The effects of selenium on methylmercury toxicity in zebrafish

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Scientific environment

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NATIONAL INSTITUTE OF NUTRITION AND SEAFOOD RESEARCH

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Abstract

Methylmercury (MeHg) is a potent neurotoxicant that remains a global concern. Selenium (Se) is an important micronutrient which is able to decrease MeHg toxicity, although the underlying mechanisms for this protection remain unclear. One hypothesis is that Se-mediated protection against MeHg toxicity occurs due to the function of Se containing proteins, termed selenoproteins. Many selenoproteins play key roles in maintaining both cellular and extracellular redox balance, and it is believed that via these roles, selenoproteins reduce MeHg-induced oxidative stress. A second hypothesis is that MeHg toxicity reduces the availability of Se for selenoprotein synthesis. Additional Se can thus be beneficial as it increases the availability of Se for selenoprotein production, reducing MeHg-induced disruption of the selenoproteome. These two hypotheses are linked, as they share a common theme suggesting that functional selenoproteins are key factors in reducing MeHg toxicity.

The aim of this thesis was to explore how dietary Se reduces MeHg toxicity. As Se status affects MeHg toxicity, an initial study aimed to identify the Se requirements of zebrafish (**Paper I**). Juvenile zebrafish were fed diets with increasing levels of Se. The Se requirements were then assessed primarily from the response of growth and the mRNA expression and activity of a key Se-dependent protein, glutathione peroxidase (GPX). The second step of this thesis was to assess how changes in Se status affect MeHg induced toxicity both at the whole organism level (**Paper II**) and then at the molecular level, with a focus on the mRNA expression of selenoprotein coding genes (**Paper III**). To do this, female zebrafish were exposed to requirement (from **Paper I**) or elevated levels of dietary Se alone or in combination with elevated levels of dietary MeHg in a 2×2 factorial experimental design. These diets were fed to fish (F_0 generation) for a five month period, during which the fish were crossed against untreated male fish to produce a maternal transfer exposed F_1 generation. The effects of these diets on growth, survival, element composition and reproductive outcomes were explored in the adult generation (**Paper II**). The F_1 generation were analysed during the embryonic stage to examine the underlying mechanisms of the Se×MeHg interactions on the expression of selenoprotein coding genes during development

(**Paper III**). The 30 selenoprotein coding genes analysed cover most of the selenoprotein families; including several members of the *gpx*, *thioredoxin reductase*, *iodothyronine deiodinase* and *methionine sulfoxide reductase* families, along with *selenophosphate synthetase 2*, *selenoprotein h*, *j-p*, *t*, *w*, *15*, *fep15* and *fam213aa*. As functional selenoprotein levels determine the Se-mediated antioxidant response, and the primary site of MeHg toxicity is the central nervous system, the total GPX activity and locomotor activity were also analysed in the F_1 generation.

The Se requirements of zebrafish were found to be 0.3 mg Se/kg DM based on growth, similar to other fish species. Meanwhile, maximum GPX activity did not correspond to zebrafish Se requirements, a controversial finding (**Paper I**). Elevated dietary Se reduced MeHg-induced decreases in growth and survival of adult fish, as found previously for vertebrates. However, elevated Se and MeHg had a synergistic negative affect on reproductive outcomes, such as embryo survival (**Paper II**), a novel finding in fish. Analyses of the mRNA expressions of selenoprotein coding genes demonstrated that only a subset of these genes were affected by MeHg. The affected genes coded for selenoproteins primarily from antioxidant pathways, and were downregulated by elevated MeHg. Meanwhile, MeHg also decreased GPX activity and induced larval hypoactivity. Elevated Se prevented the MeHg-induced downregulation for most of the affected genes. However, elevated Se only partially prevented the MeHg-induced decreases in GPX activity and larval locomotor activity (**Paper III**). As MeHg primarily affected antioxidant selenoproteins, which are also affected by Se deficiency, the response of selenoproteins to Se deficiency were then analysed in zebrafish embryos from parents fed diets deficient or replete in Se (**Thesis Supp. Material**). Considerable overlap was observed between the antioxidant selenoprotein genes downregulated by Se deficiency and those downregulated by MeHg toxicity. Overall mRNA downregulation of antioxidant selenoprotein genes by both MeHg toxicity and Se deficiency were prevented by elevating the Se status, suggesting that MeHg regulates the selenotranscriptome mainly via Se status, and that Se deficiency is a factor in MeHg toxicity.

List of publications

Paper I Penglase, S., Hamre, K., Rasinger, J.D., Ellingsen, S., 2014. Selenium status affects selenoprotein expression, reproduction, and F1 generation locomotor activity in zebrafish (*Danio rerio*). *British Journal of Nutrition*. 111, 1918-1931.

Paper II Penglase, S., Hamre, K., Ellingsen, S., 2014. Selenium and mercury have a synergistic negative effect on fish reproduction. *Aquatic Toxicology*. 149, 16-24.

Paper III Penglase, S., Hamre, K., Ellingsen, S., 2014. Selenium prevents downregulation of antioxidant selenoprotein genes by methylmercury. *Free Radical Biology and Medicine*. 75, 95-104.

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Background

1.1 Methylmercury is an environmental contaminant of global concern

Inorganic mercury (Hg) occurs ubiquitously and naturally in the environment. However, anthropogenic activities have increased both background levels of Hg in the biosphere, and created localised areas that are heavily contaminated with Hg (Driscoll et al., 2013). In anaerobic environments, such as those found in marine and fresh water sediments, Hg is methylated to methylmercury (MeHg) by sulphate and iron reducing bacteria (Driscoll et al., 2013). From this microbial starting point, MeHg readily bioaccumulates up the food chain (Fig. 1), with increased levels found at each trophic level (Driscoll et al., 2013). As such, all seafood contains some MeHg, while apex predators; such as marine mammals, sharks and swordfish; generally have the highest (>0.5 mg Hg/kg DM) MeHg levels (Wagemann et al., 1998; Mahaffey, 2004).

Concern over MeHg originated chiefly from a tragic MeHg poisoning epidemic that occurred in the 1950's, referred to as Minamata disease. This localised epidemic occurred in the seafood consuming human populations around Minamata bay, Japan (McAlpine and Araki, 1958). The bay was industrially contaminated with Hg, which resulted in seafood containing MeHg levels up to 170 fold higher than in uncontaminated areas (Hachiya, 2012). More recently, MeHg toxicity in seafood eating human populations as a consequence of localised Hg anthropogenic contamination events have been identified in Brazil (Berzas Nevado et al., 2010; Lemire et al., 2011; Fillion et al., 2013) and Sicily (Ausili et al., 2008) among others. Additionally, MeHg toxicity cases have been identified in the Faroe Islands, from consumption of naturally MeHg-contaminated whale meat (Rice, 2000; Murata et al., 2004).

However, seafood also contains elevated levels of important nutrients, such as long chain polyunsaturated fatty acids (e.g. DHA and EPA), elements (e.g. selenium and iodine) and vitamins (e.g. A and D) that can have positive effects on health (Berry and Ralston, 2008; EFSA, 2014). The human population of Seychelles has an elevated

MeHg status due to high seafood consumption, but the beneficial nutrients in the seafood appear to negate any MeHg-induced toxic effects (Wijngaarden et al., 2012). In contrast, other findings suggest that the negative effects of MeHg may actually be more widespread than currently determined and include numerous seafood consuming human populations with more moderate MeHg intakes (Karagas et al., 2012). The uncertainty surrounding the MeHg toxicity threshold level in seafood has led to conflicting views regarding seafood safety and on the benefits of seafood consumption, in both the scientific and public arena (Mahaffey, 2004). Currently, Hg remains listed by the World Health Organisation (WHO) as one of the 10 chemicals of major public health concern (WHO, 2010). Recently, the European Food Safety Authority (EFSA) decreased its recommended tolerable weekly intake (TWI) levels for MeHg from 1.6 to 1.3 µg Hg/kg body weight (EFSA, 2014).

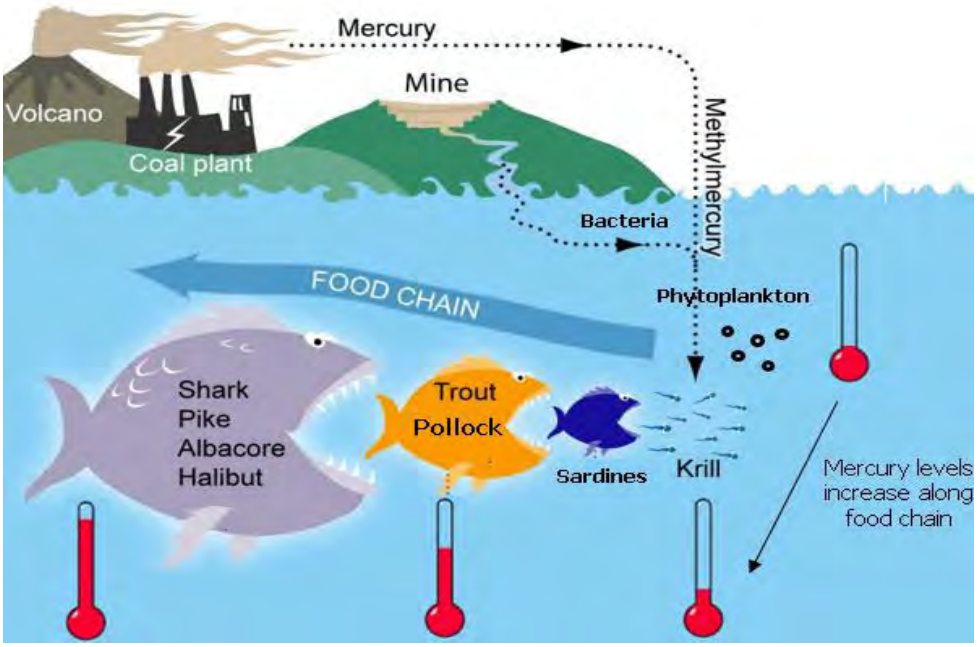

Figure 1. Methylmercury bioaccumulates in the food chain. Mercury is released into the environment from both natural sources, such as volcanoes, and human activities, such as mines and coal combustion. Once mobilized, anaerobic bacteria can methylate inorganic Hg into methylmercury. Methylmercury then bioaccumulates at each trophic level, in both fresh and salt water ecosystems. High methylmercury concentrations can be found in top end predators, such as large fish and marine mammals. Figure modified from http://groundtruthtrekking.org/Graphics/MercuryFoodChain.html.

1.2 Uptake and distribution of methylmercury in the body

The majority (>90%) of Hg present in fish is present as MeHg (Amlund et al., 2007; Lemes and Wang, 2009). In turn, the MeHg in fish is largely bound in a 1:1 ratio to thiol groups (R-SH) of mainly protein incorporated cysteine (Cys) residues, in a complex termed methylmercury-L-cysteinate (MeHg-Cys) (Harris et al., 2003; Lemes and Wang, 2009). This MeHg-Cys is transported into cells and across membranes by the L-Type amino acid transporters, LAT1 and LAT2 (Simmons-Willis et al., 2002), found throughout the body (Prasad et al., 1999; Rossier et al., 1999). It is thought that MeHg-Cys transport by the LAT's occurs as MeHg-Cys structurally mimics another LAT substrate, methionine, but this mimicry hypothesis is controversial (Hoffmeyer et al., 2006; Asaduzzaman and Schreckenbach, 2011). Irrespectively, MeHg-Cys is efficiently (>95%) absorbed (Smith and Farris, 1996; Nobuhiro et al., 2012) in the intestine (Clarkson et al., 2007) and transported throughout the body; including across the placental (Suzuki et al., 1984) and blood brain barriers (Kerper et al., 1992) in a concentration dependent manner (reviewed by Newland et al. (2008)).

1.3 Methylmercury toxicity targets the developing nervous system.

With maternal intake of MeHg, the foetal brain accumulates higher concentrations of Hg than the maternal brain (Watanabe et al., 1999). During the Minamata tragedy, it became evident that the developing foetal central nervous systems (CNS) was particularly vulnerable to MeHg-induced toxicity, as severe impairment of neurological function was observed in infants born to mothers without symptoms (Harada, 1995). Subsequently, motor skill deficits, such as ataxia and loss of balance, sensory deficits such as reduced visual sensitivity, and overall reductions in IQ, have been reported in human infants exposed to MeHg by maternal transfer (Mahaffey, 2004; Farina et al., 2011; Sheehan et al., 2014). Many aspects of MeHg-induced neurological damage during foetal development are irreversible and thus persist into adulthood (Mahaffey, 2004; Johansson et al., 2007; Weber et al., 2008; Smith et al., 2010).

Adult exposure to MeHg can also disrupt the CNS, but the primary target of chronic MeHg exposure appears to be the cardiovascular system (Karagas et al., 2012). Low level chronic exposure to MeHg has been associated with increased risk of myocardial infarction and mortality from cardiovascular disease (Karagas et al., 2012). Furthermore, a latency period can occur between the time of exposure until symptoms arise; termed late onset symptoms. For instance, three decades of aging in Minamata disease patients after MeHg exposure resulted in additional CNS related symptoms (Kinjo et al., 1993).

1.4 The biological interactions underlying methylmercury toxicity.

1.4.1 Methylmercury-induced disruption of proteins and small molecular weight compounds.

At the chemical level, the toxicity of MeHg occurs primarily because of its tendency to form covalent bonds with sulphur (S; MeHg-S) and Se (MeHg-Se), via the Hg atom (Nuttall, 1987; Dyrssen and Wedborg, 1991; Asaduzzaman and Schreckenbach, 2011). The biological ramifications of this are that both S and Se are critical components in biological systems. In particular, S is found in the thiol group of Cys, and Se in the selenol group of selenocysteine (Sec; amino acid symbol "U"), both of which are proteinogenic amino acids. With a single exception, protein incorporated Sec is always found at the active site of Se dependent proteins, termed selenoproteins (Papp et al., 2007). Cysteine is also found at enzyme active sites, but additionally is critical in exposed side chains that undergo post translational modifications that affect protein activity or function (Barford, 2004; Papp et al., 2007). Furthermore, Cys is the functional component of all the major cellular redox couples; glutathione/glutathione disulphide (2GSH/GSSG), Cys/Cystine (2Cys/CySS) and reduced/oxidised thioredoxin ($Trx(SH)_2$/TrxSS); and thus essential for the maintenance of redox balance in cells (Hussey et al., 2009). The subsequent binding of these critical Cys/Sec residues with MeHg can impair the functions of proteins and small molecular weight compounds ((Fig. 2A-B), reviewed by Farina et al. (2012)). Functionally critical

proteinogenic Cys (or Sec) residues are often the most exposed and reactive (Weerapana et al., 2010) which may further exaggerate their susceptibility to MeHg binding.

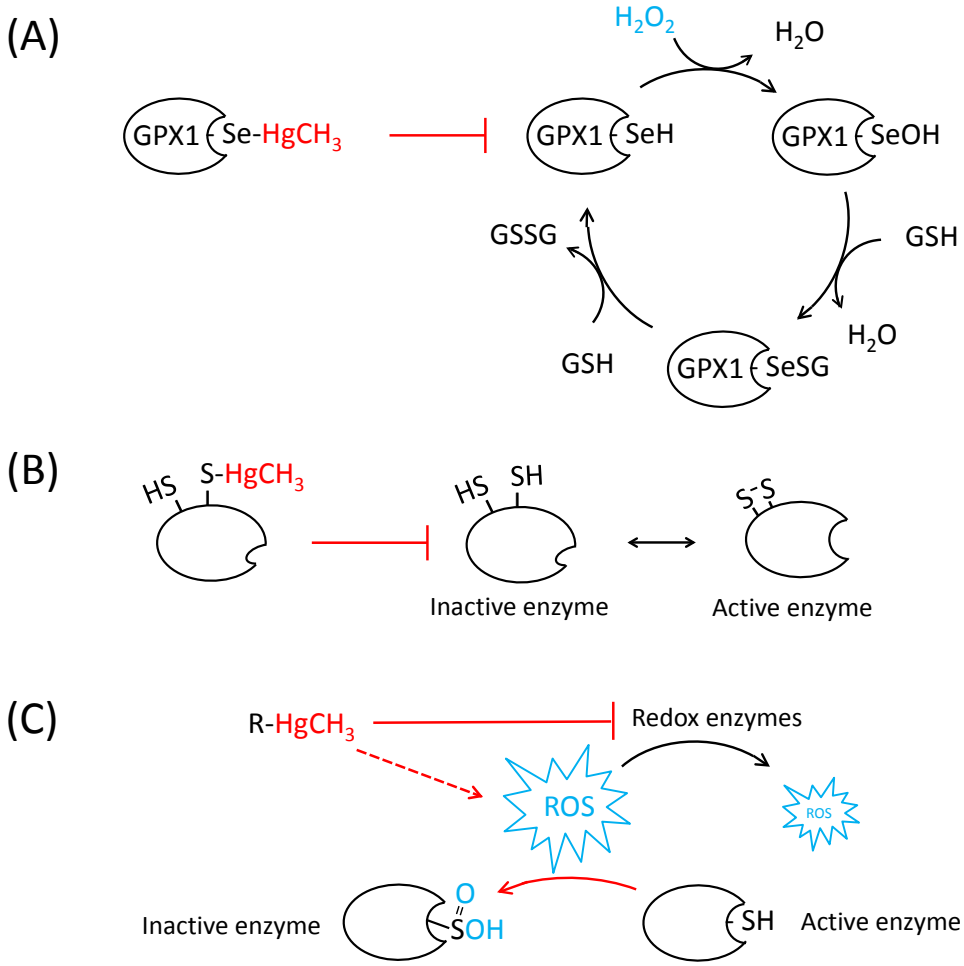

Figure 2. Mechanisms of MeHg-induced protein disruption. MeHg (-$HgCH_3$) can disrupt (red pathways) the cellular environment by **(A)** direct binding to thiol or selenol groups at the active site of proteins, herein depicted as MeHg preventing GPX1 from reducing a reactive oxygen species (ROS; coloured blue) H_20_2 to water; **(B)** direct binding to thiol groups important for post translational modification of proteins, herein depicted as MeHg preventing a disulfide bond between two thiol groups critical for correct protein structure and function; or **(C)** indirectly via disruption of downstream pathways, herein depicted as MeHg-induced increases in ROS (via direct antioxidant enzyme inhibition) resulting in the oxidation of an enzyme active site thiol group sulfur to sulfinic acid, (an irreversible modification for certain enzymes (Rhee et al., 2005)). The examples provided serve to illustrate potential direct and indirect MeHg-induced disruption of protein function and are in no way exhaustive.

In addition to direct binding to primary targets at thiol and selenol groups, or to their biological intermediates, MeHg may also induce secondary effects, whereby pathways relying on these primary targets are themselves disrupted (reviewed by Farina et al. (2012)). For instance, the function and activities of many proteins are regulated by the cellular redox balance (Barford, 2004). Reduced function of primary targets in antioxidant pathways by MeHg can increase cellular levels of reactive oxygen/nitrogen species (ROS/RNS) and shift cellular redox potentials, disrupting redox sensitive proteins and pathways that may not be affected directly by MeHg binding (Fig. 2C) (Farina et al., 2012). In neural cells, MeHg appears to induce a similar cascade effect which starts with MeHg binding and disrupting proteins involved in cellular calcium homeostasis, which then leads to disruption of calcium dependent pathways (Farina et al., 2012).

The final consequence of MeHg-S or Se binding can be impaired cellular function and cell death. Consistent with the MeHg induced neurological symptoms, MeHg toxicity can result in brain lesions, and several types of neural cells appear particularly sensitive to MeHg-induced apoptosis (Chang, 1977; Kaur et al., 2012).

1.4.2 Factors affecting the interactions between selenium, sulfur and methylmercury in biological systems.

Mercury will form Hg-Se in preference to Hg-S bonds in simple chemical solutions (Dyrssen and Wedborg, 1991). Correspondingly, MeHg has a tendency to form MeHg-Se-R in preference to MeHg-S-R bonds in biochemical solutions (Sugiura et al., 1978). Additionally, MeHg forms a promiscuous covalent bond that it will readily break to form a new bond with lower entropy, which favours S bound MeHg redistributing to Se (Sugiura et al., 1978; Asaduzzaman and Schreckenbach, 2011). The higher affinity of MeHg for Se than for S, coupled with the functional use of Se at enzyme active sites, are key reasons why selenoproteins appear particularly vulnerable to MeHg induced disruption (Farina et al., 2012).

However, despite the dominance of MeHg-Se bonds compared to MeHg-S bonds in chemical solutions, several other factors must be considered to explain the interactions between MeHg, S and Se in biological systems. For one, there is an equilibrium between the ratios of protonated thiol/selenol (R-SH or R-SeH) versus the deprotonated thiolate/selenoate ($R\text{-}S^-$ or $R\text{-}Se^-$) forms in Cys or Sec, respectively. This equilibrium is dictated largely by the cellular pH, which increases the ratio of protonated to deprotonated Cys and Sec residues as it decreases. The pH at which half the S/Se atoms are protonated (pKa) is ≈ 8.3 for Cys and ≈ 5.2 for Sec (Huber and Criddle, 1967). Thus at physiological pH of around 7.0, the equilibrium favours the Se of Sec being in the exposed $R\text{-}Se^-$ form and susceptible to MeHg binding, while the S of Cys is more likely to be in the more protected protonated R-SH form.

Interestingly, it appears that in biological systems the pKa of thiol/selenol groups is a larger factor determining the probability of their binding to MeHg than the presence of Se or S *per se*. For instance, even among two proteins containing exposed Cys residues, one can be preferentially targeted by MeHg (Farina et al., 2012). The pKa value of a protein incorporated amino acid residue side chain is affected by its neighbouring residues (Gilbert, 1990), and hence so is the exposure of the S/Se atom of Cys/Sec residue to MeHg. For instance, MeHg severely decreases the activity of the non-selenoprotein creatine kinase (Glaser et al., 2010). This probably occurs as a result of the high affinity of MeHg for the Cys thiol group at the active site that has a pKa of 5.4 (Wang et al., 2006). It is reasonable to assume that along with its physical accessibility to MeHg, the pKa's of Se in Sec residues also differ between selenoproteins, affecting their susceptibility to MeHg binding.

Another factor influencing MeHg bonding in the cellular environment is the overwhelming molar dominance of S over Se. For instance, fish tissue contains around 7 g of S and 1 mg of Se/kg DM, a ratio of 7000:1 (Waagbø et al., 2001). More specifically, nutritionally replete mammals have around 0.25 µg Se/g in Sec (3.2×10^{-6} molar Se) (Hill et al., 2012) and 260 µg S/g in Cys (8.3×10^{-3} molar S) (Wu et al., 1999) on a wet weight basis, a 2500 fold S:Se ratio. This ratio is consistent with the small number of selenoproteins, for example 25 in humans (Kryukov et al., 2003),

versus the ubiquitous presence of Cys throughout much of the proteome. The S:Se ratio may explain why despite the higher affinity of the MeHg-Se bond, MeHg is largely found bound to Cys (for example as MeHg-Cys in fish tissue, discussed in Section 1.2) and MeHg-induced disruptions at the cellular level appear to largely originate from MeHg-S bonding (Yang et al., 2007; Cuello et al., 2012; Farina et al., 2012; Ho et al., 2013; Zayas et al., 2014).

The above discussion regarding MeHg binding to amino acid side groups is based primarily on evidence from studies on MeHg-Cys, and inferred for MeHg-Sec. While MeHg will directly inhibit selenoprotein function, and thus presumably directly bind Sec within proteins (Hirota et al., 1980), MeHg can also bind a biological intermediate of Se metabolism/Sec synthesis, H_2Se (Iwata et al., 1982; Masukawa et al., 1982). It remains unknown what Se species, such as proteinogenic Sec residues, or biological intermediates such as H_2Se, are the cellular Se species most susceptible to MeHg binding. Irrespective of this, once bound by MeHg, Se is presumed to have entered the biologically unavailable Se pool (Ralston et al., 2012).

1.4.3 Selenium's antagonism of methylmercury toxicity.

Paradoxically, it is well documented that Se has a protective effect against MeHg toxicity. This effect was first reported in the early 1970's when it was found that both inorganic Se and Se naturally present in food (Se found in tuna) protected against MeHg-induced decreased growth and increase mortality (Ganther et al., 1972). However, the underlying mechanism/s for the Se-mediated protection against MeHg toxicity still remain unclear. There are currently two main hypotheses. Hypothesis 1) is that selenoproteins help to prevent oxidative stress induced when MeHg directly disrupts many key proteins involved in cellular redox system pathways (Fig. 3A). For example, glutathione peroxidases (GPX) are key antioxidant selenoproteins, and overexpression of GPX1 *in vitro* provides protection against MeHg induced oxidative stress (Farina et al., 2009). Hypothesis 2) is that MeHg induces a cellular Se deficiency by binding Se, and this Se deficiency targets selenoproteins (Fig. 3B). As such MeHg toxicity pathologies have been suggested to actually be Se deficiency pathologies

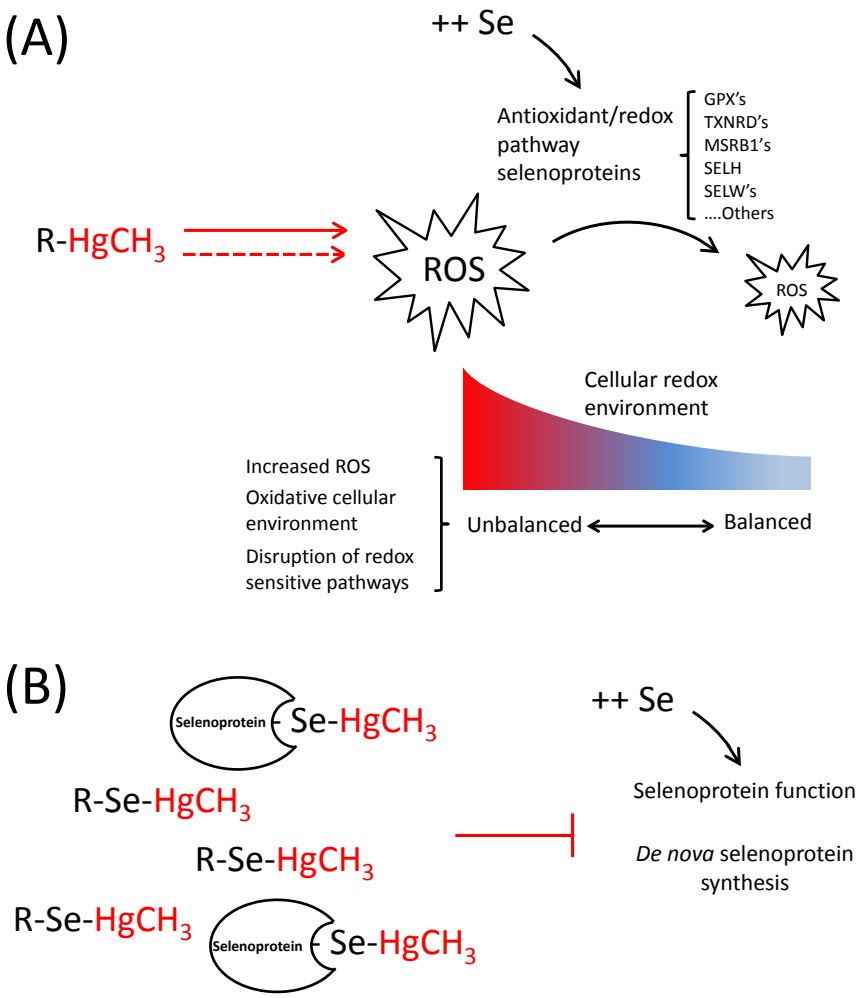

Figure 3. The proposed mechanisms for the biological interactions of selenium (Se) and methylmercury (MeHg). Elevated Se status may help to prevent MeHg-induced toxicity via two main mechanisms. The first **(A)** is by the function of selenoproteins in antioxidant and/or redox pathways. These selenoproteins help to counteract the MeHg-induced increases in reactive oxygen species (ROS) and disruption to cellular redox balance, which can occur via direct binding of MeHg to redox proteins (solid red arrow) or indirectly via the disruption of pathways requiring MeHg bound proteins (segmented red arrow). Many selenoproteins are induced by oxidative stress, and additional Se enables an increased capacity for selenoprotein translation under oxidative conditions. The second **(B)** mechanism is that elevated Se status helps to maintain bioavailable levels of cellular Se, which in turn helps to maintain the functional selenoproteome, in the face of direct binding of Se in non-bioavailable complexes (either as intermediates of Se metabolism (R-Se-$HgCH_3$), or direct binding to Se in selenoproteins) by MeHg. A specific example of the general concept of MeHg inhibiting the function of a selenoprotein **(B)** is found in Fig 2A. See Table 1 for full selenoprotein names.

(Ralston et al., 2008). In agreement, GPX activity is negatively affected by either Se deficiency or MeHg toxicity, and in both cases additional Se supplementation restores activity (Chang and Suber, 1982; Weiss et al., 1996). The two hypotheses are not conflicting, and also have a common theme, in that functional selenoproteins are required to result in the protective effect of Se against MeHg toxicity.

1.5 Selenium as an essential nutrient.

Selenium was first reported as an essential trace element in the late 1950's (Schwarz and Foltz, 1957), and as an essential component of what became the first known selenoprotein, GPX1, two decades later (Flohe et al., 1973; Rotruck et al., 1973). At the time, GPX activity was already known to be an important part of the cellular antioxidant system (Mills, 1957; Cohen and Hochstein, 1963), and the link between Se and the prevention of disease associated with oxidative stress became clear (Hafeman et al., 1974). Among others, the discovery of phospholipid hydroperoxidase (GPX4) (Ursini et al., 1985), iodothyronine deiodinase 1 (DIO1) (Berry et al., 1991a), selenoprotein W (SEPW) (Vendeland et al., 1993) and thioredoxin reductase 1 (TXNRD1) (Gladyshev et al., 1996; Tamura and Stadtman, 1996) linked Se nutrition to such areas as male fertility (GPX4), thyroid hormone metabolism (DIO1), muscular pathology (SEPW) and cellular redox signalling (TXNRD1).

In livestock, severe Se deficiency can result in necrotic liver damage, muscular dystrophy, exudative diathesis and increase mortality among others, and symptoms are often exaggerated in tandem with Vitamin E deficiency (Combs and Combs, 1986b). In humans, severe Se deficiency is rare. However, low Se status is common in Eastern Europe and areas of China among others, and is associated with increased risk of mortality, poor immune function and cognitive decline (Rayman, 2012). Clinical Se supplementation trials have demonstrated that the optimal Se status differs greatly depending on the outcomes measured. For instance the window for Se status that is associated with a decrease risk of some cancers is higher than that to fulfil maximum selenoprotein expression, but also extends into the window associated with an increased risk of type-II diabetes (Rayman, 2012). Added to this the overall window of

optimal Se status is small, and Se supplementation is only associated with benefits for the population with a Se status below this window. Ironically, individuals with an optimal Se status before supplementation can become more at risk of certain diseases with supplementation (Rayman, 2012). Currently, the recommended dietary Se intakes differ between authority bodies, but range from 40 to 85 µg/day for men and 30 to 70 µg/day for women, and are based around Se levels which result in maximum blood plasma GPX (GPX3) activity (Thomson, 2004).

Total Se concentrations in plant derived foods are largely dictated by the levels of plant bioavailable Se found in the soil (Combs and Combs, 1986a). Typical plant based foods have between 0 and 0.8 mg Se/kg (Rayman, 2012). Animal based foods are generally higher in Se than plant based foods. Organ meats and seafood generally have the highest Se levels that typically range between 0.4 and 1.5 mg/kg (Combs and Combs, 1986b; Rayman, 2012). Food Se is found in a multitude of chemical species (Dumont et al., 2006). Inorganic Se species, including selenite (SeO_3^{2-}) and selenate (SeO_4^{2-}), are commonly used in dietary supplements and experimental diets, and are the predominate Se species found in soil and water (Combs and Combs, 1986b). Organic Se species, such as the protein-incorporated amino acid selenomethionine (SeMet), predominate in the natural food chain (Dumont et al., 2006) and in commercially produced Se-enriched yeast (Se-yeast)(Polatajko et al., 2006). Both inorganic and organic Se sources can be utilised for *de novo* synthesis of Sec, and hence selenoprotein synthesis (Papp et al., 2007).

The relatively high levels of Se in seafood has been suggested to play a major role in negating toxic effects from the MeHg also found in seafood. As such, it has been suggested that seafood MeHg concentrations should be considered in context with their ratio to Se levels. This is because lower seafood Se:MeHg ratios, such as those found in many apex predators such as sharks, may provide a better indicator of the increased likelihood of MeHg-induced toxic effects than MeHg levels alone (Berry and Ralston, 2008).

1.6 Vertebrate selenogenomes

As for terminology, selenogenes are genes coding for selenoproteins, while the selenoproteome/selenotranscriptome/selenogenome are the complete sets of selenoproteins/expressed selenogenes/selenogenes of an organism. With the rise of bioinformatics the first genome wide analyses for selenogenes were performed, whereby it was determined that humans contain 25 selenogenes, while rats and mice have 24 (Kryukov et al., 2003) and zebrafish 38 (Mariotti et al., 2012). The higher number of selenogenes found in zebrafish is mainly the result of a whole genome duplication event in a common ancestor of bony fish, after the split from the lineage that led to amphibians, reptiles, birds and mammals (Amores et al., 1998; Meyer and Schartl, 1999). Subsequently, the main difference between humans and zebrafish is that several genes found as single copies in humans (7 out of 25 selenogenes) are found as two copies (paralogs) in zebrafish (i.e. *gpx1a* and *gpx1b*; For complete list see selenoproteins marked with ** in Table 1). However, the majority (15 out of 25 selenogenes; Table 1) of selenogenes in humans are also found as single copies in zebrafish (Mariotti et al., 2012).

Currently, selenoproteins can be classified into six functional groups; antioxidant, redox signalling, thyroid hormone metabolism, Sec synthesis, Se transport/storage or protein folding (Papp et al., 2007). However, *in silico* knowledge of the selenotranscriptome far exceeds that of functional studies on the selenoproteome, and around half (11 out of 25) of the human selenoproteins remain largely uncharacterised (Table 1). Table 1 lists the primary, or putated, function of vertebrate selenoproteins.

Table 1. Gene abbreviations, names and function of selenoproteins. Underlined selenoproteins were not investigated in this thesis. Modified from **Paper III.**

Abbr.*	Name	Primary function and cellular location***	Mutant/knockout***
DIO1	Iodothyronine deiodinase	Thyroid hormone metabolism, located in plasma membrane	DIO1$^{-/-}$ develop normally (Moghadaszadeh and Beggs, 2006).
DIO2		As above, located in ER membrane	DIO2$^{-/-}$ are viable, but have disrupted energy metabolism (Marsili et al., 2011).
DIO3**		As above, located in plasma membrane (Moghadaszadeh and Beggs, 2006)	ND

EPT1	Ethanolaminephospho-transferase (aka SELI)	Phospholipid synthesis (??), cytosolic (Horibata and Hirabayashi, 2007)	ND
FEP15	Fish sep15-like protein	Fish specific, unknown function. Localised in ER and Golgi (Novoselov et al., 2006)	ND
GPX1**	Glutathione peroxidase	Reduces H_20_2, cytosolic.	GPX1$^{-/-}$ develop normally (Moghadaszadeh and Beggs, 2006)
GPX2		As above, but highly expressed in gastrointestinal tract	GPX2$^{-/-}$ develop normally (Moghadaszadeh and Beggs, 2006)
GPX3**		As above, but localised to most extracellular fluids	GPX3$^{-/-}$ develop normally in both mice and zebrafish (Olson et al., 2010; Kettleborough et al., 2013).
GPX4**		Reduces H_20_2 and phospholipid hydroperoxides, associated with cell and organelle membranes, also cytosolic. Specific splice variant (Pfeifer et al., 2001) essential structural component of sperm (Ursini et al., 1999)	GPX4$^{-/-}$ is embryonic lethal (Moghadaszadeh and Beggs, 2006)
MSRB1**	Methionine sulfoxide reductase B1	Reduces oxidised methionine and may play a role in redox status. Localised in the cytosol and nucleus	MSRB1$^{-/-}$ are viable, but have elevated oxidative stress (Fomenko et al., 2009)
SPS2	Selenophosphate synthetase 2	Catalyses step in Sec biosynthesis, cytosolic (Moghadaszadeh and Beggs, 2006)	ND
SELH	Selenoprotein H	Antioxidant (??), localised to the nucleoli (Novoselov et al., 2007)	SELH$^{-/-}$ is embryonic lethal in zebrafish (Amsterdam et al., 2004).
SELJ	J	Fish specific, eye structural protein (??), localisation unknown (Castellano et al., 2005)	ND
SELK	K	Chaperone like function in a pathway degrading misfolded proteins (??), and/or Ca^{2+} homeostasis in immune cells (??). Localised to ER (Shchedrina et al., 2011)	SELK$^{-/-}$ are viable, but are immunocompromised (Saguna et al., 2011).
SELL	L	Fish specific, antioxidant (??), cytosolic (Shchedrina et al., 2007)	ND
SELM	M	Unknown function, localised to the ER and Golgi.	SELM$^{-/-}$ are viable, but have disrupted energy metabolism (Pitts et al., 2013)
SEPN	N	Function unclear, but critical for muscle development. Localised to the ER membrane.	SEPN$^{-/-}$ in humans is not embryonic lethal, but patients exhibit congenital muscular dystrophy (Petit et al., 2003)
SELO	O	Kinase (??), unknown localisation (Dudkiewicz et al., 2012).	ND
SEPP1**	P1	Primary Se transport protein, extra and intracellular.	SEPP1$^{-/-}$ viable when fed a high Se diet (Moghadaszadeh and Beggs, 2006).
SELT**	T (fish; T1)	Cellular Ca^{2+} homeostasis (??). Localised to the ER, Golgi and cytosol (Grumolato et al., 2008).	ND
SEPW	W (fish; W1)	Cytosolic antioxidant (Whanger, 2009)	ND

SEP15	15	Protein folding (??). Localised in the ER.	SEP15$^{-/-}$ are viable, but have elevated cataract levels (Kasaikina et al., 2011)
TXNRD1	Thioredoxin reductase	Reduces thioredoxin, cytosolic.	TXNRD1$^{-/-}$ is embryonic lethal (Moghadaszadeh and Beggs, 2006)
TXNRD2		As above, mitochondrial. Not found in fish	TXNRD2$^{-/-}$ is embryonic lethal (Moghadaszadeh and Beggs, 2006)
TXNRD3		As above, mainly expressed in testes (Arnér, 2009).	ND
VIMP	VCP- interacting membrane protein (aka SELS)	Protein degradation pathway (??), located in the ER (Christensen et al., 2012)	ND

* Several largely uncharacterised selenoproteins are not present in the table. These are the fish specific selenoproteins SELO2, SEPW2a, SEPW2b, FAM213aa (aka SELU1a), FAM213ab (aka SELU1c) and SELT2, and the placental mammal specific selenoproteins GPX6 and SELV.
** These selenogenes are found as paralogs (a and b) in zebrafish (Mariotti et al., 2012). However the characterisation of the individual paralogs are largely unknown, and functions and cellular locations of the single mammalian ortholog are provided.
*** For mice/rats unless otherwise stated.
Abbr, abbreviation; ER, endoplasmic reticulum; ??, indicates function suggested by homology and/or *in vitro* experiments, but further characterisation is required to confirm.

1.7 Zebrafish as a model for studying selenoproteins

Overall, zebrafish have copies of all but three of the known non-piscine vertebrate selenogenes; *txnrd2* (aka TG), *gpx6* and *selv* (Mariotti et al., 2012). Combined with other features of zebrafish, such as the short generation time, genetic traceability and transparent embryos that allow the study of early vertebrate development (Kahn, 1994), the zebrafish is an exciting model for investigating selenoproteins at an organism level in vertebrates.

Being fish, zebrafish have several major differences to mammals that are important to be aware of when they are used as a model to explore selenoproteins. The first is that unlike mammals, fish can obtain Se directly from water over their gills (Hodson and Hilton, 1983), and hence have multiple exposure routes to Se. The second is that maternal transfer of nutrients in fish occurs for a short period, development occurs after maternal nutrient transfer is complete and among others the major protein transferred to oocytes is vitellogenin (Bobe and Labbé, 2010), conditions of which all are absent

in non-monotreme mammals. The third is that unlike common rodent models, the nutrient requirements of zebrafish remain largely unknown. As such there is currently no standard diet for zebrafish, and unknown nutrient effects may confound results within the zebrafish research field (Penglase et al., 2012).

1.8 Selenoprotein synthesis

The mRNA of selenogenes have several distinct features, a) the presence of a UGA stop codon/s within the open reading frame, and b) a specific stem-loop structure in the 3' untranslated region termed the SECIS element (Sec insertion sequence)(Fig. 4). Normally, UGA codons within the open reading frame (premature stop codons) are detected and the mRNAs are rapidly degraded by nonsense-mediated decay (NMD), a pathway that minimises the translation of truncated proteins (Rebbapragada and Lykke-Andersen, 2009). However in the selenotranscriptome, NMD is utilised as a regulatory mechanism in response to Se availability. Under Se replete conditions, the premature stop codon/s of selenogenes are recoded to Sec during translation by means of a protein complex recruited to the SECIS element (Berry et al., 1991b; Papp et al., 2007). Meanwhile, under Se deficient conditions, it is thought that the recruitment of this protein complex is disrupted, and the within frame UGA codon in the mRNA of a subset of selenogenes is identified as a stop codon and the mRNA enters the NMD pathway (Moriarty et al., 1998). As a result, Se status strongly affects the selenotranscriptome and ultimately the selenoproteome. Suboptimal dietary Se levels result in decreased mRNA and protein levels primarily of antioxidant selenoproteins (Sunde and Raines, 2011) and this targeted effect may be because of functional redundancy with non-Se dependent antioxidant proteins (Wirth et al., 2010). Overall this effect is referred to as the selenoprotein hierarchy (Brigelius-Flohé, 1999), and is thought to allow Se to be preferentially allocated to selenoproteins that are more critical for cell survival when Se supply is limited (Schweizer et al., 2004).

Compared to the tight regulatory control of Sec during protein translation, SeMet is not distinguished from methionine by tRNA, and as such is substituted as methionine into

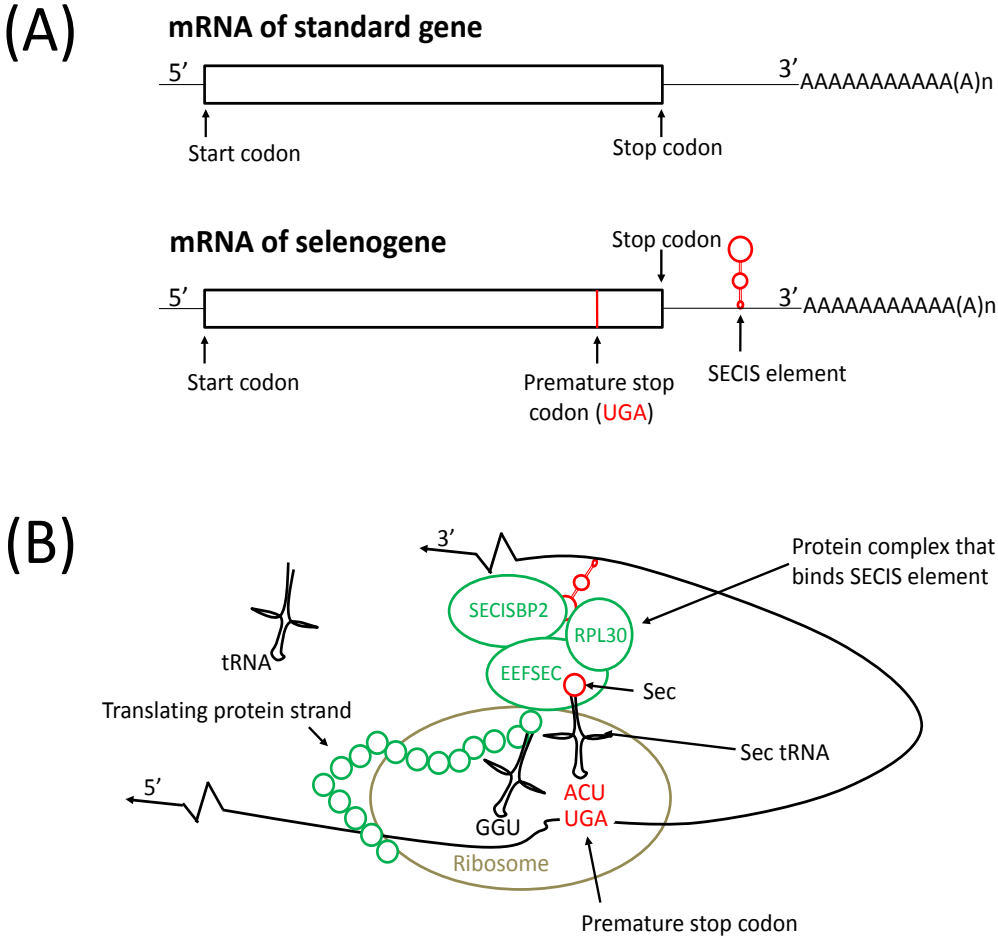

Figure 4. Unique features of selenogene mRNA's. The mRNA of selenogenes have several unique features (coloured red in **(A)**) that are essential for the insertion of selenocysteine (Sec) residue/s during protein translation. The first is a premature stop codon (nucleotide sequence UGA) which is present within the translated region of the mRNA (open black box), and the second is a stem loop structure in the 3' untranslated region of the mRNA termed the Sec insertion sequence (SECIS element). Variations on this them exist, as for instance *sepp1* mRNA contains two SECIS elements, and multiple premature stop codons. During translation **(B)**, a protein complex containing Sec tRNA that is recruited to the SECIS element interacts with the ribosome allowing Sec to be incorporated into the nascent protein chain, effectively retranslating the premature stop codon as a Sec codon. Figure **(B)** is based on the mammalian model and adapted from (Papp et al., 2007), and additional components required for mRNA translation in general are omitted for simplicity. Abbreviations; SECISBP2, SECIS binding protein 2; RPL30, Ribosomal protein L30; EEFSEC, Eukaryotic elongation factor, Sec-tRNA-specific.

proteins in a concentration-dependent manner (Waschulewski and Sunde, 1988). Thus all proteins with methionine residues may contain Se, but only those where Sec is genetically coded for are classed as selenoproteins.

Selenoprotein synthesis is metabolically costly, relatively slow and relies on a supply of an additional nutrient (Se) (Papp et al., 2007). Thus, the evolutionary retention of selenogenes implies that Sec, which is nearly always a functional residue at the active site of selenoproteins, confers a unique catalytic advantage. In agreement, compared to Cys, its closest analogue, Sec appears to increase protein stability (Nauser et al., 2014), and allow enzyme activity to occur over a wider range of physiological conditions, including changes in pH (Gromer et al., 2003) and at elevated levels of H_2O_2 (Rocher et al., 1992). Additionally, Sec can often, but not always, result in higher catalytic rates than Cys containing protein isoforms. For example $GPX^{Sec} \rightarrow GPX^{Cys}$ (GPX where the Sec is replaced by Cys) decreases activity by 1000 fold (Rocher et al., 1992), while $TXNRD1^{Sec}$ and $TXNRD1^{Cys}$ isoforms can have similar activity levels (Gromer et al., 2003). Overall, the benefit of using Sec is probably different between selenoproteins (Nauser et al., 2014), and may include additional unidentified factors.

1.9 Selenium toxicity targets the embryonic and larval stages in fish

The toxicity of Se was known well before its essentiality. For example, in the early 20th century, Se-induced toxicity from elevated Se levels that accumulated in plants growing on selenifourous soils was found to be the cause of the disease “blind staggers” in livestock (Combs and Combs, 1986b). Selenium-induced toxicity appears to be mediated predominately by Se-induced oxidative stress (Spallholz, 1994). Metabolism of small Se containing compounds, including both inorganic (e.g. selenite) and organic species (e.g. SeMet) in the presence of GSH produces ROS such as superoxide (O_2^-) and/or hydrogen peroxide (H_2O_2) in a concentration dependent manner (Spallholz, 1994). With elevated Se intake, the cellular antioxidant system is overwhelmed by Se-metabolism induced increases in ROS, leading to oxidative stress. Oxidative stress

from the metabolism of Se species is proposed to account for the cell cycle arrest and apoptosis that accounts for both the toxicity and carcinostatic properties of Se (Spallholz, 1994; Spallholz et al., 2004).

The embryonic and larval stages of oviparous species; which includes fish, amphibians, reptiles and birds; appear particularly vulnerable to Se toxicity (Janz et al., 2010). For instance, adult fish in Se contaminated water sources may be unaffected while at the same time larval fish can have Se-induced increased rates of deformities and mortality (Lemly, 1997). This difference appears to be because embryonic and larval stages in fish rely heavily on amino acids as an energy source compared to later stages (Kamler, 2008; Conceição et al., 2011), which inevitably results in oxidative stress via SeMet catabolism (Fig. 5; (Palace et al., 2004)). Coupled with this, early life stages are more sensitive to disturbances in cellular redox balance (Ufer and Wang, 2011), and thus more sensitive to Se-mediated oxidative stress in general.

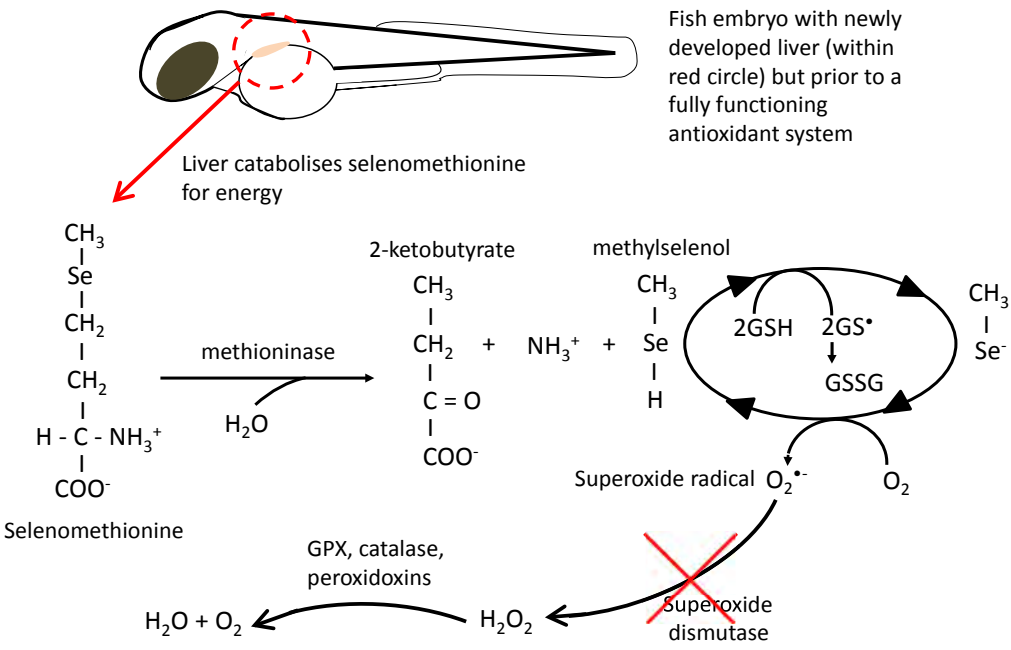

Fig. 5. See figure caption on next page

Figure 5 (previous page). A proposed mechanism of reactive oxygen specie (ROS) generation when selenomethionine is catabolised by the methioninase enzyme in hepatocytes. Methioninase cleaves selenomethionine into several products including methylselenol. The subsequent redox cycling of methylselenol oxidises glutathione (GSH) to glutathione disulfide (GSSG), which can reduce the levels of GSH, an important molecule for maintaining cellular redox balance. At the same time oxygen (O_2) is reduced to the superoxide free radical ($O_2^{\bullet -}$). The combination of decreased protection against ROS (reduced GSH levels) and the concomitant increase in ROS generated ($O_2^{\bullet -}$) is thought to be particularly harmful during a critical window in embryogenesis just after organogenesis of the liver. In this critical window the liver has methioninase, but not superoxide dismutase, activity. Henceforth, $O_2^{\bullet -}$ levels increase dramatically as they cannot enter the antioxidant chain and oxidative stress occurs. The critical period determined in trout (*Oncorhynchus mykiss*) was around 29 days post fertilisation at 8° C (Palace et al., 2004). A similar development stage occurs around 42 hours post fertilisation at 28.5° in zebrafish (Kimmel et al., 1995). Adapted from Palace et al. (2004) with permission.

Study aims

The primary aim of this study was to explore how Se and MeHg interact at both the organism and molecular level using zebrafish as the model. Specifically we aimed to assess the interactive effects of Se and MeHg on reproduction, and then on selenogene mRNA expression during development. There were three steps to complete these aims;

1. Identify key molecular responses to Se status and the Se requirements of zebrafish (**Paper I**).

   Selenium status affects many cellular process and is a factor in MeHg toxicity (Ralston et al., 2012). Therefore, uncontrolled differences in Se status may affect outcomes when exploring MeHg toxicity, which is preventable by including Se as a controlled variable. We therefore had to determine the Se requirements of zebrafish before exploring Se×MeHg interactions in the following steps.

2. Determine the interactive effects of Se and MeHg on vertebrate reproductive outcomes (**Paper II**).

   Reproduction is affected by either Se or MeHg toxicity, but little is known about how Se and MeHg interact to affect reproduction. The control diet level of Se in this step would be based on the Se requirements determined in **Paper I**, to prevent any tandem cellular effects of a deficient or toxic Se status. This experiment was also required to obtain a maternally exposed F_1 generation for the next step.

3. Use the F_1 generation generated at step (2) to identify selenogenes that are affected by the interactive effects of Se and MeHg during development (**Paper III**).

   The main concern of MeHg-induced impacts in humans are those caused by maternal transfer of MeHg during foetal development, a scenario modelled in **Paper III** by using the maternally exposed F_1 generation of zebrafish. Several

selenoproteins are molecular targets of MeHg, while Se can protect against MeHg toxicity. However, the effect of MeHg has only been investigated on a small number of selenoproteins. Thus, a more comprehensive overview of the Se×MeHg interactions on the selenotranscriptome is needed. Thus, in **Paper III** we investigated 30 out of the putative 38 selenogenes in zebrafish, which covers most vertebrate selenoprotein families and functional groups.

Results summary

2.1 Selenogene expression in response to selenium and/or methylmercury status

In total, the mRNA expressions of 30 out of the 38 putative selenogenes were analysed in zebrafish embryos maternally exposed to elevated Se and/or MeHg (**Paper III**), while four selenogenes were analysed in juvenile zebrafish in response to Se status (**Paper I**). The 30 selenogenes (**Paper III**) cover most of the selenoprotein families; including members of the *gpx*, *thioredoxin reductase*, *iodothyronine deiodinase* and *methionine sulfoxide reductase* families, along with *selenophosphate synthetase 2*, *selenoprotein h*, *j-p*, *t*, *w*, *15*, *fep15* and *fam213aa*; and include members from all six known functional groups of selenoproteins; antioxidant, redox signalling, thyroid hormone metabolism, protein folding, Se transport/storage and Sec synthesis. The four selenogenes analysed in **Paper I**; *gpx1a*, *gpx1b*, *sepp1a* and *sepp1b*; were selenogenes whose single mammalian orthologs (*Gpx1* and *Sepp1*) respond to Se status, and a subset of those analysed in **Paper III.**

The results of the analyses in **Paper III** demonstrate that a) only around one in four (8 out of 30, $p<0.05$) selenogenes respond to elevated MeHg status and these selenogenes mainly coded for antioxidant proteins, b) MeHg-induced regulation of selenogenes is exclusively via downregulation, and c) elevated Se status can prevent the MeHg-induced downregulation for most (5 out of 8) selenogenes.

Of particular note were *gpx1a* and *gpx1b*. In mammals, GPX1 is an antioxidant protein regulated by Se status, and the most common selenoprotein by quantity (Gross et al., 1995; Hill et al., 2012). The mRNA of the zebrafish *gpx1* paralogs responded to Se status in juvenile zebrafish (**Paper I**), and were downregulated by elevated MeHg in zebrafish embryos (**Paper III**). In juvenile fish, the lowest *gpx1a* and *gpx1b* expression coincided with low GPX activity, and occurred in groups that had the highest growth rates, suggesting a role of Se in regulating growth, perhaps via cellular redox status (**Paper I**).

Along with the *gpx1* paralogs, several other selenogenes that were downregulated by MeHg; *selh*, *gpx4a* and two members of the *sepw* family; *sepw1* and *sepw2a*; also code for selenoproteins from antioxidant families. Additionally, MeHg downregulated selenogenes from redox signalling (*txnrd1*, *msrb1a* and *1b*), thyroid hormone metabolism (*dio1*) or incompletely classified (*selt1b* and *fam213aa*) families. However, the antioxidant coding selenogenes were downregulated to the greatest extent by MeHg. Overall, the response of zebrafish selenogenes to MeHg had similarities to their responses to their mammalian orthologs during Se deficiency. Further analyses specifically in zebrafish embryos demonstrate that the selenogenes most downregulated by MeHg (**Paper III**) were also downregulated by Se deficiency (unpublished data, **Thesis Supp. Material**), further supporting the MeHg-induced Se deficiency hypothesis.

With the addition of the **Thesis Supp. Material** to data from the **Papers I** and **III** we could further conclude that a) low Se status regulates the expression of a small subset of selenogenes (6 out of 30, $p<0.05$) b), this subset overlaps considerably with those affected by MeHg (4 out of 6, $p<0.05$), and c) the expression of the majority of selenogenes (20 out of 30, $p<0.05$) were unaffected by Se and/or MeHg.

2.2 The interactive effects of methylmercury and selenium are life stage dependent

Elevated dietary Se prevented MeHg-induced decreases in growth and increases in mortality in adult zebrafish, as previously reported in vertebrates (**Paper II**). In contrast, reproductive outcomes; fecundity, embryo survival and the number of viable offspring produced (reproductive success); were reduced by elevated Se, and this effect was enhanced by elevated MeHg (**Paper II**), a novel finding in fish. Meanwhile, elevated Se protected against MeHg-induced decreases in selenogene mRNA expressions (see previous section) and GPX activity in zebrafish embryos (**Paper III**). The protection against MeHg-induced disruptions extended into the larval phase, whereby elevated Se was able to partially prevent MeHg-induced hypoactivity (**Paper III**). Thus, while the results show that Se can protect against MeHg-induced toxicity in

general, a critical window, hypothesised to be during embryogenesis, exists whereby elevated Se exerts toxic effects. The results suggests that the MeHg-induced synergism with Se toxicity in this critical period is due to a MeHg-induced increase in Se levels transferred to the oocytes (**Paper II**).

Discussion

This thesis aimed to address some of the open questions in relation to the protective effects of Se against MeHg toxicity. As all papers in this thesis are published, many themes have already been discussed in detail. Thus along with an expansion on concepts put forward in the papers, several key concepts developed post publication will also be discussed.

3.1 A comparison of glutathione peroxidase activity as a biomarker for selenium requirements in fish versus the rodent model.

In the rodent model (rats/mice), GPX activity is a good biomarker for Se requirements. For instance, as Se status in rodents shifts from deficient to replete, total hepatic GPX activity first increases sigmoidally, and then shifts to a plateau like response at ≈0.1 mg Se/kg DM (Fig. 6). The shift to a plateau response indicates that the Se status is adequate to fulfil maximum GPX1 protein expression (Weiss et al., 1996; 1997; Barnes et al., 2009) the main contributor to total GPX activity in mammals (Brigelius-Flohe et al., 2002) and the dominant selenoprotein in the mammalian body (Hill et al., 2012). Furthermore, at the 0.1 mg Se/kg dietary level, signs of Se deficiency or toxicity are absent and animals are in general good health (NRC, 1995; Weiss et al., 1996; 1997; Barnes et al., 2009). As a result of these indicators, 0.1 mg/Se kg is defined as the rodent model Se requirement (Weiss et al., 1996). The success of defining Se requirements based on the minimum dietary Se level that results in maximum GPX activity in rodents has led to this methodology being utilised to determine the Se requirements in fish.

However, there is now sufficient evidence that fish GPX activity does not respond to Se status in a similar manner to rodents, as discussed in **Paper I**. The re-analysed data (with models) from the five other published fish Se requirement studies helps to demonstrate this point (Fig. 7). As can be observed, an average of 6 or 4 fold higher levels of Se are required for maximum plasma (GPX3) or hepatic GPX activity, than

for growth, in fish (Fig. 7). Unlike in rodents (Fig. 6), GPX activity increases over a broader range of dietary Se levels in fish (Fig. 7). In the extreme cases, GPX activity can continue to increase even when the dietary Se level has negative effects on growth, as observed for both zebrafish and the marine fish, grouper (*Epinephelus malabaricus*) (Fig. 7D, F and H). Thus in contrast to rodents, maximum GPX activity appears to be a poor biomarker for optimal Se status in fish.

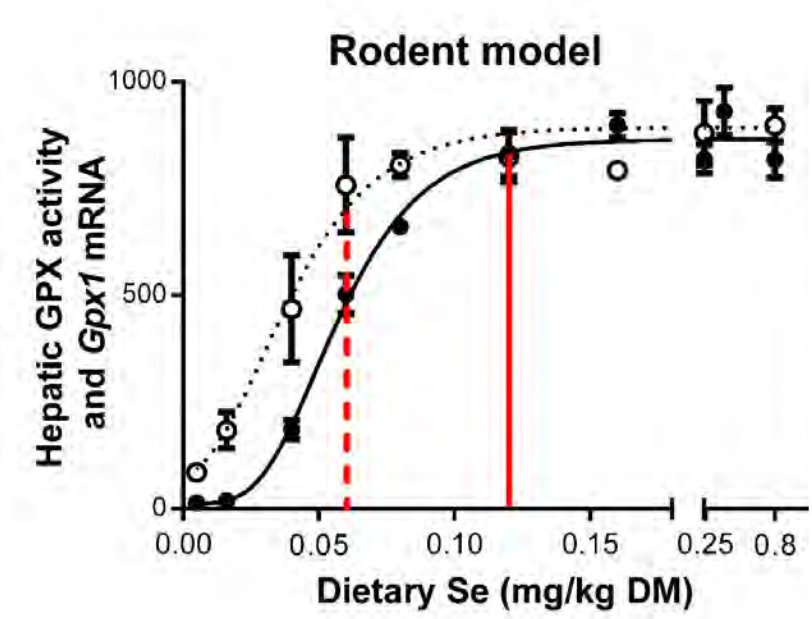

Figure 6. The rodent model response of hepatic GPX activity (●) and the mean normalised *Gpx1* mRNA levels (○) to Se status. Male rats (21 days old) were fed diets with increasing levels (0.005 to 0.80 mg Se/kg DM) of inorganic Se (sodium selenite) for 28 days. Red lines indicate the Se requirement for *Gpx1* mRNA (segmented) and GPX activity (solid) (one-way ANOVA, $p<0.05$). Adapted from Barnes et al. (2009) with permission.

Why fish and rodents, and thus presumably fish and mammals, differ in respect to their GPX activity response to Se status is unclear. The upregulation of GPX in response to elevated Se status is unlikely to be the underlying cause for the decreased growth observed in grouper and zebrafish (Fig. 7), as GPX1 overexpression does not decrease growth in mice (Cheng et al., 1997). To speculate, fish may utilise GPX as a Se storage protein, an idea previously proposed as a secondary function of mammalian GPX1

Fig. 7

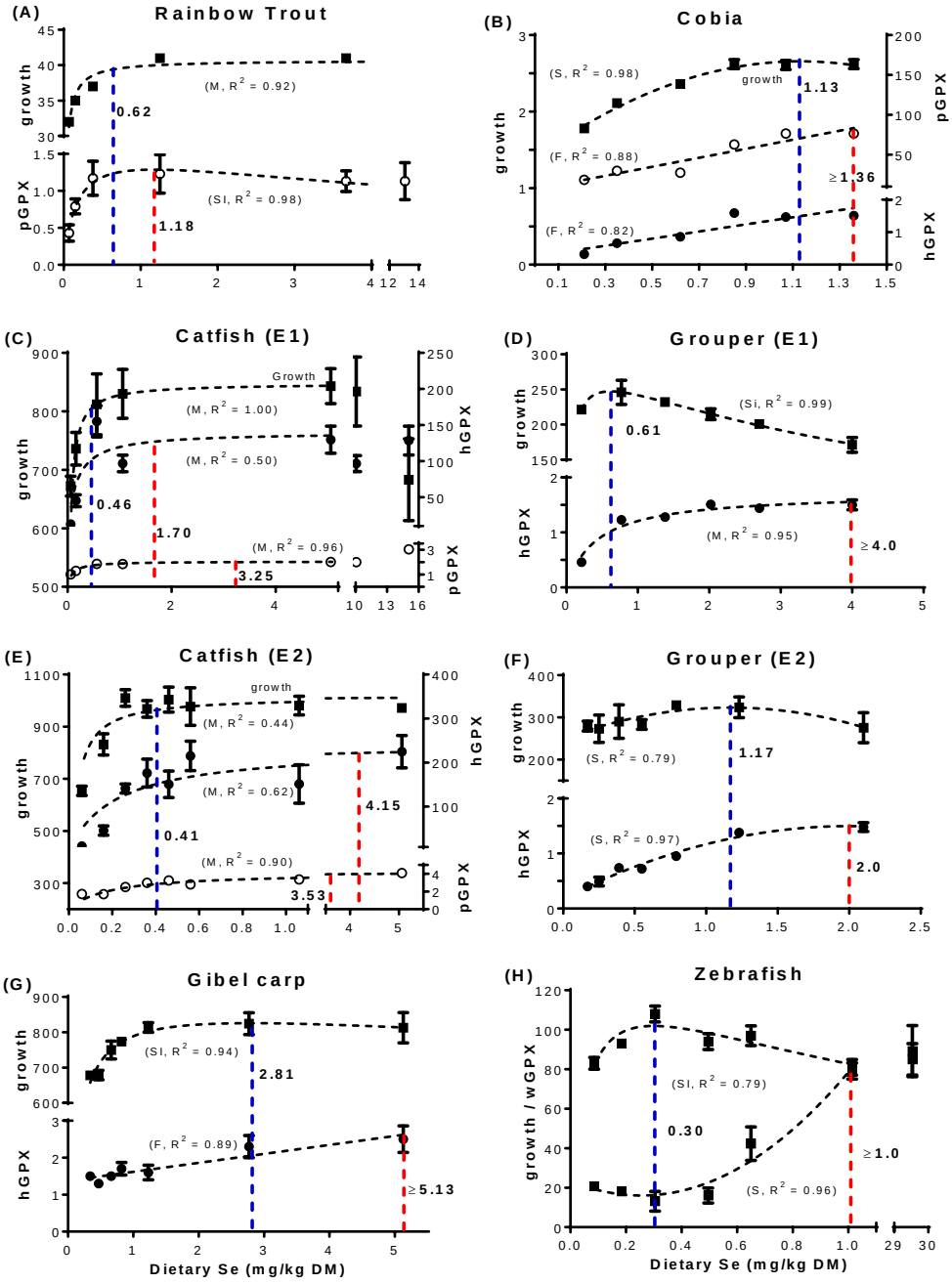

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Figure 7. (previous page). Dietary Se levels required for maximum growth (blue segmented lines) versus maximum GPX activity (red segmented lines) in fish. Growth and GPX activity data obtained from the five published studies were re-analysed with regression analysis, and included alongside results from **Paper I**. Several of these studies include two separate feeding experiments (Experiment 1 (E1) and 2 (E2)). Growth data were presented in a range of different units between studies; graph (**A** and **H**) as final weight/fish (g) (Hilton et al., 1980; **Paper I**), graphs (**C**- **G**) as % weight gain (Gatlin and Wilson, 1984; Lin and Shiau, 2005; Han et al., 2011) and graph (**B**) as specific growth rate/day (SGR %/d)(Liu et al., 2010). Broken blue or red lines and the associated numbers indicate Se requirement for maximum growth or GPX activity (h, hepatic; p, plasma; w, whole body GPX activity), respectively. The coefficient of determination (R^2) along with the fitted model; first order polynomial (F), second order (S), substrate inhibition (SI) or Michaelis-Menten (M) are found within figures. Data were tested against the null hypothesis (horizontal line) versus alternative hypothesis ($p<0.05$). The Se requirements for data fitted with the Michaelis-Menten model were based on 95% of the theoretical maximum. One data point is absent from graph **(A)**; final weight of fish fed 13 mg Se/kg was 14 g (Hilton et al., 1980).

(Burk, 1991; Gross et al., 1995). As a consequence of seafood being a rich source of Se (Combs and Combs, 1986b; Rayman, 2012), fish diets are naturally relatively high in Se. This Se rich diet may require the presence of a greater capacity or additional mechanism to prevent Se toxicity. Incorporating Se into a selenoprotein, such as GPX, that can be rapidly upregulated in response to elevated Se would help to prevent Se toxicity due to oxidative stress from uncontrolled redox cycling of Se metabolites (Spallholz, 1994). If $KO^{-/-}$ of the dominant GPX types in fish decreases their Se toxicity threshold remains to be tested, but it would support a role of GPX as a Se buffer in fish. The ability to test this hypothesis may be proximate as a result of the zebrafish mutation project (Kettleborough et al., 2013), which has made $GPX3^{-/-}$ mutants available recently, and expects to make several more ($GPX4a^{-/-}$ and $GPX1b^{-/-}$) available this year (http://www.sanger.ac.uk/sanger/Zebrafish).

As discussed in **Paper I**, zebrafish growth peaked when GPX activity/mRNA expressions were at their lowest (0.3 mg Se/kg diet, Fig. 7). It is unknown why this occurred. Perhaps in fish fed 0.3 mg Se/kg, GPX activity was at an optimal level. Meanwhile, in fish fed above 0.3 mg Se/kg, GPX activity may have responded positively to excessive Se levels to either store excess Se as discussed previously, or to counteract increased ROS generated by Se metabolism. Alternatively, low GPX

activity may have resulted in elevated cellular H_20_2 levels, an important intracellular signalling molecule that can influence cellular proliferation, and hence growth (Gough and Cotter, 2011). However, total and oxidised glutathione, redox potential and TBARS remained relatively stable with changing Se status (**Paper I**), suggesting that any changes in H_20_2 were small, or did not occur throughout the entire body.

The reason why the initial, but small, decrease in GPX activity (and mRNA expression) observed in **Paper I** was not observed in other fish studies may reflect methodology. In **Paper I**, whole zebrafish bodies were analysed, while other fish studies analysed specific organs such as the liver and plasma. As the requirement for a nutrient is determined by the entire organism, whole body analyses are a justifiable trade off against the increase in clarity of the underlying molecular mechanisms for such changes that are obtained by organ specific analyses.

3.2 Fish selenium requirements change with age

During embryogenesis the expressions of selenogenes/proteins are highly dynamic (Thisse et al., 2003; Ufer and Wang, 2011; Skjærven et al., 2013; Timme-Laragy et al., 2013). This suggests that the underlying need for the Se present in these selenoproteins must also change with development. This concept is supported by this thesis, wherein the Se requirements for juvenile growth appear to be over two fold less than those required for both reproduction and the resulting F_1 generation yolk sac larvae (**Paper I**).

3.2.1 Selenium status and reproduction

Aspects of F_0 generation Se and/or MeHg status on reproduction and the subsequent F_1 generation outcomes were explored in all three papers of this thesis. In **Paper I** we found that Se requirements for male mating success (≥0.65 mg Se/kg) were over two fold higher than the Se requirements of juvenile zebrafish based on growth (0.3 mg Se/kg). In mammals, testes are a major Se sink, where like the brain, Se is preferentially maintained under conditions of Se deficiency (Hill et al., 2012). This is likely because

sperm requires a splice variant of GPX4, termed sperm nuclei GPX (snGPX4) (Pfeifer et al., 2001), as a major structural component of the sperm mid piece (Ursini et al., 1999). The increased mating success of male fish fed 0.65 mg Se/kg DM correlated with a larger gonad somatic index, and may indicate increased sperm levels or production capacity (**Paper I**). In contrast, elevated Se levels (30 mg Se/kg DM) decreased the gonad somatic index (**Paper I**), an effect likely due to Se-induced oxidative stress in an organ that already has high Se levels under normal physiological conditions (Hill et al., 2012).

Female specific reproductive effects from elevated Se status, such as decreased fecundity and embryo survival were observed in one experiment (**Paper II**) but not the other (**Paper I**). The different diets and Se sources used in the two experiments may be a contributing factor to this; **Paper I** used a yeast/casein based diet supplemented with Se-yeast, while **Paper II** used a casein based diet supplemented with SeMet. However, the 3 fold higher dietary Se levels employed in **Paper I** than **II** suggests the weaker experimental design of **Paper I** lacked the statistical power to find differences. For example, the biggest differences in mean embryo survival relative to the high Se group were similar between the studies (≈2 fold; 44 versus 86% **Paper I**, 29 versus 53% **Paper II**). However, the negative effect of elevated Se on embryo survival was statistically significant (main effect, $p<0.05$) in **Paper II**, where the experimental design allowed the analysis of data from 108 matings (all single pair-wise matings), but not in **Paper I** which only had data from 24 matings (from groups with highest and lowest embryo survivals). Thus, the high natural variation in reproductive outcomes of fish (Bobe and Labbé, 2010) dictates that high numbers of pair or group-wise matings are desirable to obtain meaningful reproductive data, even when the mean differences between treatments are high.

3.2.2 Selenium status and larval locomotor activity.

Larval locomotor activity was affected by Se status (**Paper I** and **III**). Little is known about larval fish locomotor activity in regard to maternal nutrient status. Nutrient requirements generally decrease as weight and age increase (Baker, 1986). Thus larval

zebrafish Se requirements (via maternal nutrition) are probably higher than the 0.3 mg Se/kg required by juvenile zebrafish. For this reason, the lower locomotor activity levels (hypoactivity) of yolk sac larvae from parents fed >0.3 mg/Se kg found in **Paper I** may reflect an optimal state of behaviour.

But how could hypoactivity be a positive attribute? The answer possibly lies in energy conservation. Locomotor activity in **Paper I** and **III** were assessed in ≤5 dpf zebrafish, which utilise endogenous nutrients from the yolk as a sole source of energy (Jardine and Litvak, 2003). In terms of energy, yolk sac larvae are essentially closed systems, investing energy in either tissue formation (growth) or respiration, which is increased by activity (Kamler, 2008). The 40% lower activity levels of larvae from parents fed 0.65 versus ≤0.3 mg Se/kg would thus theoretically increase the energy available for growth.

However, in **Paper III** we describe how larvae from parents fed elevated dietary MeHg were also hypoactive. Alongside this, we also found that elevated Se (10 mg Se/kg) induced transient hypoactivity (at 3 dpf, but not present at later ages) (**Paper III**). Behavioural changes such as hypoactivity induced by MeHg are attributed to the negative effects of MeHg on neuron survival and patterning (Kidd and Batchelar, 2011; Hassan et al., 2012). This CNS disruption can impair the ability of fish larvae to capture prey (Fjeld et al., 1998; Alvarez et al., 2006), a vital behavioural transition that is required for survival when yolk sac nutrients are depleted. Overall, entirely different situations; one being the exposure to toxic levels of MeHg/Se, the second being small shifts in Se status within a nutritionally relevant range; can both lead to hypoactivity. This in itself rejects the notion that reduced activity *per se* can be defined either as a positive or negative attribute.

In zebrafish the yolk sac is depleted by <6.9 dpf at 28.5 ˚C (Jardine and Litvak, 2003). When this occurs, the larvae are entirely dependent on capturing prey for nutrition, and hence growth and survival. Like observed with MeHg, if Se-induced hypoactivity found in the >0.30 mg Se/kg groups continues past the yolk sac stage, prey capture ability could be negatively affected. Thus, two missing elements in this thesis; the

underlying molecular mechanisms for the Se-induced hypoactivity observed in **Paper I,** along with later effects such as larval prey capture ability, would help to define whether the Se-induced yolk sac stage hypoactivity is beneficial or negative.

3.3 Selenium and methylmercury have a synergistic negative effect on oviparous vertebrate reproduction.

The synergistic negative effect of Se and MeHg on zebrafish reproduction (**Paper II)** mirror findings in mallard ducks (*Anas platyrhynchos*) (Heinz and Hoffman, 1998). Interestingly, this effect in both species was associated with MeHg-induced increases in eggs Se levels. In **Paper II** we suggest that this increase in Se led to a concentration dependent increase in Se-mediate oxidative stress, the primary mechanism of Se-induced toxicity in developing embryos and fish larvae (Palace et al., 2004; Janz, 2011). Embryo survival (**Paper II**) was measured at 24 hpf, but not fertilisation rate. However, similar to Se toxicity in fish (Crane et al., 1992), embryogenesis, but not egg fertilisation rate, is targeted by the Se and MeHg interaction in ducks (Heinz and Hoffman, 1998). Embryogenesis may be particularly sensitive to Se-induced oxidative stress, as it requires a finely balanced progression of cellular proliferation, differentiation and apoptosis, which in turn is affected by cellular redox balance (Ufer and Wang, 2011). Furthermore, in fish it has been demonstrated that mismatches between enzymatic activities can lead to SeMet-induced oxidative stress during embryogenesis (See Fig. 5; (Palace et al., 2004; Spallholz et al., 2004))

Whether Se and MeHg have synergistic negative effects on reproduction in non-oviparous species is open to speculation. If MeHg-induced mediated increases in Se toxicity are the primary reason for the synergistic effect, then it is less likely to occur in mammals. This is because the Se-induced teratogenic affects observed in fish (Lemly, 1997) and birds (Heinz and Hoffman, 1998) are less prevalent in mammals, possibly because of protection provided by the placental barrier (Usami and Ohno, 1996).

A positive effect of MeHg on zebrafish mating success, fecundity and overall reproductive success occurred when exposure periods occurred for >100 days (**Paper II**). Similarly, MeHg laden diets (4-9 mg Hg/kg DM) increased mating success when fed to adult fathead minnows (*Pimephales promelas*) during the mating, but not juvenile, period (Hammerschmidt et al., 2002). In ducks, low levels (0.5 mg Hg/kg DM) of dietary MeHg increases the percent of fertilised eggs that hatched and the overall reproductive success (Heinz et al., 2010). The reasons for this hormetic effect on reproduction after short or low level exposure to MeHg are unclear.

3.4 Selenogene regulation

3.4.1 Selenium does not completely prevent methylmercury induced disruption of the selenotranscriptome.

The findings in **Paper III** provide evidence that the selenotranscriptome is regulated by MeHg induced Se deficiency, as many of the selenogenes downregulated by MeHg were rescued by elevated Se. This is in line with a recent mechanistic study demonstrating MeHg induces nonsense-mediated decay (NMD) of *gpx1* mRNA probably via inducing Se deficiency (Usuki et al., 2011).

However, Se status is only one of many factors that regulate the expression of selenogenes. Response elements (RE) are regions of DNA found upstream of a gene in the promoter region (Atkinson and Halfon, 2014). The REs recruit proteins termed transcription factors (TFs); which in turn are regulated by the cellular environment; that can up or down regulate gene expression (Todeschini et al., 2014). *In silico* analyses of selenogene promoter regions has identified between 100 and 230 putative RE's per human selenogene, with higher numbers found in selenogenes, such as *Sepp1* and *Txnrd1*, that are widely expressed (Stoytcheva and Berry, 2009). Common selenogene RE's were those bound by nuclear factor-kB (NFkB), metal-regulatory transcription factor-1 (MTF-1) and homeobox (HOX) TF's. Henceforth, the expression of selenogenes probably respond to the diverse range of factors that these latter TF's respond to; such as ROS, oxidised low density lipoproteins, bacterial or viral antigens,

hypoxia, heavy metals, and developmental stage, among others (Stoytcheva and Berry, 2009). Mammalian *Txnrd1* provides an example of how these regulatory mechanisms compete to control gene expression. Both ROS and Se status can regulate mammalian *Txnrd1* expression (Sunde and Raines, 2011). However MeHg induced ROS is a stronger regulator of *Txnrd1* mRNA levels than Se status (Usuki et al., 2011). In **Paper III**, none of the selenogenes downregulated by MeHg and not rescued by elevated Se; *gpx1b*, *txnrd1*, *selt1b*, *msrb1a* (p=0.07), *msrb1b* (p=0.053) and *dio1* (p=0.08); were regulated by Se status in zebrafish embryos (see **Thesis Supp. Material**). Thus it is likely that MeHg regulated these genes primarily via Se status independent pathways.

3.4.2 Zebrafish selenogenes and nonsense-mediated decay

Nonsense mediated decay (NMD) is discussed in both **Paper I** and **III** due to its importance as a regulatory mechanism for the selenotranscriptome and subsequently the selenoproteome. The characteristics of mRNAs that are susceptible to NMD are a) a premature stop codon located a minimum of 50 to 55 nt upstream of an exon junction (EJ), and b) a protein complex, termed the exon junction complex present 20-24 bp upstream of the exon-exon junction (EJ); which limits NMD to the first round of translation (Maquat, 2005). Figure 8 illustrates the mRNA features of zebrafish *gpx1a* compared to *msrb1a* and *txnrd1* that make the former but not the latter two, susceptible to NMD.

In the wider genome, NMD's main role is likely to prevent translation of truncated proteins, which may have reduced or no function, or be cytotoxic (Cowan et al., 2003; Pan et al., 2006). To further explore zebrafish mRNA in regard to NMD, the selenogene database (SelenoDB 2.0) and ensembl were searched for the exon position of the Sec coding UGA codon (Table 2).

As can be seen in Table 2, considerable overlap exists between those selenoproteins that were downregulated by low Se status, elevated MeHg, and that are theoretically susceptible to NMD based on the position of the UGA/Sec codon in the mRNA. Several selenogenes that were regulated by low Se status, but did not confirm to the rules of

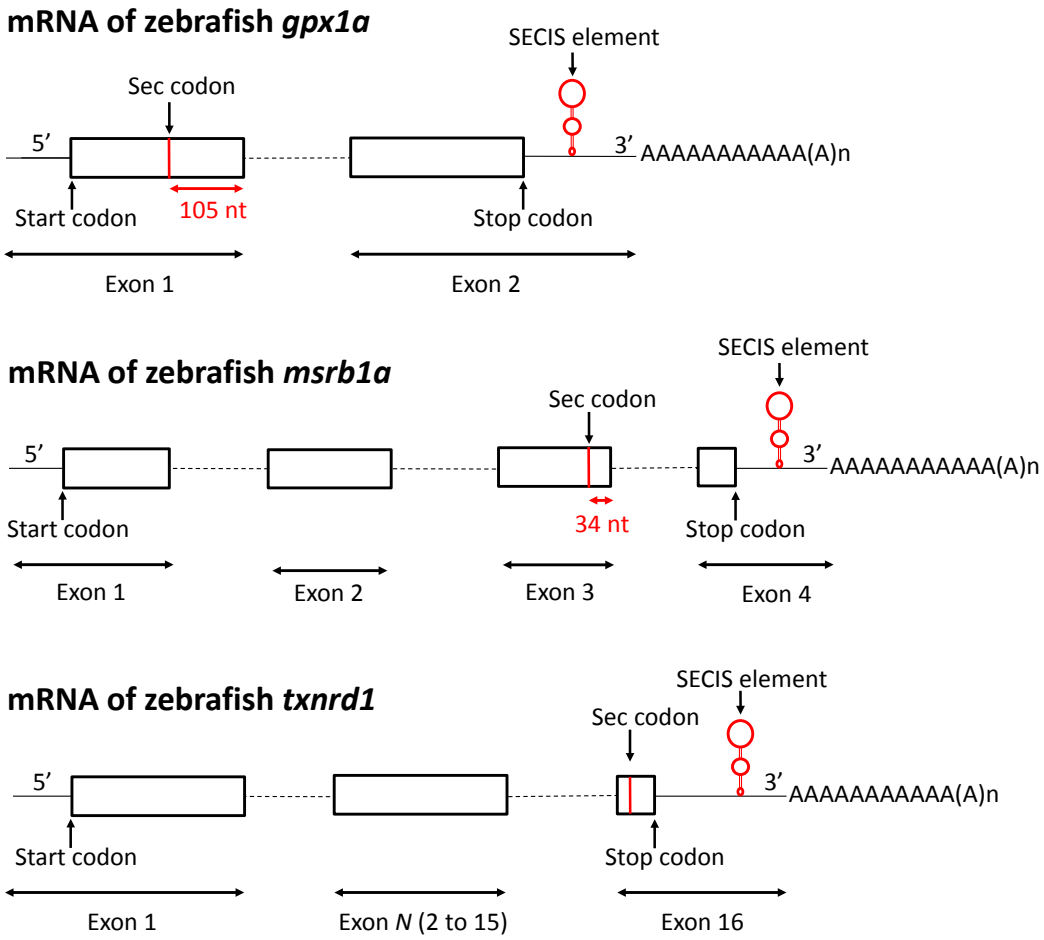

Figure 8. Selenogene mRNA susceptibility to Se-deficiency induced nonsense-mediated decay (NMD). Like mammalian *Gpx1*, zebrafish *gpx1a* mRNA contains the selenocysteine (Sec) codon 105 base pairs (bp) upstream of an exon/exon junction (EJ; dashed lines between exons), making it susceptible to NMD. In contrast, the Sec codon in *msrb1a* mRNA is <55 bp upstream of an EJ, and in *txnrd1* has no downstream EJ, in both zebrafish and mammals. These mRNA´s should therefore not be susceptible to NMD.

NMD; *gpx3*, *sepp1a* and *sepw1*; are also susceptible to Se-deficiency induced downregulation in mammals, but the regulatory mechanisms for this remain unclear (Sunde and Raines, 2011).

Table 2. The exon positions of the Sec codons in zebrafish selenogenes, the subsequent predicted susceptibility of the selenogenes to NMD, and the effect of Se or MeHg status on the expression of selenogenes determined *in vivo* in this thesis.

Gene	No. of exons in gene	Exon no. containing Sec codon	Distance (nt) to downstream EJ	NMD[1]	Regulated by ↓ Se	Regulated by ↑ MeHg
gpx1a	2	1	105	Y	↓J	↓
gpx1b	2	1	105	Y	↓J	↓
gpx3	5	2	22	N	↓	
gpx4a	3	1	105	Y	↓	↓
gpx4b	4	1	105	Y	↑ (0.09)	
txnrd1	16	16	-	N		↓
txnrd3	12?	12	-	N		
dio1	4	2	103	Y		↓ (0.08)
dio2	2	2	-	N		
Selh	1	1	-	N		↓ (0.055)
Selj	9?	7	1	N		
selk	4	4	5	N		
sell	9	6	42 and 33	N		
selm	5	2	21	N		
sepn	12	9	1	N		
Selo	9	9	-	N		
sepp1a[2]	4	1, 4[2]	26	N	↑↓J	
sepp1b	4	1	26	N		
selt1a	5	2	101	Y		
selt1b	5	2	101	Y		↓
selt2	5	2	101	Y		
sepw1	5	2	15	N	↓	↓
sepw2a	4	2	88 or 92?	Y	↓ (0.08)	↓
sepw2b	4	2	88	Y		
sep15	4	2	28	N		
fep15	4	2	56	Y		
msrb1a	4	3	34	N		↓ (0.07)
msrb1b	4	3	34	N		↓ (0.053)
sps2	8	1	151	Y		
fam213aa	6	3	15	N		↓

Abbreviations; nt, nucleotide; EJ, exon junction; J, regulation by Se status observed in juveniles but not in embryos; -, Sec codon in last exon; ?, gene annotation unclear.
Numbers in parentheses are p values for data with a statistical trend

[1.] Theoretically susceptible to nonsense-mediated decay based on position of Sec codon

[2.] Additional Sec residues are found in the last exon of *sepp1a* (mammalian *Sepp1*).

Most of the selenogenes which were downregulated by MeHg but not low Se status; *txnrd1*, *dio1*, *selh*, *selt1b*, *msrb1a*, *msrb1b* and *fam213aa*; were also among the group not rescued when elevated Se and MeHg were present together, and the majority are not theoretically susceptible to NMD. As pointed out in **Paper III**, most of these genes are not classified as antioxidants. Thus these genes appear to be a subset of genes that may be regulated by MeHg-induced effects other than MeHg-induced Se deficiency.

However the overall methodology in the table is not without exceptions. For one the selenogenes *gpx1a*, *gpx1b* and *sepp1a* were only found to be regulated by Se status in whole juvenile zebrafish (**Paper I**) and not in zebrafish embryos (**Thesis Supp. Material**). This may reflect temporal or spatial differences in gene regulation. For example, these genes may be regulated by developmental TF's in the embryos, which may be stronger regulators than Se-mediated mechanisms at this stage.

Zebrafish selenogene mRNAs provide an ideal model to further elucidate how small changes in the mRNA nucleotide sequence can affect NMD. For instance *gpx4a* and *gpx4b* share sequence identities of 85% (NCBI nblast) and both have the Sec encoding TGA codon 105 bp upstream of the EJ, but only *gpx4a* is downregulated by Se deficiency directly (**Thesis Supp. Material**), or as a consequence of MeHg (**Paper III**).

3.4.3 The glutathione peroxidase system in fish

This thesis found that zebrafish *gpx4a* was a highly inducible gene, unlike its mammalian ortholog *Gpx4* which demonstrates minimal regulation by Se (Sunde and Raines, 2011) or MeHg (Kim et al., 2005) status. Alongside this, *gpx4a* also appears to be an abundant transcript. For example, Zheng et al. (2013) found that *gpx4a* was the most abundant selenogene transcript, and one of the most abundant transcripts overall in both female and male zebrafish livers. Compared to the *gpx1* paralogs, both *gpx4a* and *gpx4b* are higher in abundance by around 10 fold during zebrafish development (0-50 dpf) (Timme-Laragy et al., 2013), and 3 fold in rainbow trout (*Oncorhynchus mykiss*) liver (Pacitti et al., 2013). This contrasts with mammals, where hepatic *Gpx1* mRNA abundance is around 7 fold higher than that of *Gpx4* (Weiss Sachdev and Sunde, 2001). Thus, at the transcriptome level fish *gpx4a* appears to be a key selenogene, possibly reflecting the highly abundant *Gpx1* transcript found in mammals.

The same contrast appears true at the functional protein level. In fish, GPX4 activity can account for over a third of total GPX activity (Grim et al., 2011; Wang et al., 2012). However, in mammals GPX1 protein contains around 50% of hepatic selenoprotein

incorporated Se (Hill et al., 2012) and hepatic GPX activity is dominated by GPX1, while GPX4 may account for less than 2% of total GPX activity (Barnes et al., 2009).

So why is the GPX system dominated by GPX1 in mammals, while in fish it appears to be shared between GPX1 and GPX4? The answer may lie in the broader substrate range of GPX4. Like GPX1, GPX4 can reduce hydrophilic hydroperoxides such as H_2O_2 and free lipid peroxides, but additionally can also reduce hydrophobic hydroperoxides such as phospholipid and cholesterol hydroperoxides incorporated in cell membranes and lipoproteins (Ufer and Wang, 2011). Compared to terrestrial vertebrates, fish are high in long chain polyunsaturated fatty acids, such as DHA and EPA (Douglas and Douglas, 1988; Mahaffey, 2004; Cladis et al., 2014), which in turn are sensitive to oxidation (Holman, 1954). All else being equal, this suggests a greater requirement for controlling oxidation in the hydrophobic cell membranes where PUFA are incorporated, which requires GPX4 activity. In line with this, a positive correlation is observed between GPX4 activity and the levels of unsaturated fatty acids across fish species (Grim et al., 2011).

However, like fish, birds also appear to have a shared GPX system. In turkey (*Meleagris gallopavo*) for instance, GPX4 accounts for half of the total GPX activity in liver, and around a third in muscle, testes and heart (Sunde and Hadley, 2010). The *gpx4* gene is the ancestor of all other *gpx* genes (Mariotti et al., 2012). Perhaps factor/s linked to terrestrial environments relaxed the requirements for GPX4 specifically in mammals or a mammalian ancestor after the split from the lineage leading to birds, allowing the dominance of the GPX1 in the GPX system, as observed in extant mammals today. Overall, the importance of GPX4 to the GPX system of fish was not evident when **Paper I** was published. In relation to the GPX system, future fish (and bird) studies should increase focus on the response of *gpx4a* (*Gpx4* in birds) mRNA expression, and GPX4 activity, in addition or in priority to the GPX1's.

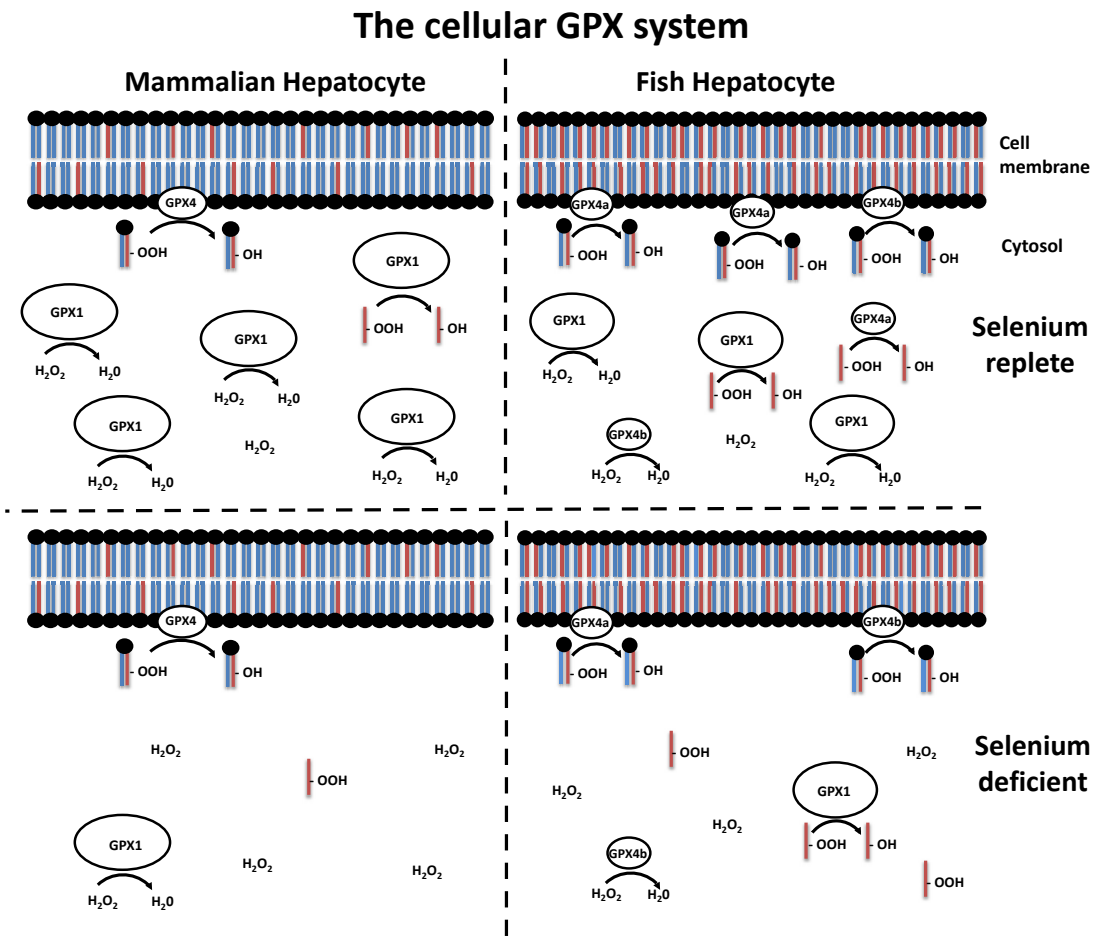

Figure 9. A comparison of the proposed mammalian versus fish GPX systems under both selenium replete (top half) and deficient (bottom half) conditions using hepatocytes. Compared to mammalian cells, fish cells have elevated long chain polyunsaturated (LCPUFA; red) versus shorter chain PUFA, monounsaturated and saturated (blue) fatty acid levels. Among others, fatty acids can be membrane incorporated as phospholipids, or found in free form in the cytosol. LCPUFA are sensitive to oxidation, which if left uncontrolled will lead to a chain reaction of oxidation, disrupting the cell membrane which leads to cell death. GPX4, but not GPX1 can prevent this by reducing oxidised fatty acids in cell membranes. Both GPX1 and GPX4 isoforms can reduce hydrophilic peroxides, such as hydrogen peroxide (H_2O_2) and free fatty acid hydroperoxides found in the cytosol. The presence of a more diverse range of peroxides in fish cells compared to the hydrophilic peroxide dominated environment in mammalian cells favours a fish GPX system shared between GPX1 and the multifunctional GPX4 paralogs. Under selenium deficient conditions, selenium supplies are diverted to maintaining GPX4 (GPX4b in fish) levels in both cell types. This helps to maintain cell membrane integrity and prevent cell death, at the expense of the GPX1's, (and GPX4a in fish), which may lead to elevation of hydrophilic ROS species in the cytosol. Note; the a and b paralogs of GPX1 in fish are not differentiated between due to their similar response to selenium status, in contrast to the a and b paralogs of fish GPX4.

Conclusions

1. **Paper I.** In contrast to rodents, maximum GPX activity was found to be an inadequate biomarker for Se requirements in zebrafish, and as discussed, for fish in general. The Se requirements of juvenile zebrafish were found to be 0.3 mg Se/kg based on growth. The Se requirements were found to be higher for reproducing male adult fish (≥0.65 mg Se/kg), as indicated by the tandem increase in mating success and gonad somatic index. Although further investigation is required, yolk sac larvae, and thus indirectly the reproducing female fish, may also require dietary Se levels of ≥0.65 mg/kg.

2. **Paper II.** As reported with other vertebrates, Se had a protective effect against MeHg-induced toxicity in adult fish. However, Se and MeHg had a synergistic negative effect on fish reproduction. This was hypothesised to be because of an elevation in Se toxicity due to the MeHg-induced increases in egg Se levels.

3. **Paper III.** Selenoproteins are overrepresented as cellular targets for MeHg induced disruption at the mRNA level, and this appears to be chiefly because of a MeHg-induced Se deficiency. A molar excess of Se to MeHg was unable to fully prevent MeHg-induced disruptions, which supports a significant role of non-selenoprotein cellular components, such as Cys containing proteins, in MeHg toxicity.

Future perspectives

As with all science, more questions were raised than answered in thesis. Several areas of research that may further understanding in the field of Se nutrition and the effect of Se status on MeHg toxicity are;

1. This thesis supports a significant role of non-selenoprotein cellular components in MeHg toxicity, as a molar excess of Se to MeHg was unable to fully prevent MeHg-induced disruptions. This is probably because Cys containing proteins are also molecular targets for MeHg (Farina et al., 2012). Several species of insects which lack selenoproteins (Chapple and Guigó, 2008; Lobanov et al., 2008) provide interesting animal models to analyse the molecular mechanisms of MeHg-toxicity in the absence of Se.

2. While MeHg-induced Se deficiency appears to be a major factor regulating the selenotranscriptome, many selenoproteins are not regulated by Se status. However, if MeHg does induced a Se deficiency, or bind selenoproteins directly, then the functional levels of selenoproteins may be reduced regardless of effects at the selenotranscriptome level. Thus, future studies should assess the effects of MeHg on the functional selenoproteome, in particular for those critical in the developing CNS, a major target of MeHg toxicity.

3. The synergistic negative effect of Se and MeHg on fish reproduction warrants further investigation. Previous to this thesis, Se has been thought of mainly as an antagonist of MeHg. Human activities are increasing Se (Lemly, 2004) and Hg (Driscoll et al., 2013) levels in the biosphere on a global scale. The existence of synergistic effects of Se and MeHg on aquatic associated animals will require changes to environmental policy and management, such as considering the localised levels of both elements simultaneously. Furthermore, the interactive effect of Se and MeHg on mammalian reproduction are poorly investigated. A large percentage of the human population rely heavily on seafood as a protein source, and hence are at risk of simultaneous intakes of high Se and MeHg

levels. This in itself warrants further investigation into Se×MeHg interactions during mammalian reproduction.

4. Overall the difference in GPX systems between fish and mammals calls into question the use of total GPX activity, or the mRNA expression of the *gpx1* paralogs, to predict fish Se requirements; the methodology used in **Paper I**. Future Se status related studies in fish should consider evaluating the response of the GPX4's alongside the GPX1's, due to their apparent importance in fish. In particular GPX4a appears to be a dominant selenoprotein in fish, and perhaps should be utilised in preference to the GPX1 paralogs in studies investigating stress response to environmental factors in fish.

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Supplementary material

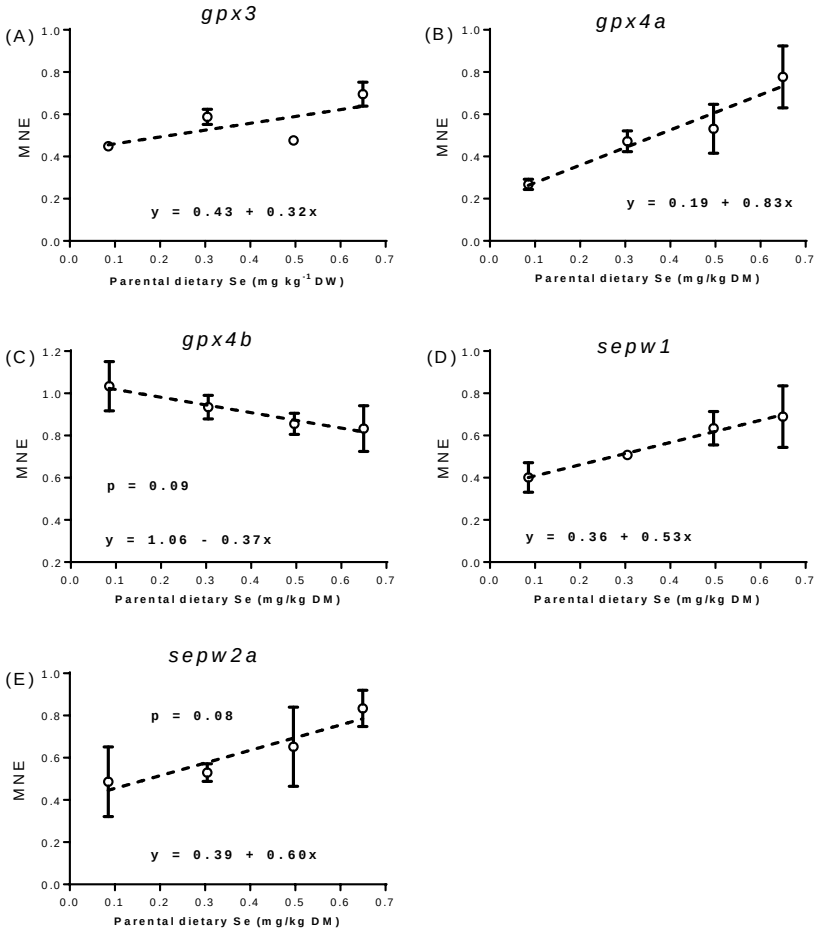
1.1 Supplementary material background

In **Paper III**, we hypothesised that selenogenes downregulated by MeHg were done so as a secondary effect of a MeHg-induced Se deficiency. To test this hypothesis, the response of selenogenes to changes in Se status were analysed in zebrafish embryos. These embryos were obtained from parents fed diets ranging from deficient to replete from the experiment described in **Paper I**. Briefly, adult zebrafish (Wild type AB strain) were fed yeast/casein based semi-purified diets. The four diets had graded levels of Se containing yeast (Se-yeast, Sel-Plex® 2000, 2000 mg Se/kg, Alltech, Lexington, KY, USA), and contained 0.09, 0.30, 0.50 or 0.65 mg Se/kg DM, and resulted in embryos with 0.34 ± 0.04, 0.61 ± 0.10, 0.97 ± 0.37 and 1.13 ± 0.13 mg Se/kg DM, respectively (**Paper I**, Supp. Table 8). These dietary Se levels were deemed in **Paper I** to range from deficient to adequate for zebrafish. The Hg levels in these diets were below the limit of quantification (<0.005 mg Hg/kg DM). These embryos were maintained until ≈ 48 hpf in petri dishes at 28.5 °C as described in **Paper I**, under identical conditions to those utilised in **Paper III**. These embryos were analysed at the same stage (48 hpf), with an identical method (RT-qPCR with identical primers), and for the same 30 selenogenes and two non-selenogenes, as described in **Paper III**. The data were analysed with multiple regression in Graphpad Prism (GraphPad Software, San Diego, CA, USA, V. 6.02). Data were first normalised with Box-Cox (Osborne, 2010) and then tested against the null hypothesis that parental dietary Se concentrations had no effect on outcome (horizontal line), or if first or second order polymomial curves explained the response more adequately. Data are presented as mean ± SEM, n=2-3. Each replicate consisted of embryos obtained from a single pairwise cross, with the 0.09 and 0.3 mg Se/kg groups having 2n, and the other groups 3n, equalling a total of 10 observations. Differences among regression models were considered significant at $p<0.05$.

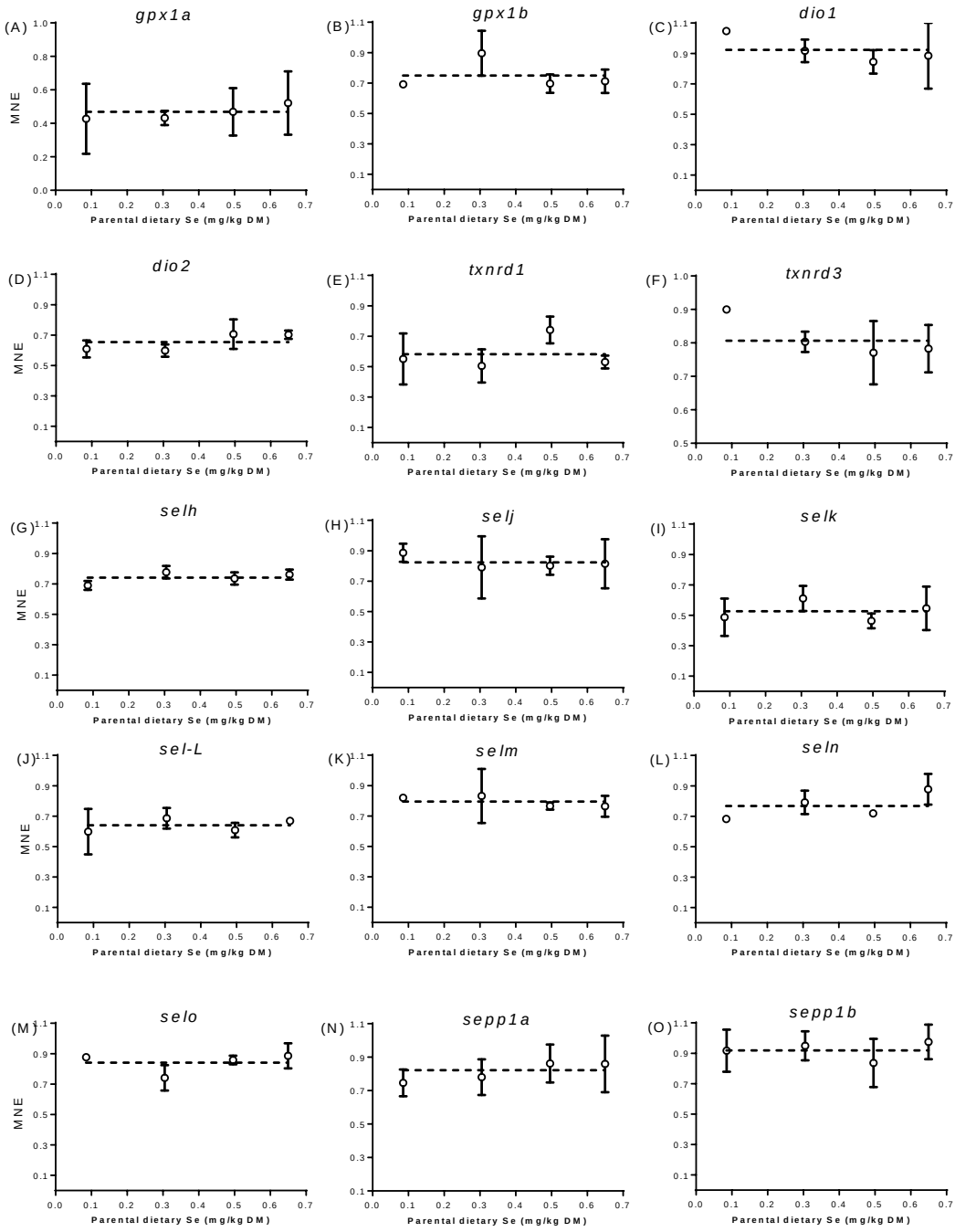
1.2 Supplementary results and discussion

The majority of the selenogenes (25 out of 30), and the two non selenogenes (*trnau1apa* and *trnau1apb*) did not respond to changes in Se status (Supp. Fig. 2). Three selenogenes had positive linear responses ($p<0.05$) to Se status; *gpx3*, *gpx4a* and *sepw1*; while a fourth; *sepw2a*; demonstrated a similar response but only as a statistical trend ($p=0.08$, Supp. Fig 1). One selenogene; *gpx4b*; had a negative linear response to Se status as a statistical trend ($p=0.09$, Supp. Fig 1). The mammalian orthologs for selenogenes regulated by Se status in zebrafish embryos are all from antioxidant families (Papp et al., 2007). Mammalian *Gpx3* and *Sepw1*, but not *Gpx4* mRNA are downregulated by sub optimal Se status (Sunde and Raines, 2011), while *sepw2a* is relatively uncharacterised and not found

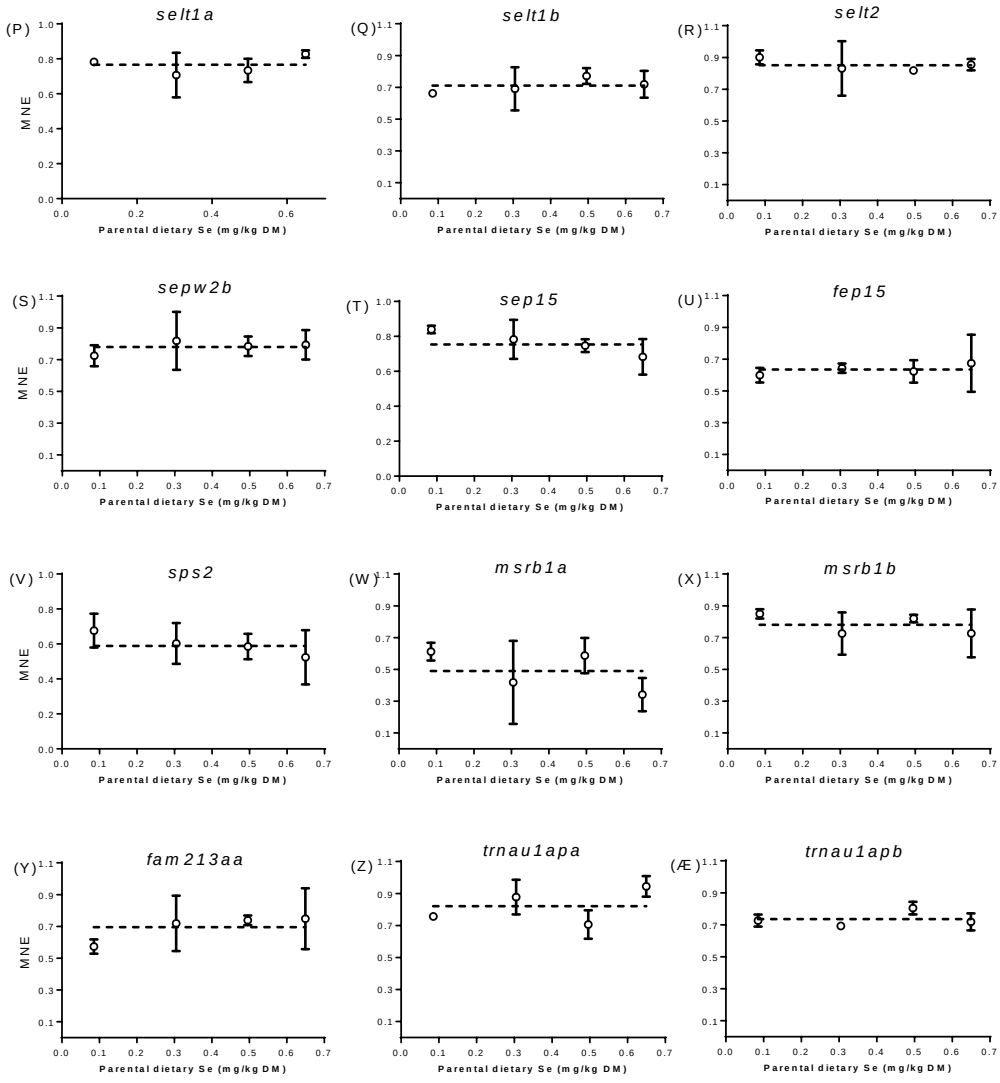
in mammals (Mariotti et al., 2012). Three of the four selenogenes downregulated by Se deficiency; *gpx4a*, *sepw1* and *sepw2a*; were also downregulated by MeHg (**Paper III**). These genes were also rescued from MeHg-induced downregulation by elevated Se (**Paper III**). Thus, the effect of Se deficiency on the selenotranscriptome had similiarities with the effect of MeHg on the selenotranscriptome. However, overall a greater number of selenogenes were affected by MeHg. It may be that MeHg induced a greater Se deficiency (**Paper III**) than the low Se group (0.09 mg Se/kg) in this experiment. For instance there was a seven to one molar excess of Hg to Se in the embryos from the (-)Se/(+)Hg group (**Paper III**), which may have resulted in large amounts of Se being bound in non-bioavailable MeHg:Se complexes (as discussed in **Paper III**), and overall less bioavailable Se than in the 0.09 mg Se/kg group in the current experiment. It is also unknown why several selenogenes that responded to Se status in juvenile fish; *gpx1a*, *gpx1b* and *sepp1a*, **Paper I**; did not respond to Se status in zebrafish embryos. It is possible that other factors were stronger regulators, such as developmental transcription factors, than Se status in zebrafish embryos. In line with the discussion in this thesis (section 3.44), *gpx4a* was the most responsive selenogene to Se status.



Supp. Figure 1. Expression of selenoprotein genes that responded to Se status. Mean normalised mRNA expressions of selenogenes; *gpx3* (A), *gpx4a* (B), *gpx4b* (C), *sepw1* (D) and *sepw2a* (E); that were up or down-regulated in 48 hpf zebrafish embryos as parental dietary Se levels increased. Data are mean ± SEM (n=2-3) where each replicate is a pool of embryos spawned from a single pairwise mating. Lines represent best fit models of data after Box Cox transformation (Linear regression, R^2; graph A = 0.42; B = 0.62; D = 0.61, $p<0.05$; C = 0.32; E = 0.34; $p=0.05$ - 0.10; equations shown in figures).



Supp. Fig. 2 Continued next page



Supp. Figure 2. Expression of selenoprotein (and *trnaup1ap*) genes that did not respond to Se status. Mean normalised mRNA expressions of selenogenes (and the *trnaup1ap* paralogs) in 48 hpf zebrafish embryos that did not respond as parental dietary Se levels increased. Data are mean ± SEM (n=2-3) where each replicate is a pool of embryos spawned from a single pairwise mating. Data were tested with regression analysis using the data mean, lines represent best fit models of data (Horizontal line; Se status had no effect on gene expression).