacillus, Enterococcus, Bacteroides*, and *Corynebacterium (*Xiao *et al.*, 2017). Furthermore, *Bacteroides* was shown to be the dominant group in caecum, while *Lactobacillus* was predominant in the small intestine sections (duodenum, jejunum and ileum; Xiao *et al.*, 2017). The microbiota's various bacterial members are involved in a physiological network of cooperation and competition within the gut (Stecher, 2015). From the one hand, the microbiota is shaped by environmental factors. On the other hand gut environment including redox balance is shaped by microbiota. Redox signalling in the chicken gut regulates many physiological functions, including self-renewal, proliferation, migration and differentiation of epithelial cells (Perez *et al.*, 2017). By modulating NADPH oxidases commensal microbiota substantially contribute to this signalling.

In fact, the normal commensal microbiota creates a so called colonisation resistance, causing hostile/disfavourable conditions against the of enteric pathogens (Stecher, 2015). Interestingly, the same nutritional (e.g. antibiotics and other drugs) or environmental (changes in diet, disease challenge) factors can affect antioxidant-prooxidant balance (Surai and Fisinin, 2016e) and disrupt microbiota in the gut (Stecher, 2015). For example, experimental infection with *Cryptosporidium parvum* in immunocompromised Swiss albino mice caused an increase in lipid peroxidation and decrease in GSH, CAT and SOD at the peak of infection in the intestine and liver (Phagat *et al.*, 2017). Important consequences of redox balance and microbiota disturbances are inflammatory host responses and activation of immune cells resulting in further production of ROS in the gut and deepening the problem. Furthermore, under inflammatory conditions, the microbial community could shift from obligate

to facultative anaerobes (Rigottier-Gois, 2013). For example, the gut microbiota was shown to contribute to the control of *Compilobacter jejuni* colonisation and could prevent lesion development (Han *et al*, 2017).

Of note, the intestinal microbiota is shown to play an important role in mucosal immunity. Dysbiosis is associated with the pathogenesis of inflammatory diseases (She *et al.*, 2017). In the complex intestinal ecosystem, a range of microbes can facilitate oxidation/reduction reactions and the maintenance of the redox balance. Indeed, a redox based response within cells is emerging as an important and conserved element of host cell and symbiotic microbe interaction (Jones and Neish, 2017). It has been suggested that in the gut commensal microflora, *Lactobacillus* species in particular are able to stimulate rapid, non-pathogenic levels of ROS production, which will oxidise reactive cysteine residues within proteins controlling cell signalling pathways. Indeed, proteins that harbour reactive cysteine residues, for example Keap1 or IκB, could function as redox sensors and transducers of signalling initiated by increased ROS concentrations (Jones and Neish, 2017). It has been shown that ROS production by intestinal epithelial cells is mediated by receptors and enzymatic processes similar to those employed by phagocytic cells to induce microbial death (Neish, 2013). Indeed, reversible oxidation of cysteine residues is responsible for adaptive responses to fluctuating levels of ROS. In fact, ROS signalling in the gut is suggested to represents an ancient evolutionary form of host cell and microbe cross-talk (Jones *et al.*, 2012; Neish and Jones, 2014). Free radical production in the gut due to commensal microbiota could also oxidise thioredoxin and glutathione, changing redox balance in the cell and inducing the transcription factors such as the Nrf2 and NF-κB. This could lead to modulation of gut inflammation and other cellular processes. It seems likely that the aforementioned microbiota-host interactions represent a universal mechanism used by bacterial communities to affect a variety of signalling and homeostatic processes in the host (Lee, 2008).

Of interest, it was shown that the loss of mucus due to pathogenic bacteria is associated with significantly increased ROS production (Alam *et al.*, 2016) further aggravating disturbances in the redox balance of the gut. Therefore, two important balances in the gut namely antioxidants/prooxidants and commensal/pathogenic microbiota works in synergy. They maintain gut redox balance via transcription factors (e.g. Nrf2 and NF-κB) and vitagene modulation, and are the main protective mechanisms of the healthy gut (Figure 8.4). Interestingly, probiotics may modulate the redox status of the gut via their metal ion chelating ability, antioxidant systems modulation, regulating signalling pathways, and enzymes producing ROS (Wang *et al.*, 2017). However, the authors raised several questions as to efficacy of probiotics in modulation of gut health. For example, the incapability of probiotic bacteria to colonise the gut and their elimination shortly thereafter substantially restrict their long term action. Interestingly, *Bacillus amyloliquefaciens* SC06 was shown to alleviate the oxidative stress of intestinal porcine epithelial cells via modulating Nrf2/Keap1 signalling pathway and decreasing ROS production (Wang *et al.*, 2017a). Clearly there is a need for more detailed elucidation of the microbial population in the poultry gut depending on the diet, feed supplements, including pre- and probiotics and vitagene-

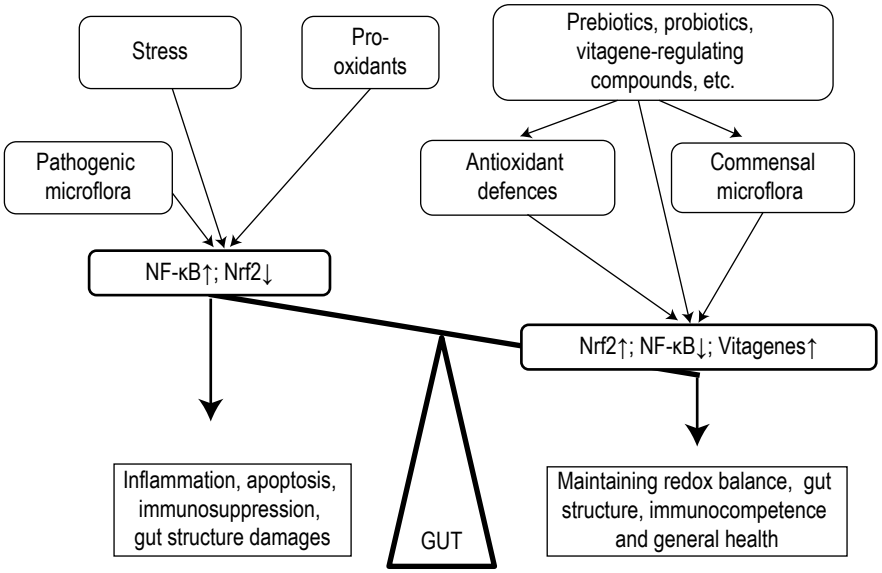

Figure 8.4. Antioxidant-prooxidant balance and microbiota in the gut (adapted from Surai *et al.*, 2017). NF-κB = nuclear factor kappa beta.

modulating nutrients. The characterisation of the stress factors leading to intestinal dysbiosis and the identification of the microbial taxa contributing to pathological effects present a new direction of future research to better understand the impact of the microbiota on health and disease (Weiss and Hennet, 2017).

8.8 Conclusions

The data presented above clearly indicated that some problems with antioxidant defences of the gut of the newly-hatched chickens could be observed (Surai, 2002, 2006; Surai and Fisinin, 2014). In this regard, usage of antioxidant blends is shown to have beneficial effects in decreasing such detrimental changes in the gut. However, it is necessary to take into account that antioxidant systems of the gut is quite complex and regulated at the levels of vitagenes operating in close relationship with a range of transcription factors, including Nrf2 and NF-κB. Therefore, the strategy of improving antioxidant defences of the gut of the newly-hatched chicks should include several points:

- regulation of mitochondria function to decrease free radical production (carnitine, betaine, vitamin E, etc.);
- activation of vita-gene network, responsible for synthesis of a range protective compounds, including HSPs, elements of the thioredoxin system, sirtuins, etc. (carnitine, betaine, vitamins A and E, etc.);

- activation of Nrf2, responsible for synthesis of antioxidant and detoxification enzymes;
- suppression of NF-κB, responsible for synthesis of pro-inflammatory cytokines;
- provision of vitamin E and elements of its biological recycling in the cell (vitamin C, selenium, vitamin B2, etc.), since vitamin E is major membrane antioxidant, which cannot be replaced by other antioxidants;
- provision of minerals necessary for additional synthesis of SOD and GSH-Px (Zn, Mn, Se).

Indeed, a designed and effective usage of the complex antioxidant composition to overcome oxidative stress related to chick placement is an important task for nutritionists. Furthermore, new frontiers in understanding the effect of microbiota on antioxidant-prooxidant balance in the gut, as well as interactions between gut redox status and microbiota await further investigation.

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Chapter 9
Looking ahead

All is well that ends well

Selenium is considered to be one of the most controversial trace elements. From the one hand, it is toxic at high doses and there is a great body of information related to environmental issues of Se contamination. On the other hand, Se deficiency is a global problem and Se supplementation with premix has become a common procedure for commercial feed producers. The importance of Se is related to the quickly developed understanding of the role of free radicals in biology and medicine, including redox balance maintenance and signalling. Indeed, detrimental effects of free radicals are well established and their involvement in the development of various diseases is proven. It is also generally accepted that mitochondria are the major source of free radicals in biological systems. Therefore even in physiological conditions free radicals are produced and they can damage all types of biological molecules, including DNA, lipids and proteins. In fact, lipid peroxidation is shown to be an important detrimental process in biological systems. Indeed, various calculations of electrons escaping from the electron-transport chain in mitochondria gave an estimation of about 20 billion free radicals in every cell every day to be produced in physiological conditions. Therefore, an old perception that free radicals are produced in stress conditions was corrected to that stress conditions increase free radical production, which is already substantial in physiological conditions. In recent years it has been established that damage to DNA and proteins are also of great importance from the point of view of understanding the pathology of many different changes in the body caused by free radicals. During evolution an integrated antioxidant system was developed in the body and in particular in every cell to deal with those free radicals. In fact, there are three major levels of antioxidant defence and Se participates in all of them.

The first level is based on the activity of antioxidant enzymes with SOD to be the main device to detoxify superoxide radicals, the most important radicals produced in biological systems. Se-dependent GSH-Px, catalase and metal-binding proteins are also integral part of the first level of antioxidant defence. It is important to underline, that GSH-Px also belongs to the second level of antioxidant defence, since it is responsible for the detoxification of lipid hydroperoxides formed as a result of the reaction of lipid peroxides with antioxidants, such as vitamin E. Indeed, an optimal interaction between vitamin E and GSH-Px provides an important mechanism of antioxidant defence. Recently, it has been shown that SeMet could affect activity of DNA repairing enzymes, while selenoproteins MsrB and TrxR are responsible for repairing oxidised proteins. This means that Se also belongs to the third level of antioxidant defence. The understanding that all antioxidants in the body are working as a team, called the antioxidant system, creates an additional interest in antioxidant interactions. Indeed, vitamin E recycling is shown to be the most important mechanism of the antioxidant

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defences. This means that if recycling is effective, even low levels of vitamin E could provide a substantial antioxidant protection. For example, in the brain, the vitamin E level is usually comparatively low and levels of polyunsaturated fatty acids are high, but in physiological conditions it is very difficult to detect any products of peroxidation there. On the other hand, if the recycling process is broken, even high vitamin E supplementation would not provide the adequate antioxidant protection. There are various additional mechanisms of antioxidant protection, including redox signalling and changes in vitagene expression, stress-protein synthesis as well as apoptosis.

Recent understanding of essentiality of free radicals in various physiological processes shifted research to the antioxidant-prooxidant balance rather than studying simply the lipid peroxidation. For example, free radicals are synthesised in phagocyte cells and used as a weapon to kill pathogens. This process is under strict metabolic control, since an excess of the free radical production could escape from the phagosome and damage various biological molecules including damages to phagocytes themselves. It seems likely that some toxicants, such as mycotoxins could affect this regulation causing an excessive free radical production and creating oxidative stress. In general, ingestion of excessive amounts of antioxidants is presumed to shift the oxidant-antioxidant balance toward the antioxidant side. This is supposed to be beneficial; however, this may also adversely affect key physiological processes that are dependent on free radicals, including prostaglandin production, cell division and differentiation. This is not the case for Se, since its activity depends on selenoprotein synthesis, a metabolically expensive process and therefore selenoproteins are synthesised as needed in response to stress-conditions and the synthesis requires an adequate Se supply. Free radicals are important signalling molecules in spermatozoa capacitation and they also are involved in neurone communications, blood vessel tone maintenance and in some others important physiological processes. Furthermore, recent evidence suggests that cellular oxidation can induce changes in gene expression during normal development. Conversely, antioxidants, such as ascorbate, glutathione, α-tocopherol or carotenoids are inhibitory to differentiation in many types of cells.

New data provides better understanding of the important role of the antioxidant-prooxidant balance in gene expression, and it seems likely that antioxidants can affect gene expression by mechanisms independent of their antioxidant activity. For example, it has been demonstrated that GSH, in addition to its antioxidant and protective function against oxidative stress, has a specific signalling role in redox regulation. Indeed, it has been shown that 2,016 genes are regulated by H_2O_2 (Fratelli *et al.*, 2005). Of these, 215 genes showed GSH-dependent expression changes, classifiable into four clusters displaying down- or up-regulation by H_2O_2, either potentiated or inhibited by GSH depletion. In particular, the biological process categories overrepresented in the largest cluster (genes whose up-regulation were inhibited by GSH depletion) were NF-kappaB activation, transcription, and DNA methylation. Furthermore, this cluster also included several cytokine and chemokine ligands and receptors, the redox regulator thioredoxin interacting protein, and the histone deacetylase sirtuin. The cluster of genes whose upregulation was potentiated by GSH depletion included two heat shock proteins (HSP40 and HSP70) and the AP-1 transcription factor

components Fos and FosB (Fratelli *et al.*, 2005). Indeed, interactions of selenoproteins and various transcription factors (Nrf2, NF-κB, AP-1, etc.) and their participation in redox balance maintenance and signalling are of great importance for future research.

In 1973 GSH-Px was described as a selenoprotein and antioxidant role of Se was established. Since then a family of selenoproteins in animals/poultry was shown to include 25 different selenoproteins. In fact, many of new selenoproteins have been described only recently and therefore the knowledge of their functions and regulation is far from being complete. Traditionally selenoproteins have been considered to function as antioxidants. This is particularly true for GSH-Px, but also for TrxR and MSR. In fact, there are four different forms of GSH-Px in chicken and they are located in different parts of the cell, in different tissues and in some cases are differently regulated. It seems likely that this is an adaptive mechanism to deal more effectively with free radical production. For example, PH-GSH-Px is specifically located in membranes and is able to deal with lipid peroxides directly, without the necessity to release them from membranes by phospholipases. GI-GSH-Px is considered to be the most important defensive mechanism against lipid hydroperoxide absorption. In fact, GI-GSH-Px destroys lipid peroxides in the GI tract preventing them from absorption. The role of GI-GSH-Px in the maintenance of the antioxidant-prooxidant balance in the GI tract needs further investigation. For the question if GSH-Px is the main selenoprotein in chickens, my answer would be, probably not. A great body of information related to the role and functions of GSH-Px accumulated for the last 40 years put this enzyme at the top of the selenoprotein list. However, TrxR, described as a selenoprotein 23 years later than GSH-Px and methionine sulfoxide reductase B (MsrB), described as a selenoprotein only in 2003 are going to change that view on the importance of different selenoproteins. TrxR is involved in regulation of processes, such as DNA synthesis, cell proliferation and apoptosis, as well as in vitamin E recycling. At the same time, understanding functions of MsrB is going to change our perception to lipid peroxidation. Indeed, free radicals induce oxidation of not only polyunsaturated fatty acids (PUFAs), but also oxidise proteins and DNA. It is generally accepted now that MsrB is an enzyme responsible for the prevention of protein oxidation. Indeed, methionine molecules located in active centre of various enzymes are considered to be 'bodyguards' for active cysteine molecules. When free radicals attack proteins, methionine would be oxidised first, thus protecting cysteine from oxidation thereby maintaining enzymatic activity. Msr is responsible for reducing oxidised methionine back to an active form. Therefore, the more we understand the function of Msr, the more we realise the importance of protein oxidation and its prevention in biological systems. Taking into account the possible roles of all 25 known selenoproteins from which about 18 members are involved in antioxidant defences, maintaining redox balance of the cell and cell signalling, it was suggested that Se is 'the chief executive of the antioxidant system'. This means that Se is responsible for regulation of the major antioxidant protections via direct antioxidant activities of GSH-Px, TrxR, Msr, or the other less studied selenoproteins (Sel H, K, M, N, O, P, S, T, W and Sel15) as well as indirectly via vitamin E recycling and other important interactions. Therefore, Se deficiency could compromise many important physiological functions as a result of the detrimental changes in the antioxidant defence system.

The plant absorbs Se from the soil in the form of selenite or selenate and synthesises selenoamino acids with SeMet representing more than 50% of the Se in cereal grains and other feed ingredients. Organic Se, which can be found in grains, forages and other feed ingredients, is primarily in the form of SeMet and is metabolised in the same way as methionine. It is actively transported through intestinal membranes during absorption and actively accumulated in tissues such as the liver and muscle. It is well known that methionine is not synthesised by animals/poultry and therefore it is an essential amino acid. The same is true for SeMet, which is not synthesised in animals/poultry and must be derived from feed sources. In fact, SeMet is considered to be a storage form of Se in the body. Indeed, when organic Se is used in the diet, the Se reserve is built in muscles in the form of SeMet. These reserves can be used in stress conditions when the Se requirement increases but feed consumption decreased. The skeletal muscles are the major Se-storage organ, accounting for about 46.9% of the total Se in the human body, while the kidney contains only 4% of the Se reserves. In humans, whole body Se depends on the regional location and varies from 3-6 mg up to 13-20 mg. In stress conditions protein catabolism by proteasomes can release SeMet, which could serve as a source of Se for newly synthesised selenoproteins, such as GSH-Px, thioredoxin reductase and methionine sulphoxide reductase. Those enzymes can deal with overproduction of free radicals and prevent a decrease in productive and reproductive performance of farm animals/poultry. It was proven that Se from both selenite and SeMet is readily available for synthesis of the selenoenzyme GSH-Px in animal tissues. There are also several lines of evidence confirming the idea that Se accumulated in tissues in the form of SeMet can be available for selenoprotein synthesis.

It should be noted that only a small proportion of the methionine pool can be replaced by SeMet, since only a very small part of methionine could be replaced by SeMet in the diet. Furthermore, the protein turnover prevents accumulation of SeMet to toxic levels in the organism. There is a great discrepancy in results of comparative evaluation of Se bioavailability from various sources. The problem is that it is difficult to choose a proper endpoint for such an evaluation. A range of techniques has been used for this purpose, including prevention of specific Se-responsive diseases in animals, measurement of the uptake of Se in different tissues after ingestion of a test compound, activity of GSH-Px in plasma, red blood cells, whole blood, liver and other tissues and selenium excretion with urine. However, none of those techniques can give an ultimate answer as for Se bioavailability or Se status of animals/poultry. It seems likely, that a combination of various techniques needs to be used for such an evaluation. In general, absorption of various forms of Se is quite high. However, the main difference between organic and inorganic forms of Se is in its accumulation in tissues and transfer to the egg or meat. Since SeMet is the main form of Se in muscles and eggs, and animals cannot synthesise it, only organic selenium can substantially increase Se concentrations in those substrates. This can give advantages to the developing animals/poultry in terms of improvement of their Se status, as well an important tool for the production of Se-egg and Se-meat. It is well appreciated that via selenoprotein expression Se is involved in regulation of cell growth, apoptosis and modifying the action of cell signalling systems and transcription factors and therefore

its adequate dietary supply is a crucial factor for many physiological processes in the animal/poultry body.

In general, there are three generations of Se supplements on the market. The first generation of Se supplements for poultry included selenite and selenate. These supplements have been used in poultry diets for the last 40-45 years and helped to get rid of global Se deficiency in poultry production. However, they are not suitable to meet the exact Se requirement of commercial poultry at stressful conditions of egg and meat production. This resulted in a second generation of organic Se for poultry, including Se-yeast, SeMet and Zn-SeMet products. Their advantages and disadvantages are mentioned above. During the last few years an effective technology of producing organic selenium in the form of Se-yeast has been developed and commercialised. Indeed, yeast as other plants can take selenite from the medium and synthesise SeMet and other organic Se-compounds. Since Se and S are chemically very similar, yeast cannot distinguish between them and in a medium deficient in S, but supplemented with Se they will synthesise SeMet instead of Met. As a result, such yeasts are enriched with organic selenium to such an extent (1000-3,000 mg/kg) that it can be used as a selenium source for human and animal consumption. Indeed, the animal/poultry industry is looking for most effective sources of organic Se to be commercially used. Se-yeast received substantial attention and commercial applications with it have been developed as reliable sources of organic Se. However, the active Se component of the yeast, SeMet, comprises only 50-70% of the total Se and there is no proof that the rest (30-50%) of the Se in the form of SeCys, MeSeCys, etc. has any additional benefits for poultry in comparison to sodium selenite. From the production point of view it is difficult to guarantee a certain percentage of SeMet in the Se-yeast, since there is a range of factors (yeast strain, medium composition and selenite concentration in it, temperature, etc.) affecting the aforementioned parameter. Analytical difficulties in the precise determination of the SeMet amount in Se-yeast further complicate the issue. Stability issues with pure SeMet and ZnSeMet in the poultry diets restrict their wide usage. It seems likely that a recently developed product (hydroxyl-SeMet) combines the advantages of both – Se-yeast (SeMet stability) and pure SeMet (high proportion of SeMet) – and can be considered as a next step in Se application in poultry nutrition. Indeed, OH-SeMet can be considered as the next, third generation of Se supplements for poultry on the market.

Selenium has important applications in poultry nutrition. In particular, commercial poultry production uses modern chickens characterised by high egg production and high growth rate. However, the price for such improvements is related to high sensitivity of birds to various stresses. Therefore, in modern poultry production there is an important move from preventing Se deficiency to meeting Se requirement. The data presented in Chapter 5 indicate that organic selenium is a choice for diets designed to maintain high productive and reproductive performance of poultry. In particular, replacement of sodium selenite by organic selenium in the breeder diet is related to improvement of fertility, hatchability and viability of chicks in early postnatal development. Indeed, organic selenium (SeMet) is more effectively transferred from the diet to the egg and further to the developing embryo. This improves antioxidant

defences and helps chickens to overcome the oxidative stress of hatching leading to an improvement of hatchability. It is well known that when a chick is hatched, many physiological systems, including immune system, are not mature and continue to develop during at least 2 weeks post-hatch. Therefore, this is the most vulnerable period of ontogenesis of the chicks.

Our data indicate that Se transferred from the egg to the embryo as a result of organic Se supplementation of the maternal diet had positive effect on Se status of the developing chicks up to 4 weeks post-hatch. This data showed the importance of the maternal diet not only for newly-hatched chicks, but also for the developing chicken in early postnatal life. In a recent experiment conducted in China with yellow broiler breeders, it was shown that the Se concentrations in serum and tissues (liver, kidney, and breast muscle) of 56 days old offspring were significantly increased by maternal SeMet intake compared to maternal sodium selenite (SS) intake. Furthermore, the antioxidant status of the 56 days old offspring was significantly improved by maternal SeMet supplementation in contrast to maternal SS supplementation. It was evidenced by increased GSH-Px activity in serum and breast muscle, GSH concentration in serum, and total antioxidant capability in pancreas, as well as cytosolic GSH-Px mRNA abundance in breast muscle, liver, and pancreas. It is important to mention that the maternal SeMet treatment significantly reduced the 48 hour drip loss of 56 days old offspring in comparison to maternal SS treatment (Zhang *et al.*, 2014). It seems likely that selenium in a breeder diet could have epigenetic effects on the progeny chick (Fisinin *et al.,* 2016).

Advantages of organic selenium for commercial laying hens are related to better shell quality and improvement of egg production. Our data on Se content in the shell and possibility its manipulation by inclusion of organic Se in the laying hen diet are a background for further research. Indeed, it is well recognised that eggshell consists of about 95-97% of mineral and 3-5% of organic matrix. Recent evidence indicates that the organic matrix is responsible for regulation of crystal formation in the developing shell. This means that 3-5% of the organic matrix determines shell quality. Since organic Se is an integral part of the organic matrix it was suggested that organic Se could affect shell quality and information is accumulating to substantiate this claim. The second advantage of organic selenium for laying hens is related to egg production maintenance at the peak of production. The problem is that even low stress in commercial egg production can affect the peak of egg production, and once egg production is decreased it is almost impossible to return to previous high level. Since Se provides additional antioxidant protection, this could help to overcome these commercially-relevant stresses and maintain high egg production at the peak. An additional benefit of organic Se for commercial layers is related to egg freshness during storage. Indeed organic selenium transferred from the diet to the egg stimulates GSH-Px in the egg yolk, white and probably perivitelline membrane leading to decreased lipid and protein oxidation and helping to maintain high Hough units during egg storage. It is not clear at present if MsrB, a major enzyme protecting from protein oxidation, can be found in eggs and this topic awaits further investigation.

The advantages of organic Se for broilers include improvement of growth rate, feed conversion ratio (FCR), decreased mortality and decreased drip loss during meat storage. This could be related to antioxidant Se action, activation of thyroid hormones, as well as improvement of immunity. Indeed, it is very expensive to maintain activated immune system. Many nutrients are distributed from growth and development to the immune system. Therefore, immunomodulating properties of Se could help to use nutrients properly and not losing them for unnecessary stimulated immune system.

Chicken Se requirement in physiological conditions is quite low and in many cases can be met by the Se found in the main feed ingredients (in Europe it is about 0.1 mg/kg and in the USA it is about 0.2-0.3 mg/kg). However, commercial poultry production is associated with a range of stresses which substantially increase the Se requirement. It is impossible to predict the strength and frequency of stresses and therefore dietary Se supplementation is considered to be an 'insurance policy', ensuring the best Se status for maximum expression of the main selenoproteins and antioxidant protection. Therefore, the commercial level of Se supplementation is about 0.3 mg/kg, and when supplemented in organic form the producer can get highest benefit of it, a 'comprehensive insurance policy'. Unfortunately, recently the European Union implemented a directive restricting organic Se supplementation of poultry at 0.2 mg/kg. In this scenario, producers are looking for Se supplements with the highest efficacy of transfer to the egg and to muscles.

The analysis of the current literature indicates that an enrichment of eggs and meat with Se is a valuable option to improve Se status of the general population. Such eggs are currently being produced in many countries worldwide delivering approximately 50% recommended daily allowance (RDA) in Se with a single egg. There are also various other egg brands enriched with a combination of various nutrients, including omega-3 PUFAs, vitamin E, carotenoids, iodine, etc. Commercial technologies for the production of Se-meat and Se-milk are under the development in various countries. As it was mentioned above it is proven that the major form of Se in Se-enriched egg (albumin) and meat is SeMet and therefore incorporation of organic Se into the diet provides an effective accumulation of SeMet in eggs and meat. It has been suggested that for the past 150 years our diet has changed substantially, while our genes have not changed. In particular, animal-derived food composition has dramatically changed as a result of using cheap feed ingredients. The meat from animals in the wild and chicken eggs produced under complete natural conditions contains higher amounts of omega-3 fatty acids compared to commercial ones. Indeed, analyses of egg composition from various wild avian species conducted at the Scottish Agricultural College indicate a great difference in fatty acid, vitamin E and carotenoid profile of eggs between commercial laying hens and wild birds. In fact, designer eggs enriched with omega-3 fatty acids are very similar in fatty acid profile to eggs produced in wild or so-called Greek eggs, produced by free range birds having unlimited access to various greens, worms, insects, etc. The same is true for selenium and carotenoids. Indeed, decreased Se levels in feeds and foods in many cases reflect the consequences of our agricultural practises. Therefore, eggs or meat produced by free-range poultry/animals fed on natural feed sources grown on well-balanced soils 100-200 years ago

would contain a much higher Se concentration than we currently have in many European and Asian countries. Again, by supplementing animal diet with natural organic sources of Se we are returning eggs back to nature. Our recent data on the Se profile of eggs from various avian species in wild confirmed this idea: Se concentration in eggs collected in wild nature is in many cases much higher than that in commercial poultry production. Our results may imply that the Se requirement for birds breeding in captivity will vary among species.

An appropriate guideline could be provided by considering the yolk Se concentration displayed by the free-living counterparts of that species. The Se level in chicken eggs after organic Se supplementation only raised the yolk Se level into the lower end of the range achieved by avian species in the wild, suggesting there may be scope for much higher levels of supplementation for poultry. It seems likely that Se level which is considered to be the norm for the commercial eggs is too low to be physiological in commercial stressful conditions and this should be studied more in detail in the future. Similar evidence of high Se concentrations in wild water birds were related to eggs of little egrets, black-crowned night herons and bridled terns from coastal areas of Hong Kong (Lam *et al.*, 2005). In tissues of the seabirds from the Barents Sea (Savinov *et al.*, 2003), from Alaska and arctic Russia (Stout *et al.*, 2002) as well as in bald eagles from Adak Island (Alaska; Stout and Trust, 2002) selenium levels were also several-fold higher in comparison do domestic chickens. Furthermore, high Se concentrations were reported in eggs from tree swallow, bank swallow and house wren (Dickerson *et al.*, 2002). Therefore, Se-enrichment of eggs, meat and milk is nothing else but production of naturally-designed food ingredients. Indeed, production and commercialisation of organic Se sources opened a new era in Se supplementation of animals and gave a real chance for producers to meet growing requirements of consumers. What is more, production of these kind of animal-derived foodstuffs is a natural way to health promotion. Indeed, it is possible to provide consumers with a range of animal-derived products with nutritionally modified composition in such a way that they can deliver a substantial amount of health-promoting nutrients, such as selenium to improve the general diet and help to maintain good health. Therefore, without changing habits and traditions of various populations ('habit is a second nature') it is possible to solve problems related to the deficiency of various nutrients, in particular selenium. The consumer will go to the same supermarket to buy the same animal-derived products (egg, milk and meat), cook and consume them as usual. The only difference will be in the amount of specific nutrients delivered with such products.

The most important application of the dietary Se supplementation is related to its immunomodulating properties. In fact, the immune system is the most complex system in the body and until now it is still not known how this system is regulated at the molecular level. It seems likely, that an effective communication between different types of the immune cells (e.g. neutrophils, macrophages, B- and T-lymphocytes) is a key element in immunocompetence. It has been suggested that receptors on the surfaces of these cells are working like mobile phones receiving and sending various signals using so called signalling molecules (e.g. cytokines, eicosanoids, etc.). These

receptors are extremely sensitive to low concentrations of communicating molecules, but they are also sensitive to free radicals, which could damage those receptors. In fact, under stress conditions free radical production can exceed the ability of the antioxidant system to detoxify them leading to oxidative stress. Those radicals can damage communication between immune cells and these many millions of immune cells will be useless without effective communication and immunocompetence would be substantially decreased. In fact, phagocytic cells produce free radicals and use them as a weapon to kill pathogens. This process is very strictly regulated. It could be comparable to a nuclear power station where the control chain reaction produces energy. However, if this control is broken (e.g. Chernobyl power station) the power station could become an atomic bomb. A similar scenario could happen when the regulation in phagocytic cells is broken. An example of such a process would likely be the action of mycotoxins on immune cells.

The free radicals, produced by phagocytes could be considered as a 'chemical weapon' used to kill pathogens. However, the problem is that the chemical weapon is not specific and could kill anything and anywhere. Protection of the immune cells in general and the receptors in particular from their own weapon by antioxidants is of great importance for the immunocompetence. Therefore, selenoproteins expressed in immune cells together with other antioxidants could be considered as a protective mechanism maintaining the integrity of receptors and preventing declining immunocompetence in stress conditions. It has been shown that selenium affects all components of the immune system, including the development and expression of non-specific (innate), humoral, and cell-mediated (adaptive) responses. In general, a deficiency in Se appears to result in immunosuppression, whereas supplementation with low doses of Se appears to result in augmentation and/or restoration of immunologic functions. In fact, a deficiency of Se has been shown to inhibit resistance to microbial and viral infections, neutrophil function, antibody production, proliferation of T and B lymphocytes in response to mitogens, and cytodestruction by T lymphocytes and NK cells. On the other hand, Se supplementation has been shown to stimulate the function of neutrophils, production of antibodies, proliferation of T and B lymphocytes in response to mitogens, production of lymphokines, NK cell-mediated cytodestruction, delayed-type hypersensitivity reactions and allograft rejection, and the ability of a host to reject transplanted malignant tumours.

When considering immuno-modulating properties of selenium, it is necessary to take into account several important points. Individual antioxidants in the body interact with each other (vitamin E, C, carotenoids, Se) and with prooxidants (iron, high level of PUFAs, mycotoxins) which themselves have immunostimulating or immunosuppressive effects. Therefore, in every experiment the results reflect a sum of all these interactions and if background dietary concentrations of those nutrients differ, the results could be completely different. This could explain the inconsistency of some results published during the last 20 years. Furthermore, antioxidants can suppress respiratory burst, however, it is not clear at present if there is a limit of this suppression after which the phagocyte antimicrobial activity would be compromised. Immunomodulating effects of antioxidants are usually shown to be maximal when

supplementation is above the requirement for maximal growth and maintenance of reproduction. It could well be that the Se and vitamin E doses that are adequate for maximal productivity in healthy, unchallenged birds are not optimal for immunocompetence and disease resistance. There is no data available on the effect of antioxidants on intestinal immune structures, which could be crucial barriers to external pathogens. Since free radicals can be damaging to intestinal structures, antioxidant functions of selenium are responsible for the prevention of damages to the intestinal lymphoid structure as well as to intestinal enterocyte membranes. This could be especially important in relation to digestive immunosuppression caused by toxins/mycotoxins, nutritional deficiencies and infectious agents. Selenium source (organic vs inorganic) seems to be an important element of its immunomodulating properties. Organic selenium appears to be an advantage because it is better assimilated from feed and better accumulated in tissues. Indeed, with the same dose of supplementation, organic selenium can deliver more of the element to the target tissues and because of toxicity of high selenium levels this could be a solution to avoid adverse effects of selenium overdose. If the body has selenium reserves in the form of SeMet in muscles, it would be liberated during the acute phase response as a result of skeletal muscle protein catabolism by proteosome action, and used for re-synthesis of new selenoproteins which could decrease oxidative stress. In chickens, the first two weeks post-hatch represent the most important period of immune system development and the maternal diet is shown to have a profound effect on this process. In particular, the first week of chick life is a period of rapid expansion of the leukocyte population, seeding of lymphoid organs and other events ultimately leading to the production of unique lymphocyte clones that will mediate immunity in postnatal development. In this respect, the effects of various combinations of natural antioxidants and n-3 PUFA await investigation. As a result of antioxidant (selenium) deficiency increased oxidative stress of a host can lead to increased virus mutation rate, and changes in a viral pathogen can result in emerging viral pathogens with new pathogenic properties. Se deficiency has been associated with a change to the viral genotype, converting the virus from a benign to a virulent strain. This possibility has not been exploited in animal/poultry production, but seems important to study more extensively. There is a need to study antioxidant composition and fatty acid profile of immunocompetent tissues depending on animal age and nutritional supplementation of antioxidants and n-3 PUFAs.

Chapter 8 of the book is devoted to antioxidant-prooxidant balance in the digestive tract which is considered to be a major determinant of animal/poultry health. Analysis of the literature related to chicken diet showed that on the one hand, there is a range of harmful compounds in the diet, including oxidised PUFAs, traces of mycotoxins, heavy metals and other environmental pollutants. A combination of such compounds could potentially stimulate lipid peroxidation and cause DNA and protein damages in the intestine leading to decrease absorption efficacy of various nutrients and deteriorating FCR. On the other hand, the diet contains a range of antioxidant compounds, including vitamin E, carotenoids, vitamin A, ascorbic acid, coenzyme Q, flavonoids, etc., which potentially can deal with those free radicals produced in the digestive tract as a result of aforementioned toxic compounds action. Therefore

a balance between antioxidants and prooxidants in the digestive tract is a key for general health. Furthermore, some nutrients which are not absorbed could have health-promoting properties. For example, it is well known that various flavonoids are not well absorbed in the small intestine. As a result, many of them will be available in the large intestine providing there an important antioxidant protection, preventing lipid peroxidation, damages to DNA and cancer.

There is a specific place for selenocompounds in the digestive tract. On the one hand sodium selenite is a reactive compound which in combination with reduced glutathione (which can be found in the digestive tract) could potentially produce free radicals contributing to lipid peroxidation. On the other hand, organic selenium in the form of SeMet possesses antioxidant properties per se and could have a completely different protective effect. Furthermore, a beneficial effect of SeMet on DNA repairing enzymes could be relevant to the anticancer effect of Se in the intestine. Furthermore, a specific gastro-intestinal GSH-Px is located in the intestine and responsible for decomposition of lipid peroxides. In fact, if the activity of this enzyme is optimal, the lipid peroxides found in the digestive tract will not be able to be absorbed and as a result will not be found in the blood. However, if the activity of this enzyme is low, lipid peroxides can escape pre-absorptive detoxification leading to their incorporation into plasma lipoproteins. Clearly, healthy gut is one of the most important determinants of the general health of human and animals. Until now, major attention to the gut was related to the bacterial population and various pre- and probiotics were used to improve the number of beneficial microbes in the gut. However, the antioxidant-prooxidant balance in the digestive tract should be studied more in detail. In fact, antioxidant potential of the chicken intestine has been characterised already (McLean *et al.*, 2005).

Selenium is shown in high doses to be toxic. Historically, Se toxicity was discovered earlier than its essentiality. That is why Se toxicity for many years was firstly considered in different textbooks. There are also various environmental issues related to accumulation of Se in various water reservoirs up to toxic levels. This led authorities in many countries to restrict Se supplementation in animal diets. For example, in the EU countries legal Se level in the complete feed should not exceed 0.5 mg/kg. In the US legal limit of Se supplementation is set at 0.3 mg/kg of supplemental Se. In recent years a large amount of information has been accumulated to show, firstly, that Se toxicity starts at doses, which are at least 10-fold higher than those commercially relevant. Secondly, in most of cases organic selenium is less toxic than sodium selenite, independently of the Se concentration in tissues. This means that Se concentration in tissues is not necessarily related to Se toxicity. The form of Se is a more important factor and promotion of lipid peroxidation by high doses of sodium selenite is considered to be an important mechanism of Se toxicity. When organic Se is included into the diet more Se is retained in the body and less released with manure. This means that legal limits of dietary Se supplementation, developed based on sodium selenite data, should be reconsidered and increased. This would give more flexibility for feed/premix producing companies, as well as for poultry, pig and dairy producers who wish, for example, to produce Se-enriched eggs, meat and milk.

The analysis of the literature presented in this volume reinforces the importance of Se in poultry nutrition and health. Indeed, optimisation of Se nutrition of poultry and farm animals will allow to increase egg and meat production efficiency, and what is more important, increase their quality. From the data presented above it is clear that organic selenium is an effective choice to achieve this goal.

Really prevention is better than cure.

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Index

D

E

F

G

H

I

R

S

T

U

V

X

Z