Thesis for the degree

Master of Science in Aquamedicine

Establishing biomarkers of dietary selenium toxicity in Atlantic salmon (*Salmo salar* L.)

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Department of Biology
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National Institute of Nutrition and Seafood Research
(NIFES)

May 2016

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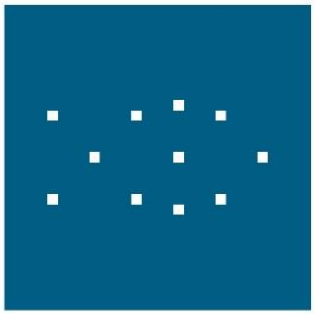

N I F E S

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ACKNOWLEDGEMENTS

The work presented in this thesis was performed at the National Institute of Nutrition and Seafood Research (NIFES, Bergen, Norway) and at EWOS Innovation (Dirdal, Norway). This thesis is a part of the research project “Evaluation of the maximum limits for selenium in Atlantic salmon feeds”, project no. 10086000, funded by The Norwegian Seafood Research Fund (FHF).

First, I would like to state my gratitude to my main supervisors Robin Ørnsrud and Heidi Amlund. I am sincerely grateful for your guidance and encouragement throughout this year. Thank you for always having time for my questions, for your good answers and for always meeting me with a smile and good spirits. To Anne-Katrine Lundebye, my co-supervisor, thank you so much for reviewing my thesis. I do really appreciate you all.

I would also like to thank all the great people who provided me with technical assistance in the labs. Jaap, thank you for guiding me through methods and for good times in the lab. To the people at the “prøvemottak”, thank you for freeze-drying my samples. A special thanks to Vidar, for giving of your time when trying to figure out how to homogenize those tough parts of the fish, it was a pleasure working with you. Thanks to the staff at the elemental lab for introducing me to their lab, and Helen, thank you for great laboratory guidance and good company. A big thank you also goes to Ståle, for helping with the Confocal Microscope. You all made my stay at NIFES a pleasure.

Furthermore, I would like to thank the staff at EWOS Innovation for the husbandry of the fish during the feeding trial, and Heidi, Robin, Pål and Siri for a good time during the sampling.

Finally, to my friends and family, thank you for always being encouraging and supportive. And to my second family, my fellow students at UiB, thanks for five amazing years! Five years ago, I had never imagined that the people at the faculty of mathematics and science really are the best of people.

Bergen, May 2016

Tone Kristin Sundal

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ABBREVIATIONS

Abbreviation	Full name
2-AG	2-arachidonoylglycerol
ARA	Arachidonic acid
CB_1	Cannabinoid receptor 1
CB_2	Cannabinoid receptor 2
CoA	Coenzyme A
DHA	Docosahexaenoic acid
DMSe	Dimethylselenide
DNA	Deoxyribonucleic acid
EPA	Eicosapentaenoic acid
EU	European Union
FCR	Feed conversion ratio
GCL	Glutamate-cysteine ligase
GP1	Growth period one (day 0-42 of feeding)
GP2	Growth period two (day 43-82 of feeding)
GPx	Glutathione peroxidase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
LA	Linoleic acid
LNA	Linolenic acid
MMSe	Methylselenol
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
PUFA	Poly-unsaturated fatty acids
Redox	Reduction-oxidation reaction
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rpm	Revolutions per minute
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
Se	Selenium
SeCys	Selenocysteine
SelP	Selenoprotein P
SeMet	Selenomethionine
SGR	Specific growth rate
TBARS	Thiobarbituric acid-reactive substances
TMSe	Trimethylselenonium
tRNA	Transfer ribonucleic acid
TRx	Thioredoxin
TrxR	Thioredoxin reductase
ww	Wet weight

ABSTRACT

To preserve animal welfare, consumer- and user safety, and to reduce environmental impact, the European Union (EU) has established maximum limits for essential minerals in animal feeds. For selenium (Se), the maximum limit in feed, when provided as a feed additive, is 0.5 mg total Se/kg feed, of which 0.2 mg/kg feed can be provided as an organic form of Se. As Se is an essential mineral, there is also a nutritional requirement, which for salmonids is 0.3 mg Se/kg feed, thus close to the maximum limit. The main source of Se in salmon feeds is marine ingredients such as fish-meal, which usually provides sufficient Se to cover the requirement. However, as the trend to reduced use of fish-meal in salmon feeds continues, the levels of naturally occurring marine-based Se in feeds will decrease. Consequently, there will be a necessity for Se supplementation to the feeds in order to meet the nutritional requirements of the fish and hence avoid negative consequences for fish health. Considering the maximum limit for Se in feed, the current legislation does not allow for supplementation to the natural levels of Se. However, regulatory maximum levels may be revised if new knowledge emerges. If both the requirement and the upper safe level of intake of Se is known for salmon, a more suitable maximum limit for Se that ensures good fish health may be proposed. The present study therefore aims to generate knowledge required for a scientifically-based maximum limit for Se in salmonid feed, through establishing biomarkers of Se toxicity in Atlantic salmon.

A 12 week feeding trial was performed with Atlantic salmon fed a basal diet supplemented with either inorganic Se (selenite) at 1.1 or 15 mg Se/kg, or organic Se (selenomethionine, SeMet) at 2.1 or 15 mg Se/kg, or two non-supplemented diets; a negative control consisting of the basal feed (0.35 mg Se/kg) or a fish-meal based positive control (0.89 mg Se/kg). Fish fed the High organic Se diet showed the highest Se accumulation in whole fish, and Se accumulation in all tissues were higher in fish fed the Low organic Se diet than in fish fed the Low inorganic Se diet. Metabolomic screening showed reduced levels of glutathione (GSH) and a depletion of intermediates in the S-adenosylmethionine (SAM) cycle in fish fed the High inorganic Se diet. The same group also showed reduced levels of vitamin C and vitamin E, and elevated levels of TBARS. Fish fed the High organic Se diet, also showed a reduction in GSH levels, however no changes were observed in levels of vitamin C, vitamin E or TBARS. Furthermore, signs of increased energy expenditure were seen in fish fed the High inorganic Se diet, as metabolomics screening showed reduced levels of free fatty acids, mono- and diacylglycerols, and coenzyme A. The same group also tended to have lower amounts of body fat and significantly reduced body weight, compared to the other treatment groups. In conclusion, the toxic mode of action caused by excessive Se, appears to have been oxidative stress, where inorganic Se showed a higher toxic potential than organic Se. From this study, markers of oxidative stress were found to be suitable markers of Se toxicity.

SAMMENDRAG

EU har etablert øvre grenseverdier for viktige mineraler i dyrefôr for å beskytte dyrevelferd, sikre forbrukertrygghet og for å redusere miljøpåvirkning. For det essensielle mineralet selen (Se) er den øvre grenseverdien satt til 0.5 mg Se/kg fôr for alle husdyrarter, hvorav 0.2 mg/kg kan tilsettes som organiske Se former. Den øvre grensen ligger nært opp til den nedre behovsgrensen (0.3 mg Se/kg fôr) for laksefisk. Hovedkilden til Se i fiskefôr har tradisjonelt vært marine ingredienser som fiskemel og dette har gitt Se konsentrasjoner som har vært tilstrekkelig for å dekke behovet. I utviklingen av nye laksefôr blir fiskemel imidlertid erstattet med alternative fôrråvarer som kan gi redusert Se innhold i laksefôr. For å dekke Se behovet hos laks kan det bli nødvendig å tilsette Se for å unngå konsekvenser for fiskens helse, men det er ikke tillatt med dagens regelverk. Når man kjenner den nedre behovsgrensen og den øvre grensen for inntak, kan man fastsette en mer hensiktsmessig grenseverdi der fiskens helse blir ivaretatt. Målet i dette masterprosjektet var derfor å finne egnede markører for toksisitet av Se i Atlantisk laks som kan brukes til å etablere en vitenskapelig basert grenseverdi.

Det ble utført et 12 ukers langt fôringsforsøk med laks, bestående av seks eksperimentelle dietter. En negativ kontroll bestående av et basalfôr uten tilsatt Se (0.35 mg Se/kg), to dietter tilsatt uorganisk Se (selenitt) til 1.1 eller 15 mg Se/kg, to dietter tilsatt organisk Se (selenometionin, SeMet) til 2.1 eller 15 mg Se/kg, og en fiskemelsbasert positiv kontroll (0.89 mg Se/kg). Akkumulering av Se ble målt i helfisk og i flere organ, der fisk fôret med organisk Se viste den høyeste Se akkumuleringen i både helfisk og i alle organ. I fisk fôret med de høyeste nivåene av uorganisk Se, viste screening av metabolitter i lever en nedgang i nivåer av antioksidanten glutathion (GSH) og en tapping av intermediater i S-adenosylmethionin (SAM) syklusen, som produserer GSH *de novo*. Den samme gruppen viste også reduserte nivåer av vitamin C og vitamin E og forhøyede nivåer av TBARS, sammenliknet med de andre gruppene. Fisk fôret med de høyeste nivåene av organisk Se, viste også reduksjon i GSH konsentrasjon, men ingen endringer i vitamin C, vitamin E eller TBARS. Disse resultatene viste at både organisk og uorganisk Se påvirket antioksidantsystemet og indikerte at oksidativt stress var den toksiske virkemåten til Se. Comet assay ble brukt for å undersøke om Se førte til DNA skade, uten å gi noen indikasjon på det. Videre viste screening av metabolitter i lever en nedgang i nivåer av frie fettsyrer, mono- og diacylglycerol og Koenzym A, i fisk fôret med de høyeste nivåene av uorganisk Se, noe som indikerte et økt energiforbruk i denne gruppen. Den samme gruppen viste også delvis redusert kroppsfett og redusert sluttvekt sammenlignet med de andre gruppene. Det ble derfor konkludert med at oksidativt stress var den toksiske virkemåten for Se, og at uorganisk Se har et større toksisk potensiale enn organisk Se. Markører for oksidativt stress vil derfor være gode markører for Se toksisitet.

1. INTRODUCTION

1.1 Selenium

Selenium (Se) is an essential mineral required for preservation of optimal health in all animals, including fish (Koller and Exon, 1986, Watanabe et al., 1997). The major role of Se is to function as an antioxidant, where it protects biological compounds against attack from free radicals produced during normal metabolism (Arteel and Sies, 2001). Selenium may also have a protective role against disorders related to oxidative stress in fish, as a consequence of physical stressors (Rider et al., 2009). In being an essential element, deprivation of Se will lead to deficiency symptoms (Poston et al., 1976). However, at elevated levels, Se may also exert toxicity, and there is a narrow margin between essentiality and toxicity in fish (Janz, 2012).

Selenium is a non-metallic element that exists in four oxidation states (-II, 0, IV, VI), as inorganic compounds such as selenite and selenate, and in organic forms as the selenoamino acids selenocysteine (SeCys) and selenomethionine (SeMet) (Suzuki, 2005, Janz, 2012). In the periodic table, Se belongs to the same group as sulfur, thus these two elements show several similar properties. Selenite and selenate are analogues of the sulfur compounds sulfite and sulfate respectively, and in SeCys and SeMet, Se substitutes sulfur in the amino acids cysteine and methionine (Johansson et al., 2005). In animals, most Se is associated with protein in the forms of either SeCys or SeMet residues (Suzuki, 2005), and most of the biological activity of Se is due to its essentiality for the functioning of selenoproteins (Hawkes and Alkan, 2010). In selenoproteins, Se occurs in the form of SeCys, where it is specifically incorporated into amino acid sequences (Suzuki, 2005). Fish are able to synthesize SeCys *de novo* from both organic and inorganic Se, but cannot utilize dietary SeCys directly (Thiry et al., 2012). Selenomethionine can not be synthesized in fish, but is taken up in the body and non-specifically incorporated into general proteins in competition with the sulfur analogue methionine (Schrauzer, 2000). This competition is due to the organism's inability to distinguish between these amino acids (Ochoa-Solano and Gitler, 1968).

Although both organic and inorganic Se can be utilized as nutrients, the chemical form of Se in the diet may have a great influence on the bioavailability and its impact on the organism (Thiry et al., 2012). Based on studies of absorption and accumulation of Se in different organisms, including fish, it is found that organic Se forms have a higher bioavailability compared to inorganic Se forms (Wang and Lovell, 1997, Ørnsrud and Lorentzen, 2002).

1.2 Sources of selenium

Fish can absorb Se from both the ambient water and the diet (Janz, 2012). However, in the aquatic environment, background concentrations of Se are usually fairly low, ranging between 0.01 and 0.1 µg/L. In addition, the absorption of Se from water in fish is limited, making the diet the primary source of Se (Janz, 2012). Thus, in aquaculture, it will be of great importance that the feeds contain sufficient amounts of Se to meet the requirements of the fish (Sweetman et al., 2010).

Selenium in food and feed derives from both plant material and animal products (Combs, 2001). The amount of Se in such products show a large variation due to both production methods and the areas where animals are reared and plants are grown (Combs, 2001). The Se concentration in plants will vary with the concentration of Se in the soil, which again varies throughout the world (Janz, 2012). Most regions however, are characterized by low to moderate levels of Se in the soil, thus plants will generally be low in Se (Janz, 2012)

Feeds fed to farmed Atlantic salmon (*Salmo salar* L.) have traditionally been based on fish-meal and fish oil, ingredients that contain ample amounts of Se (Julshamn et al., 2004, Gatlin et al., 2007). Fish meal and fish oil are ingredients extracted from wild catches (Tacon and Metian, 2008), and the growth in aquaculture and in aquaculture feed production, is expected to exceed the supply of wild fish. There has thus been a need to develop feeds less reliant on these marine ingredients (Gatlin et al., 2007). Plant ingredients are alternatives to those of marine origin, however their use reduces the levels of Se in the feed (Sanden et al., 2014).

1.3 Metabolism of selenium

1.3.1 Absorption

Since the major route of Se exposure in fish is through the diet, the central uptake route is through the gastrointestinal tract (Janz, 2012). In general, the absorption of SeMet and SeCys is greater than the absorption of selenite and selenate (Barceloux, 1999). Due to the similar properties of Se and sulfur, some forms of Se can share pathways with their sulfur analogues (Thiry et al., 2012). This is the case in the absorption of SeMet, which occurs via the same active transport mechanism as methionine (Nickel et al., 2009). The absorption of selenate takes place via an active transport mechanism shared with sulfate, while selenite is absorbed via passive diffusion (Wolffram et al., 1986, Mykkanen and Wasserman, 1989). The mechanism behind SeCys absorption is not known, but SeCys and cysteine does not appear to share transporters (Thiry et al., 2012).

1.3.2 Distribution

Not much is known regarding transport of Se in the bloodstream of fish. However, in mammals, Se is transported with help of the plasma protein albumin or Selenoprotein P (SelP) (Motsenbocker and Tappel, 1982, Thiry et al., 2012). Most of the Se will be transported to the liver, as it is the primary site for selenoprotein synthesis and catabolism (Burk and Hill, 2009). Although all tissues accumulate Se, the liver will be the primary site for Se accumulation (Janz, 2012). In Atlantic salmon, Lorentzen et al. (1994) found a higher accumulation of Se in liver compared to muscle, and in rainbow trout (*Oncorhynchus mykiss*), Rider et al., (2010) found the highest concentrations of Se in liver, followed by kidney, spleen, gills and muscle.

1.3.3 Intermediate metabolism

Hydrogen selenide (H_2Se) is the common metabolic intermediate of both organic and inorganic forms of Se, and is the precursor of selenoproteins (Figure 1.1) (Thiry et al., 2012). The formation of H_2Se from selenite has been observed to occur in red blood cells, where selenite is directly reduced to H_2Se by glutathione (GSH) immediately after absorption. Hydrogen selenide is further transported, usually to the liver, for selenoprotein synthesis. Selenate however, is transported directly to the liver before being reduced to selenite and again to H_2Se. Selenium in the form of SeMet can be metabolized primarily in two ways; it can be incorporated into general proteins, or it may follow the trans-selenation pathway where it is transformed into SeCys (Figure 1.1). Moreover, although selenoproteins contain Se in the form of SeCys, intact SeCys from dietary sources can not directly be used for selenoprotein synthesis. Even SeCys has to be transformed into H_2Se to allow for synthesis of *de novo* molecules of SeCys, which further will be incorporated into selenoproteins (Thiry et al., 2012).

The formation of SeCys starts with the phosphorylation of H_2Se into selenophosphate (Figure 1.1) (Ogra and Anan, 2009). Selenophosphate is further made available to seryl-$tRNA^{SeCys}$, where SeCys is synthesized by selenocysteine synthase. Selenocysteine synthase converts seryl-$tRNA^{SeCys}$ to selenocysteyl-$tRNA^{SeCys}$, which transports SeCys to the SeCys translation complex on mRNA. Selenocysteine is then incorporated into proteins by the UGA codon (Squires and Berry, 2008, Ogra and Anan, 2009). As the UGA codon normally serves as a stop codon, specific secondary structures in DNA, named selenocysteine insertion (SECIS) elements, specify coding of SeCys instead of termination by the UGA codon (Squires and Berry, 2008).

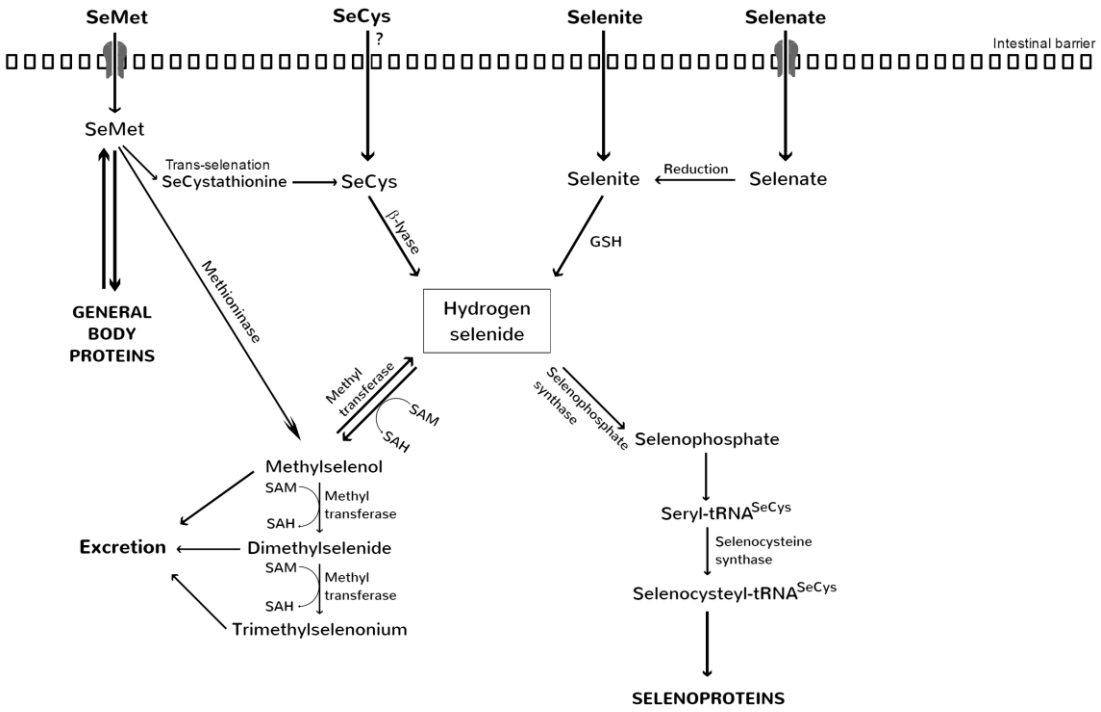

Figure 1.1 Selenomethionine (SeMet) and selenate are actively transported across the intestinal membrane, while selenite is absorbed via passive diffusion. The absorption mechanism of selenocysteine (SeCys) is not known. Selenomethionine can be incorporated into general body proteins, be directly reduced to methylselenol, or be transformed into hydrogen selenide. Hydrogen selenide is the common intermediate metabolite for both organic and inorganic forms of Se, and the precursor of selenoproteins. Hydrogen selenide is also methylated stepwise to methylselenol, dimethylselenide and trimethylselenonium before being excreted. The methyl donor is S-adenosylmethionine (SAM) which is converted into S-adenosylhomocysteine (SAH). *GSH; Glutathione*

When Se is present in excess, H_2Se is methylated stepwise to methylselenol (MMSe), dimethylselenide (DMSe) and trimethylselenonium (TMSe) before being excreted, as shown in Figure 1.1 (Suzuki, 2005). Selenomethionine may also, at excessive intake, be transformed directly into MMSe by the enzyme methioninase (Thiry et al., 2012). S-adenosylmethionine (SAM) is an important methyl group donor mediating the major methylation reactions catalyzed by methyltransferase enzymes, including methylation of H_2Se (Bottiglieri, 2002, Nakamuro et al., 2000). Methylation is an important reaction in many biological processes, which includes synthesis of lipids, modification of proteins, and gene expression (Zhang et al., 2012). Methylation of Se is considered a mechanism of detoxification, as methylated forms of Se are considered less toxic compared to the non-methylated Se forms (Nakamuro et al., 2000).

The SAM cycle (also called the methionine cycle) is important in the methylation of Se, and together with the trans-sulfuration pathway it provides precursors for the synthesis of GSH, depicted in Figure 1.2 (Nakamuro et al., 2000, Lu, 2012). The SAM cycle starts with methionine, which is converted to

SAM via the enzyme methionine adenosyltransferase (Lu, 2012). After donating its methyl group, SAM is converted to S-adenosylhomocysteine (SAH). Through a reversible reaction, SAH hydrolase catalyzes the cleavage of SAH into homocysteine and adenosine. Homocysteine may further be remethylated to methionine by vitamin B_{12}, folate and methionine synthase, or by betaine and betaine-homocysteine methyltransferase. A third alternative for homocysteine is to enter the trans-sulfuration pathway where it is converted to cysteine in a two-step process (Figure 1.2). First, cystathionine β-synthase, a vitamin B_6-dependent enzyme, catalyzes the formation of cystathionine. Then cystathionine is cleaved in a reaction catalyzed by γ-cystathionase, also dependent on vitamin B_6, which releases cysteine for GSH synthesis (Lu, 2012).

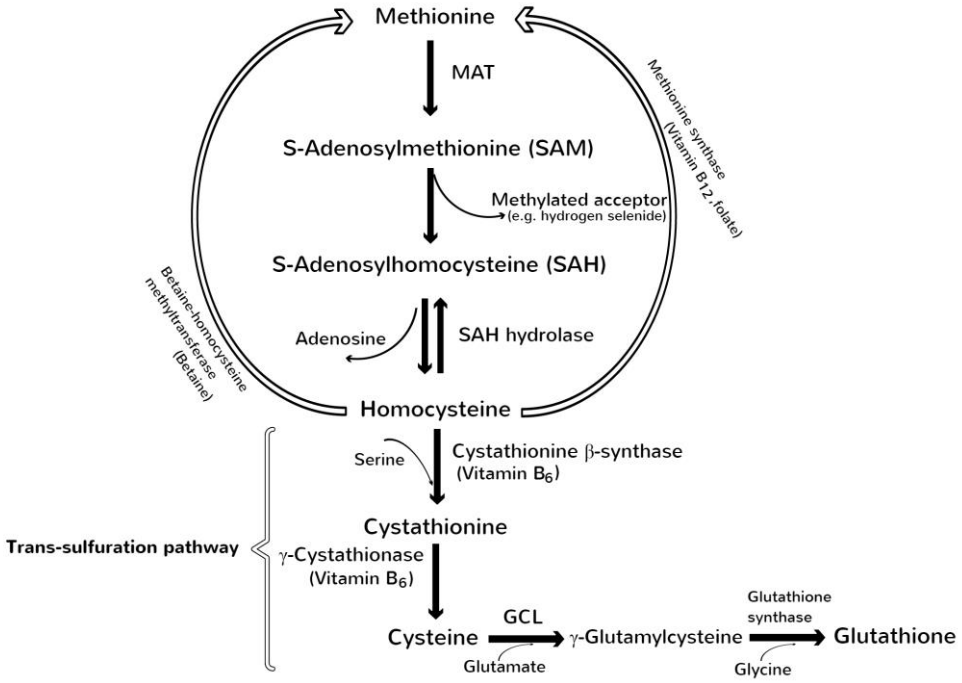

Figure 1.2 The S-adenosylmethionine (SAM) cycle and the trans-sulfuration pathway provides precursors for the production of the non-essential amino acid cysteine and for the synthesis of glutathione. SAM is also an important methyl-donor for hydrogen selenide. *MAT; Methionine adenosyltransferase*, *GCL; glutamate-cysteine ligase*

1.3.4 Excretion

The Se status is regulated through excretion of Se. In mammals, urine is the principal way of excretion, while the importance of urinary excretion of Se in fish is not known (Janz, 2012). The two major urinary metabolites are MMSe and TMSe, and the ratio in which they are excreted depends on the Se dose. Under conditions of low Se exposure, Se is excreted mostly as MMSe, while at higher exposures Se is also excreted as TMSe (Suzuki, 2005).

1.4 Functions of selenium

1.4.1 Selenoproteins

Most of the biological activity of Se is, as already mentioned, due to its essentiality for the function of selenoproteins (Hawkes and Alkan, 2010). In selenoproteins, Se is the catalytically active component, mediating, among other things, antioxidant protection and thyroid hormone production (Papp et al., 2007).

The amount of selenoproteins varies among organisms, and the selenoproteomes of fish are among the largest known, consisting of 30-37 selenoproteins (Lobanov et al., 2009). As a comparison, 25 selenoproteins have been identified in mammals (Kryukov et al., 2003). The same core selenoproteins are found in mammals and fish, and fish also possess several species-specific selenoproteins (Lobanov et al., 2009).

The first selenoprotein identified in mammals was glutathione peroxidase (GPx) (Flohe et al., 1973, Rotruck et al., 1973). In mammals, eight GPxs are so far identified, in which five of them contains Se (Brigelius-Flohé and Maiorino, 2013). In fish, four Se-containing GPxs have been identified (Kryukov and Gladyshev, 2000, Thisse et al., 2003). Glutathione peroxidase is a well known antioxidant that will be described in section 1.4.2.

Thioredoxin reductase (TrxR) is a selenoprotein that is a part of the thioredoxin (TRx) system, with important roles in the antioxidant defense (described in section 1.4.2) (Nordberg and Arner, 2001). In addition, the system plays a role in cell signaling (Saitoh et al., 1998), in cell growth, apoptosis and in the immune system (Lu and Holmgren, 2014, Pacitti et al., 2014).

Other well-characterized selenoproteins are the iodothyronine deiodinases. The deiodinases are involved in thyroid function by controlling the levels of triiodothyronine (T_3) and thyroxine (T_4). These selenoproteins converts the prohormone T_4 to the active thyroid hormone T_3, and also control the degradation of T_3 (Arthur and Beckett, 1999).

Selenoprotein P is a major selenoprotein found in plasma, and a proposed role of SelP is in the transport of Se to remote tissues (Burk and Hill, 2009). Selenoprotein P may also possess antioxidant properties, in that it is found to directly reduce phospholipid hydroperoxides *in vitro* (Takebe et al., 2002). Of the remaining selenoproteins that have been identified, most are poorly characterized and little information exist on their function (Janz, 2012).

1.4.2 The antioxidant defense and role of selenium

Oxidation is one of the two reactions in a reduction-oxidation (redox) reaction, which regulates normal physiological functions. Oxidation is the reaction where a molecule either associates with

oxygen or loses hydrogen or electrons, resulting in a higher oxidation number (Halliwell and Poulsen, 2006). Atoms or molecules with unpaired electrons are called free radicals, and are formed by losing an electron or gaining an electron from a non-radical (Martínez-Álvarez et al., 2005). With an unpaired electron the chemically reactivity of a molecule increases (Betteridge, 2000). When free radicals react with biological macromolecules, like lipids, proteins and DNA, it results in a chain reaction generating new radicals, which further can react with new macromolecules. If there is an imbalance between the production of free radicals and antioxidants, the result is oxidative stress, a state which may cause damage to cellular structures (Figure 1.3) (Betteridge, 2000).

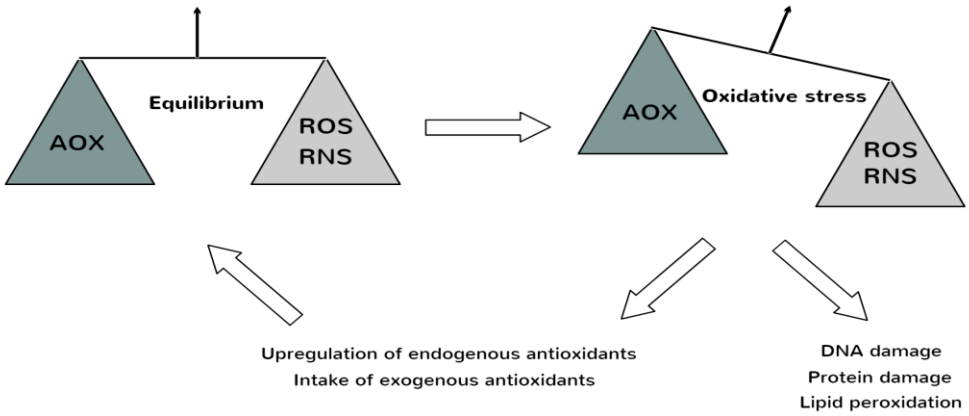

Figure 1.3 Oxidative stress is a result of an imbalance between production of reactive oxygen species (ROS) and/or reactive nitrogen species (RNS), and protective antioxidants (AOX). Oxidative stress causes damage to DNA, proteins and lipids. Equilibrium can be recovered by an up-regulation of endogenous antioxidants and/or by intake of exogenous antioxidants.

Reactive oxygen species, such as superoxide anion ($O_2\bullet^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH•), are produced by different biochemical processes within the body during normal cellular metabolism (Betteridge, 2000). "*Reactive oxygen species (ROS) is a collective term used for a group of oxidants, which are either free radicals or molecular species capable of generating free radicals*" (Kunwar and Priyadarsini, 2011). Mitochondria are the major source of ROS in most cells (Hawkes and Alkan, 2010), and low concentrations of ROS are required for ordinary physiological functions, including gene expression, cellular growth and of the immune system (Kunwar and Priyadarsini, 2011).

ROS may also be generated in response to several environmental factors, and uncontrolled generation and accumulation of ROS may lead to oxidative stress in cells (Kunwar and Priyadarsini, 2011). Excessive generation of ROS can lead to peroxidative damage to lipids, protein carbonylation

and DNA strand breakage, eventually leading to various clinical consequences (Santhosh Kumar and Priyadarsini, 2014).

Nitrogen-containing oxidants termed reactive nitrogen species (RNS), such as nitric oxide (NO•) and peroxynitrite ($ONOO^-$), also play important roles in both oxidative physiology and pathology (Pacher et al., 2007). Nitric oxide is an important factor in cell signaling, regulating critical cellular functions. However, NO• is also a mediator of cellular damage as it may react with $O_2•^-$ to form the highly reactive species $ONOO^-$. Peroxynitrite may, like ROS, react with, and damage various biological components of the cell (Pacher et al., 2007).

To protect the cells against oxidative damage mediated by ROS and RNS, organisms possess defense mechanisms, which include antioxidant systems, to keep the generation of ROS and RNS under control (Droge, 2002, Kunwar and Priyadarsini, 2011). An antioxidant can be defined as "*any substance that, when present at low concentrations compared with those of the oxidizable substrate, considerably delays or inhibits oxidation of the substrate*" (Gutteridge, 1995).

The antioxidant system consists of both endogenous and exogenous antioxidants that generally work in three different ways. They may prevent the formation of reactive species, scavenge them, or repair damaged molecules (Kunwar and Priyadarsini, 2011). Furthermore, antioxidants may be classified as enzymatic or non-enzymatic. Enzymatic antioxidants catalyze the conversion of ROS and RNS to less reactive or inert species, and thus act as the first line of defense. The secondary defense is built up of the non-enzymatic antioxidants, which function by scavenging ROS and RNS directly (Kunwar and Priyadarsini, 2011). The non-enzymatic antioxidants can also inhibit the production of ROS. This can be done by eliminating redox active metals, like iron and copper, that can change oxidation states and further promote the production of ROS (Fisher and Naughton, 2003, Kunwar and Priyadarsini, 2011). Fish possess many, if not all, of the basic antioxidant defenses that have been characterized in mammals (Di Giulio and Meyer, 2008).

Selenium plays important roles in the antioxidant defense by being a constituent of the enzymes GPx and TrxR. These enzymes are essential components of the cellular GSH and TRx systems respectively, and thus are important regulators of the intracellular redox environment (Hawkes and Alkan, 2010). Selenium is a functional component of the active center of GPx, an enzyme that protects cells and membranes from oxidative damage (Arteel and Sies, 2001). Glutathione peroxidase catalyzes the reduction of H_2O_2 and hydroperoxides, which are responsible for various cellular damage. Hydrogen peroxide are reduced to water, and hydroperoxides are reduced to their corresponding alcohols, with the subsequent oxidation of GSH to glutathione disulfide (GSSG) (Lu, 2012, Brigelius-Flohé and Maiorino, 2013). To maintain the levels of reduced GSH for continued antioxidant protection, a redox

cycle is formed, as GSSG is reduced back to GSH by the NADPH-dependent enzyme glutathione reductase (Figure 1.4) (Lu, 2012). Glutathione peroxidase also prevents both oxidation and nitration reactions caused by $ONOO^-$, thereby acting as a peroxynitrite reductase (Sies et al., 1997).

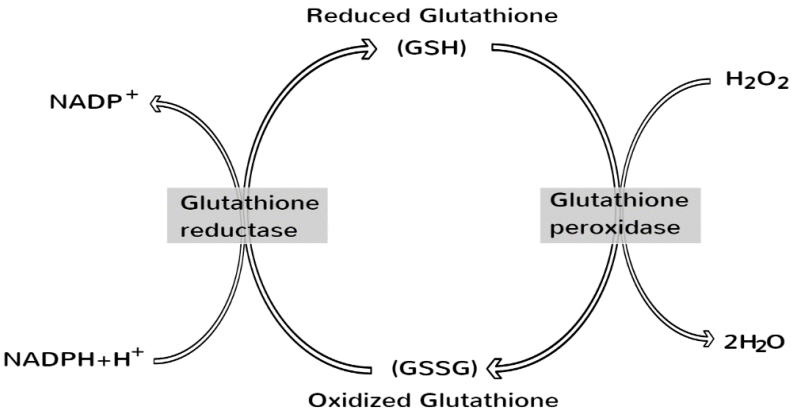

Figure 1.4 Glutathione peroxidase catalyzes the reduction of H_2O_2 with the subsequent oxidation of GSH to GSSG. In order to maintain levels of GSH for antioxidant protection, NADPH-dependent glutathione reductase catalyzes the reduction of GSSG to GSH.

The TRx system represents another central system in the regulation of the redox-environment in cells, a system based on a reductive compound, TRx, and the selenoprotein TrxR, in addition to NADPH (Nordberg and Arner, 2001). The TRx system plays a fundamental role in antioxidant defenses by maintaining a reductive environment through its redox-active cysteine residues (Fernando et al., 1992, Nordberg and Arner, 2001). Thioredoxin may act as a scavenger of ROS, and in regeneration of proteins that have been damaged by oxidative stress. To maintain the TRx system in a reduced state, TRx is reduced by TrxR, using NADPH (Fernando et al., 1992, Nordberg and Arner, 2001). Thioredoxin reductase may additionally directly reduce peroxides, including lipid peroxides and H_2O_2 (Björnstedt et al., 1995).

1.4.3 The antioxidant system plays an important role in Atlantic salmon

Fish, and particularly fatty fish such as salmonids, have a high level of poly-unsaturated fatty acids (PUFAs), which are essential in cell membranes (Martínez-Álvarez et al., 2005). Poly-unsaturated fatty acids are prone to lipid peroxidation, thus, to protect them against oxidative damage, salmonids have a relatively high requirement for antioxidants (Martínez-Álvarez et al., 2005). Farmed fish are continuously exposed to physical and environmental stressors like sub-optimal stocking densities and handling. Exposure to stressors are often followed by an increase in metabolic rate and respiration (Wendelaar Bonga, 1997), stress responses that may lead to an increased production of ROS (Liu and

Mori, 1999). Thus, when continuously exposed to stressors, there is a high requirement for antioxidant enzymes, including GPx and TRx, which in turn gives a high demand for Se. Küçükbay et al. (2009) showed that rainbow trout fed practical diets while exposed to confinement stress, required higher levels of Se for the preservation of optimal antioxidant status. Moreover, Rider et al. (2009) found that Se from an un-supplemented diet appeared to maintain Se status in fish before being exposed to stressors. However, exposure to husbandry related stressors resulted in reduced whole body Se and elevated GPx levels, suggesting an increased requirement for Se during stress. To maintain an adequate antioxidant status, it is thus essential that sufficient Se reserves are available to the fish (Rider et al., 2009).

1.5 Effects of selenium deficiency

As with many other micronutrients, intake of too low or high levels of Se, will result in adverse consequences (Huang and Hoffmann, 2013). The severity of the deficiencies will however depend on a variety of factors, including the duration and degree of deprivation (Watanabe et al., 1997).

White muscle disease, a myopathy occurring in lambs and calves, was the first reported disease related to deficiency of Se, in association with deprivation of vitamin E (Muth et al., 1958). In humans, two diseases have been related to severe Se deficiency, both occurring in rural regions of China and Russia; Keshan disease, a potentially fatal cardiomyopathy, and Kashin-Beck disease, a chronic osteoarthropathy (Combs, 2001).

In fish, symptoms of Se deficiency include reduced growth, increased mortality, exudative diathesis, anemia and degradation of liver and muscle tissue. In addition, increased red blood cell fragility and decreased packed cell volume has been observed (Poston et al., 1976, Bell et al., 1985, Bell et al., 1987).

The nutritional requirement for Se in Atlantic salmon was documented in 1976, when it was found that Se deficiency, either occurring alone or combined with deprivation of vitamin E, reduced survival of Atlantic salmon fry (Poston et al., 1976). In fish deficient in both nutrients, severe muscular dystrophy was seen, which was prevented by dietary supplementation of Se and vitamin E in combination. Selenium-deficient fish also showed reduced GPx activity in plasma (Poston et al., 1976).

Deficiency of Se is associated with reduced antioxidant protection, as the synthesis and function of GPx will be reduced. Especially the GPx form found in plasma responds rapidly to an alteration in Se status (Poston et al., 1976, Bell et al., 1986). Due to a decreased ability to protect against oxidative

stress, Se deficiency may lead to a variety of clinical disorders as a consequence of cellular damage (Santhosh Kumar and Priyadarsini, 2014).

1.6 Selenium toxicity

The toxicity of Se has been known since the late 1930s, when chronic exposure to Se was linked to the condition "alkali disease", occurring in livestock in the United States of America (Spallholz, 1994). Lack of vitality, deformed and sloughed hooves, lameness and anemia were some of the symptoms in the affected animals (Koller and Exon, 1986). "Blind staggers", another condition associated with toxic levels of Se, also occurs in livestock and is characterized by impaired vision, abdominal pain, anorexia and ataxia (Koller and Exon, 1986). Although Se toxicity in humans rarely occurs, similar symptoms of Se toxicity are found in animals and man (Spallholz, 1994).

The toxicity of Se is dependent on the dose and duration of exposure (Mézes and Balogh, 2009), and an important aspect regarding Se toxicity, is the narrow margin between essentiality and toxicity (Janz, 2012). In fact, Se is the essential element with the narrowest range between essential and toxic concentrations (Fordyce, 2013), and toxic effects in fish have been seen at dietary concentrations only 7-30 times higher than those considered essential (i.e. > 3 mg Se/kg) (Hilton et al., 1980, Hodson and Hilton, 1983). In addition to the dose and duration of exposure, the toxicity of Se is also dependent on its chemical form (Mézes and Balogh, 2009).

1.6.1 Mechanisms of selenium toxicity

Se toxicity has been attributed to the ability of Se to substitute sulfur during protein assembly. Disulfide bonds are important components of proteins, required for their structure and function. As SeMet can substitute methionine in proteins, the theory is that SeMet alters the disulfide linkages, thus affecting the structure and functions of proteins, and further impairs normal cellular biochemistry (Lemly, 2002).

Another mechanism of Se toxicity is attributed to its pro-oxidant activity, by which Se generates oxidative stress (Mézes and Balogh, 2009). The pro-oxidant activity of Se is thought to arise from its ability to oxidize thiols, and a variety of Se compounds have been studied to see if they react with GSH to produce $O_2\bullet^-$ *in vitro* (Mézes and Balogh, 2009).

Selenite has been observed to react with GSH and other thiols to form selenotrisulfides (Mézes and Balogh, 2009). In mammalian tumor cells, reduction of selenotrisulfides, by excess thiols or cellular GR, forms highly reactive selenopersulfides, which ultimately generates $O_2\bullet^-$ in the presence of oxygen (Lin and Spallholz, 1993). The selenopersulfides may also react with additional GSH to produce H_2Se. When H_2Se is present at elevated levels, and not used for synthesis of SeCys or

methylated, this form can also be involved in redox cycling and the production of ROS (Lin and Spallholz, 1993, Janz, 2012). In addition, the conversion of H_2Se to elemental Se gives rise to $O_2\bullet^-$ (Lin and Spallholz, 1993).

Production of $O_2\bullet^-$ has also been observed during the metabolism of SeMet, and oxidative stress as a result of elevated SeMet levels has been seen in isolated hepatocytes of rainbow trout (Misra et al., 2012). Oxidative stress as a result of elevated SeMet levels is thought to arise from the transformation of SeMet to methylselenol by methioninase. When methylselenol has been formed, it has been observed to react with GSH, leading to the production of $O_2\bullet^-$ (Spallholz et al., 2004). Moreover, organic diselenides, such as selenocystine and selenocystamine, are in the presence of thiols converted into selenols, which results in the generation of $O_2\bullet^-$ and H_2O_2 (Mézes and Balogh, 2009). However, selenium compounds that do not readily form H_2Se, such as elemental Se and selenate, have not been observed to react with thiols and will only cause toxicity after being reduced to selenite or selenol (Mézes and Balogh, 2009).

1.6.2 Effects of selenium toxicity

Toxic effects of Se in fish includes reduced growth, reduced feed efficiency, and increased mortality (Hilton et al., 1980). In addition, development of cataracts, skin lesions, swollen gill filament lamellae, myocarditis, and necrosis in liver and kidney have been observed (Lemly, 1997, Lemly, 2002). However, the most significant and overt toxic effects are teratogenic deformities in larvae (Lemly, 1997, Lemly, 2002).

Teratogenic deformities are a permanent pathological marker of Se toxicity in fish. The malformations are innate and occur due to elevated Se levels in eggs (Lemly, 1997). Excess Se levels in the diet of the parent fish causes elevated concentrations of Se to be deposited in egg yolk during vitellogenesis. Consequently, the larval fish are exposed to Se during yolk absorption (Lemly, 1997, Janz, 2012). The most conspicuous deformities are found in the skeleton, fins, head and mouth. These deformities involves lordosis, scoliosis and kyphosis, missing or deformed fins, gills or opercula and deformed head, eyes or mouth (Lemly, 1997).

1.6.3 Biomarkers of selenium toxicity

In toxicology, biomarkers are essential as they allow for assessment of health effects following exposure to potentially toxic compounds (Timbrell, 1998). The International Programme on Chemical Safety of the World Health Organization, has defined biomarkers as *"any substance or its product, structure or process that can be measured in the body and that can influence or predict the incidence of disease outcome"* (WHO, 2001). An ideal biomarker will provide a sensitive, informative and

reproducible indication of potential toxicity, before overt negative effects have appeared (Gatzidou et al., 2007).

When designing and establishing biomarkers, identification of toxicity pathways and insight into the biological disturbance affected in the pathways, is critical (Andersen et al., 2010). Genomics (profiling of RNA and DNA), proteomics (profiling of proteins) and metabolomics (profiling of biochemicals or metabolites) can identify many possible biomarkers of toxicity (Shaw, 2006), and these "omics" technologies have become important in detecting new biomarkers (Milburn et al., 2013). These technologies allow for identification of genes, proteins or metabolites within a sample, such as blood and tissues, where the aim is to identify and quantitate differences between comparative samples, such as treated versus non-treated (Veenstra, 2010).

1.7 Selenium in feed for Atlantic salmon

1.7.1 Maximum limit of selenium in feed

To preserve animal welfare, consumer and user safety and to reduce environmental impact, the European Union (EU) has established maximum limits for essential minerals in animal feeds. For Se, the current maximum limit in feed for all species is 0.5 mg total Se/kg feed of which 0.2 mg/kg feed can be provided as an organic form of Se (Council Directive 70/524/EEC and amendments). Farmed Atlantic salmon have traditionally been fed diets mainly based on fish-meal and fish oil (Gatlin et al., 2007), which contain ample levels of Se. In 2003, results from the annual surveillance program on fish feed, showed that the analyzed fish-meal samples contained an average concentration of 2.4 mg Se/kg (Julshamn et al., 2004). In fish feed, surveillance data in the period 2004-2013 show average Se concentrations of 0.8-1.4 mg/kg feed, with minimum and maximum concentrations of 0.2 and 4.1 mg/kg, respectively (Sanden et al., 2014). However, the maximum limit for Se is only valid when Se is added as a feed additive. As ingredients of plant origin replace marine ingredients, the trend is decreasing levels of Se in fish feeds, from 1.3 mg/kg in 2004 to 0.72 mg/kg in 2013 (Sanden et al., 2014).

1.7.2 Practical supplementation of selenium in feed

The dietary requirement of Se in Atlantic salmon is not known, but in rainbow trout the requirement is 0.35 mg/kg (Hilton et al., 1980). The natural levels of Se in feeds based on marine feed ingredients thus clearly covers the requirement.

However, due to the limited availability of marine feed ingredients, there is a need to develop new feeds using alternative ingredients for salmonids in aquaculture (Gatlin et al., 2007). Plant ingredients are among the best suited alternatives to fish oil and fish meal, yet these are feed ingredients that

will lead to a reduction of the marine based Se level and thus the Se level in general. In 2013, results from the annual surveillance program on fish feed showed that the analyzed plant feed samples contained an average Se concentration of 0.11 mg/kg, and plant oils contained concentrations <0.008 mg/kg feed (Sanden et al., 2014). Due to the lower natural levels of Se in plant ingredients, there may be a need for Se supplementation to meet the requirements of the fish. However, considering the maximum limit for Se in feed, the current legislation does not allow for supplementation to the natural levels of Se.

The current maximum limit for Se in fish feed is generic for all animal categories and not specific for fish. In order to establish scientifically-based maximum limit for Se in feed for salmonids, data are required for risk assessment on dietary toxicity of Se in Atlantic salmon. The generation of such data is the main goal of the ongoing research project, which this thesis is a part of, namely to provide knowledge on the maximum limits for Se in Atlantic salmon feed. The aim of this study was to establish biomarkers of dietary Se toxicity in Atlantic salmon.

2. MATERIAL AND METHODS

2.1 Fish experiment

The feeding trial was carried out at EWOS Innovation located in Dirdal, Norway. The fish was fed the basal diet from October 24, 2014 to acclimatize to the feed, before switching over to experimental feeds for twelve weeks, from December 2, 2014 to February 25, 2015. A total of 540 Atlantic salmon were randomly distributed into 18 fiberglass tanks (V = 0.49 m^3), with 30 fish per tank. Environmental parameters in tanks were measured five times a week, showing a salinity of 28.4 ± 0.3 ‰, temperature of 8.9 ± 0.3 °C, and oxygen levels of 87 ± 6 % at the outlet. During the feeding trial the fish were reared under constant light.

2.1.1 Feed

During the feeding trial, fish in triplicate tanks were fed one of six experimental diets (Table 2.1, Table 2.2). A basal diet with a low level of Se (high plant meal inclusion) was used as a negative control, and a diet with a naturally high level of Se (high fish-meal inclusion) was used as a positive control. Based on the basal diet, two diets were supplemented with either high or low levels of inorganic Se (selenite, DSM, Heerlen, Netherland). Additionally, two diets were supplemented with high or low levels of organic Se (SeMet) supplied as inactivated whole cell yeast (*Saccharomyces cerevisiae*) containing L-selenomethionine (AlkoSel®, Lallemand, Malvern link, England). For the diets low in Se, the target level of Se were levels close to those in commercial fish feeds (~1.4 mg/kg), while in the high Se diets the target was 13 mg/kg, a level previously seen to cause toxicity.

Table 2.1 Ingredients in the experimental diets (g/100 g).

	Negative control	Low inorganic Se	High inorganic Se	Low organic Se	High organic Se	Positive control
Fish meal[1]	8.0	8.0	8.0	8.0	8.0	15.0
SA Krill	2.0	2.0	2.0	2.0	2.0	7.0
Soy protein concentrate	18.0	18.0	18.0	18.0	18.5	10.0
Wheat Gluten	18.0	18.0	18.0	18.0	19.0	18.0
Maize Gluten	4.0	4.0	4.0	4.0	5.0	4.0
Pea Protein 50	6.1	6.1	6.1	6.1	2.6	
Pea Protein 72						4.0
Wheat	6.0	6.0	6.0	6.0	6.0	8.0
Peas	6.4	6.4	6.4	6.4	7.2	7.0
Fish Oil[2]	8.3	8.3	8.3	8.3	7.9	8.0
Rapeseed Oil	16.0	16.0	16.0	16.0	16.8	14.9
Monosodium-phosphate	2.8	2.8	2.8	2.8	2.8	2.1
Micro-nutrients[3]	4.0	4.0	4.0	4.0	4.0	2.3
Selenite		0.0022	0.028			
Selenomethionine (as a Se-yeast)				0.05	0.63	

South American Superprime fish meal[1], North Atlantic Capelin oil[2], mineral and vitamin premixture[3]
All of the diets contained 0.05 mg/kg yttrium

Table 2.2 Analytical composition of the experimental feeds, presented as mean ± standard deviation (n=2).

	Negative control	Low inorganic Se	High inorganic Se	Low organic Se	High organic Se	Positive control
Se measured (mg/kg dw)	0.35 ± 0.02	1.10 ± 0.03	15.0 ± 0.5	2.1 ± 0.1	15.0 ± 0.2	0.89 ± 0.03
Fat (g/100g ww)	23.6 ± 0.4	25.1 ± 0.3	25 ± 1	25 ± 1	26 ± 1	27.6 ± 0.3
Protein (g/100g ww)	43 ± 1	44.3 ± 0.4	45 ± 1	45 ± 1	44 ± 1	45 ± 1
Ash (g/100g ww)	5.81 ± 0.01	5.7 ± 0.1	5.75 ± 0.02	5.7 ± 0.1	5.7 ± 0.1	6.09 ± 0.02
Energy (J/g ww)	23088 ± 322	23426 ± 217	23767 ± 40	23340 ± 285	23788 ± 104	24213 ± 246
Dry matter (g/100g)	92.60 ± 0.01	93.37 ± 0.02	94.0 ± 0.3	93.491 ± 0.002	94.1 ± 0.1	94.7 ± 0.1
TBARS* (nmol/g ww)	8.23 ± 0.02	10 ± 1	11.0 ± 0.3	4 ± 1	12.0 ± 0.2	13.97 ± 0.01
Alpha-tocopherol (mg/kg ww)	278 ± 35	308 ± 4	329 ± 1	310 ± 2	335 ± 1	317 ± 3

** Thiobarbituric acid-reactive substances, dw; dry weight, ww; wet weight*

2.1.2 Sampling

The mid-sampling was carried out January 14 and 15, 2015 and the final sampling on February 24 and 25, 2015. Fish were starved for 24 hours prior to sampling. At the final sampling, ten fish from each tank were randomly sampled and anesthetized before being weighed and measured. The fish were further killed by a blow to the head.

Liver biopsies were sampled, in a standardized manner, from three fish per tank, weighed, flash frozen and transported on dry ice for subsequent metabolomics analyses. Five fish from each tank were homogenized as whole fish, frozen at -20 °C and analyzed for minerals and fat. One fish from each replicate of the Low inorganic, Low organic and Positive control were sampled for analysis of Se distribution in tissues. In these nine fish, gills, eyes, brain, heart, liver, spleen, kidney and the gastrointestinal tract, were dissected out. Muscle tissue was removed from skin and bones, and head, skin, fins and bone were sampled together as carcass. All tissues were weighed, and these samples were transported on ice, and stored in a - 80°C freezer until further analysis.

2.2 Homogenization

Prior to the Se analysis, the samples were freeze-dried and homogenized. The samples, except the carcass, eyes and liver, were homogenized in a knife mill (Grindomix, GM200, Retch GmbH, Haan, Germany). When homogenizing the carcass and the eyes, the samples were pre-homogenized in the knife mill, before being subjected to a mortar and pestle in liquid nitrogen. Homogenizing the liver was done using a Polytron® PT-MR2100 homogenizer (Kinematica AG, Luzern, Switzerland). Whole fish was homogenized using an industrial meat grinder (Dadaux Titane, Nordic supply, Aalesund, Norway).

2.3 Analytical methods

2.3.1 Determination of selenium in tissues

Total content of Se was analyzed in tissues from fish fed the Low inorganic Se, Low organic Se and Positive control diets. The samples were analyzed by inductively coupled plasma with mass spectrometry (ICP-MS) following microwave-assisted acid decomposition by the method described by Julshamn et al. (2007). When preparing the samples for the microwave-assisted acid decomposition, 0.5 mL deionized water was first pipetted into a 15 mL quartz digestion vessel (Milestone Srl, Sorisole, BG, Italy). 0.20-0.25 g of freeze-dried sample were then weighed accurately into the vessel, before adding 2 mL HNO_3 (≥ 69 %, Trace SELECT®, Sigma-Aldrich, St.Louis, MO, USA). The vessels were capped and placed in the UltraWAVE (UW) (SRC, Milestone Srl, Sorisole, BG, Italy). The gas pressure in the UW was set to 40 bar and the temperature increased incrementally to 260°C. Following digestion in the UW, the samples were diluted with deionized water to 25 mL in a

volumetric flask. The exception was the brain samples, which, due to small sample size, were diluted to 10 mL.

Total Se concentrations were determined using ICP-MS (iCapQ™, Thermo Scientific™, Bremen, Germany) equipped with an autosampler (SC-4Q DX FAST, Elemental Scientific, Omaha, NE, USA). In the ICP-MS, the sample solution is nebulized and carried into argon plasma, where the sample is vaporized and the elements are ionized. The selected masses are detected as the amounts of hits per second as the ions are brought forward to a mass sensitive detector. The elements were quantified using an external standard curve made up by a multi element standard (Spectrascan, Teknolab, Ski, Norway) and a mercury standard (Spectrascan, Teknolab, Kungsbacka, Sweden) in 5 % HNO_3. In addition, gold (Spectrascan, Teknolab, Kungsbacka, Sweden) was added to stabilize the mercury ions.

The samples were analyzed using rhodium, thulium and germanium as internal standards. Germanium was also used as an internal standard for Se. Blank samples were analyzed to account for background mineral contamination, and the certified reference materials Oyster tissue (OT, CRM 1566, National Institute of Standards and Technology, Gaithersburg, MD, USA) and Lobster hepatopancreas (Tort-3, National Research Council, Ottawa, Canada) were analyzed as a measure of analysis accuracy. The certified values of OT and Tort-3 are 2.06 ± 0.15 and 10.90 ± 1.00, respectively. The samples were analyzed for Se in two rounds, and the analyzed values of OT were 2.28 and 1.83. Both of these values were thus outside the standard deviation on OT. However, as the values were within the upper and lower control limits of the control chart they were found satisfactory. The values obtained for Tort-3 were 10.83 and 10.41, thus in good agreement with the certified values.

Most of the samples were analyzed with two parallels. The exceptions were brain and heart, which consisted of single samples due to small sample size.

2.3.2 Metabolomic screening for biomarkers

"Metabolomics measures metabolites to allow for a direct evaluation of health and all of its influences" (Metabolon, 2015). Homeostatic alterations that underlie health, disease and response, are reflected in the metabolome of a sample. The metabolome of a sample is also central in the understanding of biological processes, and in the understanding of health impacts that operate by altering these processes (Metabolon, 2015). Non-targeted screening methods may be used in the identification and quantification of a high number of metabolites. Through such screening methods, metabolites involved in particular pathways may be identified, and this is made possible through chromatographic separation and mass spectrometry, combined with statistical analysis and detailed mathematical modeling (Lawton et al., 2008). In the present study, the goal was to identify differences in global metabolite profiles in liver from Atlantic salmon fed the six experimental diets.

The work was carried out by Metabolon Inc., USA, and samples were analyzed using a combination of reverse phase ultra performance liquid chromatograpy mass spectrometry (RP/UPLC-MS/MS) with positive and negative ion mode electrospray ionization, and hydrophilic interaction (HILIC) UPLC-MS/MS. In the liver samples, about 500 metabolites were detected. Data included in this study were metabolites illustrating consistent changes in specific pathways; the SAM cycle, vitamin C, and a selection of lipid and protein metabolites. The raw data of each biochemical were rescaled by dividing each sample value by the median value for the specific biochemical. The resulting data are thus semi-quantitative with arbitrary units.

2.3.3 Vitamin E

Vitamin E refers to the lipid-soluble antioxidants tocopherols and tocotrienols (Meeteren et al., 2005). The method determines α-, β-, γ- and δ-tocopherol isomers and α-, β-, γ- and δ- tokotrienol by high-performance liquid chromatography (HPLC), and was performed according to the method described by Hamre et al. (2010). In short, the homogenized liver samples were saponified (20 minutes at 100 °C) using ethanol, potassium hydroxide, pyrogallol, ascorbic acid and EDTA, before the samples were extracted three times with hexane. The solvent was subsequently evaporated under nitrogen and the samples were diluted with a standard volume of hexane before injection into the HPLC.

2.3.4 Thiobarbituric acid-reactive substances (TBARS)

Thiobarbituric acid-reactive substances (TBARS) are products of lipid peroxidation and were determined by the method described by Hamre et al. (2001). By use of Bligh and Dyer extraction, fat and water-soluble components in the liver samples were separated, and the aldehydes were extracted from the sample in the methanol:water phase. Thiobarbituric acid (TBA) were added in excess to an aliquot of the methanol:water phase and then heated to form a colored complex between aldehydes in the sample and TBA. The absorption was measured at 532 nm, and the concentration of TBARS were quantified using a standard curve.

2.3.5 Total body fat

In order to determine the total amount of body fat in the fish, fat were determined gravimetrically after ethyl acetate extraction and solvent evaporation. Homogenized and freeze-dried material was weighed and ethyl acetate was added. The sample was then shaken, filtered and transferred to a glass dish for evaporation. The fat was weighed when the solvent had evaporated. “Fat” in this method is defined as the fraction that is soluble in ethyl acetate, thus primarily non-polar lipids such as triacylglycerol and not e.g. phospholipids.

2.3.6 Comet Assay

The Comet assay, also known as single cell gel electrophoresis assay, allows for detection of DNA damage in individual cells. In response to an electrophoretic field, damaged cellular DNA is separated from undamaged DNA and drawn through an agarose gel. The migrated DNA yields a shape of a "comet tail", with the nucleus being the "comet head" (Figure 2.1). The size and shape of the comet, in addition to the distribution of DNA between the "head" and the "tail", corresponds to the amount of DNA damage in the cell (Kumaravel et al., 2009).

The OxiSelect™ 96-Well Comet Assay Kit (Cell Biolabs, Inc., San Diego, CA, USA) was used and the assay was carried out according to the product manual. Liver tissue from 45 fish, fed the Low and High inorganic Se, Low and High organic Se, and Negative control diet, were analyzed for DNA damage. The assay was performed in two rounds. During the first round, samples were analyzed with three parallels. When preparing the slide, the parallels were placed next to each other while the diets were randomly distributed. To include all of the samples, two OxiSelect™ 96-Well Comet Slides were prepared. To be able to evaluate the slides independently, all samples from the Negative control group were included on both slides. In the second round, the samples were analyzed with two parallels.

2.3.6.1 Preparation of reagents

All of the reagents were prepared prior to performing the assay, and stored at 4°C.

2.3.6.2 Tissue preparation

Small pieces of liver tissue, of approximately the same size, were placed in 2.0 mL Eppendorf tubes® (Eppendorf AG, Hamburg, Germany), and carefully minced with sharp tweezers. The procedure was carried out on dry ice and the samples were stored in an 80°C freezer until further analysis.

2.3.6.3 Alkaline Comet Assay

When performing the assay, 1 mL ice cold PBS containing 20 mM EDTA (without Mg^{2+} and Ca^{2+}) was added to each Eppendorf tube® containing liver tissue. The tissue suspension was allowed to stand for some minutes before the supernatant was transferred to a new Eppendorf tube® and placed in a centrifuge (5804R, Eppendorf AG, Hamburg, Germany) at 1500 revolutions per minute (rpm) for 15 minutes. Following centrifugation, the cells were resuspended in 3 mL ice-cold PBS (without Mg^{2+} and Ca^{2+}), giving approximately 1 x 10^5 cells/mL.

In a pre-warmed 96-well plate, cell samples were mixed with molten agarose at a 1:10 ratio, before application of 20 µL/well to the OxiSelect™ 96-Well Comet Slide. After the application, the slides were allowed to gel at 4°C for 15 minutes. The slides were then immersed in pre-chilled lysis buffer

for 50 minutes at 4°C before being treated with alkaline solution for 30 minutes. The slides were subsequently transferred to a horizontal electrophoresis chamber filled with alkaline electrophoresis solution. The chamber was connected to 22 volt (1 volt/cm) and 300 mA for 25 minutes. Following electrophoresis the slides were washed with deionized water and 70 % ethanol, before being dried over night.

When running the electrophoresis, the choices were between Tris-borate-EDTA electrophoresis and alkaline electrophoresis. Based on the assay sensitivity, the alkaline electrophoresis was chosen. Tris-borate-EDTA electrophoresis is preferred for analysis of apoptosis and will detect single-stranded and double-stranded DNA breaks. In addition, it may detect a few apurinic/apyrimidinic sites. The alkaline electrophoresis however, is more sensitive as it detects single-stranded and double-stranded DNA breaks, the majority of apurinic/apyrimidinic sites, in addition to alkali labile DNA adducts.

2.3.6.4 Image analysis

In order to visualize the DNA in the samples, 50 µL of diluted Vista Green DNA Dye was added to each well, and the slides were incubated at room temperature for 15 minutes. The slides were subsequently examined under a confocal microscope (C2, Nikon, Tokyo, Japan) at a magnification of x10, and pictures were taken with the NIS-Elements Confocal software (Nikon, Tokyo, Japan, Ver. 4.30). When scoring the wells, cells near the edges of the gel, cells in the edges of the picture, superimposed comets, and cells covered by artefacts, were excluded. All of the wells were scored blindly.

The DNA damage is determined by measuring the extent of migration of the genetic material from the nucleus. The two most common parameters used to analyze Comet assay results are the “tail moment” and the “tail DNA %”. As the tail moment considers both the migration of the DNA and the relative amount of DNA in the tail, it is considered an appropriate index of induced DNA damage. In this study, the tail moment was measured as the “extent tail moment”.

- Extent tail moment = tail DNA % x length of tail
- Tail DNA % = 100 x tail DNA intensity / cell DNA intensity

The mean intensity of the cell and the tail, in addition to the length of the tail, was measured using the NIS-Elements Confocal software (Nikon, Tokyo, Japan, Ver. 4.30).

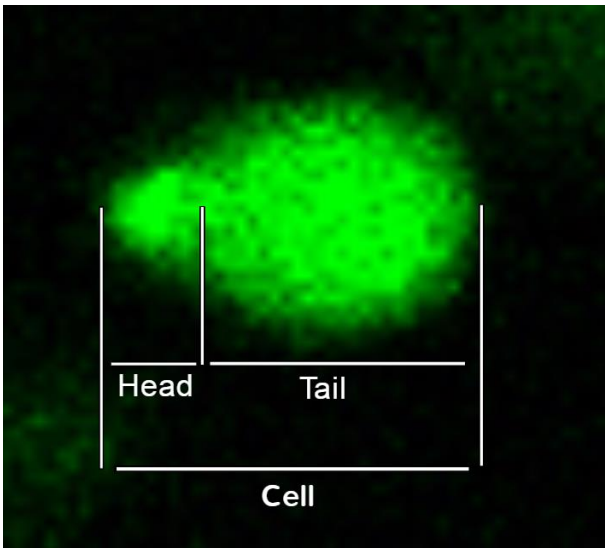

Figure 2.1 A cell showing DNA damage in the Comet assay. The migrated DNA yields a shape of a "comet tail", with the nucleus being the "comet head". The size and shape of the comet, in addition to the distribution of DNA between the "head" and the "tail", corresponds to the amount of DNA damage in the cell.

2.4 Calculations

Specific growth rate (SGR) of Atlantic salmon was calculated with the following equation;

- $SGR = ((\ln W_f - \ln W_i) \times 100) / t$,

Where W_f and W_i are the final weights (tank means) respectively, and t is the time (days) between W_f and W_i.

Feed conversion ratio (FCR) was calculated using the equation

- FCR = feed intake (g) / fish weight gain (g),

where both the feed intake and weight gain were tank means.

Total feed intake for each treatment was calculated from weekly data on feed fed and uneaten feed per tank

The relative distribution of Se among different tissues was calculated as;

- Tissue Se accumulation (%) = (tissue Se (mg) / sum Se in all tissues) * 100

where tissue Se (mg) was calculated as;

- Tissue Se (mg) = concentration (mg/kg dry weight) x weight of tissue (kg).

2.5 Statistical analyses

Graphpad Prism (GraphPad software, Inc., 2015, San Diego, USA, Ver.6) was used for statistical analysis of the data, with the exception of the metabolomics data. In Graphpad Prism, one-way ANOVA was used to test for significant differences among groups, followed by Tukey's multiple comparisons test. Differences were considered significant at $P<0.05$.

For the metabolomics data, nested design ANOVA was used to test for differences among treatments (Ruohonen, 1998) with Statistica software (StatSoft Inc., 2013, Tulsa, USA, Ver.12). In these analyzes, data for each individual fish were nested in replicate treatments. Data were tested for normal distribution, and Levene's test was used to check for homogeneity of variance ($P<0.05$). Furthermore, Tukey's honest significant difference (HSD) was used to test for significant differences among groups.

3. RESULTS

3.1 Growth performance

With the exception of the High inorganic Se group, there were no major differences in final weights and weight gain among treatments after 82 days of feeding (Table 3.1). The fish which were fed the High inorganic Se diet however, showed final weights and weight gain significantly lower than all other groups ($P<0.05$). The feed intake in the High inorganic Se group was also significantly lower than in the groups receiving the other diets.

There were no major differences among treatments in the FCR (Table 3.1) in either growth period one (GP1; day 0-42 of feeding), or two (GP2; day 43-82 of feeding). Fish fed the High inorganic Se diet had significantly lower SGR in GP1 ($P<0.05$) than all of the other groups, except the positive control. In GP2 however, there were no major differences in SGR among any of the treatments.

Table 3.1 Initial and final body weight (g), weight gain (g), FCR (feed conversion ratio), SGR (specific growth rate) and feed intake (kg), presented as mean ± SD (n=3).

	Negative control	Low inorganic Se	High inorganic Se	Low organic Se	High organic Se	Positive control
Initial weight	572 ± 7	581 ± 16	567 ± 21	583 ± 34	583 ± 37	571 ± 35
Final weight	1211 ± 32^{a}	1226 ± 54^{a}	1008 ± 5^{b}	1242 ± 76^{a}	1242 ± 74^{a}	1230 ± 93^{a}
Weight gain	647 ± 52^{a}	631 ± 88^{a}	430 ± 57^{b}	648 ± 72^{a}	637 ± 79^{a}	639 ± 22^{a}
Feed intake	14 ± 1^{a}	15 ± 1^{a}	10 ± 1^{b}	14 ± 1^{a}	14 ± 1^{a}	14 ± 1^{a}
FCR GP1	0.77 ± 0.03	0.78 ± 0.03	0.86 ± 0.01	0.77 ± 0.04	0.8 ± 0.1	0.8 ± 0.1
FCR GP2	1.2 ± 0.4	1.6 ± 0.7	1.4 ± 0.5	1.3 ± 0.3	1.2 ± 0.1	1.0 ± 0.3
SGR GP1	1.15 ± 0.04^{a}	1.18 ± 0.04^{a}	0.88 ± 0.04^{b}	1.2 ± 0.1^{a}	1.2 ± 0.1^{a}	1.1 ± 0.2^{ab}
SGR GP2	0.7 ± 0.1	0.58 ± 0.03	0.5 ± 0.1	0.6 ± 0.2	0.6 ± 0.1	0.7 ± 0.1

Lowercase letters indicate a significant difference between treatment groups ($P<0.05$). Data were analyzed using one-way ANOVA. Selenium (Se) concentration in diets: Negative control: 0.35 mg Se/kg (non-supplemented), Low inorganic Se: 1.1 mg Se/kg (selenite, supplemented), High inorganic Se: 15 mg Se/kg (selenite, supplemented), Low organic Se: 2.1 mg Se/kg (SeMet, supplemented), High organic Se: 15 mg Se/kg (SeMet, supplemented), Positive control: 0.89 mg Se/kg (non-supplemented). SeMet; selenomethionine, GP1; day 0-42 of feeding, GP2; day 43-82 of feeding.

3.2 Hepatic biomarkers of selenium toxicity

3.2.1 Intermediates of the SAM cycle

When considering the SAM cycle and its metabolite intermediates (Table 3.2), a significant reduction ($P<0.05$) was seen in relative levels of methionine, SAH, homocysteine, cysteine and GSH in fish fed the High inorganic Se diet. In addition, levels of cysteine and GSH were significantly reduced in fish fed the High organic Se diet.

Regarding the group receiving the High inorganic Se diet, methionine levels were significantly reduced compared to the Low inorganic Se group and both of the organic Se groups. Levels of SAH

were reduced compared to the Low inorganic Se and Negative control group. Homocysteine showed lower levels in the High inorganic Se group, compared to all the other treatments, except the High organic Se group, while levels of cystathionine were reduced compared to the Positive control. Regarding levels of cysteine, they were significantly lower in both of the High Se groups, compared to all other treatments. In addition, GSH levels in the High inorganic Se group were lower than in all other treatments, except the High organic Se group, while the High organic Se group showed reduced GSH levels compared to the Positive and Negative control. No differences were seen in levels of GSSG among treatment groups.

Table 3.2 Relative levels of the metabolite intermediates in the SAM cycle (scaled intensity values). Presented as mean ± SD (n=3, with 3 fish nested in each replicate tank using nested design ANOVA).

	Negative control	**Low inorganic Se**	**High inorganic Se**	**Low organic Se**	**High organic Se**	**Positive control**
Methionine	1.0 ± 0.1^{ab}	1.1 ± 0.4^{a}	0.8 ± 0.1^{b}	1.3 ± 0.3^{a}	1.2 ± 0.1^{a}	1.0 ± 0.1^{ab}
SAM	1.0 ± 0.3	0.95 ± 0.02	1.2 ± 0.5	1.0 ± 0.6	0.9 ± 0.5	1.1 ± 0.4
SAH	1.3 ± 0.3^{a}	1.4 ± 0.2^{a}	0.7 ± 0.3^{b}	1.0 ± 0.3^{ab}	1.0 ± 0.1^{ab}	1.2 ± 0.3^{ab}
Homocysteine	1.1 ± 0.2^{ac}	1.2 ± 0.2^{a}	0.3 ± 0.1^{b}	1.0 ± 0.3^{ac}	0.8 ± 0.2^{bc}	1.12 ± 0.04^{ac}
Cystathionine	1.3 ± 0.3^{ab}	1.1 ± 0.1^{ab}	0.6 ± 0.2^{b}	1.5 ± 0.5^{ab}	1.2 ± 0.8^{ab}	1.6 ± 0.4^{a}
Cysteine	1.5 ± 0.2^{a}	1.2 ± 0.3^{a}	0.3 ± 0.1^{b}	1.2 ± 0.1^{a}	0.6 ± 0.1^{b}	1.4 ± 0.1^{a}
GSH	1.4 ± 0.4^{a}	1.2 ± 0.5^{ac}	0.2 ± 0.1^{b}	1.2 ± 0.6^{ac}	0.7 ± 0.4^{bc}	1.5 ± 0.3^{a}
GSSG	1.1 ± 0.2	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.1

Lowercase letters indicates significant differences between treatment groups (P<0.05). Selenium (Se) concentration in diets: Negative control: 0.35 mg Se/kg (non-supplemented), Low inorganic Se: 1.1 mg Se/kg (selenite, supplemented), High inorganic Se: 15 mg Se/kg (selenite, supplemented), Low organic Se: 2.1 mg Se/kg (SeMet, supplemented), High organic Se: 15 mg Se/kg (SeMet, supplemented), Positive control: 0.89 mg Se/kg (non-supplemented). SeMet; selenomethionine, SAM; S-Adenosylmethionine, SAH; S-Adenosylhomocysteine, GSH; reduced glutathione, GSSG; oxidized glutathione.

3.2.2 Biomarkers of oxidative stress

After GP1, there were no major differences among treatments in concentrations of any of the tocopherols (Table 3.3). After GP2 however, fish receiving the High inorganic Se treatment, showed levels of α-tocopherol significantly lower (P<0.05) than the other groups, except from the Positive control. The High inorganic Se group also showed the lowest levels of β- and γ-tocopherols. Levels of β-tocopherol were significantly reduced compared to the Low inorganic Se group and the Negative control, while levels of γ-tocopherol were reduced compared to the same groups, in addition to the High organic Se treatment.

Furthermore, no major differences in TBARS concentrations were seen among treatments after GP1 (Table 3.3). After GP2 however, the highest TBARS levels were measured in the High inorganic Se group, significantly higher than the other treatments, except from the High organic Se group.

Fish fed the High inorganic Se diet, additionally showed the lowest levels of ascorbate (Vitamin C) (Table 3.3). These levels were significantly lower than in the other treatments, with the exception of the groups receiving organic Se diets.

Table 3.3 Hepatic levels of α-, β-, and γ-tocopherol (mg kg^{-1} ww), TBARS (nmol g^{-1} ww) and ascorbate (scaled intensity values), after growth period (GP) 1 and 2, presented as mean ± SD (n=3).

	Negative control	Low inorganic Se	High inorganic Se	Low organic Se	High organic Se	Positive control
α-tocopherol GP1	1130 ± 209	987 ± 131	1117 ± 104	1070 ± 281	1173 ± 280	917 ± 84
α-tocopherol GP2	1613 ± 240^{a}	1557 ± 287^{a}	753 ± 181^{b}	1453 ± 176^{a}	1413 ± 21^{a}	1240 ± 89^{ab}
β-tocopherol GP1	1.2 ± 0.4	1.2 ± 0.1	1.0 ± 0.3	1.0 ± 0.2	1.2 ± 0.4	1.0 ± 0.2
β-tocopherol GP2	1.5 ± 0.3^{a}	1.4 ± 0.4^{a}	0.3 ± 0.4^{b}	0.8 ± 0.7^{ab}	1.1 ± 0.2^{ab}	1.4 ± 0.4^{ab}
γ-tocopherol GP1	61 ± 10	52 ± 7	48 ± 6	55 ± 13	61 ± 16	41 ± 5
γ-tocopherol GP2	75 ± 12^{a}	67 ± 7^{a}	32 ± 7^{b}	60 ± 19^{ab}	64 ± 7^{a}	51 ± 2^{ab}
Ascorbate	1.2 ± 0.6^{a}	1.3 ± 0.4^{a}	0.5 ± 0.1^{b}	1.0 ± 0.3^{ab}	0.8 ± 0.4^{ab}	1.4 ± 0.7^{a}
TBARS GP1	4.1 ± 0.1	4.0 ± 0	4.4 ± 0.3	4.0 ± 0	4.1 ± 0.2	4.7 ± 0.8
TBARS GP2	4.0 ± 0^{a}	4.0 ± 0.1^{a}	5.2 ± 0.4^{b}	4.3 ± 0.3^{a}	4.4 ± 0.6^{ab}	4.1 ± 0.2^{a}

Lowercase letters indicate a significant difference among groups (P<0.05). Data on tocopherols and TBARS were analyzed by one-way ANOVA. Data on ascorbate were analyzed using nested design ANOVA (n=3, with 3 fish nested in each replicate tank). Selenium (Se) concentration in diets: Negative control: 0.35 mg Se/kg (non-supplemented), Low inorganic Se: 1.1 mg Se/kg (selenite, supplemented), High inorganic Se: 15 mg Se/kg (selenite, supplemented), Low organic Se: 2.1 mg Se/kg (SeMet, supplemented), High organic Se: 15 mg Se/kg (SeMet, supplemented), Positive control: 0.89 mg Se/kg (non-supplemented). SeMet; selenomethionine, GP1; day 0-42 of feeding; GP2; day 43-82 of feeding, TBARS; Thiobarbituric acid-reactive substances.

3.3 Biomarkers of oxidative damage

The results from the Comet assay are shown in Table 3.4. The ratio of cells with and without a comet tail from the first round of analyses show a high variation within some of the groups. When considering the extent tail moment, there was little variation among the treatments, however the high inorganic Se group showed the highest levels of DNA migration. The results from the second round of the Comet assay showed very little variation among dietary treatments, considering both the ratio between cells with and without a tail, and the extent tail moment.

Table 3.4 Ratio between cells with and without a comet tail (%) and extent tail moment (ETM) in round one (R_1) and two (R_2) of Comet assay. Data are presented as mean ± SD (n=3).

	Negative control	Low inorganic Se	High inorganic Se	Low organic Se	High organic Se
% Comets R_1	8 ± 4	17 ± 13	36 ± 6	7 ± 3	13 ± 19
ETM R_1	3555 ± 403	4550 ± 851	5213 ± 2034	4352 ± 1234	4791 ± 1423
% Comets R_2	48 ± 17	30 ± 17	45 ± 8	40 ± 10	43 ± 12
ETM R_2	5426 ± 1258	5034 ± 624	5130 ± 1299	5800 ± 738	4965 ± 1136

Selenium (Se) concentration in diets: Negative control: 0.35 mg Se/kg (non-supplemented), Low inorganic Se: 1.1 mg Se/kg (selenite, supplemented), High inorganic Se: 15 mg Se/kg (selenite, supplemented), Low organic Se: 2.1 mg Se/kg (SeMet, supplemented), High organic Se: 15 mg Se/kg (SeMet, supplemented), Positive control: 0.89 mg Se/kg (non-supplemented). SeMet; selenomethionine.

3.4 Biomarkers of energy expenditure and appetite

3.4.1 Endocannabinoids

Endocannabinoids are endogenous neuromodulators derived from fatty acids, and levels of several endocannabinoids were detected in the metabolomics screening. Results on three endocannabinoids are included here. Significantly reduced levels ($P<0.05$) of endocannabinoids were seen in fish fed the High Se treatments (Table 3.5). Oleoylethanolamide showed lower levels in the High inorganic Se treatment compared to the Low organic Se group and the Negative control. In addition, the High organic Se group showed reduced levels compared to the Low organic Se group. Levels of N-palmitoyltaurine were reduced in the High inorganic Se group compared to the Low organic Se group and the Positive control, while the organic Se group showed the same trend as with oleoyl-ethanolamide. Fish fed the High organic Se diet showed no reduction in N-oleoyltaurine, while levels were reduced in the High inorganic Se group when compared to the group receiving the Low organic Se diet.

Table 3.5 Relative levels of endocannabinoids (scaled intensity values) among the different treatments. Presented as mean ± SD (n=3).

	Negative control	Low inorganic Se	High inorganic Se	Low organic Se	High organic Se	Positive control
Oleoyl-ethanolamide	1.5 ± 0.3^{ac}	1.1 ± 0.1^{ab}	0.4 ± 0.1^{b}	2 ± 1^{a}	0.9 ± 0.3^{bc}	1.2 ± 0.4^{ab}
N-palmitoyl-taurine	1.3 ± 0.3^{ac}	1.1 ± 0.3^{ac}	0.4 ± 0.2^{c}	2 ± 1^{a}	0.6 ± 0.3^{bc}	2 ± 1^{ab}
N-oleoyltaurine	1.5 ± 0.5^{ab}	1.0 ± 0.5^{ab}	0.1 ± 0.1^{b}	2 ± 1^{a}	0.5 ± 0.3^{ab}	1.5 ± 0.5^{ab}

Lowercase letters indicates significant differences among treatments ($P<0.05$). Data were analyzed using nested design ANOVA (n=3, with 3 fish nested in each replicate tank). Selenium (Se) concentration in diets: Negative control: 0.35 mg Se/kg (non-supplemented), Low inorganic Se: 1.1 mg Se/kg (selenite, supplemented), High inorganic Se: 15 mg Se/kg (selenite, supplemented), Low organic Se: 2.1 mg Se/kg (SeMet, supplemented), High organic Se: 15 mg Se/kg (SeMet, supplemented), Positive control: 0.89 mg Se/kg (non-supplemented). SeMet; selenomethionine.

3.4.2 Lipid biomarkers of energy expenditure

The metabolomics screening detected a variety of lipid metabolites in fish fed the six experimental diets, including the selection of lipid metabolites presented here. In livers from fish receiving the High inorganic Se diet, significantly reduced levels ($P<0.05$) of the free fatty acids palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1n9) linoleic acid (LA, 18:2n6) and linolenic acid (LNA, 18:3n3) were seen (Table 3.6). In this treatment group, reduced levels of palmitic acid were found when compared to both the Positive and Negative control, and the Low organic Se group. In addition, reduced levels of palmitic acid were seen in the High organic Se group when compared to the Positive control. In the High inorganic Se group, stearic acid showed the same trend as palmitic acid, and reduced levels of both oleic acid, LA and LNA were seen when compared to all the other treatments, with the

exception of the High organic Se group. Considering eicosapentaenoic acid (EPA, 20:5n3), docosahexaenoic acid (DHA, 22:6n3) and arachidonic acid (ARA, 20:4n6) no major differences were seen among treatments. However, reduced levels of EPA were found in the High inorganic Se group, when compared to the Positive control.

Levels of the monoacylglycerol 1-olyoylglycerol (18:1) were significantly reduced in fish fed the High inorganic Se diet, compared to all of the treatments, except the High organic Se group (Table 3.6). The concentration of the diacylglycerol 1-oleoyl-2-linoleoyl-glycerol (18:1/18:2) were also significantly reduced in the High inorganic Se group, compared to the other treatments, with the exception of the High organic Se- and Positive control group. Levels of 18:1/18:2 in the High organic Se group were significantly lower than in the Low inorganic Se treatment.

Furthermore, Coenzyme A levels were significantly reduced in fish fed the High inorganic Se diet, when compared to the Positive and Negative control, in addition to the Low inorganic Se group. The High organic Se group had reduced levels of Coenzyme A, when compared to the Negative control (Table 3.6).

Table 3.6 Relative levels of free fatty acids, mono- and diacylglycerols and Coenzyme-A (scaled intensity values) among the different groups. Presented as mean ± SD (n=3).

	Negative control	Low inorganic Se	High inorganic Se	Low organic Se	High organic Se	Positive control
Palmitic acid	1.0 ± 0.1^{b}	0.9 ± 0.1^{ac}	0.6 ± 0.1^{c}	1.1 ± 0.3^{ab}	0.8 ± 0.3^{bc}	1.2 ± 0.2^{a}
Stearic acid	1.0 ± 0.1^{a}	0.9 ± 0.2^{ab}	0.5 ± 0.2^{b}	1.2 ± 0.3^{a}	0.8 ± 0.3^{ab}	1.1 ± 0.2^{a}
Oleic acid	1.1 ± 0.1^{a}	1.0 ± 0.2^{a}	0.5 ± 0.1^{b}	1.1 ± 0.2^{a}	0.8 ± 0.2^{ab}	1.0 ± 0.2^{a}
Linoleic acid	1.1 ± 0.2^{a}	1.0 ± 0.2^{a}	0.5 ± 0.1^{b}	1.1 ± 0.2^{a}	0.8 ± 0.2^{ab}	1.0 ± 0.1^{a}
Linolenic acid	1.2 ± 0.2^{a}	1.1 ± 0.3^{a}	0.5 ± 0.1^{b}	1.2 ± 0.2^{a}	0.8 ± 0.2^{ab}	1.0 ± 0.1^{ab}
Arachidonic acid	1.1 ± 0.2	1.1 ± 0.5	0.9 ± 0.4	1.2 ± 0.1	0.8 ± 0.2	1.1 ± 0.2
EPA	1.0 ± 0.1^{ab}	0.8 ± 0.2^{ab}	0.7 ± 0.2^{b}	1.0 ± 0.3^{ab}	0.9 ± 0.2^{ab}	1.2 ± 0.2^{a}
DHA	1.0 ± 0.1	0.9 ± 0.2	0.9 ± 0.3	1.0 ± 0.2	0.8 ± 0.1	1.2 ± 0.2
1-oleoylglycerol	1.2 ± 0.2^{a}	1.1 ± 0.3^{a}	0.3 ± 0.1^{b}	1.1 ± 0.1^{a}	0.8 ± 0.5^{ab}	1.4 ± 0.3^{a}
1-oleoyl-2-linoleoyl-glycerol	1.32 ± 0.04^{ac}	1.5 ± 0.5^{a}	0.6 ± 0.1^{b}	1.3 ± 0.1^{ac}	1.0 ± 0.2^{bc}	0.9 ± 0.1^{bc}
Coenzyme A	1.3 ± 0.2^{a}	0.9 ± 0.5^{ac}	0.27 ± 0.04^{b}	0.7 ± 0.5^{ab}	0.7 ± 0.3^{bc}	1.0 ± 0.2^{ac}

Lowercase letters indicates significant differences among groups (P<0.05). Data were analyzed using nested design ANOVA (n=3, with 3 fish nested in each replicate tank). Selenium (Se) concentration in diets: Negative control: 0.35 mg Se/kg (non-supplemented), Low inorganic Se: 1.1 mg Se/kg (selenite, supplemented), High inorganic Se: 15 mg Se/kg (selenite, supplemented), Low organic Se: 2.1 mg Se/kg (SeMet, supplemented), High organic Se: 15 mg Se/kg (SeMet, supplemented), Positive control: 0.89 mg Se/kg (non-supplemented). SeMet; selenomethionine, EPA; eicosapentaenoic acid, DHA; docosahexaenoic acid.

3.4.3 Lipid stores in whole fish homogenate

No major differences were found among groups in levels of total body fat in the fish after GP1 (Figure 3.1). After GP2, fish fed the High inorganic Se diet had the lowest levels of body fat, however only

significantly different (P<0.05) from the Low organic Se group, which had the highest levels of total body fat.

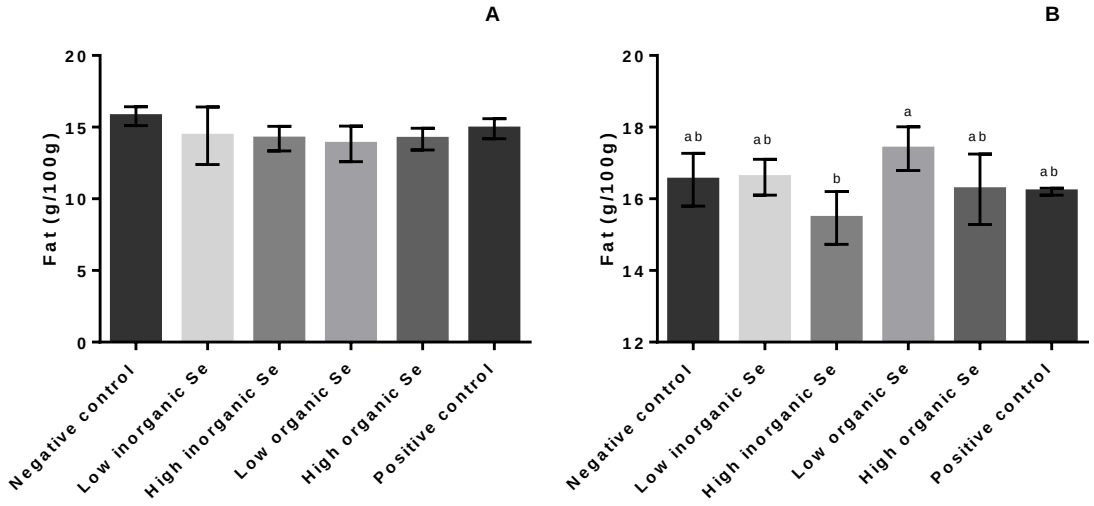

Figure 3.1 Total body fat (g/100g) in whole fish homogenate. A) GP1 (0-42 days of feeding) B) GP2 (43-82 days of feeding). Presented as mean ± SD (n=3) Lowercase letters indicate a significant difference among dietary treatments (P<0.05). Data were analyzed using one-way ANOVA. Selenium (Se) concentration in diets: Negative control: 0.35 mg Se/kg (non-supplemented), Low inorganic Se: 1.1 mg Se/kg (selenite, supplemented), High inorganic Se: 15 mg Se/kg (selenite, supplemented), Low organic Se: 2.1 mg Se/kg (SeMet, supplemented), High organic Se: 15 mg Se/kg (SeMet, supplemented), Positive control: 0.89 mg Se/kg (non-supplemented). SeMet; selenomethionine.

3.4.4 Biomarkers of protein catabolism

Many amino acids were detected in the metabolomics screening and those that were representative of protein catabolism are presented here. Regarding the intermediates in the Urea cycle (Table 3.7), both of the organic Se treatments had significantly elevated levels (P<0.05) of ornithine and citrulline. In the Low organic Se group ornithine levels were elevated compared to all of the treatments, except from the High organic Se group. The High organic Se group showed elevated levels compared to the Low inorganic Se group. Levels of citrulline were higher in the Low organic Se group, when compared to the Positive control, while the High organic Se group showed elevated levels compared to both of the control groups. No major differences were seen in levels of argininosuccinate, however, a significant difference were seen between the High inorganic Se and the Low organic Se groups, showing the lowest and highest levels of argininosuccinate respectively. Levels of arginine were significantly lower in the High inorganic Se group, compared to the Low Se groups. Moreover, levels of urea were elevated in the four groups fed diets supplemented with Se. However, levels were only significantly higher in fish fed the organic Se diets, when compared to both of the control groups.

Table 3.7 Relative levels of intermediates in the Urea cycle (scaled intensity values) among the different treatments, presented as mean ± SD (n=3).

	Negative control	Low inorganic Se	High inorganic Se	Low organic Se	High organic Se	Positive control
Ornithine	0.95 ± 0.03^{bc}	0.9 ± 0.2^{c}	0.9 ± 0.2^{bc}	1.3 ± 0.3^{a}	1.2 ± 0.2^{ab}	0.9 ± 0.1^{bc}
Citrulline	0.9 ± 0.1^{bc}	1.1 ± 0.2^{ab}	1.1 ± 0.2^{ab}	1.3 ± 0.2^{ac}	1.4 ± 0.3^{a}	0.75 ± 0.03^{b}
Argininosuccinate	1.1 ± 0.1^{ab}	1.1 ± 0.4^{ab}	0.6 ± 0.2^{b}	1.6 ± 0.5^{a}	0.9 ± 0.4^{ab}	1.0 ± 0.1^{ab}
Arginine	1.1 ± 0.1^{ab}	1.2 ± 0.5^{a}	0.7 ± 0.1^{b}	1.2 ± 0.3^{a}	1.0 ± 0.1^{ab}	1.0 ± 0.1^{ab}
Urea	0.77 ± 0.03^{b}	1.1 ± 0.7^{ab}	1.2 ± 0.3^{ab}	1.6 ± 1.1^{a}	1.5 ± 0.5^{a}	0.8 ± 0.1^{b}

Lowercase letters indicate a significant difference among dietary treatments (P<0.05). Data were analyzed using Nested design ANOVA (n=3 with 3 fish nested in each replicate tank). Selenium (Se) concentration in diets: Negative control: 0.35 mg selenium/kg (non-supplemented), Low inorganic Se: 1.1 mg Se/kg (selenite, supplemented), High inorganic Se: 15 mg Se/kg (selenite, supplemented), Low organic Se: 2.1 mg Se/kg (SeMet, supplemented), High organic Se: 15 mg Se/kg (SeMet, supplemented), Positive control: 0.89 mg Se/kg (non-supplemented). SeMet; selenomethionine

3.5 Selenium accumulation

3.5.1 Selenium in whole fish homogenate

Analyses of Se in whole fish homogenate showed a significantly higher (P<0.05) Se accumulation in fish fed High Se diets, compared to those fed the Low Se diets (Figure 3.2). The highest Se concentrations were found in fish fed the High organic Se treatment. This group also had significantly higher Se accumulation than the group receiving the High inorganic Se diet. Among the groups receiving the Low Se diets, there were no major differences in Se concentrations.

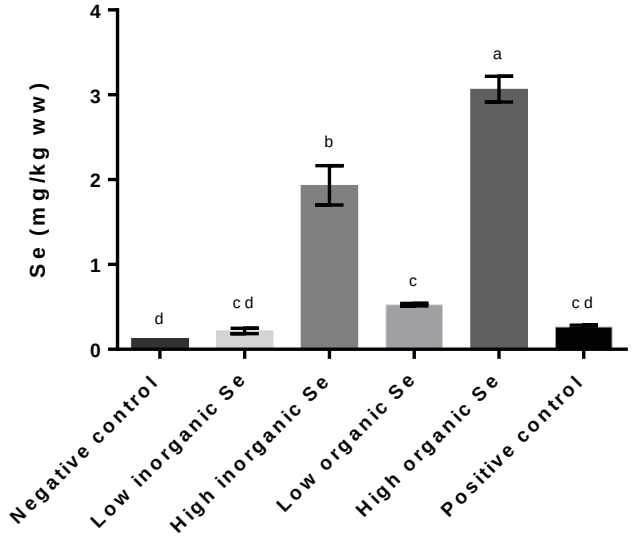

Figure 3.2 Concentrations (mg/kg ww) of selenium (Se) in whole fish homogenates for the different treatments. Presented as mean ± SD (n=3). Lowercase letters indicates significant differences among treatments (P<0.05). Data were analyzed using one-way ANOVA. Selenium concentration in diets: Negative control: 0.35 mg selenium/kg (non-supplemented), Low inorganic Se: 1.1 mg Se/kg (selenite, supplemented), High inorganic Se: 15 mg Se/kg (selenite, supplemented), Low organic Se: 2.1 mg Se/kg (SeMet, supplemented), High organic Se: 15 mg Se/kg (SeMet, supplemented), Positive control: 0.89 mg Se/kg (non-supplemented). SeMet; selenomethionine.

3.5.2 Tissue distribution of selenium

Fish fed the Low organic Se diet had significantly higher (P<0.05) Se concentrations in all tissues, compared to fish fed the Low inorganic Se diet and the Positive control (Figure 3.3). There were no major differences between the inorganic Se group and the positive control in most of the tissues, however in gills, muscle and carcass, the positive control group showed significantly higher Se concentrations. The distribution of organic Se among different tissues was liver >> kidney > intestine > spleen > heart > gills > brain > eye > muscle > carcass. For both inorganic Se and the positive control, the distribution was liver > kidney > intestine > spleen > brain > gills > eye > heart > carcass > muscle. However, the Positive control had identical concentrations in muscle and carcass.

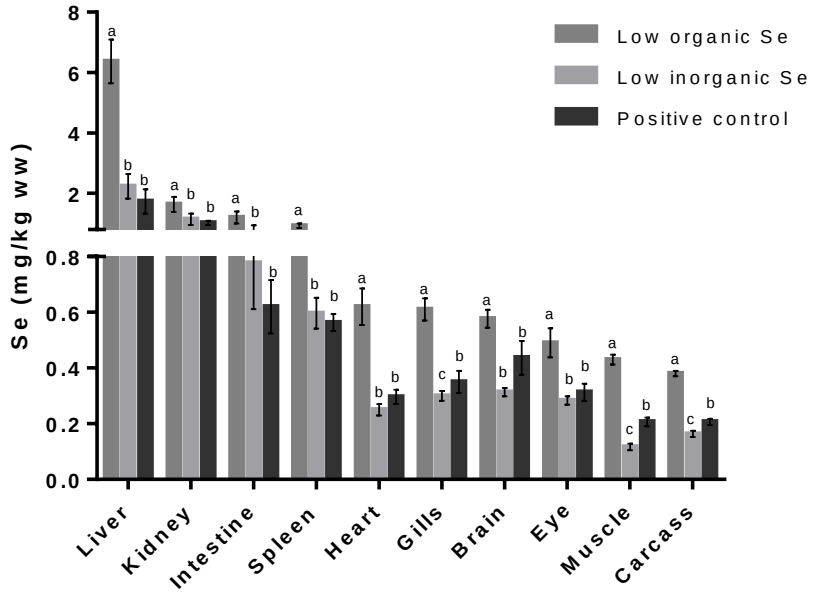

Figure 3.3 Selenium (Se) concentrations in tissues from fish fed a Low inorganic Se diet (selenite, 1.1 mg Se/kg), a Low organic Se diet (selenomethionine, 2.1 mg Se/kg) or a Positive control diet (0.89 mg Se/kg). Data are presented as mean ± SD (n=3). Lowercase letters indicates significant differences among treatments, within each tissue (P<0.05). Data were analyzed using one-way ANOVA.

The relative distribution (%) of Se among different tissues was muscle > intestine > carcass > liver >> kidney > gills >> eye > spleen > heart > gonads > brain for all dietary treatments (Figure 3.4). Fish fed the Low organic Se diet had more Se in muscle than fish fed the Low inorganic Se diet. In the intestine, kidney and spleen the order was reversed, as the relative amount of Se in these tissues was higher in the inorganic Se group, compared to the organic Se group. The Positive control group had a similar distribution to the organic Se group in muscle, intestine and spleen. Moreover, the positive control group had similar distribution to that of the inorganic Se group in intestine and eye, and levels were significantly lower than both groups in liver selenium concentration. The relative amounts of Se in the remaining tissues, showed no major differences among fish fed the Se diets.

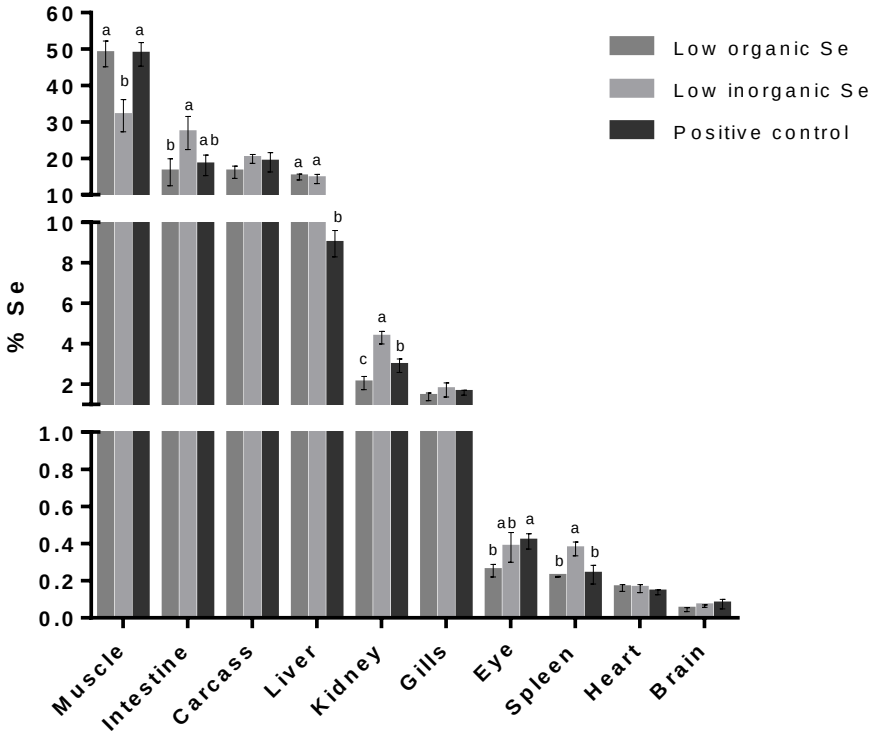

Figure 3.4 Relative distribution (%) of selenium (Se) among tissues from fish fed a Low inorganic Se diet (selenite, 1.1 mg Se/kg), a Low organic Se diet (selenomethionine, 2.1 mg Se/kg) or a Positive control diet (0.89 mg Se/kg). Data are presented as mean ± SD (n=3). Lowercase letters indicates significant differences among treatments, within each tissue ($P<0.05$). Data were analyzed using one-way ANOVA.

4. DISCUSSION

In the present study, results indicated that the highest dietary levels of Se induced a reduction in the antioxidant capacity of the fish. Fish fed the High inorganic Se diet showed decreased levels of the antioxidants GSH, vitamin E and vitamin C. The same group had elevated levels of TBARS, a lipid peroxidation byproduct, confirming impaired protection against lipid peroxidation. Furthermore, fish fed the High inorganic Se diet had the lowest body weights at the end for the feeding trial. Fish fed the High organic Se diet also had reduced levels of GSH, however this group showed no changes in levels of vitamin E, vitamin C and TBARS. It thus appears that oxidative stress was the toxic mode of action of dietary Se, and that inorganic Se had a higher toxic potential than organic Se.

4.1 Oxidative stress in aquaculture

There are many factors essential to assure good health and welfare of fish in aquaculture, and managing stress is a decisive one. Stress, defined by Wendelaar Bonga (1997) is *"a condition in which the dynamic equilibrium of animal organisms, called homeostasis, is threatened or disturbed as a result of the actions of intrinsic or extrinsic stimuli, commonly defined as stressors"*.

Farmed fish are continuously exposed to a variety of stressors, including changes in temperature and light regime, and poor water quality (Conte, 2004). Stress may be caused by factors related to feed (e.g. toxic levels of a nutrient) and feeding (Conte, 2004), and the population density may be critical, as both too low or too high densities of fish may be stressful (North et al., 2006). Moreover, stressors includes some unavoidable husbandry routines, including handling and pumping during procedures like grading, vaccination and transport (Wendelaar Bonga, 1997, Conte, 2004).

Although stressors may pose a threat to, or disturb the homeostasis, stressors also evoke a stress response making the fish able to maintain or re-establish the homeostatic equilibrium (Wendelaar Bonga, 1997). The stress response includes both behavioral and physiological responses, and although it is a complex one, there appears to be a connection between the stress response and oxidative stress (Liu and Mori, 1999). In short, a balance in, and interactions among stress mediators like hormones, neurotransmitters and oxidants, regulates the stress response. As stress increases the metabolic rate of the fish, increased production of ROS may result from an imbalance in, and over-interaction among the stress mediators, further resulting in oxidative stress. Increased production of ROS may result from catabolic processes triggered by stress hormones, as well as from oxidation of these hormones (Liu and Mori, 1999). Moreover, as both physical and oxidative stress leaves the fish more prone to infections and diseases (Wendelaar Bonga, 1997), it is of great importance to keep stressors at a minimum. However, as stressors never can be fully eliminated, it is essential that the

fish have a good nutritional status. A good nutritional status with sufficient amounts of vital antioxidants will provide the fish with a high antioxidant capacity and a robust defense against oxidative stress (Martínez-Álvarez et al., 2005).

4.1.1 The pro-oxidant activity of selenium

Selenium is an essential mineral that plays an important role in the antioxidant defense of the fish, in being a constituent of GPx (Rotruck et al., 1973). However, although sufficient amounts of Se are essential for optimal fish health, Se is known to be toxic at levels barely exceeding the requirement (Janz, 2012). In addition to its function as an antioxidant, Se can itself contribute to oxidative stress through its pro-oxidant activity (Spallholz et al., 2004, Mézes and Balogh, 2009). Possessing the paradoxical role as both an antioxidant and a pro-oxidant is not a trait exclusive to Se, as antioxidants such as vitamin C and vitamin E also have this feature (Villanueva and Kross, 2012). The pro-oxidant activity of an antioxidant will depend on its concentration and its redox potential, in addition to the presence of redox metals and other antioxidants (Villanueva and Kross, 2012). The pro-oxidant activity of Se is thought to arise from its ability to oxidize thiols, and both selenite and SeMet have been observed to cause oxidative stress in hepatocytes of rainbow trout *in vitro* (Misra and Niyogi, 2009, Misra et al., 2012). However, little is known about the pro-oxidant activity of Se in Atlantic salmon.

4.2 Biomarkers of selenium toxicity

Reactive oxygen species and other free radicals responsible for oxidative damage, are highly reactive and exists only for a short moment of time. In biological systems, OH• can survive for 10^{-10} seconds, while some radicals has a life span of seconds or minutes (Kohen and Nyska, 2002). Direct measurements of ROS to detect oxidative stress is therefore a difficult approach (Kohen and Nyska, 2002). The antioxidant capacity of the organism, as well as markers of antioxidant damage however, are useful indicators of oxidative stress. The antioxidant capacity of the fish comprises the status of exogenous and endogenous antioxidants and their capacity to withstand oxidative stress. A high antioxidant capacity will provide the fish with a sufficient and robust defense against ROS (Koivula and Eeva, 2010). The antioxidant capacity does not give a direct measure of oxidative stress, but as elevated levels of ROS can deplete antioxidants, a depletion may indicate that the antioxidants have acted in defense and scavenged ROS (Koivula and Eeva, 2010).

4.2.1 Reduced antioxidant capacity as a result of oxidative stress

Glutathione is considered one of the most important endogenous antioxidants and participates both directly and indirectly in the scavenging of ROS. Glutathione may directly react with and neutralize ROS in reactions generating glutathionyl radicals, which may further form GSSG (Ross et al., 1985).

Glutathione may also act as a reductant of disulfides and protect protein sulfhydryls, thus regulating the composition of protein thiols and disulfides, and acting as a redox buffer of the cell (Deneke, 2000). Furthermore, GSH functions as a cofactor for GPx, which catalyzes the reduction of H_2O_2 and lipid peroxides, with the subsequent oxidation of GSH to GSSG (Lu, 2012).

To maintain the levels of reduced GSH for continued antioxidant defense, a redox cycle is formed, in that GSSG is reduced back to GSH by glutathione reductase (Lu, 2012). During normal physiological conditions, the regeneration from GSSG occurrs continuously, and thus maintains levels of GSH required at normal levels of ROS (Griffith, 1999). Under such non-stressed conditions, the GSH/GSSG ratio may be above 100:1 (Zitka et al., 2012). Oxidative stress however, can rapidly reduce GSH levels in favor of GSSG. The result is an accumulation of GSSG, causing the GSH/GSSG ratio to decrease several fold, and this ratio is thus frequently used as a marker of oxidative stress (Griffith, 1999, Zitka et al., 2012).

In the present study, no changes in hepatic levels of GSSG was seen in any of the six dietary groups, indicating an absence of GSSG accumulation. Elevated levels of Se did however influence GSH, as fish fed both the High inorganic Se diet and the High organic Se diet, had significantly reduced hepatic GSH levels. The absence of GSSG accumulation, may be explained by GSSG secretion out of the cell, a consequence of GSSG accumulation observed during oxidative stress (Griffith, 1999). The loss of GSSG could further impair the regeneration of GSH, and oxidative stress would hence increase the demand of GSH synthesized *de novo* (Griffith, 1999). Another possibility is that GSH was depleted without being oxidized to GSSG. Indeed, GSH can form adducts with electrophilic intermediates directly, leading to GSH depletion without concomitant formation of GSSG (Blair, 2006). This implies that the ratio GSH/GSSG may not be the most suitable biomarker for Se toxicity, but rather that GSH alone or GSH-adducts such as thiadiazabicyclo-4-oxo-2(E)-nonenal-GSH (Blair, 2006) might be more suited.

Glutathione is synthesized *de novo* via the SAM cycle and the trans-sulfuration pathway from the amino acids cysteine, glutamate and glycine. The first step is the formation of γ-glutamylcysteine from glutamate and cysteine, before GSH is formed from γ-glutamylcysteine and glycine. The first step is rate limiting and catalyzed by glutamate-cysteine ligase (GCL), which under non-stressed conditions is regulated by feedback inhibition of GSH and the availability of cysteine. During oxidative stress however, the GCL activity increases to produce more GSH (Lu, 2012). Thus, during oxidative stress, an upregulation of GSH synthesis will occur, until GSH inhibits its own synthesis, or until the precursors becomes a limiting factor.

In the present study, fish fed the High inorganic Se diet, had a significant depletion of the intermediates in the SAM cycle, in addition to reduced GSH levels, in liver (Figure 4.1). These results indicate a shift in equilibrium, in that there may have been an increased GSH production, depleting its precursors. Fish fed the High organic Se diet also showed significantly reduced levels of GSH and cysteine, indicating elevated production of GSH to combat oxidative stress in this group as well. The fact that the High organic Se group did not show depletion of the other intermediates in the SAM cycle, may be due to higher levels of methionine present as SeMet. As there is no discrimination between methionine and SeMet (Ochoa-Solano and Gitler, 1968), there may have been more substrate available to maintain the cycle. Subsequently, a high synthesis rate of GSH as a response to higher GSH consumption, may have depleted cysteine since SeCys has an alternative pathway through β-lyase transformation to H_2Se (Figure 1.1) and probably can not be used to form cysteine.

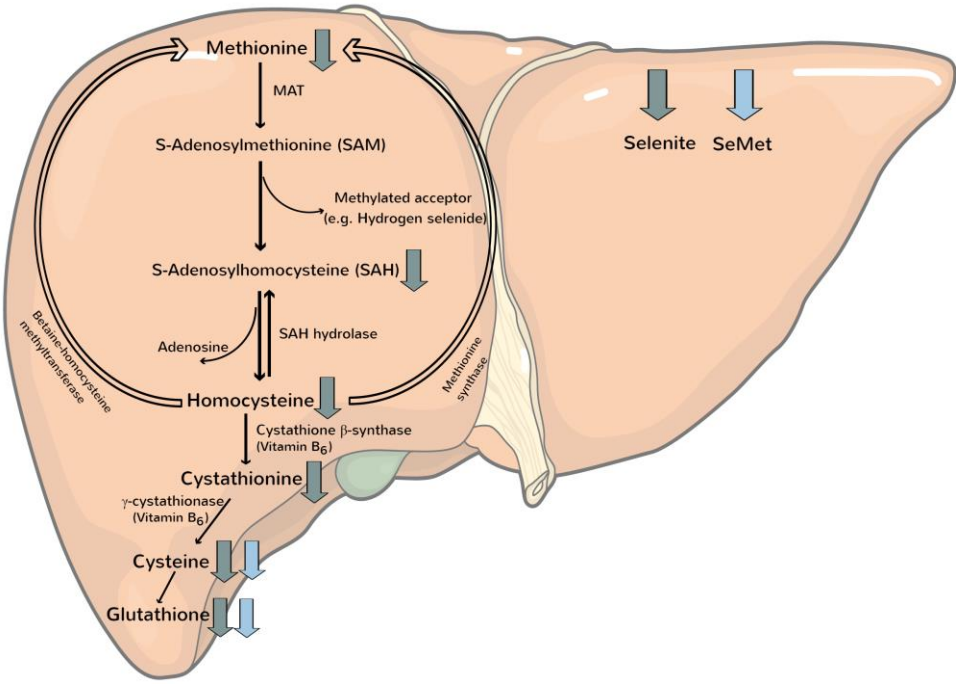

Figure 4.1 The S-adenosylmethionine (SAM) cycle provides precursors for the production of the non-essential amino acid cysteine and the antioxidant glutathione. SAM is also an important methyl-donor for hydrogen selenide. This figure shows how organic Se (selenomethionine, SeMet) and inorganic Se (selenite) affect the intermediates in the SAM cycle (here presented in a human liver). The green arrow represents reduced levels of the corresponding metabolite in fish fed the High inorganic Se diet (15 mg Se/kg). The blue arrow represents reduced levels of the corresponding metabolite in fish fed the High organic Se diet (15 mg Se/kg).

The SAM cycle also plays an important role in the methylation of Se, in that SAM serves as a methyl donor for H_2Se. As methylation of Se is considered a mechanism of detoxification (Nakamuro et al., 2000), there may be a high demand for SAM at excessive Se exposure. The utilization of SAM as a

methyl donor, may thus have contributed to the depletion of the intermediates in the cycle. In that any form of Se has to be transformed into H_2Se in order to be methylated by SAM, may also explain the difference in depleted intermediates between the High organic Se and High inorganic Se groups. The metabolism of selenite and SeMet are quite different, and while selenite is reduced to H_2Se directly and immediately after absorption, SeMet may have several metabolic fates (Figure 1.1) (Thiry et al., 2012). The fact that SeMet can be stored in general body proteins, while selenite that is not utilized for selenoproteins synthesis can not (Thiry et al., 2012), may lead to a greater need to detoxify selenite than SeMet. If that is the case, more methionine will be utilized to generate SAM for the detoxification of selenite, and may deplete the methionine stores. A depletion of methionine will further deplete the downstream intermediates in the cycle, and finally GSH as the final product.

Fish fed the High inorganic Se diet also had significantly reduced hepatic levels of vitamin E and vitamin C, and the reduced levels of GSH may partly explain the depletion of these two antioxidants. To provide protection against ROS, GSH works together with vitamin E and vitamin C, making up an antioxidant network (Figure 4.2) (Meeteren et al., 2005). In this network, GSH serves as a hydrogen donor for vitamin C. When donating a hydrogen atom to the oxidized form of vitamin C, dehydroascorbic acid, GSH causes vitamin C to continuously be regenerated, and vitamin C further serves in the regeneration of vitamin E (Meeteren et al., 2005).

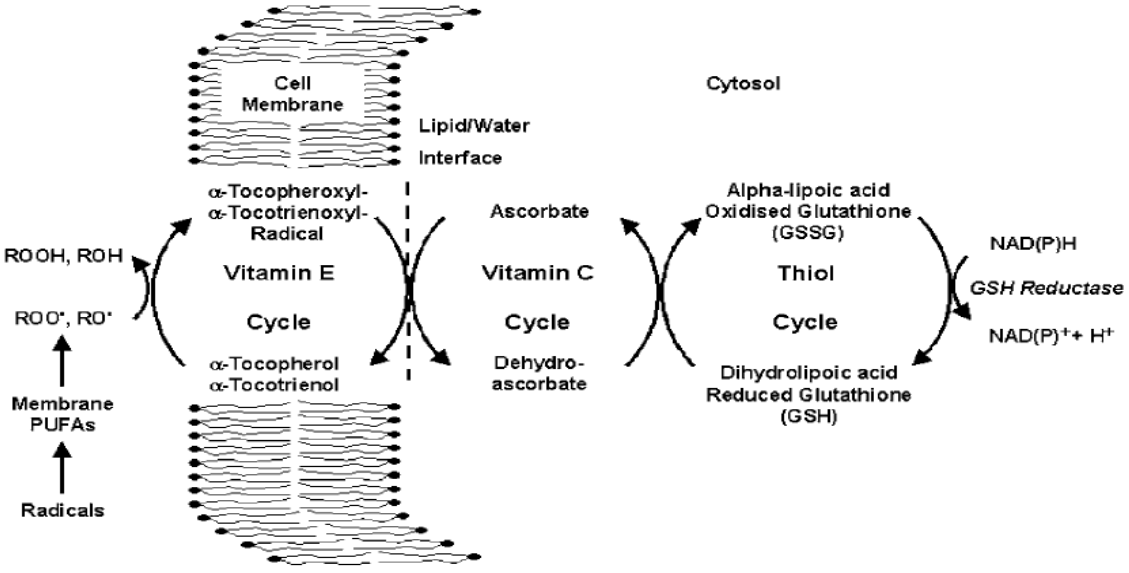

Figure 4.2 The antioxidant network consisting of glutathione (GSH), vitamin C and vitamin E. In the antioxidant system, GSH serves as a hydrogen donor for vitamin C, thus regenerating vitamin C from its oxidized form. Vitamin E is in turn regenerated from its oxidized form by vitamin C (Meeteren et al., 2005).

Due to this cooperation, low levels of GSH will result in a reduced capacity to regenerate both vitamin E and vitamin C. A combination of oxidative stress and reduced levels of GSH, may thus explain the reduced concentrations of these two vitamins in fish fed the High inorganic Se diet. Although levels of GSH were reduced in fish fed the High organic Se diet as well, this group showed no difference in levels of vitamin E and C. A possible explanation could be the difference in GSH levels between fish fed the High organic Se and High inorganic Se diets. Fish fed the High inorganic Se diet tended to have lower GSH levels (not significantly different) compared to the High organic Se group. This indicates that levels of GSH in fish fed High inorganic Se were too low to regenerate vitamin C and E, while the fish fed High organic Se maintained sufficient levels of GSH to provide for the regeneration of those antioxidants. Thus, considering the antioxidants studied, it appears that the High inorganic Se diet caused a greater reduction in the antioxidant capacity of the fish, compared to the High organic Se diet. Fish receiving the Low organic Se, Low inorganic Se and the control diets, did not have reduced levels of GSH, vitamin E or vitamin C, thus these treatments did not appear to alter the antioxidant capacity.

4.2.2 Oxidative byproducts as markers of antioxidant damage

A consequence of reduced levels of antioxidants is a reduced capacity to protect against oxidative damage to DNA, proteins and lipids. The presence of elevated levels of oxidation byproducts, may thus confirm both oxidative damage and reduced antioxidant capacity.

A common method to detect oxidative damage to lipids is by determining malonaldehyde (MDA), a product of lipid peroxidation often reported as TBARS (Del Rio et al., 2005). As reduced levels of vitamin E makes lipids more prone to peroxidation, elevated levels of TBARS can result when levels of vitamin E are low.

Indeed, the depletion of vitamin E in fish fed the High inorganic Se diet was supported by significantly elevated levels of TBARS, indicating oxidative damage to lipids, despite the slightly higher concentrations of vitamin E in the High inorganic Se diet. Fish fed the other diets, showed no alterations in TBARS levels, indicating that the levels of vitamin E were sufficient to protect against lipid peroxidation.

4.2.3 Does selenium toxicity cause DNA damage?

Indications of oxidative damage in fish fed the High Se inorganic Se diet, were evident from the reduced antioxidant capacity and elevated TBARS. The Comet assay was included in this study to determine whether Se also induced DNA damage.

The initial run of the Comet assay indicated DNA damage in fish fed the High inorganic Se diet. However, some conflicting results between independent measurements indicated that the results rather seemed to be artefacts of the assay conditions. This was confirmed by the following assay run, and the artefacts probably occurred during electrophoresis. When examining the slides, there appeared to be a correlation between the length of the comet tails in the samples and the position of the samples on the slide. The samples in the first columns showed no or little migration, whereas samples further along the slide showed longer comet tails.

When running a Comet assay, the results may be influenced by some critical parameters in the protocol. These includes agarose density, duration of the alkaline treatment, electrophoresis time and voltage, and tissue preparation (Speit et al., 2015). From the protocol used in the present study, the slides should be placed in the electrophoresis chamber for 15-30 minutes at 1 volt/cm. The electrophoresis run was set to 25 minutes, thus within the proposed interval, however it may not have been the optimal duration in this case. Moreover, the volume of the electrophoresis solution should be adjusted to produce a current setting of 300 mA. The current setting was set to 300 mA on the instrument, and the electrophoresis solution barely covered the slides, as proposed by Collins et al. (2008). However, "just barely covering the slide" is a relative volume, and too much electrophoresis solution may have been added, possibly affecting the electrophoresis run. Additionally, the protocol did not come with suggestions on time or rpm for the centrifugation, nor were any proposals found in previous publications. Thus, centrifuging for 15 minutes at 1500 rpm may not have been optimal.

Regardless of cause, the results obtained from the Comet assay were not robust enough to conclude on hepatic DNA damage. However, DNA strand-breaks as a result of Se toxicity has previously been observed *in vitro,* as a result of reactions between H_2Se and oxygen (Peyroche et al., 2012). Thus, in this study, the Se levels in the feed did not seem to cause any DNA damage in the fish, or the assay conditions were not optimized to detect potential DNA damage.

4.3 Does selenium toxicity lead to increased energy expenditure?

Symptoms previously associated with Se toxicity in fish, includes reduced growth and feed efficiency (Hilton et al., 1980, Hamilton, 2004, Vidal et al., 2005). Reduced growth is frequently seen when fish are exposed to a toxicant, and is assumed to be a result of decreased feed intake, a common response of fish to stress (Bernier, 2006), or elevated energy expenditure due to the costs of detoxification or tissue repair (Schlenk et al., 2008).

In the present study, fish fed the High inorganic Se diet had significantly lower growth than fish in all other treatments. This was also reflected by a significantly lower feed intake throughout the feeding

trial. The reduced feed intake in fish fed the High inorganic Se diet was observed at the beginning of the trial, and the feeding rate was upregulated in an attempt to increase the feed intake. As the upregulated feeding rate did not increase the feed intake in the High inorganic Se group, one can assume that the food avoidance was a result of Se toxicity. Hilton et al. (1980) also reported food avoidance in rainbow trout fed inorganic Se at 13 μg Se/g feed.

4.3.1 The endocannabinoid system – a factor in the regulation of appetite

Feed intake is influenced by appetite, and the physiological control of appetite is highly complex. Several neurotransmitters and neuropeptides are involved in the regulation of appetite and satiety, including endocannabinoids (Berry and Mechoulam, 2002). Endocannabinoids are endogenous neuromodulators, derived from fatty acids, that together with their receptors and enzymes, constitute the endocannabinoid system (Wang and Ueda, 2009). This system is involved in the homeostatic control of many physiological functions, including food intake and energy expenditure (André and Gonthier, 2010). The two most studied receptors are cannabinoid receptor 1 (CB_1) and cannabinoid receptor 2 (CB_2), where CB_1 is located in the hypothalamus, the brain area associated with feeding (Berry and Mechoulam, 2002). Although cannabinoid receptors are primarily expressed in the central nervous system, they are also found at lower levels in other tissues, including the liver (Wang and Ueda, 2009).

The ARA-derived endocannabinoids anandamide and 2-arachidonoylglycerol (2-AG) are the two most studied endocannabinoids. Endocannabinoids are involved in both homeostatic and hedonic food intakes, and the binding of anandamide and 2-AG to CB_1 and CB_2, activates pathways in several brain areas, resulting in increased appetite (Naughton et al., 2013). Moreover, administration of antagonists towards the cannabinoid receptors decreases food intake and weight gain in rats (Vickers et al., 2003). As the endocannabinoids are derivatives of fatty acids, it has been observed that changes in levels of lipid precursor molecules, regulates tissue levels of endocannabinoids (Petersen et al., 2006). Additionally, it has been found that the fatty acid composition of the diet may have an impact on the production of endocannabinoids. Alvheim et al. (2014) demonstrated that elevating the levels of LA in diets for rats, increased levels of ARA in hepatic phospholipids, and elevated levels of the ARA-derived endocannabinoids in the liver. These elevated endocannabinoid levels did not result in elevated feed intake, but increased the feed efficiency and caused higher weight gain in the rats.

In the present study, levels of several endocannabinoids were determined, including oleoylethanolamide, N-oleoyltaurine, N-palmitoyltaurine. Fish fed the High inorganic Se diet, was the only group which had significantly reduced levels of endocannabinoids. Oleoylethanolamide and N-

oleoyltaurine are derivatives of oleic acid, while N-palmitoyltaurine is derived from palmitic acid (André and Gonthier, 2010), and both fatty acid precursors also showed reduced levels in fish fed the High inorganic Se diet. Although there may be a connection between the reduced levels of endocannabinoids and the reduced appetite in fish fed the High inorganic Se diet, the regulation of appetite is complex and controlled by several factors, including a network of hormones. It can thus not be concluded that the reduced levels of endocannabinoids was the reason for the reduced feed intake in this study.

4.3.2 The roles of lipids, carbohydrates and proteins in energy production

As exposure to toxicants may initiate energy demanding processes such as detoxification and tissue repair, the toxicants may alter the metabolic rate, and raise the energy expenditure of the organism. In such cases, "trade-offs" may become apparent, as more energy will be utilized for vital processes to combat the toxicant, than on investment activities like growth (Wendelaar Bonga, 1997).

4.3.2.1 Utilization of lipid stores to meet the energy requirements

The energy requirement in fish is provided by the metabolic oxidation of lipids, carbohydrates and proteins. The main energy reserves in fish are lipids stored as triacylglycerols, which are made up of three fatty acids esterified to a glycerol (Torstensen et al., 2001). To generate energy, the triacylglycerol needs to release its fatty acids for β-oxidation. In order to do that, triacylglycerols are hydrolyzed to diacylclycerols and monoacylglycerols, with the simultaneous release of free fatty acids (shown in Figure 4.3) (Nes et al., 2004). Further, the oxidation of fatty acids starts with the coupling of coenzyme A (CoA) to the carboxyl group of the fatty acid molecule, forming acyl-CoA. Acyl-CoA then undergoes β-oxidation where two carbon atoms are cleaved off as acetyl-CoA, which then enters the Krebs cycle, the final common pathway for the oxidation of fatty acids, carbohydrates and amino acids (Nes et al., 2004, Berg et al., 2002).

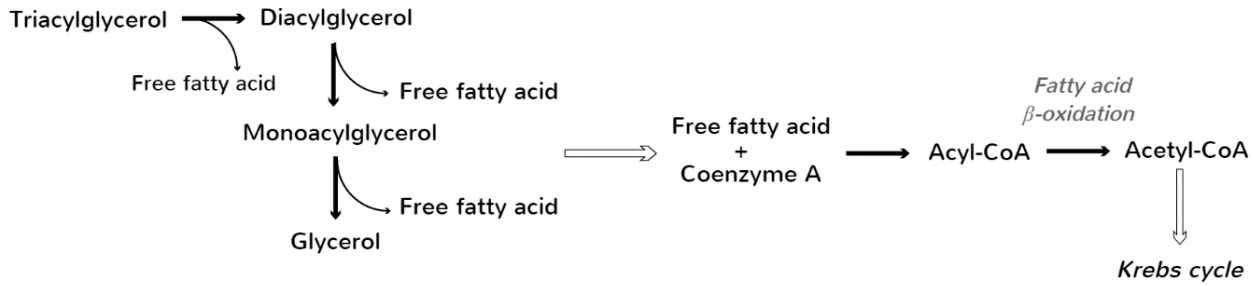

Figure 4.3 In order to release fatty acids for β-oxidation, a triacylglycerol is hydrolyzed to diacylglycerol, monoacylglycerol and glycerol. The fatty acids further couples with Coenzyme A (CoA), forming Acyl-CoA, which again forms Acetyl-CoA when it is β-oxidized. Acetyl-CoA further enters the Krebs cycle in order to generate energy.

In this study, fish fed the High inorganic Se diet was the only group with changed levels of lipid metabolites. This group had significantly reduced levels of both free fatty acids, monoacylglycerol, diacylglycerol and CoA, compared to the other groups. These results indicate an increased utilization of lipids as the substrate for energy production, which again indicate an increased energy expenditure as a result of Se exposure and subsequent detoxification. The reduction in these lipid metabolites was further partly supported by an indication of reduced body fat in fish fed the High inorganic Se diet.

Reduced levels following exposure to High inorganic Se were not seen in all fatty acids studied. The saturated and monosaturated fatty acids palmitic acid (16:0), stearic acid (18:0) and oleic acid (18:1n9), in addition to the 18C PUFAS LA and LNA, were significantly reduced. Moreover, the long chained PUFA EPA (20:5n3), were partly reduced, while DHA (22:6n3) and ARA (20:4n6) were not effected. From these results, it appears that the 16C and 18C fatty acids have been utilized for β-oxidation and energy generation, while the long chained PUFAs appear to have been preserved.

These results are in accordance with previous studies on Atlantic salmon and rainbow trout, suggesting that there is a selective utilization of fatty acids for β-oxidation (Henderson and Sargent, 1985, Kiessling and Kiessling, 1993, Torstensen et al., 2004). This selectivity appears to be based on chain length and degree of unsaturation, as saturated and monosaturated fatty acids, in addition to shorter chained PUFAs, are the preferred substrates for β-oxidation (Henderson and Sargent, 1985, Kiessling and Kiessling, 1993). Since long chained PUFAs (≥20C) are vital in maintaining the functions and structures of cellular membranes (especially DHA), the selective utilization of fatty acids in β-oxidation, seems to be a mechanism where long chained PUFAs are saved for such functions, rather than being utilized for energy production (Kiessling and Kiessling, 1993, Tocher, 2010). Palmitic acid, oleic acid and LA have been observed to be preferred for β-oxidation, while ARA and DHA are found to be oxidized at low rates, if oxidized at all (Henderson and Sargent, 1985, Kiessling and Kiessling, 1993). Furthermore, EPA has been observed to be a preferred substrate over DHA (Torstensen et al., 2004). The results of the fatty acid levels in this study, are thus consistent with previous findings, and it appears that selective utilization of fatty acids for β-oxidation occurred in fish fed the High inorganic Se diet.

4.3.2.2 Carbohydrates - no major energy source in fish

The breakdown of glucose results in formation of acetyl-CoA and generation of energy by the Krebs cycle (Nes et al., 2004). Fish however, have limited capacity to utilize glucose as a substrate in energy production. This is due to low activity and partly deficient induction of hexokinase, the first regulatory step in the glucose catabolism (Hemre, 2001). This is also most likely the reason why none

of the metabolites studied suggested elevated utilization of carbohydrates for energy production *(data not shown).*

4.3.2.3 Excess methionine may have increased nitrogen turnover

In order to utilize proteins as an energy source, they are broken down to their respective amino acids. The amino group of the amino acids are removed and replaced by O_2 through oxidative deamination, and the amino acid is turned into a keto acid. The keto acid may be utilized to generate energy when it is transformed to acetyl-CoA, which in turn enters the Krebs cycle. Moreover, the amino groups resulting from the deamination, may be converted to ammonia and transported to the liver for detoxification. Through the urea cycle, ammonia is transformed into urea, before being transported to the kidneys and excreted through urine (Figure 4.4) (Nes et al., 2004).

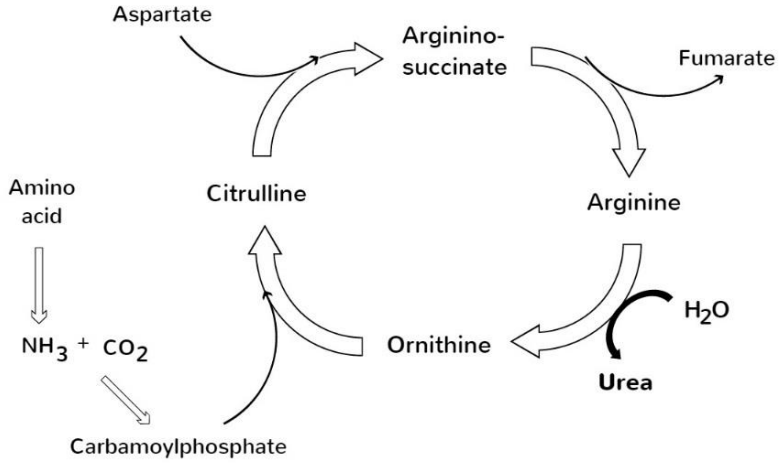

Figure 4.4 The urea cycle serves in the detoxification of ammonia resulting from deamination of amino acids. The final product in the cycle is urea, which is excreted via urine.

In the present study, fish fed the two organic Se diets showed significantly elevated levels of ornithine, citrulline and argininosuccinate, compared to the other groups. The two organic Se groups also showed significantly elevated levels of urea when compared to the control groups, however not when compared to the inorganic Se groups. When amino acids are present in excess levels of those required for protein synthesis, they will be deaminated and transformed into urea (Walton and Cowey, 1982). Thus, the elevated production of urea in the two groups receiving SeMet, may indicate excessive levels of methionine in these fish. Moreover, when SeMet is present in excess levels of those incorporated into general proteins, SeMet will follow the trans-selenation pathway and be transformed into H_2Se (Thiry et al., 2012). If present in excess, H_2Se may contribute to the production

of ROS (Lin and Spallholz, 1993). Consequently, this may be reflected in the oxidative stress seen as reduced levels of GSH in fish fed the High organic Se diet.

Fish fed the High inorganic Se diet showed no changes in levels of intermediates in the urea cycle, thus proteins do not seem to have been a major substrate for energy production in this treatment. However, the High inorganic Se group had significantly reduced levels of hepatic lipid metabolites. Since it appears that inorganic Se had a higher toxic potential than organic Se, it is likely that lipids was the major energy source in combating Se toxicity. However, although the results indicated an elevated utilization of energy reserves, energy expenditure was not measured directly in this study. Moreover, with the reduced feed intake being a complicating factor, the indications of increased energy expenditure are not sufficient to suggest increased energy expenditure as a specific marker of Se toxicity.

4.4 Organic and inorganic selenium species showed different distribution among organs

Although fish can utilize both organic and inorganic Se as nutrients, the chemical form in the diet may have a great influence on its impact on the fish. Organic and inorganic Se species possess different features and are metabolized in different ways (Thiry et al., 2012). In this study, this was reflected in their toxicity, but also in how the different forms were distributed in the fish.

Several studies based on the bioavailability of different Se forms, have shown that SeMet is the species with the highest bioavailability in fish (Wang and Lovell, 1997, Rider et al., 2010). Compared to selenite, SeMet appears to be absorbed to a greater extent and show a higher accumulation in both blood and tissues (Wang and Lovell, 1997, Rider et al., 2010).

In the present study, Se accumulation was determined in whole fish homogenate of fish receiving the six experimental diets. In addition, the distribution of Se among tissues was determined in fish receiving the Low inorganic, Low organic Se, and the Positive control diet. In whole fish homogenate, fish fed the High organic Se diet showed the significantly highest Se accumulation compared to the other groups, including the High inorganic Se group. Higher whole fish accumulation of SeMet compared to selenite have previously been observed in Atlantic salmon fed diets at 1 and 2 mg Se/kg (Lorentzen et al., 1994), and in rainbow trout fed diets at 2, 4 and 7 mg Se/kg (Rider et al., 2009). In the present study, the Low inorganic and Low organic Se diets, and the Positive control, showed similar accumulation of Se in whole fish.

Differences were observed between the Low inorganic and Low organic diets, and the Positive control, in terms of Se accumulation in tissues. Fish fed Se as SeMet showed significantly higher Se accumulation in all tissues, compared to fish fed selenite and the Positive control. The high

accumulation of SeMet compared to selenite, both in whole fish and in tissues, is likely due to SeMet being non-specifically incorporated into general body proteins (Schrauzer, 2000). Other forms of Se, including selenite, are transformed to SeCys before being incorporated in to selenoproteins in a specific and stoichiometric manner. Selenite can not be stored, and administration of Se levels beyond adequate Se status have not shown increased levels of selenoproteins (Patching and Gardiner, 1999, Thiry et al., 2012).

The highest Se concentrations in all three groups (Low organic Se, Low inorganic Se and Positive control), were seen in the liver, confirming this organ's central role in Se metabolism in salmon. The high accumulation in this tissue is likely due to the role of the liver in Se metabolism and in selenoprotein synthesis (Burk and Hill, 2009). The kidneys had the second highest Se concentration, which may be explained by the kidneys function as a clearance organ, as well as their role in selenoproteins synthesis (Thiry et al., 2012). In rainbow trout, Rider et al., (2010) also found the highest Se accumulation in liver and kidney, followed by spleen, gills and muscle, similar to the results obtained in this study. Compared to the other tissues, a relatively low Se concentration was found in muscle. However, SeMet showed significantly higher accumulation in this tissue compared to selenite, confirming incorporation of SeMet into muscle proteins. Although Se concentrations in muscle were among the lowest compared to the other tissues, nearly 50 % of SeMet was found in muscle, compared to about 30 % of selenite.

Regarding the relative distribution of SeMet and selenite in liver, they were found in this tissue at the same levels (about 14 %). Lorentzen et al. (1994) found a higher Se concentration in liver of fish fed selenite, compared to fish fed SeMet, confirming the livers role in the metabolism of selenite. In this study, the finding of a significantly higher Se concentration in liver of fish fed SeMet than in fish fed selenite, indicate that SeMet not only followed the methionine pathway into proteins, but also the Se-pathway to the liver. This is supported by the elevated production of urea, in fish fed the Low organic Se diet, suggesting excess levels of methionine for incorporation into proteins.

Based on the hepatic biomarkers on reduced antioxidant capacity and elevated TBARS, selenite showed a higher toxic potential than SeMet. The fact that selenite showed a lower accumulation in liver compared to SeMet, in turn indicate that selenite becomes toxic as lower accumulated levels. Moreover, the diverse metabolic fates of SeMet, and the fact that it has the property of being incorporated into general body proteins, may explain the lower toxic potential of SeMet.

5. CONCLUSION

In conclusion, it was found that oxidative stress was the toxic mode of action caused by excessive Se, confirmed by reduced antioxidant capacity and oxidative damage measured as elevated levels of TBARS. Moreover, since inorganic Se appeared to affect the antioxidant capacity to a higher degree, in addition to causing reduced body weight, it can be concluded that inorganic Se had a higher toxic potential than organic Se.

Thus, although Se is an essential element required in the diet for Atlantic salmon, elevated dietary Se levels (15 mg Se/kg) may result in toxicity. Biomarkers are valuable in order to obtain information on potential toxicity before overt negative effects of exposure occur. From this study, biomarkers of oxidative stress were found to be suitable biomarkers of Se toxicity. These biomarkers include altered levels of the antioxidants GSH, vitamin E and vitamin C, in addition to elevated levels of TBARS. This study did not find altered GSH/GSSG ratio, DNA damage, energy expenditure or appetite to be specific biomarkers of Se toxicity. However, although the results obtained in this study were not robust enough to conclude on DNA damage, DNA damage has previously been found as a result of Se toxicity. Further method optimization is required in order to conclude whether the Comet assay can be used to detect DNA damage as a result of Se toxicity in Atlantic salmon.

FUTURE PERSPECTIVES

Nutrients have the potential to exert both health benefits and health risks, hence it is important to establish a safe level of intake of nutrients. A fish feeding trial is to be conducted at NIFES in 2016 to establish the threshold for Se intake in Atlantic salmon by modelling the toxicological response in a bench-mark model. Low, but detectable increases in an adverse response using specific and sensitive biomarkers of Se toxicity will be essential when modelling the dose threshold for toxic effects.

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