

EFFECTS OF CHRONIC EXPOSURE TO SELENIUM ON
SOCIAL BEHAVIOUR AND SOCIAL LEARNING
IN ZEBRAFISH (*Danio rerio*)

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By

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Abstract

Elevated levels of contaminants from human activities have become a major threat to animals, particularly within aquatic ecosystems. Selenium (Se) is a naturally occurring element with a narrow range of safe intake. Excessive selenium has toxicological effects, while too little causes nutritional deficits. The adverse neurobehavioural effects of Se have been investigated in both humans and fishes, but little is known about its effects on social behaviour and social learning or serotonin signalling in the brain. Considerable evidence shows that excessive exposure to Se causes toxic bioaccumulation in female fish, with Se transferring to the developing embryos leading to a diverse range of adverse health in offspring. Therefore, in this thesis, I investigated dietary exposure and transgenerational effects of chronic exposure to Se on different aspects of social behaviour including antipredator response, group preference, and social learning in zebrafish (*Danio rerio*), with a particular focus on alterations in the serotonergic pathway. In the first experiment of chapter one, I documented that exposure of adult zebrafish to the highest concentration (31.5 µg Se/g dry weight) of dietary selenomethionine (Se-Met), caused elevated levels of baseline fear behaviour, with fish swimming lower in the water column and in tighter shoals compared to fish in the other treatments. With this high level of baseline stress, these fish did not significantly intensify antipredator (fear) behaviours in response to exposure to chemical alarm cues. In group preference tests, when individual fish were given an opportunity to shoal with groups of different sizes, fish exposed to the highest Se-Met concentration spent significantly less time in groups. In the social learning test, I found that zebrafish exposed to the highest concentration of Se-Met (34.1 µg Se/g dry weight) displayed significantly lower escape responses compared to fish in control and lower exposure groups (3.6 and 12.8 µg Se/g dry weight). These impaired behaviours were associated with higher oxidative stress and dysregulation in mRNA expression of 5-HT receptors (*htr1aa*, *htr1b*, *htr1d*, *htr2aa*, *htr2b*, and *htrcl1*), 5-HT synthesis (*tph2*), its reuptake (*slc6a4a*) as well as monoamine oxidase (*mao*), an important enzyme in the degradation of 5-HT. In my final experiment, female zebrafish were treated with different concentrations of Se-Met and bred with untreated male zebrafish to produce the F1-generation required to investigate the transgenerational effects of dietary Se on social behaviour and social learning in zebrafish. Offspring were raised to adulthood (6-month-old) without Se exposure. Then, in a series of behavioural tests, I found offspring that were maternally exposed to high levels

of Se showed behavioural signs of stress (although no physical impairment), had weaker group preferences, and also demonstrated impaired social learning. These neurobehavioural deficits appear to be linked to perturbations in the serotonergic system in the brain, as maternal exposure to high Se concentrations led to dysregulation of this neurotransmitter (e.g., altered transcription of 5-HT receptors). Overall, my study highlights that Se contamination impairs multiple social behaviours and social learning, and it has important trans-generational consequences even in the absence of direct Se exposure.

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List of Abbreviations

%	Percent
~	Approximately
<	Less than
>	Greater than
±	Denotes error of a statistic
µg	Microgram
µl	Microliter
µM	Micromolar
·OH	Hydroxyl radical
5-HT	Serotonin
5-HTP	5-hydroxytryptophan
5-HIAA	5-hydroxyindoleacetic acid
AAAD	L-aromatic amino acid decarboxylase
ANOVA	Analysis of variance
°C	Degree Celsius
Ca ²⁺	Calcium cation
CAT	Catalase enzyme
cm	Centimetre
CNS	Central nervous system
Cu/Zn-SOD	Copper/zinc superoxide dismutase
DNA	Deoxyribonucleic acid
dpf	Days post-fertilization
dw	Dry weight
g	Gram
<i>g</i>	Gravitational force
GABA	Gamma-aminobutyric acid
GPX	Glutathione peroxidase

GPX1a	Glutathione peroxidase 1a
GSH	Reduced glutathione
GSSG	Oxidized glutathione
H ₂ O ₂	Hydrogen peroxide
hpf	Hours post-fertilization
hrs	Hours
htr1	Serotonin receptor syptype 1
htr2	Serotonin receptor syptype 2
kg	Kilogram
l	Liter
LOOH	Lipid hydroperoxide
LPO	Lipid peroxidation
M	Molar
MAO	Monoamine oxidase
mg	Milligram
min	Minute
ml	Milliliter
Mn-SOD	Manganese superoxide dismutase
mRNA	Messenger RNA
N	Normal
n	Sample size
O ₂ ⁻	Superoxide anion
OH ⁻	Hydroxide
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	Potential of hydrogen
PNS	Peripheral nervous system
RNA	Ribonucleic acid

RNS	Reactive nitrogen species
ROS	Reactive oxygen species
s	Second
Se	Selenium
Se ⁰	Elemental selenium
Se ²⁻	Selenide
SECIS	Selenocysteine insertion sequence
Se-Cys	Selenocysteine
SEM	Standard error of the mean
Se-Met	Selenomethionine
SeO ₃ ⁻²	Selenite
SeO ₄ ⁻²	Selenate
SLC6A4a	serotonin transporter gene
SOD	Superoxide dismutase enzyme
S-S	disulfide linkages
tRNA	Transfer ribonucleic acid
TPH	tryptophan hydroxylase
UGA	Opal suppressor
v/v	Volume/volume
vs.	Versus

Chapter 1: General Introduction

1.1. Social behaviour

Individuals in a variety of species require frequent interactions with others, especially their own conspecifics, in order to survive and reproduce successfully (Whishaw et al., 2006). The set of behaviours used in these social interactions are identified as social behaviours (Robinson et al., 2008). Living in a social group provides individuals with multiple benefits, including access to mates, access and transfer of information, defending territories, efficient foraging, and protection against predators (Hoppitt and Laland, 2013). Even those species that have solitary living also at some point in their life will join a group or engage in social behaviours like mating. Many neuropsychiatric disorders, including autism, schizophrenia, depression, and social anxiety disorder, may interfere with social functioning and are linked to social defects and social dysfunctions (Geng and Peterson, 2019). For example, autism is characterised by challenges with social skills and deficits in processing social cues. Studying and developing animal models with social deficits has far-reaching implications for many neuropsychiatric diseases, and studying these behaviours requires developing specific behavioural assays.

One major advantage of living in a group is to have more protection against predators. The effects of predation on prey population dynamics can be complex and unpredictable, and it depends largely on their ability to avoid predation (Scott and Sloman, 2004). One way that a group may increase its defences against predators is via “the many eyes hypothesis”, in which more individuals are detecting predators (Lima, 1995). This task of scanning the environment for predators can be distributed over many individuals. This collaboration presumably provides a higher level of vigilance, which subsequently lets individuals have more time to do other tasks like foraging (Lima, 1995; Olson et al., 2015). Another way that being in a group helps individuals reduce the chance of being captured by predators is known as “the dilution effect”. In this hypothesis, each individual is just one of many in a group, so they are diluting their risk of attack. A third hypothesis for an antipredator response to predators in group-living animals like fish and birds is “the predator confusion effect” (Milinski and Heller, 1978). It can be difficult for a predator to focus on individual prey from groups when many targets are moving at the same time (Olson et al., 2013). For example, the attack success of three-spined stickleback (*Gasterosteus aculeatus* L.)

was reduced by an increase in *Daphnia magna* group size due to the poor neural mapping of targeted prey, that led to an increase in the spatial error of each attack (Ioannou et al., 2007). Group tendencies and behavioural homogeneity within groups are important for reducing the risk of predators, and individuals that act differently or leave the group would be at increased risk.

1.2. Social learning

Animals living in social groups have the opportunity to learn from the behaviour of others, which is known as social learning. In social learning theory, Albert Bandura proposed that the learning process and social behaviour offer new behaviours that can be acquired by observing and imitating others (Bandura and Walters, 1977). Social learning is associated with observation, extraction of information from those observations, and making decisions about the behaviour's performance (Lyons and Berge, 2012). It can arise by observing a behaviour and the consequences of that behaviour, and it does not always occur as a result of first-hand experiences (Bandura and Walters, 1977; Lyons and Berge, 2012). Reinforcement plays a key role in the learning process; once a particular behaviour is constantly rewarded, it will most likely persist; however, if a behaviour is punished regularly, it will probably desist. Impaired social learning is associated with diminished social engagement motivation, and it can be seen in individuals with major depressive disorders and autism (Frey and McCabe, 2020b).

There are a variety of examples of social influences on the adaptive modification of behaviour, ranging from food selection and predator avoidance to the learning of songs, routes, and motor skills (Hoppitt and Laland, 2013; Huber, 2012). Living in social groups also can lead to synchrony in showing an established behaviour and the transmission of new behaviour patterns throughout a group. These social influences are mainly beneficial to the observer, either instantly or in the long term (Huber, 2012). In animal social learning, the response of the observers is relatively similar to that shown by demonstrators (Huber, 2012).

Prey animals can collect potentially life-saving information by using conspecific cues that indicate predator activities (social information use) as well as learning about those predators as a result of those cues (social learning) (Lindeyer and Reader, 2010). In predation ecology, observers learn to recognise a specific habitat or species as a threat, or they can learn to display particular behaviours from models which are known as tutors or demonstrators (Crane, 2017). This phenomenon is

widespread among different taxa, including mammals, birds, fish, and amphibians (Ferrari et al., 2007a; Griffin, 2004). Before learning, subjects probably show little or no response to a given stimulus, while after that stimulus is presented with an alarm signal, it can evoke the avoidance response (Griffin, 2004). Socially learned and socially facilitated antipredator responses to novel predator stimuli have been shown in different taxa (Griffin, 2004). For example, information on predators is socially transmitted in European blackbird, *Turdus merula*, by observation of mobbing (Curio, 1978). Guppies also showed a significantly higher likelihood of surviving encounters with a predator when paired with a conspecific that have some experience with a predator versus a predator-naïve partner (Alfieri and Dugatkin, 2009). In zebrafish (*Danio rerio*), information on predators is socially transmitted by visual observation of alarmed conspecific and by alarm cues passively released from injured skin (Hall and Suboski, 1995).

1.3. Serotonin Neurotransmitter

Neurotransmission is the foundation of neuronal communication and is essential for brain development, behaviour, learning and memory, and even maintenance of life. Neurotransmitters are endogenous metabolites that act as messengers in intracellular signalling across synapses in the CNS (Tufi et al., 2016). They are released from presynaptic neurons and diffuse across the synaptic cleft, where they bind to their specific receptor-binding site. Different enzymes, transporters, and receptors regulate the function of neurotransmitters in the nervous system, and many of these neurotransmitters are synthesised from simple precursors such as amino acids. Neurotransmitters are also essential stimulants for proper development of the CNS during early development. They are produced at different levels in the developing organisms, which can impact the modulation and formation of synapses. Alterations in the levels and function of these neurotransmitters during development might lead to the improper development of the CNS and impair normal neurological functionality (Tufi et al., 2016). There are different neurotransmitter systems, including the serotonergic system that regulate social behaviour, as well as learning and memory.

Serotonin (5-hydroxytryptamine; 5-HT), one of the highly conserved central neurotransmitters, is a monoamine widely distributed throughout the animal kingdoms with important roles in the vertebrate nervous system (Charnay and Léger, 2010). 5-HT is one of the major neurotransmitters

in the CNS, but is also found in the pineal gland as a precursor of melatonin as well as in enterochromaffin cells, myenteric cells in the digestive system, beta-cells in the pancreas, parafollicular cells in the thyroid, ovarian cumulus cells, dorsal root ganglia and taste buds (Trowbridge et al., 2011). 5-HT and its receptors are essential in the regulation of almost all brain functions and physiological processes, including learning and memory, social behaviours, defensive behaviours, aggression, mood, food intake, reproduction, sleep, circadian rhythm, pain, and vascular function (Berger et al., 2009). 5-HT may modulate these functions by impacting neuroplasticity and thereby the sensitivity to environmental factors (Kiser et al., 2012). Dysregulation of the serotonergic system has been associated with the pathogenesis of many psychiatric and neurological disorders (Roth, 1994; Roth and Xia, 2004).

During brain development, all of the monoamine neurotransmitter systems are present comparatively early; however, serotonin likely occurs earliest in the most terminal regions even prior to the time of its assumed role as a neurotransmitter (Whitaker-Azmitia, 2001). Serotonin plays several fundamental roles during development and in adults by acting as a neurotransmitter and neuromodulator as well as regulating cell proliferation, neuronal differentiation, neurite growth, and synaptogenesis (Côté et al., 2007). Therefore, 5-HT regulates processes such as synaptic plasticity in the sensory cortex, migration of cranial neuronal crest cells, and neurogenesis (Gross et al., 2002; Lillesaar, 2011). In human and animal models, the correlation between abnormalities in the serotonergic system and various pathologies, including depression, autism, schizophrenia, stress, anxiety, affective disorders, migraine, and addiction to psychostimulant drugs, has been demonstrated. For example, increased levels of serotonin have been found in the plasma of autistic patients (Singh et al., 1997).

1.3.2. Serotonin synthesis, uptake, and metabolism

L-tryptophan is the base of the 5-HT production, with dietary protein being its major source (Horzmann and Freeman, 2016). Serotonin is produced in a two-step reaction. First, the rate-limiting enzyme tryptophan hydroxylase (L-tryptophan-5-monooxygenase; TPH) converts tryptophan to 5-hydroxytryptophan (5-HTP). There are two genes *TPH1* and *TPH2*, in mammals that code for TPH. In the second step, the enzyme L-aromatic amino acid decarboxylase (AAAD) converts 5-HTP quickly to 5-HT (Figure 1.1). Subsequently, 5-HT is transported into synaptic

vesicles by vesicular transporter SLC18A2 and released from the vesicle into the synaptic cleft via neuronal depolarisation. Then, serotonin can bind to its receptors either on postsynaptic neurons or the membrane of presynaptic neurons. Binding of serotonin to the autoreceptors acts as negative feedback against the further release of serotonin (Cerrito and Raiteri, 1979). The highly selective serotonin transporter (SERT or 5-HTT), which is encoded by the *SLC6A4* gene, located on the presynaptic membrane reuptakes 5-HT from the synaptic cleft. Finally, inside the cell, 5-HT is either recycled back into presynaptic vesicles to keep it protected from metabolism or metabolised by enzyme monoamine oxidase (MAO) first to produce 5-hydroxy-indoleacetaldehyde, which is then rapidly metabolised by aldehyde dehydrogenase to form 5-hydroxyindoleacetic acid (5-HIAA). Oxidative deamination of 5-HT by MAO though leads to the production of hydrogen peroxide (H_2O_2), which is an oxyradical.

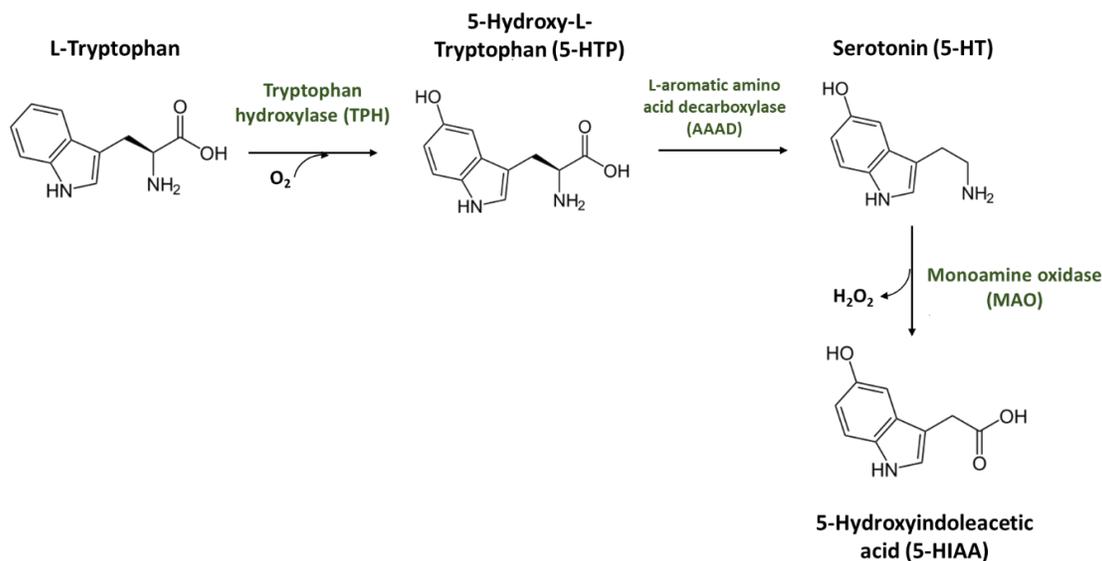


Fig. 1.1. 5-HT synthesis and metabolism processes. 5-HT is synthesised from amino acids L-tryptophan. Under the hydroxylation of TPH, L-tryptophan is converted into 5-HTP, which is then catalysed to 5-HT by the enzyme AAAD. After accomplishment of the biological function of 5-HT, it subsequently metabolised into 5-HIAA by MAO to be removed from the body. H₂O₂ is a byproduct of oxidative deamination of 5-HT, which can be converted into the highly reactive O₂^{•-} and [•]OH. Abbreviations: 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, serotonin; 5-HTP, 5-hydroxytryptophan; AAAD, L-aromatic amino acid decarboxylase; MAO, monoamine oxidase; TPH, tryptophan hydroxylase; O₂^{•-}, superoxide anion; [•]OH, hydroxyl radicals. (Figure were modified from Lv and Liu, 2017).

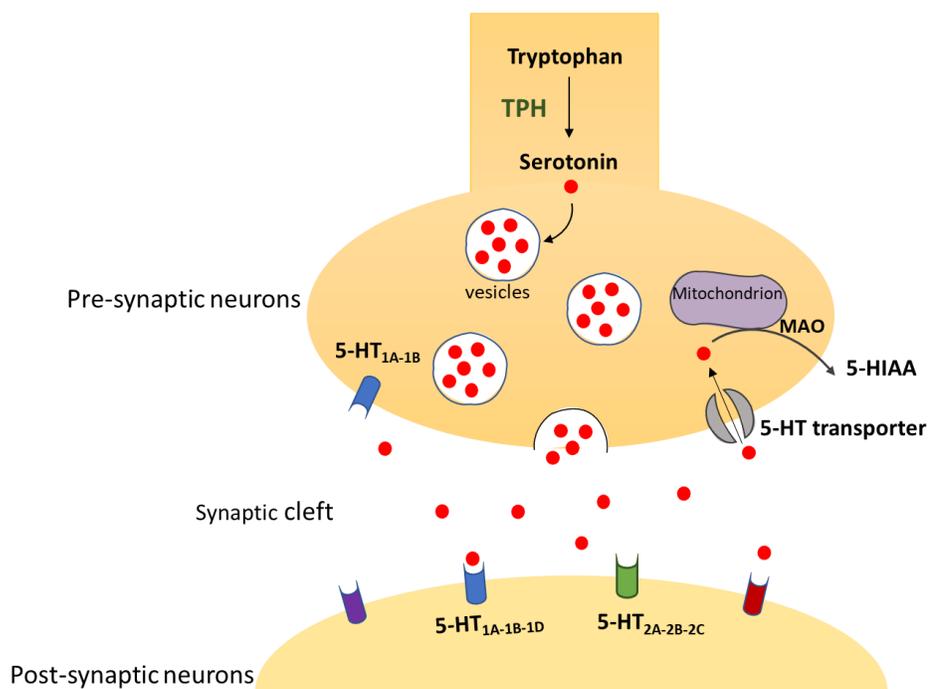


Fig. 1.2. Serotonergic neurotransmission in neurons from synthesis to reuptake and degradation. Conversion of tryptophan to 5-HT is catalysed by TPH in presynaptic neurons. 5-HT then stored into vesicles before it can be realised into the synaptic cleft. 5-HT attached to its receptors in presynaptic or postsynaptic neurons and excess 5-HT reuptake to the cell by 5-HT transporter. Subsequently, 5-HT can be metabolised to 5-HIAA by MAO. Abbreviations: 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, serotonin; MAO, monoamine oxidase; TPH, tryptophan hydroxylase.

The complex action and varied effects of serotonin are mediated via different 5-HT receptors. There are seven major subfamilies of 5-HT receptors with 14 subtypes that are found in the CNS and peripheral nervous system (PNS) (Celada et al., 2013a). The 5-HT₁, 5-HT₂, 5-HT₄, 5-HT₅, 5-HT₆ 5-HT₇, couple to G-proteins, whereas 5-HT₃ receptors are ligand-gated ion channels (Cavallaro, 2008). These receptors also regulate the release of other neurotransmitters, including dopamine, glutamate, GABA, epinephrine/norepinephrine, and acetylcholine, as well as various hormones such as oxytocin, vasopressin, cortisol, and corticotropin. 5-HT receptors also impact different biological and neurological processes, including aggression, anxiety, cognition, learning and memory, mood and appetite. Therefore, they are the target of a variety of pharmaceuticals

such as antidepressants, antipsychotics, hallucinogens and entactogens drugs (Nichols and Nichols, 2008). Some of these receptors are located both postsynaptic to 5-HT neurons and also presynaptic or on the 5-HT neurons themselves (autoreceptors). The 5-HT_{1A} receptor subtype is the first receptor to be fully sequenced. The density of 5-HT_{1A} binding sites can be found in most parts of the brain, but it is significantly high in limbic brain areas, notably hippocampus, amygdala, lateral septum, cortical areas, and also the mesencephalic raphe nuclei (Barnes and Sharp, 1999). 5-HT_{1A} receptor promotes the secretion of a growth factor and increases markers of growth in neuronal cultures, showing its neurotrophic role in the developing brain, and even in adults (Azmitia et al., 1996; Riad et al., 1994). The activity of this receptor in the brain also regulates the acetylcholine and noradrenaline release. 5-HT_{1B} and 5-HT_{1D} receptors, which are also distributed in different brain regions, have roles as both a 5-HT autoreceptor and a heteroreceptor. The 5-HT_{1B} receptor differs depending on its location, and not only it controls the release of 5-HT, but it also has an important role in the regulation GABA pathway and indirect activation of the dopamine pathway (Barnes and Sharp, 1999). The subtype 5-HT_{2A} receptor is the main excitatory receptor for 5-HT, although it may also have an inhibitory effect on certain areas in the brain (Martin et al., 1998). The 5-HT_{2C} subtype is another important receptor of 5-HT; activation of this receptor inhibits dopamine and norepinephrine release in some areas of the brain including amygdala, and hippocampus (Alex et al., 2005).

1.3.3. The role of 5-HT in social behaviours and learning and memory

In the CNS, serotonin is produced in neurons originating in the raphe nuclei located in the midline of the brainstem. These serotonin containing neurons send ascending projections that terminate in a defined and organised manner in cortical, limbic, midbrain, and hindbrain regions. In fact, all brain regions express various 5-HT receptors, and even individual neurons may express multiple 5-HT receptors (Berger et al., 2009). Therefore, nearly all the behavioural out-put can be modulated by 5-HT depending on the specific 5-HT receptors and specific brain regions. For example, the 5-HT_{1A} receptor has anxiolytic and antidepressant activities as well as cognition-enhancing of this receptor has also been shown in different studies (Charney et al., 1990; Handley, 1995). In addition, other 5-HT receptors subtype such as 5-HT_{1B}, and 5-HT_{2C} regulate mood, anxiety-like behaviours, and reward processing (Gross and Hen, 2004; Lesch, 2005). 5-HT_{2A} receptor modulates cognitive processes and attention by enhancing glutamate release (Amargós-

Bosch et al., 2004). It has also been shown that suicidal, as well as depressed patients, have higher numbers of 5-HT_{2A}, and 5-HT_{2C} receptors than normal people which is suggested postsynaptic over-density of this receptor is involved in the pathogenesis of depression (Eison and Mullins, 1995; Niswender et al., 2001). Serotonin plays a key role in shaping different social responses, and the serotonergic system itself is highly responsive to social influences (Kiser et al., 2012). Studies on macaques (*Macaca*) revealed a connection between social behaviours and 5-HT transporter. Genes encoding serotonin transporter contains a regulatory variation that has been associated with anxiety-related traits susceptibility for depreciation. Selective 5-HT reuptake inhibitors (SSRIs) have become the most prescribed medication to treat depression and to avoid its recurrence.

Several lines of evidence have revealed the role of 5-HT and its receptors in important brain regions (e.g. cerebral cortex, hippocampus, septum and amygdala) associated with different aspects of cognition, including learning and memory (Buhot, 1997; Ogren et al., 2008). On a mechanistic level, it has been suggested that 5-HT neurons contribute to the learning procedure by propagating learning signals (Frey and McCabe, 2020a). It has been indicated both depletion and increases in brain serotonin levels, and activities could impair learning performance (Frey and McCabe, 2020a; Ogren, 1985; Schmitt et al., 2006). In both humans and rodents, reduction in 5-HT levels due to low 5-HT precursor tryptophan diet was associated with impairment in memory functions (Lieben et al., 2004; Sambeth et al., 2007). Likewise, intake of a tryptophan-free diet led to an impairment in contextual fear conditioning in mice (Uchida et al., 2007). Conversely, higher activity of 5-HT receptors in both humans and rodents reduced memory performance (Ogren, 1985; Wand and Oswald, 2003). It has been revealed that 5-HT depletion can impair an individual's ability to learn from social rewards (Frey and McCabe, 2020a). Activation of different receptors like 5-HT_{1A} has been demonstrated to impair certain aspects of learning and memory by influencing or inhibiting the release of glutamate, GABA, and acetylcholine in different parts of brain like cerebral cortex and hippocampus (Ogren et al., 2008).

1.3.4. Contaminants induced 5-HT impairments

A broad range of environmental contaminants can impact serotonergic neurotransmission. For example, it has been shown that exposure to thallium led to a reduction in 5-HT levels, and an increase in ROS formation in the brain (Hasan et al., 1978). However, co-exposure to arsenic and lead increased the 5-HT levels in different brain regions in mice (Mejía et al., 1997). It has been

shown both direct, and maternal exposure to methylmercury inhibited MAO activity in vivo and in vitro in both rats and fishes (Berntssen et al., 2003; Beyrouthy et al., 2006; Chakrabarti et al., 1998). Likewise, developmental exposure to organophosphate pesticides such as chlorpyrifos has been found to cause an alteration in 5-HT by increasing its reuptake and upregulation in the expression levels of 5-HT receptors, which was also associated with changes in 5-HT dependant behaviours including learning and memory in adult rats (Aldridge et al., 2005; Aldridge et al., 2004). A reduction in 5-HT levels and an increase in 5HIAA/5HT were found in different brain regions of juvenile rainbow trout after exposure to β -Naphthoflavone and benzo(a)pyrene (Gesto et al., 2009). All of these findings point to the serotonergic system as a favourable target for a broad range of environmental contaminants.

1.4. Selenium

1.4.1. Properties, sources, and uses of selenium

Selenium (Se) is a metalloid of the same family as oxygen and sulfur with atomic number 34. It was first discovered in 1817 by Swedish chemist Jons Jacob Berzelius and named after Selene, the Greek goddess of the moon. Se is located above sulfur and below tellurium in the periodic table and has some similarities to arsenic as well. This chemical element resembles sulfur in terms of atomic size, bond energies, ionisation potential, and main oxidation states (Tinggi, 2003). The abundance of Se in the Earth's crust is very low (ranks 69th) because of its easily volatilised during crust formation; therefore, it is considered a limited and non-renewable resource on the Earth (El-Ramady et al., 2016; Janz, 2011). Geographical distribution of Se is highly variable around the world, and higher Se concentrations can be found in sedimentary rocks such as limestones, sandstones, and shales. In addition, significantly high Se levels have been reported in some phosphate-rich rocks, marine or black shales, coals, and other enriched rocks with organic carbon (Ryan and Dittrick, 2000). The central region of North America also is a significant exception, as Se creates largely from Cretaceous marine sedimentary rocks, particularly black shale, phosphate rocks (phosphorites), and coal (Maher et al., 2010; Presser et al., 2004).

Se is present in the environment in four oxidation states (VI, IV, 0, -II) and in two forms of organic or inorganic. The main organic forms are selenomethionine (Se-Met) and selenocysteine (Se-Cys). The inorganic forms are selenite (SeO_3^{-2}), selenide (Se^{2-}), selenate (SeO_4^{-2}), and the selenium element (Se^0) (Graham, 1991). The key step in the biogeochemical cycling of Se occurs when

primary and secondary producers like bacteria, algae, and plants transfer inorganic Se into organoselenides, predominantly selenomethionine (Maher et al., 2010). This transformation has critical implications for bioaccumulation and toxicity at higher trophic levels.

Se is used in the manufacture of different products. In electronics, semiconductor and photoelectric features of Se make it useful in solar energy panels, electric eyes, photographic exposure meters, rectifiers for home entertainment equipment, and light control switches (Janz, 2011). Se is also used to coat the metal cylinders from which a photographic image is transferred in xerography. This element is widely used in the glass and ceramic industries as well as in producing plastics, paints, enamels, inks, and rubber (Fishbein, 1983; Haygarth, 1994). Additionally, Se is involved in the manufacture of pharmaceuticals for human and veterinary purposes as a nutritional supplement. Se is applied in the form of selenium sulfide as a component in antidandruff shampoos, as well as an ingredient of fungicides (Fishbein, 1983; website, 2010). Se is also used in the field of agriculture and biology to amend deficient soil, in pesticide formulations, and ultimately animal feeding as a nutritional supplement (Mehdi et al., 2013). Moreover, radioactive Se (Se-75) is used in diagnostic medicine aids in visualisation of certain malignant tumours (Janz, 2011). This element is also a by-product of metallurgy. It is obtained from the sludge electrolytic refining of copper, which contains 5-25% of Se (Mehdi et al., 2013). In addition, it can be produced from the reprocessing of residues from the electrolysis of lead and nickel.

1.4.2. Essentiality of selenium

Se is recognised as an essential micronutrient in 1957 (Mayland, 1994) and is a key compound of a variety of functional selenoproteins for many living species, including human, animals, archaea, and some plants (except higher plants and yeasts) (El-Ramady et al., 2016; Hesketh, 2008). Although selenoproteins represent diverse molecular pathways and biological function, all of them contain at least one 21st amino acid selenocysteine (Sec), and most serve oxidoreductase functions. Se is encoded into nascent polypeptide chains by the UGA codon, whose regular work is to terminate translation (or of protein synthesis). Thus its effective suppression requires a specific recognition element within mRNA which is called selenocysteine insertion sequence (SECIS) and an elongation factor which is specific for selenocysteine-tRNA (Labunskyy et al., 2014).

Selenoproteins are major components of different metabolic pathways as well as enzymes, including glutathione peroxidase, thioredoxin reductase, one methionine-sulfoxide reductase, and

deiodinases. These enzymes play essential roles in antioxidation and intracellular redox regulation, reproduction, muscle function, and tumours prevention (Mehdi et al., 2013). In 1973, Se was found to be an essential component of glutathione peroxidase (GPX), which helps in intracellular defence systems against oxidative damage by preventing the production of reactive oxygen species (Rotruck et al., 1973). Thioredoxin reductase (TrxR), another selenoprotein, also has a role in redox regulation by catalysing the reduction of oxidised thioredoxin, using nicotinamide adenine dinucleotide phosphate (NADPH) as the electron donor as well as DNA synthesis, and protein repair (Arnér and Holmgren, 2000). Se in the form of selenocysteine also has been shown to be an important component of iodothyronine deiodinases, which are a subfamily of deiodinase enzymes important in the activation and deactivation of thyroid hormones. A substantial body of persuasive evidence from epidemiological, cell and animal, and human studies indicates that Se has anticarcinogenic roles (Combs Jr, 2005; Ip, 1998; Rayman, 2005; Whanger, 2004).

Se and Se-dependent enzymes are modulators of brain function, including memory and cognition, coordination, and motor performance. The majority of selenoproteins were shown to be expressed in the brain (Roth et al., 2010), particularly in cortical and hippocampal neurons (Zhang et al., 2008). Se metabolism looks to be altered in the pathology of disorders such as Alzheimer's disease, Parkinson's disease, and epilepsy, possibly, due to pro-oxidative events accompanying these states (Schweizer et al., 2004). It has been shown Se deficiency caused a significant increase in reactive oxygen species (ROS), especially lipid hydroperoxide in the cells (Saito et al., 2003). With its high oxygen consumption and lipid-rich content, the brain is highly susceptible to oxidative stress (Salim, 2017). The main role of different selenoproteins in CNS is neuroprotection through scavenging ROS and reactive nitrogen species (RNS), as well as mediating Ca^{2+} influx via ion channels and anti-inflammatory effects (Solovyev, 2015).

Se and selenoproteins have a diverse relation to brain metabolism and function, which may also show their direct role in the brain signalling pathways (Solovyev, 2015). Selenoproteins seem to be of special importance in the development and functioning of GABAergic neurons. In both organisms with Se deficiency and genetic impairment of selenoprotein biosynthesis, these neurons are affected most severely. Se deficiency or malfunction of its transporter, selenoprotein P, causes degeneration of GABAergic neurons leading to impaired neuronal function, which may result in cognitive impairment (Solovyev, 2015). Se and selenoproteins reduce the over-potential of the

excitatory glutamate system, which is a hallmark of Alzheimer's disease, epilepsy, and other neurological disorders. It has been shown that Se could potentially exert antidepressant effects through its modulatory role in different neurotransmitter systems, including serotonin and dopamine. For instance, Castano et al. (1997b) found an increase of both dopamine and serotonin turnover in the prefrontal cortex of the rats fed the Se-deficient diet. The m-trifluoromethyl-diphenyl diselenide ($m\text{-CF}_3\text{-PhSe}$)₂ modulates the serotonergic pathway by mechanisms that involve selective inhibition of monoamine oxidase A (MAO-A), resulting in an increase of 5-HT availability in the synaptic cleft (Bruning et al., 2009). The mechanism of Se interaction with the neuronal signalling is complex and maybe not be limited to the antioxidant purpose of selenoproteins.

1.4.3. Toxicity of selenium

Although Se is an essential element for different organisms, toxicity can arise at levels only slightly above those required (Janz et al., 2010). Se has become a contaminant of potential concern in several countries, including Canada, the United States of America, Australia, New Zealand, and China (He et al., 2018). A wide spectrum of human activities, ranging from the most basic agricultural practices to the most high-tech industrial processes, have hastened the release of Se from geologic sources and made it available to terrestrial environments and eventually entering aquatic ecosystems. (Hamilton, 2004). Agricultural drainage, sewage sludge, fly ash from coal-fired power plants, oil refineries, and mining of phosphates and metal ores are all sources of Se contamination of the aquatic environment (Lemly, 1985). Uptake of Se by biota can be from water or diet. Two main inorganic forms of Se selenate and selenite are soluble in water and can be absorbed by fish and wildlife via gills, epidermis, or gut. However, dietary exposure of animals to Se in the form of Se-Met is the dominant pathway of uptake as animals are typically at higher trophic levels in aquatic and terrestrial food webs (Dallinger et al., 1987). It has been shown Se-Met represents 60-80% of the total Se present in contaminated aquatic food webs (Janz et al., 2014; Maher et al., 2010).

Se is known as an element of potential concern because it can bioaccumulate within the base of the food chain, from water and sediment to aquatic plants and aquatic invertebrates, and quickly attain levels that are toxic to wildlife and fish (Hamilton, 2004; Lemly, 2004). This rapid bioaccumulation causes a marginal level between its safety and poisoning. (Lemly, 2004).

Bioaccumulation of Se leading to toxicological effects and change in aquatic communities has been investigated in lab and field studies. The most sensitive taxa for Se toxicity are oviparous vertebrates (Janz et al., 2010). A transition from no effect to reproductive disruption in fish can occur within only a few $\mu\text{g/l}$ waterborne Se. The dietary concentrations of Se that fish need to keep normal physiological homeostasis varies between 0.5-1.0 $\mu\text{g Se/g dry mass (d.m.)}$, while concentrations more than 3 $\mu\text{g Se/g d.m.}$ in the diet can accumulate and cause serious problems in both the consumer's body and be passed on to subsequent generations (Janz et al., 2010; Lemly, 1997). Different concentrations of Se have been reported in various fish species in Se-impacted ecosystems, for example, 11 to 42 $\mu\text{g Se/g dry weight (dw)}$ in juvenile fathead minnows *Pimephales promelas* and white sucker *Catostomus commersoni* (Driedger et al., 2009), 1.45 to 26.8 $\mu\text{g Se/g dw}$ lake chub (*Couesius plumbeus*), spottail shiner (*Notropis hudsonius*) and northern pike (*Esox lucius*) (Phibbs et al., 2011); 4 to 127 $\mu\text{g Se/g dry weight}$ in bluegill (*Lepomis macrochirus*) tissues (14–105 in liver, 24–127 in eggs, 4–23 in muscle, 7–38 $\mu\text{g Se/g dry weight}$ in whole-body) (Lemly, 2014); 4.89 -11.7 $\mu\text{g Se/g dry weight}$ in bluegill and largemouth bass (*Micropterus salmoides*) (Lemly, 2018). The negative effects of a high concentration of Se in fish can manifest itself in a variety of ways, such as developmental abnormalities, swim performance reduction, reproductive failure, and visual problems (Choudhury et al., 2015; Thomas and Janz, 2011a; Thomas et al., 2013).

The maternal transfer of Se is the main mechanism of its developmental toxicity. Se mostly accumulates in the yolk proteins during vitellogenesis and then affects developing larva upon yolk resorption (Janz et al., 2010), which leads to increased deformation and mortality in offspring (Thomas and Janz, 2015). High concentrations of selenium via dietary intake cause greater selenomethionine incorporation into yolk proteins in the place of methionine. Therefore absorption of yolk protein by the developing embryo consequently results in larval deformation (Janz et al., 2010).

1.4.4. Biochemical basis of selenium toxicity

One of the proposed mechanisms of selenium toxicity is a result of an error in protein synthesis (Lemly, 2002). Sulfur is an essential component of proteins, and sulfur-to-sulfur linkages between amino acids are required for the appropriate functioning of protein molecules, either as components of cellular structure like synthase of tissue or as enzymes in the metabolism of cells

(Lemly, 2002). Selenium has similar basic chemical and physical properties to sulfur, and based on studies of mammalian cells, it is not readily distinguished from sulfur upon protein synthesis. It is believed that the mechanistic properties behind selenium toxicity in mammals are the same in fish, and when the concentration of Se increases in the environment, this element is mistakenly substituted for sulfur and can form a triselenium linkage (Se-Se-Se) or a selenotrisulfide linkage (S-Se-S) (Sunde, 1984). As far as the normal tertiary structure of protein molecules depends on disulfide chemical bonds (S-S), replacement of Se for S in protein synthesis could result in improperly folded or dysfunctional proteins and enzymes, which impair the normal cellular biochemistry (Diplock and Hoekstra, 1976; Sunde, 1984). However, this suggested mechanism of Se toxicity has been criticised in different studies. The Se residue in Se-Met is protected by the terminal methyl group in the amino acid structure and thereby prevents the formation of covalent bridges. In addition, The incorporation of selenocysteine into proteins is a highly regulated process, and it requires a UGA codon, SECIS, and specific tRNA, (Janz et al., 2010). Therefore, neither Se-Met in which the Se is shielded by the terminal methyl group nor selenocysteine, which is regulated by the mRNA sequence, impact protein structure, or function.

However, there is a great agreement that oxidative stress is the primary mechanism of Se toxicity, as the oxidation of cellular thiols were generated by different selenium species (Hoffman, 2002; Lavado et al., 2012). Se-Met through the action of methioninase enzymes produces highly reactive metabolites such as methylselenol and selenide anion, then oxidation of these reactive metabolites in the presence of GSH results in the production of superoxide radicals ($O_2^{\bullet-}$) (Palace et al., 2004; Spallholz et al., 2004). Different studies have been shown that Se-Met exposure can cause toxicity via induction of oxidative stress. For example, Se exposure induced oxidative stress in the zebrafish brain, as demonstrated by a reduction in GSH:GSSG ratio increased LPO levels, and up-regulation of antioxidant genes (Naderi et al., 2018b). In rainbow trout (*Oncorhynchus mykiss*), exposure to Se-Met induced oxidative stress and loss of cell viability in isolated hepatocytes (Misra et al., 2012; Misra et al., 2010). Se bioaccumulation induced oxidative stress in liver and gill tissues of Mozambique tilapia (*Oreochromis mossambicus*) (Gobi et al., 2018). In addition, developmental and parental or maternal exposure to Se-Met has resulted in an elevated level of oxidative stress biomarkers in zebrafish and medaka embryos (Arnold et al., 2016; Lavado et al., 2012; Naderi et al., 2018a; Thomas and Janz, 2016). Oxidative stress is known as a common

pathogenic mechanism triggering a variety of neuropsychiatric disorders as well as cognitive impairments in both humans and animals (Praticò et al., 2002; Wells et al., 2009). For example, It has been shown associative and spatial learning impairment in zebrafish with elevated levels of oxidative stress (Ruhl et al., 2016).

1.4.5. Neurotoxicity of selenium

The neurotoxic effects of Se on the central nervous system (CNS) have been investigated in different studies. Se exposure dysregulated various neurotransmitters in CNS of animal model organisms. For example, exposure to different compounds of Se showed an increase of dopamine levels and its metabolites in rats and mice, respectively (Rasekh et al., 1997; Tsunoda et al., 2000). An increase in dopamine levels associated with altered expression of dopaminergic cell marker was measure in brains of zebrafish exposed to elevated concentrations of Se-Met (Naderi et al., 2018b; Naderi et al., 2017). Exposure to elevated concentrations of Se also led to inhibition of glutamate uptake (Ardais et al., 2010a; Nogueira et al., 2003; Souza et al., 2010), as well as alteration of cholinergic signalling and degeneration of its neurons in *C. elegans* (Estevez et al., 2012).

This exposure to high levels of Se may also cause neurobehavioural abnormalities in organisms. However, very few studies have addressed the effects of Se on specific behaviours in animals. Previous studies revealed that zebrafish exposed to high concentrations of dietary Se displayed impaired performance in both associative and latent learning tasks (Naderi et al., 2018b; Naderi et al., 2017). Thomas and Janz (2011) found environmentally relevant dietary Se-Met exposure impaired the swimming of adult zebrafish. There was also a significant reduction in escape response exhibited by adult zebrafish fed a Se-Met spiked diet (Raine et al., 2016). In addition, Smith et al. (2010) have reported that developmental exposure to Se-Met causes spatial learning impairment in adult zebrafish. Parental exposure to Se in the form of sodium selenite reduced brain acetylcholine and impaired active avoidance learning in mice (Ajarem et al., 2011). Maternal exposure to Se-Met also induced learning impairment in zebrafish offspring tested in a latent learning task, as well as dopaminergic system hyperfunction in the brain (Naderi et al., 2018a).

1.5. Study species

1.5.1. An introduction to Zebrafish

The zebrafish is a tropical freshwater teleost fish that belongs to the family Cyprinidae. This small-bodied shoaling species is native to southeastern Asia, where it is found in different countries, including India, Pakistan, Bangladesh, and parts of southern Nepal. Zebrafish are diurnal predators of aquatic invertebrates and live and reproduce externally in shallow and slow waters of pools, rice paddies, and seasonal streams. They were introduced to laboratories in the 1970s by George Streisinger, and colleagues recognised their potential for developmental biology and genetics research. In 1981 the first publication using zebrafish as a model animal was published (Streisinger et al., 1981).

The zebrafish possesses many features that make it particularly well-suited for experimental manipulation and controlled laboratory designs. It is an intensively studied model species, and many laboratory lines and mutants are available. Being small in size, they can be kept in large numbers in the laboratory, and their maintenance is easy (Westerfield, 2000). Females are able to spawn hundreds of eggs only in one spawning event (~ every 3 days) (Detrich et al., 1999). Unlike mammals, the eggs are transparent, which makes it possible to observe developing embryos in their natural environment. Moreover, the embryos themselves are transparent during the first few days of their lives. All major organs develop within 24 h after fertilisation, and their generation time is 3 to 4 months. The zebrafish life cycle includes the embryonic stage (0-72 hours post-fertilization; hpf), larval stage (4-29 days post fertilisation; dpf), juvenile stage (30-89 dpf), adult zebrafish (3-24 months), and aged zebrafish ($2 \pm$ years). The zebrafish has also become an important organism for the study of vertebrate gene function (Haffter et al., 1996). The ability to accelerate genetic studies by gene knockdown or overexpression has led to the extensive use of zebrafish in the detailed examination of vertebrate gene function and the study of human genetic disease (Golzio et al., 2012; Howe et al., 2013). Comparison to the human reference genome, approximately 70% of human genes have at least one zebrafish orthologue.

1.5.2. Social behaviour and social learning in zebrafish

1.5.2.1. Social preference and shoaling

The zebrafish can serve as a reliable animal model in the study of complex social behaviours because of its robust and rigid behavioural phenotype (Howe et al., 2013). Zebrafish naturally express a strong preference for their conspecifics. Social preference (the innate tendency of animals to observe, mimic, and approach a conspecific) is well conserved among social vertebrate species, including zebrafish (Geng and Peterson, 2019). In this simple social behaviour, fish exhibit a social preference for their conspecifics, and it forms a necessary foundation for later, higher-order social functions such as shoaling, schooling, and other complex social interactions. Zebrafish prefer to join more numerous groups, although this preference depends on the activity level of the stimulus shoal (Pritchard et al., 2001) and the sex of the fish in the group, as male zebrafish have a preference to shoal with females (Ruhl and McRobert, 2005). Shoaling behaviour is a prominent feature of zebrafish. Zebrafish are highly motivated to aggregate, shoal, and school in both wild and laboratory environments (Mahabir et al., 2013; Suriyampola et al., 2015). Shoaling is representing the complex interaction of individuals moving together in coordinated movements. Shoaling is associated with predation risk in which it reduces the risk of being predation by mechanisms described previously, including increased predator detection, decreasing chances of capture, and increased predator confusion (Buske and Gerlai, 2011; Giardina, 2008). Swimming as a group also provides a hydrodynamic advantage, whereby the individuals swimming at the back use less energy due to reduced water resistance (Suriyampola et al., 2015). Both group preference and shoaling behaviour in zebrafish are maintained at a relatively stable high level throughout ontogeny, beginning only 7-14 dpf (Buske and Gerlai, 2011). However, as the fish develop, shoal cohesion significantly increases (Buske and Gerlai, 2011). Exposure to different environmental chemicals and pharmaceuticals including lead, bisphenol A (BPA), 17 α -ethinylestradiol, sodium valproate (anti-epileptic drug), lysergic acid diethylamide (LSD), ibogaine (principal psychoactive component), ethanol, have been shown to impact shoaling or/and social preference in zebrafish (Cachat et al., 2013; Ellwanger et al., 2016; Gerlai et al., 2000; Grossman et al., 2010; Liu et al., 2016; Müller et al., 2017; Volkova et al., 2015; Wang et al., 2015; Wang et al., 2018).

1.5.2.2. Antipredator response

The ability to detect predators and respond to them is essential for survival in animals. The antipredator responses can be innate; for instance, they can detect predators even if they had never faced them before, or can be learned following exposure to a predatory risk (Bass and Gerlai, 2008; Ferrari et al., 2007b). Numerous fish species, including zebrafish, have been found to demonstrate fear responses to conspecific ‘alarm cues’, which are olfactory substances released from epidermal club cells when their skin is injured (Hall and Suboski, 1995). Zebrafish have been found to respond to alarm cues with a variety of behaviours, including erratic movement or zig-zagging (i.e., ‘dashing’), freezing, changes in shoaling, and increased bottom dwell time (Gerlai et al., 2000; Speedie and Gerlai, 2008). The erratic movement near the bottom is almost always followed by cessation of movement or immobility (Speedie and Gerlai, 2008). A motionless zebrafish at the bottom and under a cloud of debris may be hard for predators to detect (Speedie and Gerlai, 2008). Fear responses in zebrafish also lead to increased shoal tightness and cohesion near the bottom of the tank (Waldman, 1982).

1.5.2.3. Social learning in zebrafish

Different aspects of learning and memory in zebrafish have been of interest in recent years, becoming an important model organism for studies on the neurological basis of learning and memory (Gerlai, 2011). Learning and memory have been quantified in zebrafish using either learning tasks adapted from rodent learning tasks or zebrafish-specific assays. Zebrafish have been shown to perform well in different learning tasks including spatial learning (Karnik and Gerlai, 2012), associative learning, (Gerlai, 2011; Sison and Gerlai, 2010), latent learning (Gómez-Laplaza and Gerlai, 2010), shuttle box active appetitive conditioning (Pather and Gerlai, 2009), place conditioning (Eddins et al., 2009), appetitive choice discrimination (Bilotta et al., 2005), and active avoidance conditioning (Xu et al., 2007). Zebrafish can also learn socially about different circumstances, like finding food or avoiding predators without paying personal learning costs. Naïve fish have been found that can learn about an escape route or a source of food from a knowledgeable demonstrator fish (Lindeyer and Reader, 2010; Zala and Maattanen, 2013). Different experimental studies have also revealed exposure to environmental pollutants, and pharmaceuticals compounds can impair several behaviours and cognitive functions in zebrafish. For example, chronic exposure of zebrafish to high concentrations of Se-Met impaired associated

learning and latent learning (Naderi et al., 2018b; Naderi et al., 2017). Zebrafish exposure to various contaminants such as silver, mercury, arsenic, lead, bisphenol A, chlorpyrifos, antipsychotic drugs, nicotine, and alcohol also impacted different social behaviours and learning and memory (Eddins et al., 2009; Fernandes et al., 2014; Levin et al., 2003; Luchiari et al., 2015; Powers et al., 2011; Saili et al., 2012)

1.5.3. The serotonergic neurotransmitter in zebrafish

Zebrafish possess all major neuromodulatory systems, including neurotransmitters, their receptors, enzymes of synthesis and metabolism, and transporters, similar to those observed in the mammalian CNS (Panula et al., 2010b). Serotonin is an important modulator of normal and pathological brain mechanisms (Herculano and Maximino, 2014b; Maximino et al., 2013a; Maximino et al., 2013b). Similar to other teleost fishes, zebrafish have a well-developed serotonergic system that is functionally equivalent to mammals (Stewart et al., 2013; Winberg et al., 1997; Winberg and Thornqvist, 2016). The expression patterns, binding, and signalling properties of 5-HT receptors, and transporters also resemble those in mammals (Maximino et al., 2013a; Panula et al., 2010b). Due to a specific genome duplication 320-350 million years ago in the ray-finned fish radiation, prior to or coinciding with the appearance of teleost fishes, fish often contain two copies of genes found in other vertebrates (Christoffels et al., 2004; Meyer and Scharl, 1999).

Many of the duplicated genes that code for functional proteins were kept in zebrafish. For instance, TPH1 (*tph1a*, *tph1b*) dominates the diencephalic areas, and TPH3 in hypothalamic areas, while TPH2 is expressed in zebrafish raphe (Bellipanni et al., 2002; Teraoka et al., 2004). Thus, the rate of raphe 5-HT synthesis in zebrafish and mammals is restricted by tryptophan availability. However, 5-HT neurons expressing TPH1 and TPH2 in teleostean hypothalamic are less susceptible to tryptophan availability, but its availability still can impact hypothalamic 5-HT release by influencing on raphe 5-HT synthesis (Lillesaar, 2011). For encoding AAAD and MAO, there is only one ortholog gene (Herculano and Maximino, 2014b; Lillesaar, 2011; Setini et al., 2005). The zebrafish MAO shares about 70% nucleotide and amino acid identity with both mammalian MAOs, even though pharmacological experiments have shown that zebrafish MAO is more similar to the mammalian MAOA (Sallinen et al., 2009a). There are also two paralogs of 5-HT re-uptake transporter genes *slc6a4a* and *slc6a4b* (Norton et al., 2008). *Slc6a4a* is extensively

distributed throughout the brain, while *slc6a4b* seems to be located only in medulla and retina (Norton et al., 2008; Wang et al., 2006). Moreover, many of 5-HT receptors important in the regulation of social behaviour as well as learning and memory such as 5-HT_{1A} (*htr1aa*, *htr1ab*), 5-HT_{1B} (*htr1b*), 5-HT_{1D} (*htr1d*), 5-HT_{2A} (*htr2aa*), 5-HT_{2B} (*htr2b*), and 5-HT_{2C} (*htr2cl1*, *htr2cl2*) are possessed by zebrafish. 5-HT receptors also are present during the early development of zebrafish embryos (Panula et al., 2010b; Pei et al., 2016). For example, it has been shown *htr1aa* is highly expressed in the nervous system of zebrafish during CNS development (Pei et al., 2016).

Exposure to different contaminants has been found to impact serotonin balance in the zebrafish brain. For example, acute exposure to methylmercury led to a decrease in extracellular levels of 5-HT, and an increase in extracellular levels of tryptamine-4,5-dione, a partially oxidised metabolite of 5-HT in the zebrafish brain. In addition, it has been shown exposure of developing zebrafish to silver caused elevations of serotonin turnover in the brain without affecting basal neurotransmitter levels (Powers et al., 2011).

1.6. Research objectives

Using a zebrafish model, my research was designed as the first test of the effects of chronic exposure to environmentally relevant concentrations of dietary Se-Met on antipredator behaviour, social behaviour, and social learning, as well as its underlying mechanisms in zebrafish. We considered a representative range of Se concentrations from 0.2 (control) to 34.1 µg se/g, similar to concentrations reported in aquatic invertebrates and smaller prey fishes collected from Se-impacted sites (Driedger et al., 2009; Fan et al., 2002b; Lemly, 2014, 2018; Mackay, 2006; May et al., 2008; Muscatello and Janz, 2009; Phibbs et al., 2011). 5-HT is one of the brain's main neurotransmitters that involves regulating social behaviour, learning and memory. I also explored alteration in the serotonergic neurotransmitter in the zebrafish brain as a possible mechanism underlying Se neurotoxicity. The brain, with its high oxygen consumption and lipid-rich content, is highly susceptible to oxidative stress. Exposure to the high levels of Se can induce oxidative stress, which has a strong potential to impact normal CNS function (Janz et al., 2010; Salim, 2017). I also evaluated the impacts of exposure to Se on oxidative stress biomarkers. Moreover, exposure to dietary Se-Met led to the deposition of this element in the eggs, which can subsequently impact the early life stages of developing embryos. Serotonin is one of the earliest neurotransmitters that appear in the CNS; therefore, maternal exposure to Se-Met can impact this neurotransmitter in the

zebrafish brain. Although developmental exposure to Se-Met can impair spatial and latent learning, as well as the dopaminergic system in adult zebrafish (Naderi et al., 2018a; Smith et al., 2010), next to nothing is known about maternal exposure to Se-Met on social learning and antipredator behaviour.

Principle Hypothesis: My hypothesis is that chronic exposure to environmentally relevant concentrations of dietary Se-Met leads to disruption of social behaviour and social learning in zebrafish via the disruption of serotonergic neurotransmission and elevated oxidative stress.

Research objectives: My research had three main objectives:

- i. To investigate the impacts of chronic exposure to dietary Se-Met on group preference and antipredator behaviours, and the regulatory genes important in serotonergic neurotransmitters (Chapter 2).**

In this study, I exposed adult zebrafish to different environmentally relevant concentrations of Se-Met (control, 2.1, 11.6, 31.5 $\mu\text{g Se/g dry weight}$) for 60 days. Se concentrations were measured in diet, water, and whole-body tissues. Then I tested them for antipredator responses by measuring shoaling behaviour and vertical position before and after exposure to chemical alarm cues. I also assessed group preference by giving them a chance to choose between two different groups of zebrafish. In addition, I assessed the mRNA expression level of 5-HT synthesis, its receptors, and transporter, as well as the gene important in the degradation of 5-HT.

- ii. To study the impacts of chronic exposure to Se-Met on social learning, as well as serotonin and oxidative stress biomarkers in exposed adult zebrafish (Chapter 3).**

In this experiment, first, I tested whether zebrafish can learn socially from knowledgeable demonstrators about an escape route to avoid an oncoming trawl in a social learning task. Then I assessed whether exposure to different concentrations of dietary Se-Met for 90 days could affect zebrafish social learning. Total Se concentration was measured in fish diet, water, and whole-body tissues. I evaluated the changes in oxidative status and alterations in serotonergic neurotransmission in the zebrafish brain to uncover mechanism(s) underlying Se neurotoxicity. The lipid hydroperoxidation content of the brain and the mRNA expression of antioxidant enzymes, including glutathione peroxidase 1a (*gpx1a*), catalase (*cat*), manganese superoxide dismutase (*Mn-sod*), and copper/zinc superoxide dismutase (*Cu/Zn-sod*) were used as biomarkers

of oxidative stress. I quantified 5-HT levels of the brain as well as the associated genes in the regulation of 5-HT.

iii. To investigate the effects of maternal exposure to different concentrations of Se-Met on antipredator behaviour, group preference, and social learning in F1-generation adult zebrafish (Chapter 4).

In this study, female zebrafish treated with different concentrations of Se-Met for 90 days were paired with untreated males for breeding. Embryos were collected and raised in clean water and fed with a regular diet until adulthood (6-month-old) without Se exposure. Total Se concentrations were measured in female whole-body tissues and eggs, as well as in the diet. Then, I measured the effects of maternal exposure to Se-Met with different behavioural endpoints, including antipredator behaviour, and group preference. I also tested whether maternal exposure to Se-Met impairs social learning. The expression of genes involved in 5-HT neurotransmission, including 5-HT receptors, transporter, synthesis, and degradation, were assessed in the brain of F1-generation zebrafish.

Chapter 2: Chronic exposure to dietary selenomethionine dysregulates the genes involved in serotonergic neurotransmission and alters group preference and antipredator behaviours in zebrafish (*Danio rerio*)

Preface

The aim of this chapter is to address the first objective of my doctoral research work, which is to investigate the impacts of chronic exposure to dietary Se-Met on social and antipredator behaviours, and the genes important in the regulation of serotonergic neurotransmitters. After 60 days of exposure, the highest dose (31.5 µg/g dry wt.) caused the highest level of baseline fear behaviour, with fish swimming lower in the water column and in tighter shoals compared to fish in the other treatments. With high levels of baseline fear, these fish did not significantly intensify fear behaviours in response to predation risk in the form of exposure to chemical alarm cues. When an individual fish was given an opportunity to shoal with groups of differing size (3 vs. 4 individuals), fish exposed to the high dose (31.5 µg/g dry wt.) spent less time with groups in general, and only control fish showed a significant preference for the larger group. In the zebrafish brain, we found significant upregulation in the mRNA expression of serotonin receptors (*hrr1aa* and *htr1b*), a transporter (*slc6a4a*), and tryptophan hydroxylase-2 (*tph2*), whereas downregulation of monoamine oxidase (*mao*) gene, in the zebrafish brain. The results of this study suggest that disruption of serotonergic neurotransmission might have been responsible for Se-induced impairment of antipredator and social behaviour in zebrafish.

The content of chapter 2 was reprinted (adapted) from Environmental pollution (DOI: 10.1016/j.envpol.2018.12.090). Attaran, A., Salahinejad, A., Crane, A.L., Niyogi, S., Chivers, D.P. “Chronic exposure to dietary selenomethionine dysregulates the genes involved in serotonergic neurotransmission and alters social and antipredator behaviours in zebrafish (*Danio rerio*)”. Copyright 2019, with permission from Elsevier Inc.

Authors contribution

Anoosha Attaran (University of Saskatchewan) designed and conducted the experiment, generated and analysed the data, prepared all the figures and tables, and drafted and revised the manuscript. Arash Salahinejad and Adam Crane (University of Saskatchewan) provided technical assistance

and edited the manuscript. Som Niyogi and Doug Chivers (University of Saskatchewan) provided inspiration, scientific input and guidance, commented on and edited the manuscript, and provided funding for the research.

2.1. Introduction

Selenium (Se) is an essential micronutrient that plays crucial roles in maintaining physiological homeostasis in all vertebrates including humans (Thomas et al., 2013). This metalloid is important for maintaining normal brain function, the regulation of thyroid hormone, and for lowering the risk of heart disease and cancer through reduction of oxidative stress (Labunskyy et al., 2014; Mullur et al., 2014; Richardson, 2005). Selenium is incorporated into seleno-proteins in the form of selenocysteine, and these proteins have antioxidative functions and defend cells from oxidative stress (Labunskyy et al., 2014; Mullur et al., 2014; Plateau et al., 2017). This element enters the environment mainly as inorganic forms such as selenate and selenite, where it can be bio-transferred to the more available organic forms of seleno-amino acids, including selenocysteine and selenomethionine, by primary producers and certain bacteria (Fan et al., 2002a; Janz et al., 2014). Dietary selenomethionine (Se-Met) is the predominant source of Se available to fish, especially in Se-contaminated aquatic ecosystems (Fan et al., 2002a).

There is a narrow physiological range between Se essentiality and toxicity, as over-exposure causes bioaccumulation and subsequent toxicity (Lemly, 1997; Thomas and Janz, 2011b). Se is present in the environment in natural sources of coal, crustal rock, and phosphate soil, but anthropogenic activities such as mining, oil refining, power generation, agricultural drainage on seleniferous soils, and animal husbandry have led to increased Se release causing contamination of aquatic environments (Janz et al., 2010). High intake of Se can lead to behavioural changes, damage to the immune and reproductive systems, developmental problems, and cardiovascular problems in fish (Ammar and Couri, 1981; Lemly, 1997; Pettem et al., 2017; Wiseman et al., 2011). The major underlying mechanism for selenium toxicity is its capacity to elevate the production of reactive oxygen species (ROS), thereby inducing oxidative stress, which can cause damage to the brain and central nervous system (CNS) (Ellwanger et al., 2016; Janz et al., 2010). Neurotoxicity can occur via disturbance to the cholinergic, glutamatergic and dopaminergic neurotransmitter systems (Ardais et al., 2010b; Estevez et al., 2012; Naderi et al., 2017). However, few studies have assessed the neurobehavioural effects of Se toxicity.

The monoamine serotonin (5-hydroxytryptamine, 5-HT) is one of the primary neurotransmitters in the CNS. It is highly conserved across vertebrate species and plays an important role in regulating a range of physiological, neuroendocrine and behavioural processes, including social

behaviours (Donaldson and Young, 2008). The serotonergic neurons are primarily found in the raphe nuclei of the CNS, and they innervate almost all regions of the brain (Sallinen et al., 2009b). Tryptophan hydroxylase, and monoamine oxidase are the rate-limiting enzymes involved in the biosynthesis and degradation of serotonin inside the cell (Cooper et al., 2003). Serotonin, once released from the presynaptic neurons into the synaptic cleft, binds to its receptors expressed on the postsynaptic membrane. The serotonin receptors are divided into seven different families corresponding to 14 receptor subtypes (Celada et al., 2013b). The reuptake of serotonin into the presynaptic neuron occurs via the serotonin transporter (slc6a4a). The different functions of serotonin are regulated by its binding to the specific receptor sub-types. For example, serotonin binding to htr1a and htr2a receptors can regulate anxiety and depression in vertebrates (Celada et al., 2013b; Popova and Naumenko, 2013). In addition, alterations in serotonin synthesis, bioavailability, and expression of serotonin receptors and other molecules associated with its functioning have been found to cause problems with social interactions, aggression, stress, and fear (Cardoso et al., 2015; Carrillo et al., 2009; Herculano and Maximino, 2014a).

Toxicological studies on animals can allow us to understand whether chemicals have harmful effects on animals in the natural environment, while also serving to model potential effects on humans. Zebrafish (*Danio rerio*) are increasingly being used in neurobehavioural studies as a model test organism due to their physiological homology with humans, sharing all major brain structures and key neurotransmitters, receptors and hormones (Bretaud et al., 2004; Grunwald and Eisen, 2002; Rico et al., 2011). Zebrafish possess a complex serotonergic system featuring major genes for 5-HT synthesis, metabolism and signalling, similar to those in mammals (Maximino et al., 2013b).

For many species, social behaviours are critical for finding food and mates, defending territories, and avoiding predators (Hoppitt and Laland, 2013). For instance, many fish species, including zebrafish, live in social groups that swim together to increase their probability of predator detection and escape (Giardina, 2008; Miller and Gerlai, 2007). Indeed, fish in a shoal are a more difficult target for predators. The dilution effect decreases the probability that an individual shoal member will be captured by a predator (Scott and Sloman, 2004; Speedie and Gerlai, 2008). Moreover, multiple individuals moving in a close shoal can confuse approaching predators, making successful attacks less likely (the Predator Confusion Hypothesis) (Ioannou et al., 2008; Jeschke and Tollrian,

2007; Scott and Sloman, 2004). Fish also show preferences within a shoal. When given a choice between shoals of different sizes, they typically prefer to spend more time with the larger shoal, presumably for increased safety from predators (Agrillo et al., 2007; Bisazza et al., 2010; Piffer et al., 2012; Seguin and Gerlai, 2017b). Consequently, impaired social behaviours can have major fitness consequences for animals.

Our goal here was to investigate the effects of chronic dietary exposure to Se-Met on social behaviours in adult zebrafish with a particular focus on the serotonergic pathway. First, we exposed zebrafish to relevant concentrations of Se-Met and measured their shoaling behaviour before and after exposure to chemical alarm cues, which are released by injured conspecifics and reliably indicate a predator attack (Chivers and Smith, 1998). Furthermore, we also assessed zebrafish for group-size preferences in a second experiment. In addition, we quantified the expression of zebrafish genes encoding serotonin receptors, and transporter, as well as the genes involved in the production of serotonin in the zebrafish brain.

2.2. Material and methods

2.2.1. Fish husbandry

All methods used in this study were approved by the University of Saskatchewan Animal Research Ethics Board (protocol no. 20170037). A total of 344 experimentally-naïve adult (12 months old) female zebrafish were obtained from a stock colony housed at the RJF Smith Center for Aquatic Ecology of the University of Saskatchewan. The colony had few males, and we chose to use only females to avoid introducing sexual variation into our tests of social behaviour. Fish were housed in groups of 5 in 6-l tanks supplied with filtered and dechlorinated tap water with total hardness 150 mg/l as CaCO₃, alkalinity 120 mg/l as CaCO₃, pH 8, at 27 °C and with a 14:10 h light:dark photoperiod. Zebrafish were fed flake food (Nutrafin Max flakes, Germany) twice daily.

2.2.2. Diet preparation

To obtain diets with different concentrations of Se, we mixed either 3, 10, or 30 µg of seleno-L-methionine (purity > 98%, Sigma Aldrich, Oakville, ON, Canada) into 150 ml of distilled water with 100 g of flake food for 10 minutes. A control diet was prepared in the same manner but without Se-Met. Each mixture was lyophilised in a freeze dryer (Labconco, USA) for 48 h. These concentrations were chosen because they are environmentally relevant and comparable to

concentrations found in prey species at Se-impacted aquatic ecosystems (Driedger et al., 2009). Representative samples of each diet (1 g each, three replicates per treatment) were analysed for total Se concentration (see below for details). During the exposure period, zebrafish were fed with either the control diet or one of the Se-Met diets twice daily for 60 days, as in previous studies (Naderi et al., 2018b; Raine et al., 2016). Food portions were ~2% of body weight per day. At each feeding, fish were allowed to consume food for 30 minutes before any excess food was removed. Fish were exposed using a static renewal system, and a 75% water change was carried out in each tank on a daily basis.

2.2.3. Chemical alarm cues

To obtain chemical alarm cues, we used standard procedures for physical euthanasia rather than chemical methods that could potentially interfere with alarm cue chemistry. We euthanised 4 zebrafish with a blow to the head, and a total of 4.94 cm² skin was removed (lateral sides of the body). The skin was then homogenised in 200 ml distilled water, filtered through glass wool to remove remaining tissues, and stored in plastic bags at -20 °C until being thawed at the time of use. During trials, we injected 1 ml of this solution into tanks containing 5 litres of water. This concentration (~1 cm² of skin in 40 litres of water) was chosen based on preliminary trials demonstrating that it would induce an overt antipredator response (e.g., dashing, freezing, and vertical sinking).

2.2.4. Evaluation of antipredator response

Observational tanks (6 litres, ~28 × 22 × 22 cm) had three sides covered with opaque barriers to block visual cues from other tanks and the observer. First, a group of 5 fish from one of the treatments was moved into an observational tank with a dipnet. Each group of fish had been housed together throughout the exposure period, so the fish in each group were familiar with one another. Fish were given 30 min to acclimate before an observation began (Engeszer et al., 2007a; Wright and Krause, 2006a). The behavioural trials lasted for 16 min, first consisting of an 8-min pre-stimulus period followed by an injection of alarm cues and then an 8-min post-stimulus period, as in previous studies on fish alarm-cue responses (Chivers et al., 2013; Ferrari et al., 2006). The alarm cue was delivered to the tank via a plastic tube (5 mm diameter) attached 1.5 cm below the surface of the water (Fig. 1A). The water in the experimental tanks was replaced with new water after each trial. Behaviour was recorded with cameras (Logitech C922x Pro Stream Webcams)

from the top and side of the tanks. We evaluated shoal cohesion using 64 screenshots (top view) that were extracted with VirtualDub software every 15s during the 16-min period. Geometric landmarks (one on the head of each fish) were digitised for morphometric analyses using tpsDig232, and inter-individual distances (Fig.1B) were measured using CoordGen8.

We also quantified the vertical position of zebrafish (side view) before and after injection of alarm cues. Each individual was given a score every 15 s based on their position in reference to horizontal lines (3 cm apart) on the glass (Fig. 1A). A score of 3 was given to a fish in the top third of the 9 cm deep water column, whereas a score of 1 was given to a fish in the bottom third. We used 8-13 replicates per treatment.

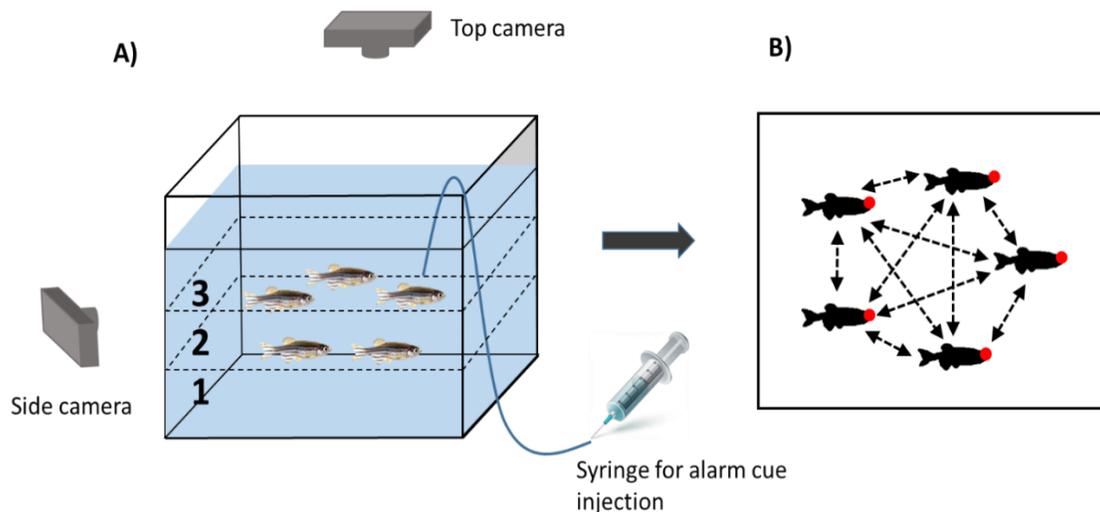


Fig. 2.1. Schematics of (A) the experimental tank for measuring zebrafish shoaling behaviour and their vertical position before and after an injection of alarm cue where dashed lines defined three vertical positions and cameras recorded side and top views, and (B) the linear inter-individual distances (between red landmarks on the head) measured to determine shoal cohesiveness.

2.2.5. Evaluation of group preference

In this experiment, the observational tank was divided into three compartments by two glass dividers where each side compartment (12.5 cm wide) served as a ‘conspecific box’ that would hold conspecifics (Fig. 2), similar to previous studies on group preference (Pritchard et al., 2001; Ruhl and McRobert, 2005; Seguin and Gerlai, 2017b). Between the conspecific boxes, the central

compartment (36 cm wide) was further divided into three zones (12 cm wide each) with lines on the glass. The outer zones that were adjacent to the conspecific boxes were designated as conspecific zones, where we considered fish to be choosing to spend time with the conspecifics. The aquarium was covered on three sides with white Plexiglas to facilitate contrast for video tracking.

Prior to each trial, a group of 3 unexposed fish was moved into one of the side compartments, whereas a group of 4 unexposed fish was moved into the opposite side. These individuals were similarly-sized females (40-42 mm) from our stock colony and were replaced with new fish after every four trials. Then a focal fish from one of the Se-Met treatments was transferred by beaker and added into an opaque holding cylinder (~10 cm diameter) within the central compartment. After a 10-min acclimation period, the holding cylinder was gently removed, and the time spent in each zone was recorded for 10 min (Wright and Krause, 2006b). We conducted 18-21 replicates per treatment with the left/right location of the conspecific group sizes being randomised across trials.

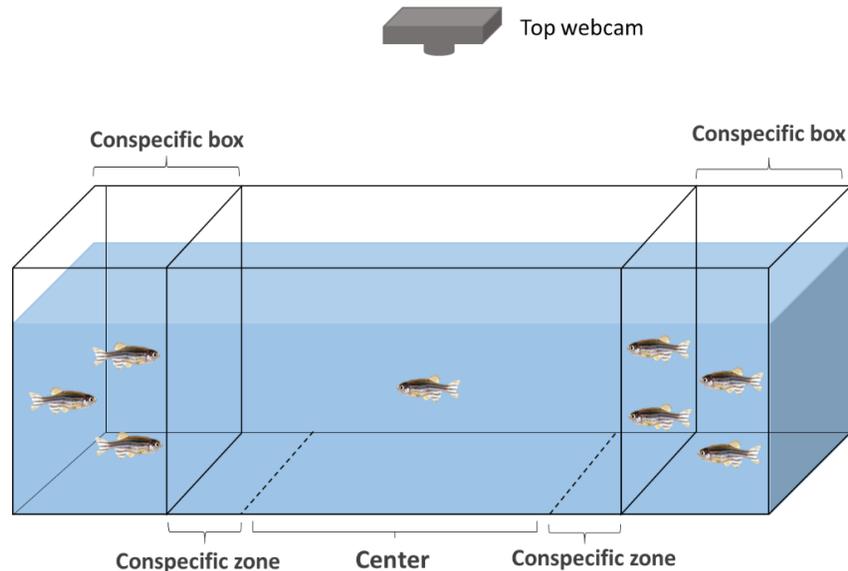


Fig. 2.2. Schematic of the experimental tank for measuring group preference in zebrafish. Two conspecific boxes were separated by glass dividers on each side. The middle section was divided into three zones: two conspecific zones and a center zone demarked with dashed lines. Behaviours were recorded by an overhead camera.

2.2.6. Quantification of total selenium concentration:

The concentrations of total Se in the whole-body of fish and diets were measured using a graphite furnace atomic absorption spectrometer (GF-AAS, PerkinElmer AAnalyst 800, USA). Prior to analysis, tissue and food samples were weighed and digested with 1 N nitric acid using 1:5 mass by volume ratio and then kept in an incubator at 60 °C for 48 h in a hot air oven. The digested samples were centrifuged at 15,000 g for 4 min, and then supernatants were collected and kept at 4 °C until analysis. Total Se concentrations were measured using appropriate dilutions, method blanks and a certified Se standard (PerkinElmer, USA). In addition, the quality control and quality assurance of our Se analysis were maintained by analysing a reference material (DOLT-4; National Research Council of Canada) using the same procedure. The recovery % of Se in the reference material was 96%, and the detection limit of our Se analysis was 0.1 ppb.

2.2.7. Gene expression measurement

Expression levels of the zebrafish genes involved in 5-HT biosynthesis (*tph1a*, *tph1b*, *tph2*), degradation (*mao*) and transporter (*slc6a4a*, *slc6a4b*), as well as 5-HT receptors (*htr1aa*, *htr1ab*, *htr1bd*, *htr1b*, *htr52b*) were evaluated by Quantitative real-time PCR. For each treatment, we extracted total RNA from a pooled sample of 3 zebrafish whole-brains using an RNeasy Mini Kit (Qiagen, Germany). The RNA concentrations and purity were quantified using a Nanodrop spectrophotometer (NanoDrop, Thermo Scientific, USA) via the absorbance ratios at wavelengths of 260 and 280 nm, respectively. Subsequently, 1 µg of total RNA was used to synthesise the cDNA by QuantiTect Reverse Transcript kit (Qiagen, Germany). The 20 µL PCR mixture consisted of 10 µL SYBR Green PCR Master Mix (SensiFAST, Bioline, USA), 2 µL of cDNA, 0.8 µL of each forward and reverse gene-specific primers (Table 3.1), and 6.4 µL nuclease-free water. The mixture was then evaluated with Quantitative real-time PCR on an iCycler Thermal Cycler (Bio-Rad, USA). The relative abundance of mRNA of target genes was determined by normalisation to β -actin as the housekeeping gene. Relative expression levels were determined by the arithmetic comparative $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001a). We replicated each sample four times (biological replication), extracted RNA from each sample three times (technical replication), and averaged the data from the technical replicates for each sample.

2.2.8. Statistical Analysis

For the antipredator experiment, we assessed shoal cohesiveness and vertical position using univariate two-way repeated-measure ANOVAs where time (pre/post stimuli periods) was the within-subjects factor and diet was the fixed factor. Post-hoc tests were paired *t*-tests for each treatment group. To assess group preferences, we analysed both the proportion of time that fish used a conspecific zone (i.e., the proportion of time not in the centre zone) and the proportion of time that fish chose the larger shoal when making a choice (i.e., the proportion of time in the conspecific zone with 4 fish/the total time in both conspecific zones). Here, we used univariate one-way ANOVAs, followed by Tukey post-hoc tests. For the proportion of time with the larger shoal, we conducted additional post-hoc tests (one-sample *t*-tests) to determine preferences compared to the random expectation (a proportion of 0.5). We adjusted alpha for these tests with a Bonferroni correction ($\alpha=0.05/4=0.0125$) to prevent this second round of post-hoc testing from inflating our experimental-wise error rate. To assess Se diet concentrations, whole-body

concentrations, and fold change in the expression of genes, we again used univariate one-way ANOVAs with Tukey post-hoc tests. Evaluations of residuals revealed that data were normal. However, when data were heteroscedastic, we instead used Welch's tests followed by Games-Howell post-hoc tests. For statistical conclusions, we used $\alpha = 0.05$, and all data were analysed using SPSS (version 23.0, IBM SPSS Inc, USA).

2.3. Results

2.3.1. Selenium concentrations

The measured total Se concentrations in the diets and in the zebrafish are provided in Table 2. The diet concentrations were different among groups ($F_{3, 4.1} = 372$, $p < 0.001$), where the control concentration was significantly lower in comparison to the other groups ($p < 0.05$). The dietary exposures also affected the whole-body concentrations ($F_{3, 8} = 114$, $p < 0.001$). Zebrafish fed with the two highest doses of Se had more than three times higher whole-body Se concentrations than those in the control group (all $p < 0.05$).

Table 2.1. Total Se concentrations in food ($\mu\text{g/g}$ dry weight) and fish ($\mu\text{g/g}$ wet weight).

<i>Nominal concentrations of Se in the diet ($\mu\text{g/g}$)</i>	<i>Measured concentrations of Se in the diet ($\mu\text{g/g}$ dry weight)</i>	<i>Measured whole-body concentrations of Se in zebrafish ($\mu\text{g/g}$ wet weight)</i>
<i>Control</i>	0.19 ± 0.15	0.83 ± 0.17
<i>3</i>	2.14 ± 0.21^a	0.95 ± 0.04
<i>10</i>	11.64 ± 0.43^a	3.20 ± 0.49^a
<i>30</i>	31.5 ± 1.01^a	8.91 ± 1.00^a

^a Significantly different from control group at $p < 0.01$. $n = 3$ for total Se concentrations per treatment.

2.3.2. Antipredator responses

For shoal cohesion, there was a significant interaction between the exposure treatment and time ($F_{3, 40} = 3.11$, $p = 0.037$; Fig. 2.3A). When exposed to predation risk, shoals became more cohesive in the control treatment ($t_{12} = 8.39$, $p < 0.001$) and in the treatments with lower Se doses (2.1 μg :

$t_{12} = 3.83$, $p = 0.002$; $11.6 \mu\text{g}$: $t_7 = 3.51$, $p = 0.010$). However, shoal cohesion did not change significantly for fish exposed to the highest Se concentration ($t_9 = 1.68$, $p = 0.126$).

For vertical position, we again found a significant interaction between the exposure treatment and time ($F_{3,34} = 3.22$, $p = 0.03$; Fig. 2.3B). Again, fish in the control treatment and the lower doses showed significant changes in behaviour in response to predation risk, significantly decreasing their vertical position (Control: $t_9 = 4.31$, $p = 0.002$; $2.1 \mu\text{g}$: $t_9 = 5.08$, $p = 0.001$; $11.6 \mu\text{g}$: $t_7 = 3.37$, $p = 0.012$). However, no significant change in vertical position was found for fish exposed to the highest Se concentration in the diet ($t_9 = 2.22$, $p = 0.053$).

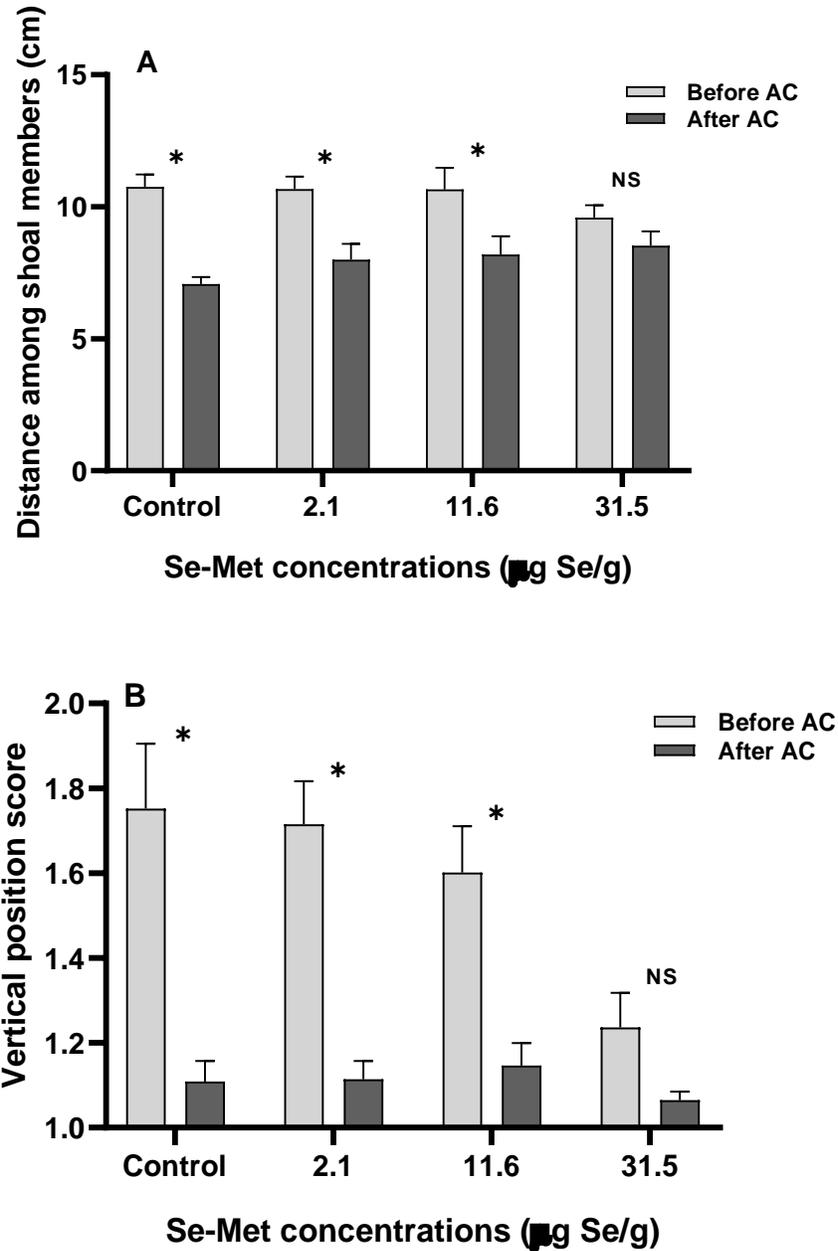


Fig. 2.3. Mean (\pm SE) distance among shoal members (A), and vertical position scores (B) before and after injection of alarm cues to zebrafish exposed to different concentrations of Se-Met. Asterisks indicate significant differences before and after injection of alarm cue, $p < 0.05$. NS = non-significant. ($n = 9 - 12$ groups per treatment)

2.3.3. Group preference performance

Exposure to Se significantly affected the proportion of time that fish spent near one of the groups ($F_{3,72} = 4.64$ $p = 0.005$; Fig. 2.4A). Fish exposed to the highest dosage spent significantly less time near the shoals compared to the control and the lowest dose ($p = 0.04$ and $p = 0.004$ respectively), while there was no significant difference between the zebrafish that were exposed to the intermediate dose and the highest dose ($p = 0.32$).

We also found that fish differed in their preference for the larger group ($F_{3,72} = 3.61$, $p = 0.017$; Fig. 2.4B), with only the control treatment differing significantly from the intermediate treatment dose ($p = 0.01$, with all other comparisons yielding $p > 0.15$). Additional post-hoc testing against the random expectation of 0.5 revealed that only the control treatment showed a significant preference for the group of 4 fish ($t_{20} = 2.86$, $p = 0.01$, $\alpha = 0.0125$, all other $p > 0.05$).

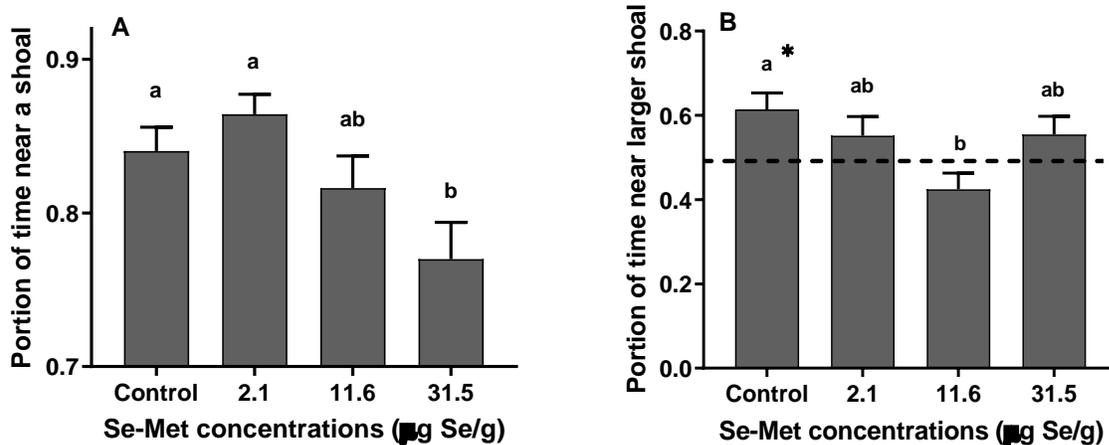


Fig. 2.4. Mean (\pm SE) proportion of time the zebrafish exposed to different concentrations of Se-Met spent in (A) both conspecific zones, and in (B) the zone adjacent to the larger shoal. Bars with different letters are significantly different from each other at $\alpha < 0.05$. The asterisk represents a significant difference from the random expectation (horizontal dashed line). ($n = 18 - 21$ per treatment)

2.3.4. Responses of serotonergic genes in the zebrafish brain

Se-Met exposure significantly upregulated expression of *htr1aa* ($F_{3,12} = 13.8$ $p < 0.001$), *htr1b* ($F_{3,12} = 10.7$ $p = 0.001$), *tph2* ($F_{3,12} = 4.70$ $p = 0.022$), *slc6a4a* ($F_{3,12} = 15.3$ $p < 0.001$), and

downregulated expression of *mao* ($F_{3,12} = 3.68$ $p = 0.044$) (Fig. 2.5). Compared to the control, all Se-Met concentrations caused significant upregulation of *htr1aa* expression in fish (2.1: $p = 0.01$, 11.6: $p < 0.001$, 31.5: $p = 0.001$, Fig. 2.5A), and the two highest concentrations increased the mRNA levels of *htr1b* and *slc6a4a* by approximately 4–5 fold (11.6: $p = 0.001$, 31.5: $p = 0.006$, Fig. 2.5B; and 11.6: $p < 0.001$, 31.5: $p < 0.001$, Fig. 2.5D, respectively), whereas only the highest concentration significantly increased the transcription level of *tph2* and by only two fold ($p = 0.045$, Fig. 2.5C). In contrast, all Se-Met concentrations caused roughly 50% decreases in the mRNA expression of *mao* (2.1: $p = 0.001$, 11.6: $p = 0.011$, 31.5: $p = 0.003$). No significant changes in transcription status were found for any serotonergic genes examined in this study (Table 1; all $p > 0.05$; data are not shown).

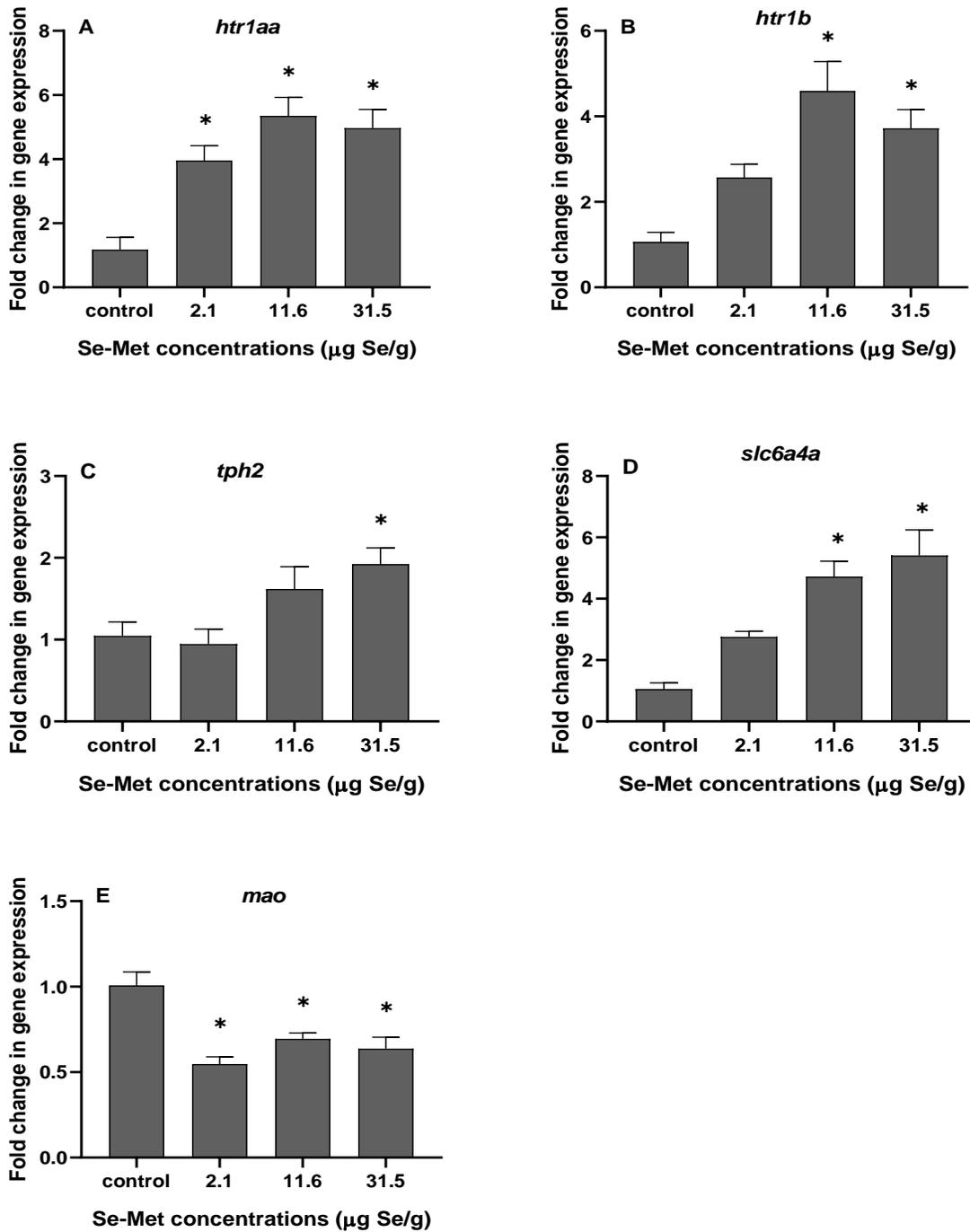


Fig. 2.5. Mean (\pm SE) fold change in the expression of genes *htr1aa* (A), *htr1b* (B), *tph2* (C), *slc6a4a* (D), *mao* (E) in the zebrafish brain. Bars with different letters are significantly different from each other at $\alpha < 0.05$. $n = 4$ per treatment.

2.4. Discussion

To our knowledge, this is the first study to investigate the effects of sublethal Se-Met exposure on both social and antipredator behaviours in zebrafish, and evaluated in conjunction with the expression of genes involved in serotonergic neurotransmission. Following exposure to different environmentally relevant concentrations (2.1 to 31.5 $\mu\text{g/g}$), the whole-body concentrations of Se in zebrafish elevated in a dose-dependent manner, similar to that observed previously in zebrafish (Pettem et al., 2017). Moreover, comparable whole-body Se concentrations were also found in wild fishes, collected from different Se-impacted aquatic ecosystems [e.g., burbot, *Lota lota*, at 11 $\mu\text{g Se/g}$ (Muscatello and Janz, 2009); fathead minnow, *Pimephales promelas*, at 3-29 $\mu\text{g Se/g}$ and white sucker, *Catostomus commersonii*, at 21-43 $\mu\text{g Se/g}$ (Driedger et al., 2009)]. Our results indicate that chronic dietary exposure to Se-Met can alter these critically important antipredator and social behaviours, and dysregulation of the genes involved in the serotonergic pathway are likely an underlying mechanism for such behavioural changes.

A particularly noteworthy finding of this study was that an elevated concentration (31.5 $\mu\text{g/g}$) of Se impaired the response of zebrafish to predation risk in terms of not properly increasing shoal cohesion or decreasing vertical space use. Clearly, these patterns were driven by individuals being in tighter shoals and at the bottom of the tank prior to any actual threat, whereas zebrafish prefer to spend most of their time foraging for food near the top of the water (Gerlai et al., 2000; Saverino and Gerlai, 2008). Such behaviour could be interpreted as having high levels of anxiety and stress as they respond to the stressors like predators by sinking to the bottom (Engeszer et al., 2007b). It is unlikely that increased bottom-dwelling was a result of abnormal swimming performance, as exposure to Se-Met has not been shown to impair locomotion activity in zebrafish (Naderi et al., 2017). However, exposure to a high concentration of Se-Met is known to induce stress (higher cortisol levels) in different fish species including zebrafish and rainbow trout (*Oncorhynchus mykiss*) (Miller et al., 2007; Thomas and Janz, 2011b). Similar sub-lethal effects are known to result from exposure to many trace elements and organic toxicants (Scott and Sloman, 2004; Weis and Weis, 1974a; Weis and Weis, 1974b).

The results of our group preference experiment clearly demonstrate that the proportion of time zebrafish spent near a shoal was lower following exposure to the highest concentration of dietary Se. This decreased tendency to join a shoal would make these fish more vulnerable to predation

threats. In the present study, we found, at least some evidence indicating, that fish exposed to higher Se-Met concentrations (11.6 $\mu\text{g Se/g}$) can fail to prefer larger groups correctly. This indicates that these fish were not able to discriminate between different sized shoals either due to not recognizing group numbers or more simply to not recognizing higher levels of overall activity in larger groups (Pritchard et al., 2001).

5-HT is one of the main neurotransmitters in the CNS that regulates social behaviours in the fish, and abnormality in its function has been implicated in several diseases like depression and anxiety disorder. The serotonergic system is a highly regulated and unified process, and it is not surprising that alteration in a single process disturbs other processes involved in 5-HT transmission. In this study, we detected an altered mRNA expression of several of the important genes involved in serotonin signalling in the brain of Se-exposed zebrafish. Although the impacts of Se deficiency on the serotonergic pathway have been assessed in rats, *Rattus norvegicus* (Castaño et al., 1997a), we are not aware of any previous literature on the effects of supra-nutritional levels of Se on serotonergic transmission in any species including fishes. Our data show that dietary exposure to Se-Met causes upregulation of two 5-HT receptor genes, *htr1aa* and *htr1b*. These two receptors, *htr1a* and *htr1b* play important roles in the regulation of social behaviours and can induce susceptibility to depression and anxiety disorders (Albert, 2012; Villafuerte et al., 2009). For example, *5-htr1a* knockout mice showed enhanced anxiety and increased responses to stress (Parks et al., 1998). These receptors are also involved in the modulation and release of other neurotransmitters such as gamma-aminobutyric acid (GABA) and dopamine which are important in the regulation of social behaviours as well as learning and memory (Barnes and Sharp, 1999; Katsurabayashi et al., 2003). Consequently, dysregulation of these two 5-HT receptors appears to be a driver of the reduced shoal cohesion, increased bottom-dwelling, and decreased group preference that we observed in this study following treatment with a high dose of dietary Se-Met.

The upregulation in 5-HT receptors parallels the upregulation of genes involved in serotonin synthesis (*tph2*) and transport (*slc6a4a*), revealing serotonin biosynthesis and reuptake are also targets of Se neurotoxicity. In humans, *tph2* and *slc6a4* are important genes for controlling anxiety-related behaviours like depression (Canli and Lesch, 2007a). In female zebrafish, upregulation of these genes causes higher aggression (Filby et al., 2010). In contrast, we found a downregulation of the gene *mao*, unlike in a previous study where upregulation of *mao* gene was observed in

zebrafish exposed to chronic dietary Se (Naderi et al., 2018b). Mao is responsible for catalyzing the inactivation of 5-HT from the neuron via removing a terminal amine group (Rybaczyk et al., 2008). Thus, exposure to Se-Met may increase intrasynaptic concentrations of serotonin by reducing the mRNA expression of *mao*, which in turn may lead to the disruption of serotonergic signalling in the brain. It has been shown that monoamine oxidase is responsible for regulating social behaviours, including schooling. In the *Astyanax* cavefish, low *mao* activity in the brain causes a lack of schooling (Elipot et al., 2014). There is a correlation between changes in social behaviours and the anti-oxidative balance in the brain (Müller et al., 2017). Antioxidant mechanisms play an important role in maintaining brain redox homeostasis; however, it has been reported chronic exposure to dietary Se disrupts redox homeostasis and induces oxidative stress in zebrafish brain (Janz et al., 2010; Naderi et al., 2018b). Hence, the alterations observed in the mRNA expressions of serotonergic genes in the present study were likely to have been mediated by Se-induced oxidative stress in zebrafish brain.

Our study reveals that dietary exposure to elevated concentrations of Se-Met can impair social and antipredator behaviours in zebrafish. Exposure to the high concentration of dietary Se-Met resulted in weaker response to predation risk because fish were already showing signs of anxiety prior to risk perception. Se-Met exposure also weakens the attraction to conspecifics and appears to impair the ability of fish to discriminate between groups of different sizes. Such behavioural changes were likely driven by altered expression of important genes involved in the 5-HT pathway. Se contamination in the environment is an increasing problem, and further investigations into the underlying mechanisms of selenium neurotoxicity could be fruitful for regulating and mitigating such impacts.

Chapter 3: Effects of Chronic Exposure to Selenomethionine on Social Learning Outcomes in Zebrafish (*Danio rerio*): Serotonergic Dysregulation and Oxidative Stress in the Brain

Preface

The aim of this chapter is to address the second objective of my research work, which is to determine the impacts of chronic exposure to dietary selenomethionine (Se-Met) on social learning in adult zebrafish. To this end, fish were exposed to environmentally relevant concentrations of Se-Met (control, control, 3.6, 12.8, 34.1 $\mu\text{g Se/g}$ dry weight) for 90 days. The results indicated that fish in the highest exposure group (34.1 $\mu\text{g/g}$) displayed significantly slower escape responses compared to fish in control and lower exposure groups (3.6 and 12.8 $\mu\text{g Se/g}$). This impaired behaviour was associated with elevated responses of oxidative stress biomarkers and dysregulation in genes that are key in the serotonergic pathway. The findings of this chapter complement the findings of chapter 2 and provide a mechanistic understanding of how Se-Met effects social learning in adult zebrafish.

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Authors contribution

Anoosha Attaran (University of Saskatchewan) designed and conducted the experiment, generated and analysed the data, prepared all the figures and tables, and drafted and revised the manuscript. Arash Salahinejad, Mohammad Naderi, and Adam Crane (University of Saskatchewan) provided technical assistance and edited the manuscript. Som Niyogi and Doug Chivers (University of Saskatchewan) provided inspiration, scientific input and guidance, commented on and edited the manuscript, and provided funding for the research.

3.1. Introduction

Animals living in groups often use available social cues to learn about their environment, from locating appropriate food sources and mates to avoiding predators and parasites (Brown and Laland, 2003; Crane and Ferrari, 2013; Hoppitt and Laland, 2013). Such ‘social learning’ has been defined as ‘instances in which the behaviour of a demonstrator, or its by-products, modifies the subsequent behaviour of an observer’ (Griffin, 2004). This learning mechanism can be critical for both individual survival and the long-term dynamics of social populations (Brown and Laland, 2001; Shier and Owings, 2007). However, social learning can be impaired by various forms of human-induced environmental change, such as habitat alterations that impede sensory acuity (Borner et al., 2015) or exposure to harmful pesticides (Lan et al., 2019) or trace elements (Lakshmi Priya and Geetha, 2011).

Selenium (Se) is a trace element that is an essential micronutrient, being incorporated into the amino acid selenocysteine, the main compound of selenoproteins (Labunsky et al., 2014). These proteins are involved in various biological functions, including normal development and neurological performance. Some selenoproteins are also important in the maintenance of redox homeostasis within cells, protecting organisms from harmful oxidative damage (Hatfield et al., 2014). Others play a vital role in the metabolism of thyroid hormones, which are essential for the growth and development of vertebrates. Their dysregulation can impair development, especially neurodevelopment. However, there is a narrow margin between an adequate concentration of Se and a potentially toxic concentration. For example, in fish, concentrations of 0.1 to 0.5 $\mu\text{g Se/g}$ dry weight (dw) are essential to maintain biological functions such as growth, whereas concentrations above 3 $\mu\text{g Se/g}$ dw can bioaccumulate and cause toxicity (Lemly, 1997; Thomas and Janz, 2011a).

Toxic levels of Se in the environment primarily come from anthropogenic activities such as mining (coal, metal, uranium, etc.), oil refining, and agricultural runoff (Janz et al., 2010). Inorganic forms of Se in aquatic ecosystems are taken up by microorganisms and primary producers. These organisms biotransform inorganic Se into organic forms like selenomethionine (Se-Met), which is the main available dietary source of Se to resident animals (Fan et al., 2002b). Se-Met can bioaccumulate in upper trophic levels and can cause teratogenic effects in fish and aquatic birds (Janz et al., 2010). Other toxic effects of Se include neurological disease, cognitive decline,

reproductive failure, developmental and cardiovascular problems, and behavioural abnormalities (Attaran et al., 2019; Naderi et al., 2018a; Pettem et al., 2017; Thomas and Janz, 2015).

At high concentrations, Se acts as a pro-oxidant, generating reactive oxygen species (ROS) that induce cytotoxicity and cell death (Plateau et al., 2017). Normally there is a balance between the production of ROS and the antioxidant capacity of the cell. However, oxidative stress occurs when the ROS level exceeds the antioxidant capacity of a cell (Chauhan and Chauhan, 2006). Se-Met is metabolized into methylselenol, either via the transsulfuration pathway or the enzyme methioninase. The subsequent redox cycling of methylselenol in the presence of glutathione produces superoxide radicals which lead to oxidative stress in fish (Kupsco and Schlenk, 2014; Misra et al., 2012; Palace et al., 2004). Oxidative stress has been known to be a critical factor in neurological impairment because it can cause damage to the brain and central nervous system (CNS). Some endogenous enzymes, such as monoamine oxidase that plays an essential role in regulating dopaminergic and serotonergic neurotransmitters in the brain, also produce ROS (Chauhan and Chauhan, 2006; Simonson et al., 1993). Antioxidant defence mechanisms within the cells include primary enzymes like superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx), which directly eliminate ROS (Chauhan and Chauhan, 2006). However, exposure to Se may lead to disruption of antioxidative enzymes and depletion of the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSH), resulting in concomitant cellular accumulation of ROS and oxidative damage (Naderi et al., 2018b; Naderi et al., 2017). Excessive oxidative stress is associated with decreased cognitive performance and several neurodegenerative and neuropsychiatric disorders such as Alzheimer disease, Huntington disease, Parkinson disease, autism, depression and anxiety (Chauhan and Chauhan, 2006; Glade, 2010; Salim, 2017).

The neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) is involved in various cognitive functions, including learning, memory, and decision making, and is closely linked to social behaviour. The complex actions of this monoamine are modulated by a large family of related receptors with 14 types of 5-HT receptors that have been classified into seven major families (Celada et al., 2013a). The rate-limiting step in the synthesis of 5-HT is the hydroxylation of the essential amino acid, tryptophan, by the enzyme, tryptophan hydroxylase. After production of 5-HT, it is released into the synaptic cleft and binds to its receptors in pre- or post-synaptic neurons. Another key regulator in this pathway is the 5-HT transporter, which removes serotonin that has

been released into the synaptic cleft. Degradation of 5-HT, an essential part of 5-HT homeostasis, is mainly served by the enzyme monoamine oxidase (MAO) where it is partly metabolized into 5-hydroxyindoleacetic (Cooper et al., 2003; Gaspar and Lillesaar, 2012). Early studies showed that both the depletion or increase of brain 5-HT activity could impair learning performance (Ogren, 1985; Ögren et al., 2008). Although the serotonergic system is a target for different environmental neurotoxicants like heavy metals and drugs, there are few studies on the effects of Se on the neurotransmitter serotonin.

The present study was designed to explore the impacts of chronic dietary exposure to Se-Met on social learning outcomes in adult zebrafish (*Danio rerio*), with a focus on serotonergic neurotransmission and oxidative stress. This species is a well-established model organism in research (e.g., developmental, molecular, neurobehavioral, drug, and toxicological) and has been used increasingly for understanding different aspects of learning and memory and for evaluating the toxicity of various agents including Se (Naderi et al., 2018a; Naderi et al., 2017). The zebrafish CNS has all the major domains found in the mammalian brain, and the same neurotransmitters (e.g., GABA, dopamine, glutamate, and serotonin) (Panula et al., 2010a). Our goal was to determine whether ecologically relevant concentrations of Se-Met would hinder socially-learned predator avoidance in an escape task. We conducted two experiments. In experiment 1, we determined whether observer zebrafish, in the absence of Se exposure, could learn from experienced individuals (i.e., demonstrators) about escape routes within a maze, compared to observers paired with untrained individuals (i.e., sham demonstrators). Following the pairings, we tested observers in the absence of demonstrators to determine whether they had learned socially. In the second experiment, we evaluated whether exposure to Se impaired social learning outcomes for observers that were paired with experienced demonstrators. In both experiments, we used behavioural tests to evaluate learning performance after 4 days of training. After completing experiment 2, we quantified the levels of lipid hydroperoxide (LPO), 5-HT, and the mRNA expression of serotonergic cell markers and antioxidant enzymes in the zebrafish brain.

3.2. Materials and methods

3.2.1. Fish maintenance

Experimentally-naïve adult (12 months old) female zebrafish (n= 150) were sourced from a stock colony housed at the RJF Smith Center for Aquatic Ecology at the University of Saskatchewan. Fish were held in groups of 8 in 6-l tanks with filtered and dechlorinated tap water (total hardness = 150 mg/L as CaCO₃, alkalinity = 120 mg/L as CaCO₃, PH = 8, temperature = 27°C and photoperiod = 14:10 h light:dark). All fish were given 3 weeks to acclimate to the laboratory conditions prior to their use in the experiments. During this time, zebrafish were fed twice daily with flake food (Nutrafin Max flakes, Germany) and frozen blood worms (Hikari Bio-Pure). The experimental methodology used in the present study were approved by the University of Saskatchewan Animal Research Ethics Board (protocol no. 20170037).

3.2.2. Social learning maze

The behavioural tests were conducted in a plastic maze (100×50×15 cm), modified from that used by Lindeyer and Reader (2010). A white plastic partition divided the maze into two main parts: a ‘trawl zone’ where zebrafish faced an approaching trawl and a ‘safe zone’ that they could reach after swimming through one of two escape holes (3×4 cm) that were 30 cm apart and 3 cm from the bottom of the tank (Figure 1.3). The escape holes were also visually distinct, with one hole outlined with yellow tape and the other with white tape. We choose yellow because previous studies have shown that zebrafish do not innately prefer this colour (Bault et al., 2015; Park et al., 2016). The trawl was made with black mesh inside a white plastic frame on two small metal rollers, which allowed it to be moved forward (to within 2 cm of the partition) and backward using a PVC handle. The safe zone was enriched with synthetic plants and a group of 5 unfamiliar zebrafish behind a transparent partition (Figure 3.1).

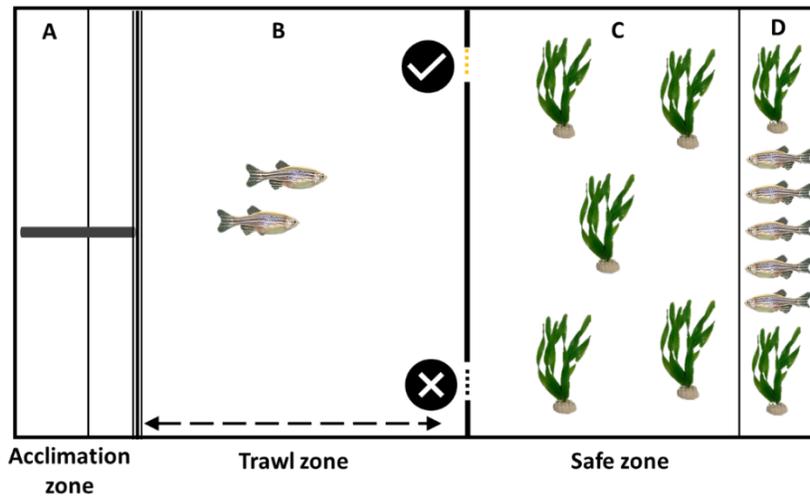


Fig. 3.1. Schematic of the experimental maze for measuring social learning of zebrafish. The trawl (B) and safe (C) zones were separated by an opaque partition that had two escape holes, one yellow (correct) and one white (wrong). The acclimation zone (A) and conspecific box (D) were separated with transparent partitions. The trawl net was moved back and forth in zone B (depicted by the dashed arrow).

3.2.3. Experiment 1: Social learning from trained vs. untrained demonstrators

Demonstrator training

In the first phase of the experiment, we prepared two groups of two zebrafish to later serve as ‘trained demonstrators’ by conditioning both groups to use the yellow escape hole while the other hole was closed. A training session consisted of a 5-min acclimation period followed by four 2-min trials where the trawl was moved forward and backward up to four times (once every 30 s) if the fish had not located the open hole. Between each trial, fish were given 2 min of acclimation after being moved back into the trawl zone with a dip net. On each occasion, we enhanced learned recognition of the specific escape route by decreasing the size of the escape hole by using partitions with smaller holes. When fish did not exit the trawl zone, we gently steered them through the correct escape hole with a dip net. To avoid any potential spatial bias, one group of fish was trained with the yellow escape hole on the right side of the partition and the other group on the left side. We trained fish repeatedly (>10 sessions) until they had used the hole in less than 30 seconds in $\geq 90\%$ of the sessions. During this phase, we also prepared four zebrafish to serve as ‘untrained demonstrators’ (i.e., ‘sham demonstrators’) later. These individuals were simply allowed to

become familiar with the maze for 2 days with both escape holes closed and without any movement from the trawl.

Social conditioning

In the next phase of the experiment, we paired two ‘observer’ zebrafish with a group of either trained or sham demonstrators. We conducted one session per day for 4 days, performed in the same manner as demonstrator training (see above) except that both escape holes were open. On the fourth day of training, we video-recorded the escape latencies of both demonstrators and observers. If fish did not escape during the 2-min trial, they were given the maximal value of 2 min.

Observer testing

One day after the final training session, observers were tested alone (i.e., no demonstrators) for their escape latencies and whether the correct route was used. Each observer was given a value of ‘1’ for a correct route choice for each trial during the session (maximal value of 8), which was converted to a correct ‘route preference’ percentage (ranging 0 to 100). All sessions were video-recorded, and all other procedures were conducted as in the conditioning phase. Sample sizes were 15 per treatment.

3.2.4. Experiment 2: The effect of Se-Met on social learning outcomes

3.2.4.1. Experimental diet and exposures

We obtained diets with different concentrations of Se by mixing 3, 10, or 30 µg of seleno-L-methionine (purity > 98%, Sigma Aldrich, Oakville, ON, Canada) with flake food following previously established methodology (Attaran et al., 2019). A control diet was prepared in the same manner but without Se-Met. Representative samples of each diet (1 g each, three replicates per treatment) were analyzed for total Se concentration (see Selenium Quantification section below for details). During the exposure period, zebrafish were fed with either the control diet or one of the Se-Met diets twice daily for 90 days. Food portions were ~2% of body weight per day. We also supplemented all treatment diets with frozen bloodworms three times per week. A 100% system-automated water change was conducted each day of the exposure period for all tanks.

3.2.4.2. Social learning tests

In this experiment, observer fish from the exposure treatments (0, 3, 10, or 30 μg) were given an opportunity to learn from trained demonstrators (no sham demonstrators in this experiment). We used four groups of two demonstrators (one group trained with each escape hole on each side of the maze) to condition pairs of observers, randomized across diet treatments (59 observer groups total, 14-15 per treatment). All other methods related to demonstrator training, social conditioning, and observer testing were the same as in experiment 1.

3.2.5. Selenium quantification

After the experiment was completed, concentrations of total Se in the diets and whole body of zebrafish were measured using a graphite furnace atomic absorption spectrometer (GF-AAS, PerkinElmer AAnalyst 800, USA) as described previously (Attaran et al., 2019). For the quantification of Se in the water, samples were treated with 0.2% (v/v) concentrated nitric acid. Food and tissue samples were digested with 1N nitric acid using 1:5 mass (g) by volume (ml) ratio and then kept in an incubator at 60 °C for 48h. The acid-treated samples were centrifuged at 15,000 g for 4 min, and supernatants were collected. After preparation of the samples, they were stored at 4 °C until further analysis. Se concentrations were quantified using the proper dilution, method blanks, and certified standard from PerkinElmer, USA. Furthermore, to validate the Se measurement procedure, the reagent blank and certified reference material (DOLT-4; National Research Council of Canada) were processed simultaneously. The recovery rate of Se was 96%, and the detection limit of 0.1 ppb.

3.2.6. Biochemical assessments

Lipid hydroperoxide (LOOH), which is the main primary product of lipid peroxidation and an important marker of oxidative stress, was measured in the zebrafish whole-brain. Lipid hydroperoxide was quantified according to the manufacture's instruction using a commercially available assay kit (Abcam, USA). This kit quantified hydroperoxides, which are highly unstable, directly utilizing the redox reaction with ferrous ions. First, we homogenized two brains (replicated 3 times) using ice-cold phosphate-buffered saline (PBS) containing no transition metal ions. Lipid hydroperoxides were extracted into chloroform, and we transferred 500 μl of that to glass tubes and added 500 μl of Extract R saturated methanol and vortexed the mixture. Then we added 1 ml

of cold chloroform to each sample and vortexed the mixture and subsequently centrifuged that at 1,500 g for 5 min at 0°C. Then we collected the bottom chloroform layer and transferred that to another tube and store on ice. 450 µl of the chloroform-methanol solvent mixture was added to 500 µl of the chloroform extract of each sample, followed by adding 50 µl of the freshly prepared Chromogen to each assay tube and vortexed properly. We kept the samples at room temperature for 5 min and then transferred 300 µl from each tube into a 96-well plate. The absorbance was then read at 500 nm using a multimode microplate reader (Varioskan Flash, Thermo Fisher Scientific, Finland).

In addition, the level of total 5-HT in the whole-brain was measured using a serotonin ELISA kit following the manufacture's protocol (Bio Vision, USA). The brain tissues (pool of 2-3 brain) were homogenized in PBS (~ 50 mg tissue in 450 µl PBS) on ice. Then the homogenates were centrifuged for 5 min at 5000 g, and the samples were transferred to new tubes. After washing the micro ELISA plate, we added 50 µl of each sample into each well before immediately adding Biotin-detection antibody-working solution and mixing gently. Afterward, we covered the plate for a 45-min incubation at 37 °C. Then we discarded the solution and washed the plate with 1X Wash Solution. 0.1 ml of HRP-Streptavidin Conjugate (SABC) working solution was added into each well, and we again covered the plate for incubation at 37 °C for 30 min. After discarding the solution, we washed the plate again. Then, we added 90 µl of TMB substrate into each well for incubation at 37 °C for 20 min. Finally, we added 50 µl of Stop Solution to each well and the results were read at 450 nm.

3.2.7. Quantitative real-time polymerase chain reaction assessments

The mRNA expression of genes associated with antioxidant enzymes, including glutathione peroxidase (*gpx1a*), catalase (*cat*), manganese superoxide dismutase (*Mn-sod*), and copper/zinc superoxide dismutase (*Cu/Zn-sod*) were evaluated by Quantitative real-time PCR. In addition, we quantified the mRNA expression of genes associated with 5-HT receptors 5-HT_{1A} (*htr1aa*, *htr1ab*), 5-HT_{1B} (*htr1b*), 5-HT_{1D} (*htr1d*), 5-HT_{2A} (*htr2aa*), 5-HT_{2B} (*htr2b*), and 5-HT_{2C} (*htr2cl1*, *htr2cl2*) as well as genes involved in 5-HT synthesis (*tph1a*, *tph1b*, *tph2*), reuptake (*slc6a4a*, *slc6a4b*), and degradation (*mao*) (see Supplementary Information). We extracted total RNA using an RNeasy Mini Kit (Qiagen, Germany). The RNA concentrations and purity were quantified by a Nanodrop spectrophotometer (NanoDrop, Thermo Scientific, USA) by measuring the extinction

at 260 and 280 nm wavelengths, respectively. Subsequently, RNA samples were converted to cDNA using a SensiFAST cDNA Synthesis kit (BIOLINE, USA). The transcript levels (20 μ L reaction volume) of forward and reverse gene-specific primers (Table 3.1), cDNA, SYBR Green PCR Master Mix (SensiFAST, SYBR No-ROX Kit, Biorline, USA), and nuclease-free water were evaluated with quantitative real-time PCR on an iCycler Thermal Cycler (Bio-Rad, USA). We used the gene encoding β -actin as the housekeeping gene. The relative expression of target genes was assessed by the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001a).

Table 3.1. Nucleotide sequences of the forward and reverse primers used for qPCR.

<i>Gene</i>	<i>Gene bank accession no.</i>	<i>Forward</i>	<i>Reverse</i>
<i>Gpx1a</i>	NM_001007281.2	ACCTGTCCGCGAAACTATTG	GCTCGTTCATCTGGGTGTAATC
<i>cat</i>	NM_130912.2	GGACCTTCTACATCCAGGTTATG	CAGGAATCAGAGGGAAGCTTTTAT
<i>Cu/Zn-sod</i>	NM_131294.1	ACACAAACGGCTGCATCA	TCCGACGTGTCTCACACTAT
<i>Mn-sod</i>	NM_199976.1	AGAGCGGAAGATTGAGGATTG	CGCATGTTCCCAGACATCTAT
<i>tph1a</i>	NM_178306.3	CAGTTCAGTCAGGAGATTGG	GACAGTGCGTGCTTCAG
<i>tph1b</i>	NM_001001843.2	CAGGAATTAGGGTTGGCTTCT	CTTAGTGAACCCTCCTGCTTC
<i>tph2</i>	NM_001310068.1	GTGCTCCCATAGAGTGCTAAT	GTACTTTCTCCTCTGCCGATAG
<i>slc6a4a</i>	NM_001039972.1	CCATCTGTCCTTGCTTCCTAT T	GTCCAGAAAGGGTACAGGTAATC
<i>slc6a4b</i>	NM_001177459.1	GAATCCTCTGGGCTTGGAATG	GCTGAAGTAGACAATGGTGAAGAT
<i>mao</i>	NM_212827.3	GCAGTCAGAGCCCGAATC	CACACCCATAAACTTGAGGAATC
<i>htr1aa</i>	NM_001123321.1	GGCCGCGTTAATTCTGTTTG	AGGGAGCCGATGAGATAGTT
<i>htr1ab</i>	NM_001145766.1	TCATTAACTGGCTCGGCTATTC	CGCGCTCTGGAAGTCTTTAT
<i>htr1b</i>	NM_001128709.1	GTGTCGGTGCTCGTGATG	CAGCCAGATGTCGCAGATG
<i>htr1d</i>	NM_001145686.1	CTGGTGATGGGTGTCTGTAAA	TTGATGAGCGAGTTGAGGTATC
<i>htr2aa</i>	XM_684208.9	TGGCCGTAAATCTGGAAAGAA	CCAGAAGTCCAGAAGCATATC
<i>htr2b</i>	NM_001044743.1	GCTGCTCATTCTTCTGGTCAT	GTTAGTGGCGTTCTGGAGTT
<i>htr2cl1</i>	NM_001129893.1	AAACTTCTTCCTCCGCTCAC	CAGTGGCCAGGCATAATCATA
<i>htr2cl2</i>	XM_001339004.7	TCACCGTGTGGACCATTTC	GACACACGCGCAGTCTATAA
<i>β-Actin</i>	NM_131031.2	AGGTCATCACCATTGGCAAT	GATGTCCACGTCGCACTTCAT

3.2.8. Statistical analysis

After arcsine transforming the route preference data, all behavioural data met the assumptions for parametric testing. For both experiments, we first used univariate General Linear Models to analyze the effect of the treatment variable (experiment 1: demonstrator type; experiment 2: diet

concentration) on observer responses, while including the demonstrator group as a random factor (i.e., a blocking variable). Because the blocking variable had no effect, we removed it and re-analyzed the data. Hence, for experiment 1, we used independent t-tests to compare demonstrator types, and for experiment 2, we performed one-way ANOVAs with the diet treatment as the only factor) followed by Tukey post-hoc tests. In each case, we analyzed conditioning and testing data separately. To evaluate Se concentrations in the diet and whole-body, lipid hydroperoxide level, 5-HT level, and fold change in the expression of genes, we again used univariate one-way ANOVAs with Tukey post-hoc tests. Data were normally distributed, but when variances were unequal, we instead used Welch's tests followed by Games-Howell post-hoc tests. For statistical conclusions, we used $\alpha = 0.05$, and all data were analyzed using SPSS (version 23.0, IBM SPSS Inc, USA).

3.3. Results

3.3.1. Experiment 1: Social learning from trained vs. untrained demonstrators

During conditioning, observers paired with trained demonstrators escaped over twice as fast as observers paired with sham demonstrators ($t_{28} = 4.80$, $p < 0.005$; Fig. 3.2A). Subsequently, during testing, observers that had been paired with trained demonstrators again escaped twice as fast ($t_{28} = 3.51$, $p = 0.002$; Fig. 3.2B), revealing they had learned to find the route using prior social information. Moreover, these observers scored significantly higher (a 4-fold increase) for correct route preference ($t_{28} = 6.40$, $p < 0.001$; Fig. 3.2C).

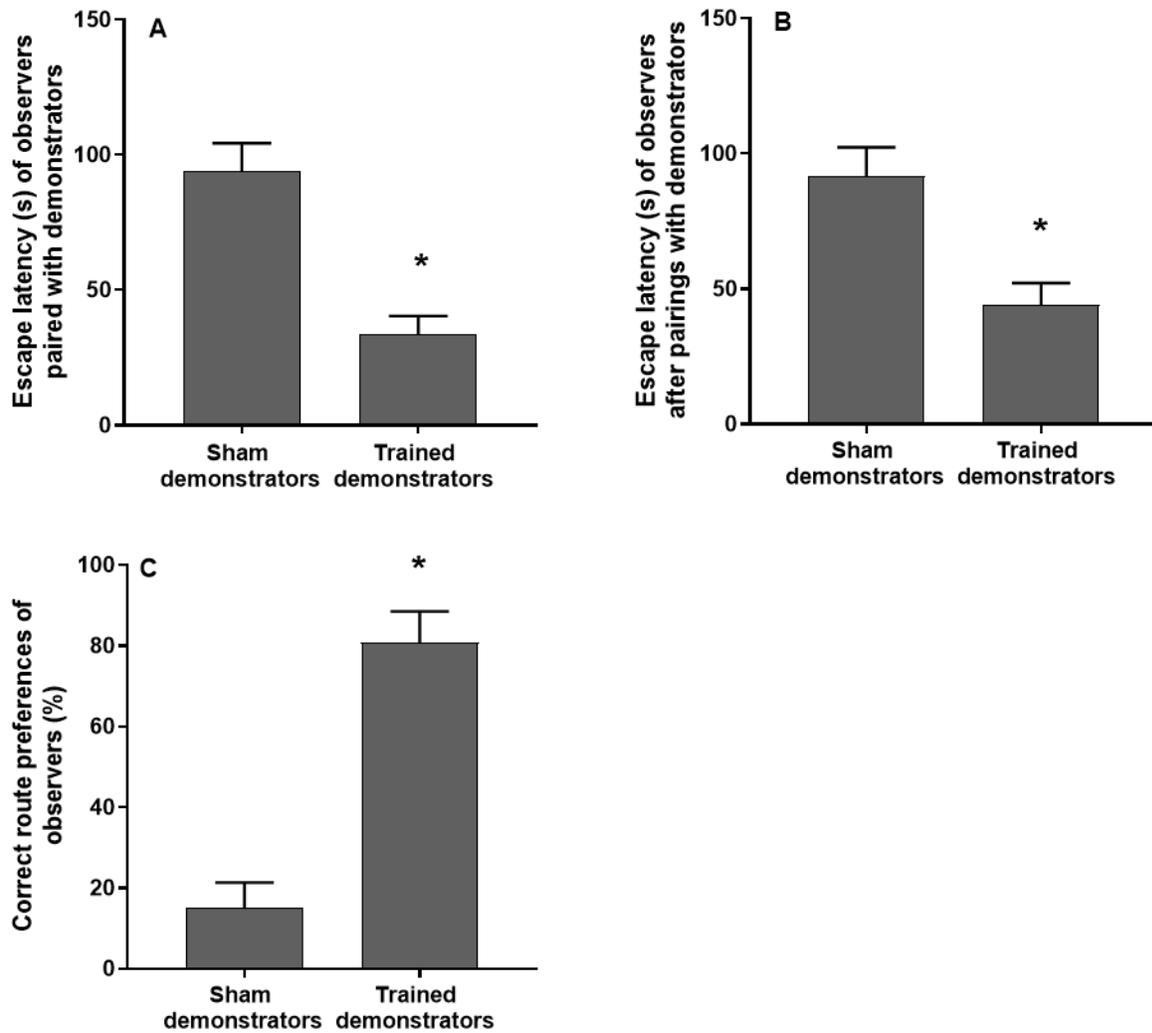


Fig. 3. 2. Mean (\pm SE) escape latency of zebrafish observers on the last day of training when they were paired with sham or trained demonstrators (A) and of observers without demonstrators (B), and percentage of correct route preference of observers (C). Asterisks above bars represent significant differences at $p < 0.05$.

3.3.2. Experiment 2: The effect of Se-Met on social learning outcomes

3.3.2.1. Selenium concentrations

The Se concentrations in the treatment diets and the associated whole-body concentrations are provided in Table 3.2. Diet concentrations differed significantly among the groups ($F_{3,8} = 588.7$, $p < 0.001$), with the medium ($12.8 \pm 0.41 \mu\text{g/g}$) and high ($34.1 \pm 1.1 \mu\text{g/g}$) treatments having

significantly higher Se than the control (0.9 ± 0.02) (both $p < 0.001$). The dietary exposure also affected the whole-body Se concentrations ($F_{3,8} = 52.54$, $p < 0.005$) with significantly more accumulation after treatment with 12.8 and 34.1 $\mu\text{g/g}$ diets compared with the control (all $p < 0.005$). The Se concentration in bloodworms used across treatments was negligible (0.1 ± 0.05 μg wet weight).

Table 3.2. Selenium concentrations in food ($\mu\text{g/g}$ dry weight) and fish body ($\mu\text{g/g}$ wet weight).

<i>Nominal concentrations in diet ($\mu\text{g/g}$)</i>	<i>Measured concentrations in diet ($\mu\text{g/g}$ dry weight)</i>	<i>Measured whole-body concentrations in zebrafish ($\mu\text{g/g}$ wet weight)</i>
<i>Control</i>	0.9 ± 0.02	0.56 ± 0.33
<i>3</i>	3.64 ± 0.11	1.00 ± 0.15
<i>10</i>	12.8 ± 0.42 *	2.20 ± 0.17 *
<i>30</i>	34.1 ± 1.1 *	4.27 ± 0.39 *

Asterisks represent significant differences from control at $\alpha = 0.05$.

3.3.2.2. Social learning performance

Exposure to dietary Se-Met affected the escape latency during conditioning trials ($F_{3,55} = 3.67$, $p = 0.02$) where control observers escaped almost twice as fast as those exposed to the highest concentration ($p = 0.01$; Fig 3.3A). In the absence of demonstrators, there was, again, a significant difference in the escape latency due to Se-Met concentration ($F_{3,55} = 4.59$, $p = 0.006$) with observers exposed to the highest concentration being significantly slower than controls ($p = 0.038$; Fig. 3.3B). However, there were no significant differences in the escape latency of zebrafish that were exposed to low or medium concentrations compared to the control exposure group, either when the observers were paired with demonstrators (3.6: $p = 0.60$, 12.8: $p = 0.28$; Fig. 3.3A) or when they were tested alone (3.6: $p = 0.99$, 12.8: $p = 0.98$; Fig. 3.3B). For route preference, we again found a significant effect of Se-Met exposure ($F_{3,55} = 4.43$, $p = 0.007$; Fig. 3.3C). Control observers in the absence of demonstrators chose the correct escape hole roughly twice as often as observers with the highest exposure ($p = 0.02$), while there were no differences between the other groups (all $p > 0.5$).

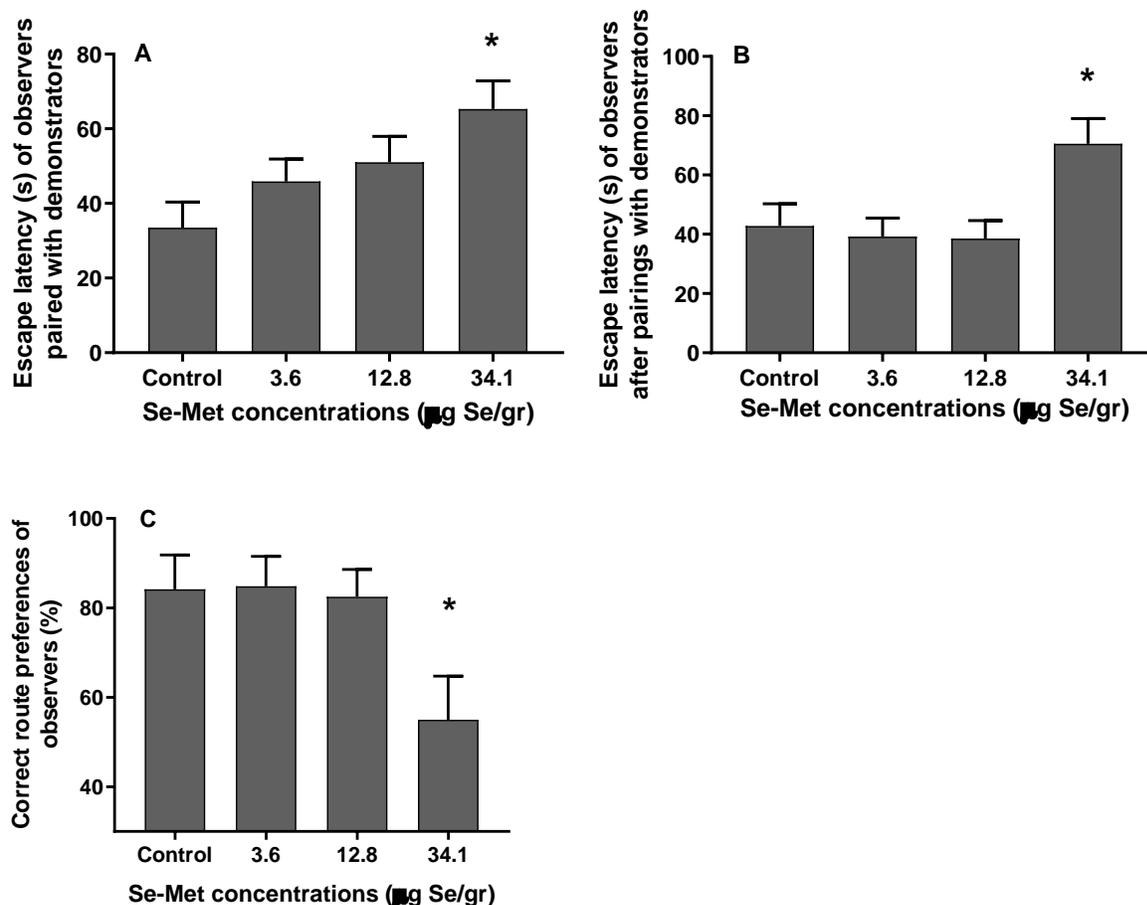


Fig. 3.3. Mean (\pm SE) escape latency of Se-Met exposed observer fish when they were paired with demonstrators (A) and without demonstrators (B), and percentage of correct route preference of observers (C). Asterisks above bars represent significant differences from control at $\alpha < 0.05$.

3.3.3. Oxidative stress markers in the zebrafish brain

Se-Met exposure significantly increased lipid hydroperoxidation content of the zebrafish brain ($F_{3,8} = 17.9$, $p = 0.001$; Fig. 3.4A). This marker of oxidative damage showed a significant increase in the highest dietary exposed group compared to all other groups (all $p \leq 0.01$). The expression of antioxidant genes was also upregulated: *gpx1* ($F_{3,8} = 5.53$, $p = 0.024$; Fig. 3.4B), *cat* ($F_{3,8} = 7.39$, $p = 0.011$; Fig. 3.4C), *Cu/Zn-sod* ($F_{3,8} = 6.60$, $p = 0.015$; Fig. 3.4D), and *Mn-sod* ($F_{3,8} = 10.6$, $p = 0.004$; Fig. 3.4E). Compared to the control, the highest Se-Met exposure concentration caused

significant upregulation of *gpx1* ($p = 0.041$), *cat* ($p = 0.022$), *Cu/Zn-sod* ($p = 0.02$), and *Mn-sod* ($p = 0.046$) expression.

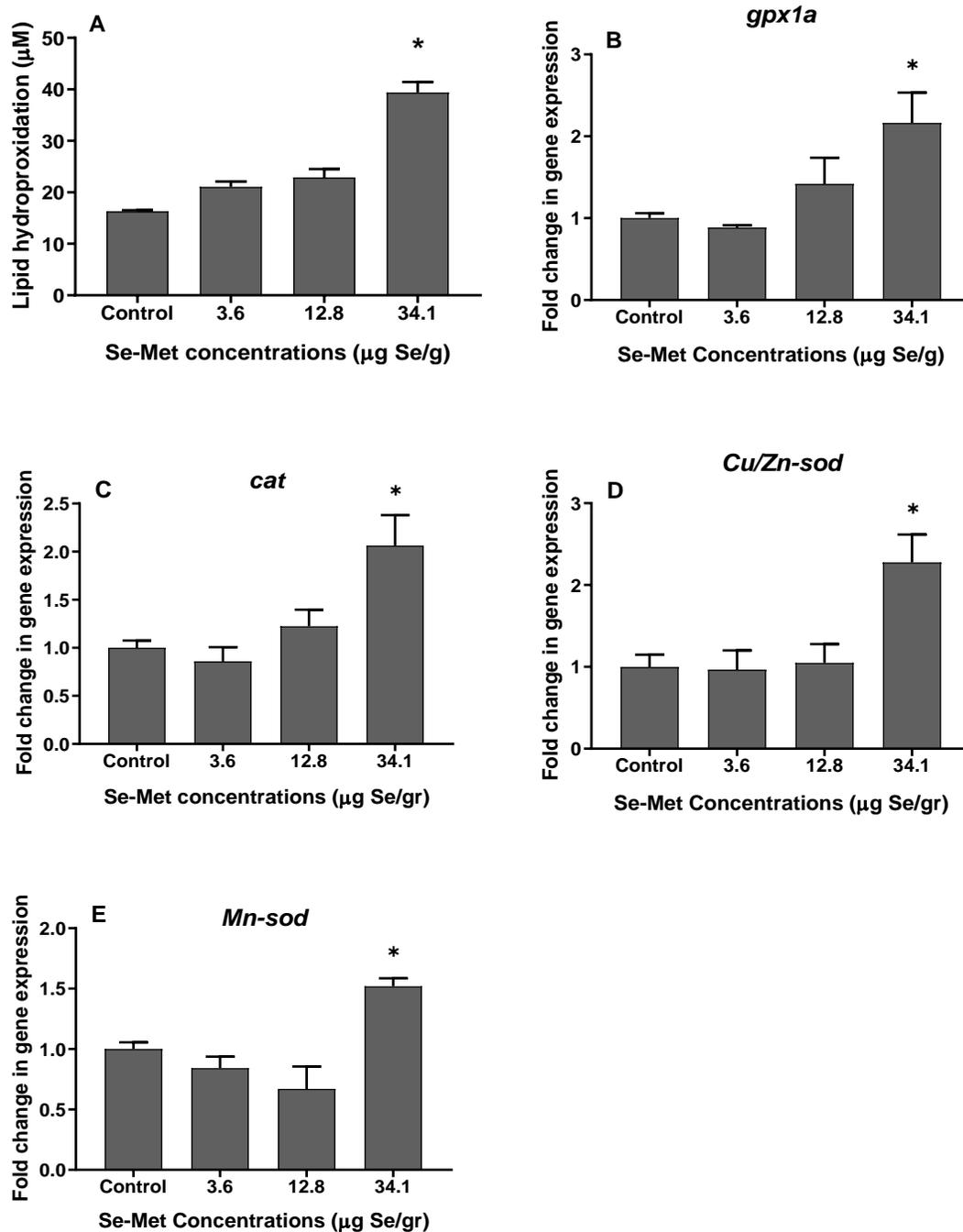


Fig. 3.4. Mean (\pm SE) lipid hydroperoxidation levels (A), and fold change in the expression level of antioxidant genes *gpx1a* (B), *cat* (C), *Cu/Zn-sod* (D), and *Mn-sod* (E) in the brains of zebrafish exposed to different concentrations of Se-Met. The asterisk represents a significant difference from control at $\alpha < 0.05$.

3.3.4. Serotonergic markers in the zebrafish brain

We found a marginally non-significant effect of Se exposure on 5-HT level in the zebrafish brain ($F_{3,8} = 3.71$, $p = 0.06$; Fig. 3.5.A), and Se-Met exposure significantly upregulated expression of *tph2* ($F_{3,8} = 14.5$, $p = 0.001$; Fig. 3.5B), 5-HT transporter *slc6a4a* ($F_{3,4.09} = 21.2$, $p = 0.006$; Fig. 3.5C), and *mao* ($F_{3,8} = 14.1$, $p = 0.001$; Fig. 3.5D). Exposure to Se-Met also elevated the transcription of 5-HT receptors including *htr1aa* ($F_{3,8} = 26.2$, $p < 0.001$; Fig. 3.6A), *htr1ab* ($F_{3,8} = 8.66$, $p = 0.07$; Fig. 3.6B), *htr1b* ($F_{3,8} = 7.81$, $p = 0.009$; Fig. 3.6C), *htr1d* ($F_{3,3.96} = 14.6$, $p = 0.01$; Fig. 3.6D), *htr2aa* ($F_{3,8} = 14.7$, $p = 0.001$; Fig. 3.6E), *htr2b* ($F_{3,8} = 9.04$, $p = 0.006$; Fig. 3.6F), and *htr2cll* ($F_{3,4.29} = 16.6$, $p = 0.008$; Fig. 3.6G). Compared to the control, all Se-Met concentrations caused significant upregulation of *htr1aa* (3.6: $p = 0.008$, 12.8: $p = 0.003$, 34.1: $p < 0.001$) and *htr2aa* (3.6: $p = 0.04$, 12.8: $p = 0.001$, 34.1: $p = 0.004$), whereas only the low and medium concentrations of Se significantly increased (~3 fold) the mRNA level of *htr2b* (all $p = 0.009$). Se exposure also increased the expression of *htr1ab* in the medium exposure group by approximately two fold ($p = 0.005$), whereas the highest concentration significantly increased the transcription level of *htr1b* ($p = 0.01$), *htr1d* ($p = 0.03$), *slc6a4a* ($p = 0.004$), *tph2* ($p = 0.005$), and *mao* ($p = 0.005$) compared to the control (50% to 250% increases). No significant changes in transcription status were found for any serotonergic genes examined in this study (all $p > 0.05$; data are not shown).

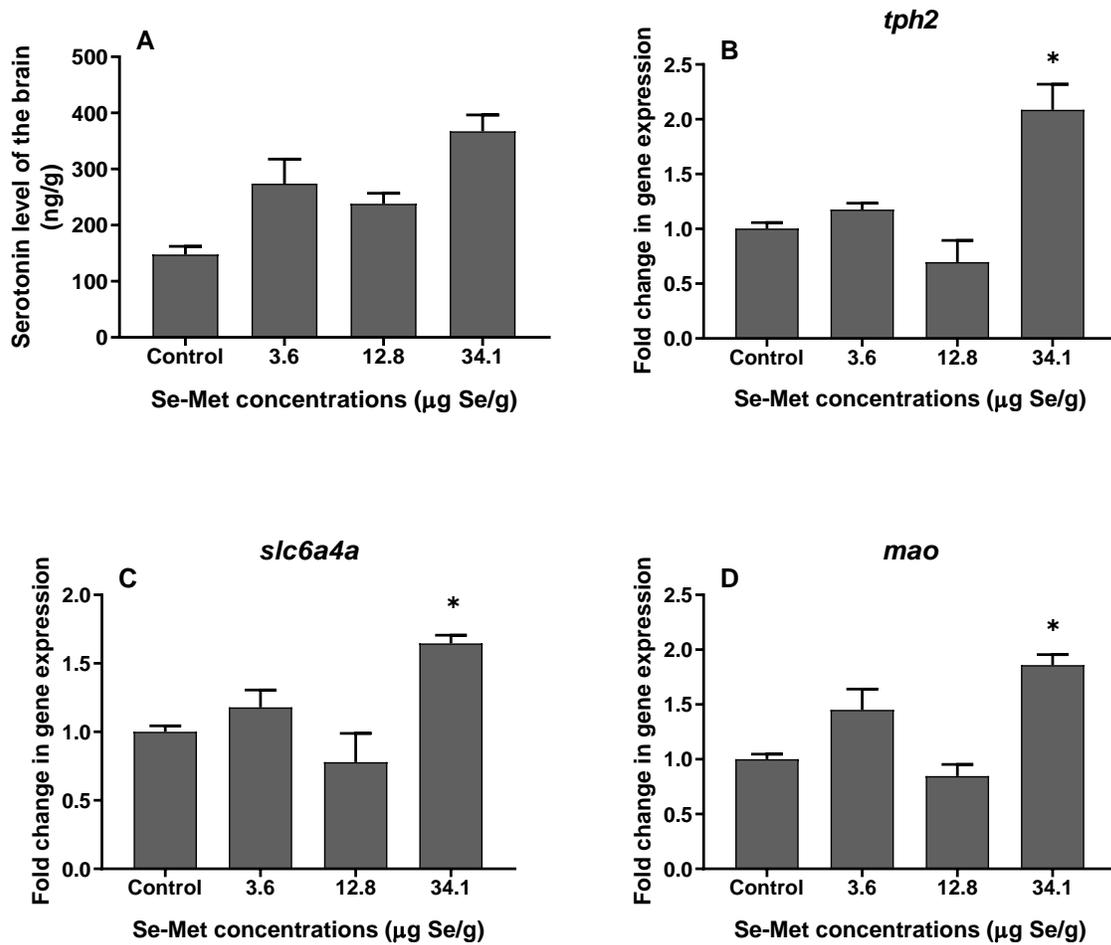
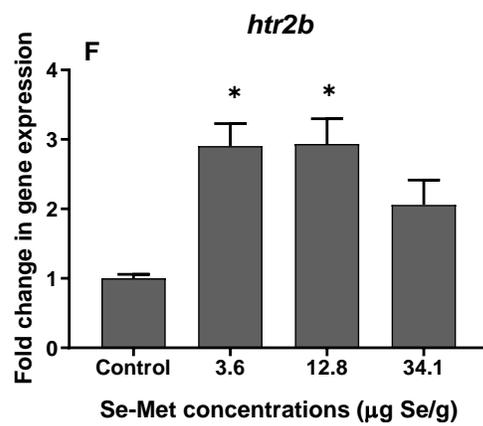
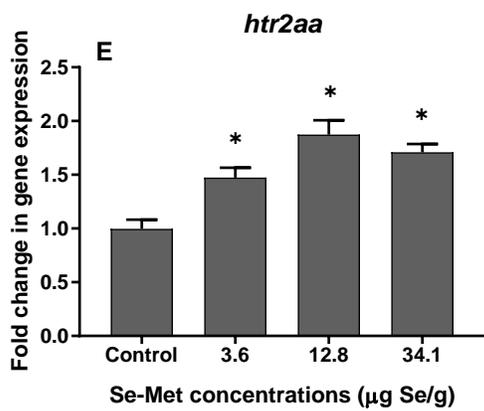
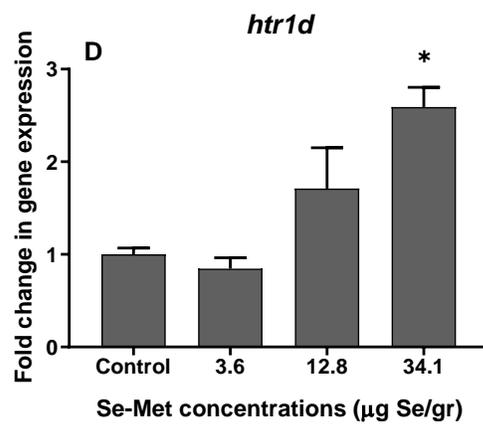
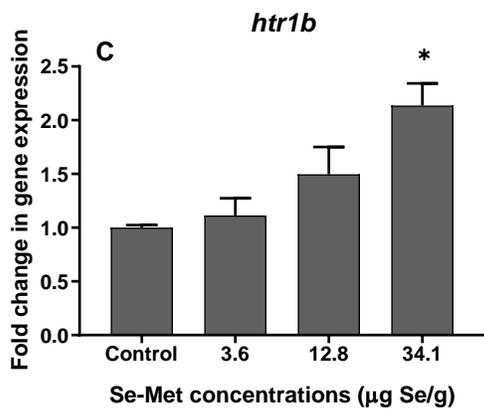
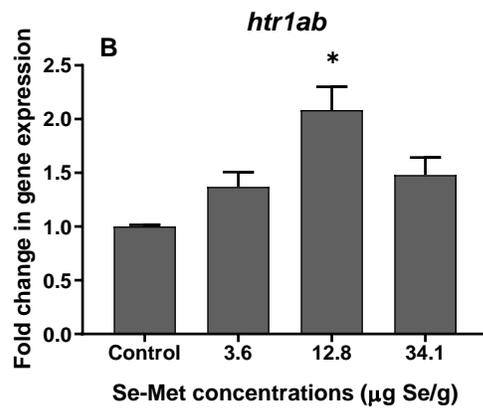
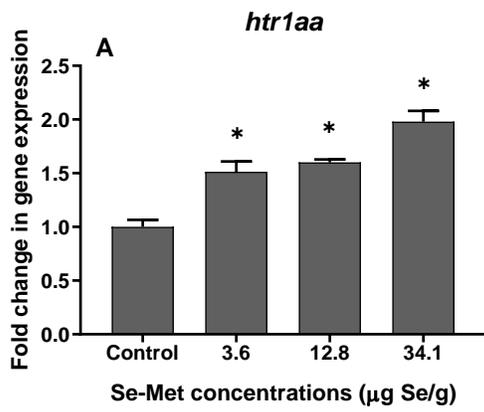


Fig. 3.5. Mean (\pm SE) and serotonin levels (A), and fold change in the expression level of genes involved in 5-HT synthesis (*tph2*; B), re-uptake (*slc6a4a*; C), and metabolism (*mao*; D) in the brains of zebrafish exposed to different concentrations of Se-Met. The asterisk represents a significant difference from control at $\alpha < 0.05$.



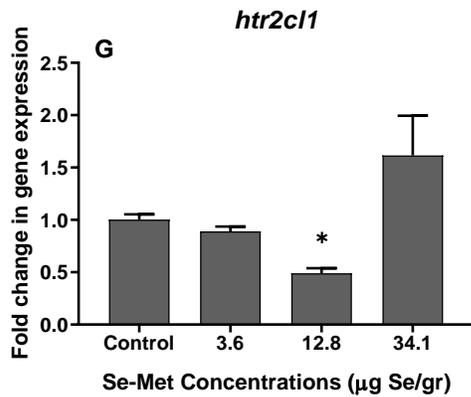


Fig. 3.6. Mean (\pm SE) fold change in the mRNA abundance of 5-HT receptors including *htr1aa* (A), *htr1ab* (B), *htr1b* (C), *htr1d* (D), *htr2aa* (E), *htr2b* (F), and *htr2cl1* (G) in the brain of zebrafish that were treated with different concentrations of dietary Se-Met. Asterisks represent a significant difference from control at $\alpha < 0.05$.

3.4. Discussion:

Despite the increasing evidence of Se neurotoxicity, few studies have addressed the impact of this element on learning and memory. This study on zebrafish is the first to link selenium exposure to social learning outcomes. A broad range of sub-lethal Se concentrations (3.6 to 34.1 μ g Se/g dw) resulted in elevated whole-body Se concentrations (2.20 to 4.27 μ g Se/g wet weight), which are comparable with previous laboratory studies on zebrafish (Attaran et al., 2019; Pettem et al., 2017; Thomas and Janz, 2011a) and other fish species collected from Se-impacted ecosystems (Driedger et al., 2009; Muscatello and Janz, 2009). Exposure to the highest Se concentration, in particular, caused a major impairment of socially-learned predator avoidance, where learned escapes were significantly less accurate and slower than those of unexposed fish. Our results also demonstrate higher LPO in the brain of exposed zebrafish, indicating oxidative damage in the zebrafish brain. Although there was some dysregulation in the important genes in the serotonergic pathway, we did not find a statistically significant effect on serotonin levels.

In the first experiment, we found that zebrafish can learn socially about predator avoidance, similar to other fish species (Brown and Laland, 2002; Brown and Warburton, 1999; Lindeyer and Reader,

2010; Reader et al., 2003). Naïve observers that were paired with experienced demonstrators learned to escape more rapidly (and more accurately) from an approaching trawl compared to observers that were paired with naïve individuals (i.e., sham demonstrators). The subjects also had the opportunity to learn directly (i.e., personal learning) about the trawl, but our results demonstrated that training with knowledgeable demonstrators significantly accelerated learning.

The result of the second experiment revealed that an elevated concentration of (34.1 µg/g) Se impaired socially-learned predator avoidance. Again, these individuals had the opportunity to learn personally, but social learning was clearly the dominant learning mechanism. When given the opportunity for personal learning in the absence of an experienced demonstrator (experiment 1), observers only managed to make correct route choices ~15% of the time and needed ~90 seconds to escape. However, the presence of experienced demonstrators (both experiments) facilitated correct escape 80% of the time and well under a minute, unless the observers were exposed to the highest Se concentration. This is unsurprising given that Se-Met exposure in higher doses is known to affect the cognitive ability of zebrafish in terms of associative and latent learning (Naderi et al., 2018b; Naderi et al., 2017).

Poor learning outcomes can result from either cognitive or physical impairments. However, we did not observe any signs of physical impairment in this study, which is consistent with prior research where similar Se-Met exposures had no effect on zebrafish locomotion (Naderi et al., 2017). Combined with our molecular results (discussed below), these observations suggest that a cognitive impairment hindered neural processes that are related to either learning or the motivation to escape. We used both positive (a group of conspecifics) and negative (trawl net) reinforcement to motivate escape responses, and even zebrafish exposed to the highest concentration escaped in over 70% of the sessions. In our previous work, similar exposures caused higher levels of stress and baseline fear, while also disrupting shoaling behaviour and social preferences (Attaran et al., 2019). Hence, such a direct effect on social cognition likely explains the impaired social learning outcomes in this study.

The brain, with its high oxygen consumption and lipid-rich content, is highly vulnerable to oxidative stress which can negatively impact normal CNS functions (Salim, 2017). There is evidence for an association between increased oxidative stress and depression-like behaviours and

cognitive impairment in rats (Patki et al., 2013), and oxidative stress appears to contribute to behavioural and cognitive impairments in zebrafish (Ruhl et al., 2016). In the current study, there was a marked induction in the LPO level in the brains of zebrafish exposed to the highest concentration of Se-Met, indicating oxidative damage. LPO toxicity occurs as a chain reaction between polyunsaturated fatty acids and ROS, which finally produces lipid peroxides and hydrocarbon polymers that are both highly toxic to cells (Horton et al., 1987). Such an effect is also known to disrupt other learning processes (latent learning and associative learning) in zebrafish (Naderi et al., 2018b; Naderi et al., 2017).

Consistent with an increase in LPO level, we found upregulation in the expression of antioxidant genes, including *gpx1*, *cat*, *Cu/Zn-sod*, and *Mn-sod* in the brain of zebrafish exposed to the highest concentration of Se-Met. These antioxidant enzymes are considered as the first line of defence against ROS (Ighodaro and Akinloye, 2019). Superoxide dismutase abolishes the free radical superoxide by converting it to peroxide, which is then catalyzed (either by catalase or glutathione peroxidase) into harmless molecules such as water and oxygen. An increase in the expression of the genes related to these enzymes may have occurred in this study due to a direct response to selenium toxicity and excess lipid hydroperoxidation radicals. Such a mechanism is consistent with previous studies on zebrafish and rainbow trout (*Oncorhynchus mykiss*) (Misra et al., 2012; Misra and Niyogi, 2009; Naderi et al., 2018b).

Serotonin is one of the primary neurotransmitters in the CNS and modulates a broad spectrum of essential neuronal functions ranging from social responses to cognitive appraisal (Canli and Lesch, 2007b). Different studies have demonstrated that abnormality in the function of the serotonergic system contributes to a broad range of neurobehavioral disorders like autism, depression, anxiety disorders (Chauhan and Chauhan, 2006; Lesch and Waider, 2012). Here, for the first time, we measured the levels of 5-HT in the brain of zebrafish after chronic exposure to different concentrations of Se-Met. Although we found higher ($\sim 3 \times$) levels of 5-HT after exposure to the highest Se-Met concentration compared to the control, this induction was not statistically significant (Fig. 3.3A), which might be due to low statistical power (3 replicates per group) as we were limited in the number of samples. Nevertheless, Se appears to have a biological impact on this neurotransmitter. In a previous study, Se deficiency in the diet increased the serotonin turnover

in the brain of rats (*Rattus norvegicus*) (Castaño et al., 1997b). Further studies are needed to illustrate the effects of exposure to high levels of Se-Met on this neurotransmitter.

5-HT signalling is regulated via several linked processes, including synthesis, release, reuptake, degradation, and receptor activities. Our data show that dietary exposure to Se-Met causes upregulation of genes important for 5-HT synthesis (*tph2*) and reuptake (*slc6a4a*) in the brain of zebrafish. Consistent with these findings, we previously found that exposure to Se-Met for 60 days also led to an increase in the mRNA expression of *tph2* and *slc6a4a* (Attaran et al., 2019). An association between anxiety-like behaviours and depression, with a dysregulation in the expression of these two genes, has been found in both humans and others animals (Donner et al., 2012; Grabe et al., 2005; Lesch et al., 2012).

MAO is a mitochondrial enzyme that catalyzes the oxidative deamination of various monoamines in the brain (e.g., 5-HT, dopamine, and norepinephrine) by producing hydrogen peroxide (J. C. Shih et al., 1999; Wiers et al., 2016). Therefore, any alteration in MAO levels might have major impacts on the brain and behaviour by lowering or raising neurotransmitter levels and inducing oxidative stress (Duncan et al., 2012). In the current study, we found upregulation in the mRNA expression of *mao* in the brain of zebrafish exposed to the highest concentration of Se-Met compared to the control. This upregulation is consistent with another study in which maternal exposure to dietary Se increased the expression of *mao* in the brain of zebrafish offspring (Naderi et al., 2018a). However, in our previous study, we found that Se-Met exposure reduced the expression of *mao* (Attaran et al., 2019). This difference might be due to factors that differed between the experiments such as age (12 vs. 6 month), experimental methods (e.g., group preference vs. social learning), and exposure length (60 vs. 90 days).

Our results indicated that the increase in the expression of 5-HT synthesis and turnover altered the transcription of 5-HT receptors. Different 5-HT receptors have significant roles in the modulation of anxiety, social behaviour, and learning and memory (Cavallaro, 2008; Donaldson et al., 2014). These receptors are also implicated in the regulation of other neurotransmitters that control social behaviour and learning and memory (e.g., dopamine and gamma-aminobutyric or GABA) (Barnes and Sharp, 1999; Katsurabayashi et al., 2003). Our data showed that Se-Met exposure causes alterations in the mRNA expression of the genes that are important in the regulation of 5-HT_{1A}, 5-

HT_{1B}, 5-HT_{1D}, 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors. Here, we found upregulation in *htr1aa* in all low, medium, and high exposure groups, whereas for *htr1ab*, we only found this upregulation for the medium Se-Met exposure. Moreover, there was a marked increase in the expression of *htr1b* and *htr1d* exposed to 34.4 µg Se/g diet. This is consistent with our previous findings where exposure to Se-Met for 60 days led to upregulation of *htr1aa* and *htr1b* (Attaran et al., 2019). The activity of 5-HT_{1A} in the hippocampus has an inhibitory influence on human memory function (Yasuno et al., 2003), and this gene also regulates several transduction mechanisms such as kinases and immediate early genes involved in memory function (Ögren et al., 2008). Therefore, dysregulations in the function of 5-HT_{1A} might affect different aspects of learning and memory, including social learning. Knock-out mice lacking *htr1a* and *htr1b* receptors showed the behavioral patterns of depression, anxiety, reduced social interaction, altered spatial learning, and memory deficits (Hoyer et al., 2002).

In the present study, we also found significantly higher mRNA *htr2aa* expression following treatment with all three Se-Met exposure concentrations, while also finding upregulation of *htr2b* in the low and medium exposed groups. In contrast, the medium exposure led to downregulation in the mRNA of *htr2c1l*. It has been reported that, in humans, alterations in the *htr2a* expression can cause mental illness related to mood and responses to stress (Abdolmaleky et al., 2011; Acevedo-Triana et al., 2017). 5-HT_{2B} antagonist drugs have been reported to reduce social behaviours (Hoyer et al., 2002), and 5-HT_{2C} has been shown to influence anxiety and cognition, as knock-out mice without this receptor suffered from cognitive impairment (Hoyer et al., 2002). Consequently, the upregulation of the primary genes in the serotonergic system might account for learning and social behaviour disruption observed in the present study.

Overall, our findings provide the first documentation of the impacts of exposure to environmentally relevant concentrations of Se-Met on social learning outcomes on zebrafish. Exposure to high concentrations of dietary Se-Met led to impaired socially-learned predator avoidance. We also found exposure to high levels of Se-Met can induce oxidative stress, which might cause neurological impairment. Exposure to Se-Met affected the serotonergic system, from synthesis to reuptake and receptors in the brain, which consequently might impact both social behaviour as well as learning and memory.

Chapter 4: Maternal exposure to selenomethionine dysregulates the serotonergic pathway and impairs antipredator behaviour, group preference and social learning in zebrafish offspring

Preface

The aim of this chapter is to address the fourth objective of my doctoral research work, which is to examine the impacts of maternal exposure to dietary Se-Met on antipredator behaviour, group preference, and social learning in F1-generation adult zebrafish. To this end, adult female zebrafish were exposed to environmentally relevant concentrations of Se-Met then bred with untreated males, and the offspring were raised to adulthood (6 months old) without Se exposure. Then, in a series of behavioural tests, we found the offspring that were maternally exposed to high levels of Se showed behavioural signs of stress (although no physical impairment), had weaker group preferences, and also demonstrated impaired social learning. These neurobehavioural deficits appear to be linked to perturbations in the serotonergic system in the brain, as maternal exposure to high Se concentrations led to dysregulation of this neurotransmitter (e.g., altered transcription of 5-HT receptors).

The content of chapter 4 was adapted from environmental pollution (DOI: 10.1016/j.envpol.2021.117289). Attaran, A., Salahinejad, A., Naderi, M., Crane, A.L., Chivers, D.P., Niyogi, S. “Transgenerational effects of selenomethionine on behaviour, social cognition, and the expression of genes in the serotonergic pathway in zebrafish”.

Authors contribution

Anoosha Attaran (University of Saskatchewan) designed and conducted the experiment, generated and analysed the data, prepared all the figures and tables, and drafted and revised the manuscript. Arash Salahinejad, Mohammad Naderi, and Adam Crane (University of Saskatchewan) provided technical assistance and edited the manuscript. Som Niyogi and Doug Chivers (University of Saskatchewan) provided inspiration, scientific input and guidance, commented on and edited the manuscript, and provided funding for the research.

4.1. Introduction

Selenium (Se) is a naturally-occurring trace element that is essential for animals to maintain physiological homeostasis. However, Se becomes toxic outside of a narrow range of safe concentrations (Janz et al., 2010). Anthropogenic activities such as mining (uranium and coal), fossil-fuel processing, and agriculture on seleniferous soils can greatly increase the release of Se into the environment. Primary producers absorb Se, which can then biomagnify in food webs and result in adverse effects in higher trophic levels (Janz et al., 2010). Selenomethionine (Se-Met) is the main dietary form of Se to which higher trophic organisms like fish are exposed in the natural environments (Chapman et al., 2010; Janz et al., 2014; Muscatello and Janz, 2009). In Se-impacted aquatic ecosystems, the range of Se concentrations reported in various prey species includes 2.7-42 and 1.5-97.7 $\mu\text{g Se/g}$ dry weight (dw) in fish and invertebrates, respectively (Driedger et al., 2009; Lemly, 2014; May et al., 2008; Muscatello and Janz, 2009; Phibbs et al., 2011; Schuler et al., 1990).

Selenomethionine is also a well-known teratogen in oviparous vertebrates, including fishes, aquatic birds, and amphibians (Lemly, 1997). In fishes, it can become incorporated into the egg-yolk protein precursor, vitellogenin, which is maternally transferred to developing embryos (i.e., maternal 'deposition') (Holm et al., 2005; Janz et al., 2010; Muscatello et al., 2006; Naderi et al., 2018a). Such deposition is thought to be the predominant origin of the pollutant in eggs (Muscatello et al., 2006; Naderi et al., 2018a; Raine et al., 2016; Thomas and Janz, 2015), where high accumulation via maternal transfer can cause both direct mortality and sub-lethal effects such as developmental abnormalities (e.g., spinal curvatures and craniofacial and fin deformities) (Janz et al., 2010; Thomas and Janz, 2015) and neurobehavioral problems via dopaminergic dysregulation (Naderi et al., 2018a; Smith et al., 2010).

Se toxicity is known to disrupt the brain's normal function via changes in monoamine neurotransmitters (Vinceti et al., 2014). Such neurotransmitter systems (e.g., dopamine, norepinephrine, and serotonin) start to differentiate during embryogenesis and are found relatively early during brain development, with serotonin (5-hydroxytryptamine; 5-HT) being the earliest in the most terminal regions, even prior to becoming a neurotransmitter (Whitaker-Azmitia, 2001). 5-HT is highly conserved across vertebrate species and plays several fundamental roles, both during adulthood and in development. It is present in the brain prior to synaptogenesis (Hansson

et al., 1998) and is involved in cell proliferation, neuronal differentiation, neurite growth, and synaptogenesis (Côté et al., 2007) by regulating synaptic plasticity in the sensory cortex, migration of cranial neuronal crest cells, and neurogenesis (Gross et al., 2002; Lillesaar, 2011). Such roles are important in reproduction, aggression, social behaviour, and cognition.

Abnormal behaviour, including depression, anxiety disorders, and autism, can result from the dysregulation of 5-HT during brain development (Lisboa et al., 2007; Walsh et al., 2008). For example, early-life 5-HT dysregulation has been found to cause a wide range of behavioural alterations in rodents, ranging from stress-related phenotypes and impaired fear extinction to social deficits (Ansorge et al., 2008; Popa et al., 2008; Wellman et al., 2007). Several 5-HT receptors and linked processes (e.g., synthesis, release, reuptake, and degradation) are involved in the regulation of 5-HT signalling. The 5-HT_{1A} receptor is one of the primary mediators of 5-HT, as it regulates 5-HT neurons and the function of several neurotransmitter systems via autoreceptors and postsynaptic receptors, respectively (Ogren et al., 2008). This receptor is highly concentrated in specific brain regions associated with memory function (cortical and limbic) in both rodents and humans (Hall et al., 1997; Pazos and Palacios, 1985). The presence of the 5-HT_{1B} receptor in the hippocampal formation suggests a potential role of this receptor in the regulation of memory as well. The stimulation of this receptor has been shown to impair spatial learning in rats (Buhot et al., 2000; Buhot et al., 1995).

Social interactions are a key part of life for many animal species, particularly for those living in groups (Wilson, 1975). In many cases, the behaviour of others is critically important for fitness activities such as finding food and mates, and for avoiding threats (Crane and Ferrari, 2013; Hoppitt and Laland, 2013). However, direct exposure to high levels of Se-Met (>30 µg Se/g) can impair social behaviour and cognition in adult zebrafish (Attaran et al., 2019; Attaran et al., 2020; Naderi et al., 2018b; Naderi et al., 2017), with such impairment being linked to different neuropsychiatric disorders, including autism, schizophrenia, depression, and social anxiety (Lisboa et al., 2007; Walsh et al., 2008). In contrast, the neurobehavioral consequences of maternal exposure to Se have received little scientific attention.

The zebrafish *Danio rerio* is a developing model for translation research on the mammalian central nervous system, as well as human neurological disorders, toxicology, and drug discovery (Saleem and Kannan, 2018). The zebrafish brain possesses the primary mammalian neurotransmitters, with

serotonin being detected as early as 1 day post-fertilization (Brustein et al., 2003). Moreover, several zebrafish traits (rapid embryonic development, short life-span, high fecundity, and small body size) generally reduce laboratory/experimental maintenance in comparison to mammalian models (Lee and Freeman, 2014; Reed and Jennings, 2011).

This study is the first to explore environmentally realistic concentrations of dietary Se-Met, with the aim of determining the maternal influences on offspring behaviours and the serotonergic system in zebrafish. First, we manipulated the exposure of adult females by exposing them to different Se-Met concentrations in their diet. The offspring were then reared in clean water with a normal diet (no added Se-Met) until 6 months of age. Then, in 4 experiments, we tested offspring populations for appropriate behavioural responses: antipredator responses toward predation risk (experiment 1), general individual activity (experiment 2), group preferences (experiment 3), and social learning of predation risk (experiment 4). These behavioural experiments were followed by analysis of the mRNA expression of serotonergic cell markers in the zebrafish brain.

4.2. Material and methods

4.2.1. Fish maintenance and exposure

All fish housing and experimental procedures used in this study were approved by the University of Saskatchewan Animal Research Ethics Board (protocol no. 20170037). Experimentally naïve adult zebrafish ($n = 150$) were sourced from the RJF Smith Center for Aquatic Ecology of the University of Saskatchewan. The zebrafish were housed in 6-l tanks (ratio of 2-female:1-male) with filtered and dechlorinated tap water (hereafter, 'water') and controlled temperature (27 °C) and photoperiod (14 h light and 10 h dark). The diet preparation and exposure regimen consisted of exposure to either control flake food (Nutrafin Max flakes, Germany) or different nominal concentrations of Se-Met (3, 10, and 30 $\mu\text{g/g}$ dry weight (dw)) that were mixed with the respective flake foods for 90 days, as it described previously (Attaran et al., 2020). Total Se concentrations in the treatment diets were confirmed analytically and reported elsewhere (Attaran et al., 2020). The measured Se concentration in all treatment diets was within $\pm 20\%$ of the nominal Se concentration. Fish were fed flake food ($\sim 2\%$ of body weight per day) and bloodworms three times per week. After 80 days of exposure, male zebrafish were removed from the exposure tanks to prevent spawning, while females continued receiving the treatment diets. Then, 10 days later, 8 female zebrafish from each exposed dietary group were paired with 4 Se-untreated male zebrafish

for breeding overnight. The following morning the eggs were collected, and the embryos were kept in clean water. Offspring were raised to 3 months old in 3-l tanks filled with clean water. We quantified Se concentrations in the diet (n=3), female whole-body (n=3), and eggs (n= 3 replicates of 100 pooled egg samples) using inductively coupled plasma mass spectrometry (ICP-MS) at the Saskatchewan Research Council (SRC; Saskatoon, Saskatchewan, Canada) (Attaran et al., 2019; Attaran et al., 2020).

4.2.2. Experiment 1: Antipredator behaviour

To assess antipredator behaviour of offspring that were maternally exposed to Se-Met, we measured the vertical position of groups of zebrafish (5 per group) in 6-l observational tanks (28×22×22 cm) in response to chemical alarm cues. These cues are released by zebrafish, as well as numerous other species when their skin is damaged during a predator attack, and thus, alarm cues are a reliable indicator of predation risk for nearby individuals that detect the cues via olfaction (Ferrari et al., 2010; Jesuthasan and Mathuru, 2008). To this end, we first obtained the alarm cues by sacrificing 4 zebrafish, extracting and homogenising their skin with distilled water, and freezing the resulting solution (-20 °C) until thawing it prior to testing (Attaran et al., 2019; Salahinejad et al., 2021). We used a concentration of ~1 cm² of skin in 40 l of water, as reported in our previous study (Attaran et al., 2019). The groups of zebrafish that were tested were familiar with their group members, having been together from the beginning of the exposure period. For testing, each group was moved into observational tanks and allowed to acclimate for 10 minutes before the trial began. Behavioural trials lasted for 17 min including an 8-min pre-stimulus period, followed by 1-min for injection of alarm cues via a plastic hose, and then an 8-min post-stimulus period. The vertical position of zebrafish was recorded using a side-view camera (Logitech C922x Pro Stream Webcams). We scored the position as 1 (to a fish in the bottom third), 2 (middle third), or 3 (top third) every 15 s. These scores were then summed for each fish and for all members of each group. We conducted 8-11 replicates per treatment group.

4.2.3. Experiment 2: General individual activity

In this experiment, we assessed the general individual activity of zebrafish that were maternally exposed to the Se-Met treatments, using an established method (Salahinejad et al., 2020). For this, we used experimental tanks (~ 20 l, 20×36×36 cm) divided into 6 horizontal and 6 vertical visual segments. First, we moved individual zebrafish to the observational tank for a 5-min acclimation.

Then, we recorded the total number of lines crossed during a 5-min observation period. We tested 20 fish (replicates) per treatment.

4.2.4. Experiment 3: Group preference

We assessed the impact of maternal exposure to Se-Met on offspring social behaviour by employing a well-established group preference paradigm (Fig. 2.2) (Attaran et al., 2019; Pritchard et al., 2001; Ruhl and McRobert, 2005). Observational tanks (~ 20 l, 20×36×36 cm) were divided into 3 main sections with glass dividers. The outer sections would serve as ‘conspecific boxes’ (12.5 cm wide) that would hold non-focal zebrafish, whereas the centre section (36 cm wide) would hold the test individual. This section was sub-divided into 6 visual zones (demarked by lines on the tank). The 2 zones adjacent to the conspecific boxes were designated as ‘conspecific zones’ (6 cm wide), and the 4 middle sub-sections (24 cm wide in total) were considered the ‘centre’ (Fig. 1S). Before each trial, a group of untreated, similar-sized (42-44 mm) zebrafish was moved into each conspecific box to serve as a social stimulus. Previous studies have demonstrated that zebrafish are attracted to such stimuli, can discriminate between conspecific groups of different sizes, and prefer to shoal with larger groups (Seguin and Gerlai, 2017a). In this study, each trial involved a group of 3 individuals in one of the conspecific boxes versus a group of 5 individuals in the opposite chamber, with the left/right locations randomised across trials. To test focal fish, we placed one individual from either the control or maternally treated groups into the centre. After a 5-min acclimation period, we started a 10-min observation period where the amount of time spent in each zone was recorded. We conducted 18-22 replicates per treatment.

4.2.5. Experiment 4: Social learning

To assess social learning in maternally-exposed offspring, we used an escape-route maze (100×50×15 cm), as in previous studies (Attaran et al., 2020; Lindeyer and Reader, 2010). The maze was divided into two main zones: a trawl zone and a safe zone. The dividing partition (white corrugated plastic) had two escape holes (3×4 cm) positioned 30 cm apart and 3 cm above the bottom of the maze. The two holes, one bordered with yellow tape and the other with white tape, could be opened and closed using a removable barrier (corrugated plastic). When zebrafish occupied the trawl zone, a trawl net could be moved forward and backward to encourage fish to escape through a hole to the safety zone. The safe zone contained a group of 5 conspecifics (behind a glass barrier) and synthetic plant habitat.

The behavioural assay had three main phases. First, we trained a group of 2 untreated zebrafish as “trained demonstrators”. A training session consisted of a 5-min acclimation period followed by four 2-min trials where the trawl net was moved forward and backward every 30 s. Following each trial, the fish was moved back to the trawl zone and given a 2 min of acclimation period before the next session. During these sessions, only the yellow escape hole was open. After 10 sessions, demonstrators had escaped through the hole in < 20 s in $\geq 90\%$ of sessions. We also designated four groups of two zebrafish as “sham demonstrators”. These individuals were placed in the maze for the same amount of time to become familiar with the apparatus while the escape routes were closed, and the trawl net was still.

The second phase of this experiment consisted of a social conditioning where we paired a group of two zebrafish, either control or treated, with trained or sham demonstrators. Together, these fish were given one session as described above, but both escape holes were open. Then we removed the demonstrators from the maze, and the final phase (observer testing) began. First, a 10-min acclimation period was provided, and then we used the trawl to test the observers for escape latency and route preference. We also recorded whether the correct route was chosen and converted this value to a percentage of correct escapes out of the total.

4.2.6. Serotonin biomarkers: Quantitative real-time polymerase chain reaction

We measured the mRNA expression of the genes associated with 5-HT receptors, including 5-HT_{1A} (*htr1aa*, *htr1ab*), 5-HT_{1B} (*htr1b*), 5-HT_{1D} (*htr1d*), 5-HT_{2A} (*htr2aa*), 5-HT_{2B} (*htr2b*), and the genes involved in synthesis (*tph2*) and degradation (*mao*) of 5-HT, as well as its transporters (*slc6a4a*, *slc6a4b*). Total RNA was extracted from whole-brain tissue (pool of 2 brains) using an RNeasy Mini Kit (Qiagen, Germany). The purity and concentrations of RNA were determined using a Nanodrop spectrophotometer (Thermo Scientific, U.S.A). Subsequently, the cDNA was produced using a SensiFAST cDNA Synthesis Kit (Bioline, U.S.A). Finally, the transcript levels of certain genes were measured ($n = 4$ per treatment) in triplicate in 20 μ l reaction volumes on iCycler Thermal Cycler (SensiFAST, SYBR No-ROX Kit, Bioline, U.S.A) (Attaran et al., 2019). The gene encoding β -actin was considered as the housekeeping gene, and the relative expression of target genes was assessed by the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001b). See Table 3.1. or Attaran et al. (2020) for primer sequences.

4.2.7. Statistical analysis

To assess the vertical position of zebrafish in experiment 1, we conducted a two-way repeated-measures ANOVA where time (pre/post stimuli period) was the within-subject factor and diet was the fixed factor. For post-hoc testing, we split the data by time and performed separate one-way ANOVAs with Tukey tests. General individual activity in experiment 2 was analysed using a one-way ANOVA with post-hoc Tukey tests. We also used this approach for group preferences in experiment 3, where we analysed the proportion of time individuals spent in a conspecific zone, as well as the ratio of time in the zone near the larger shoal. To assess social learning in experiment 4, we conducted two-way ANOVAs on the observers' escape latency and route preference probabilities. In each case, we tested the effects of maternal exposure, the training treatment (either with knowledgeable or sham demonstrators), and their interaction. Route preference data were logit transformed for normality prior to analysis. For post-hoc tests, we split the data by the training treatment and then performed separate one-way ANOVAs with Tukey tests. Finally, we evaluated Se concentrations in the diets, the water, the whole-body of adult females, and their eggs, as well as for the fold change in the expression of genes in the serotonergic pathway (one-way ANOVAs with Tukey tests). In some cases, data were heteroscedastic, so we used Welch's tests followed by Games-Howell post-hoc tests rather than parametric ANOVAs. We used $\alpha = 0.05$ unless noted otherwise, and all analyses were conducted using SPSS (version 23.0, IBM SPSS Inc, U.S.A).

4.3. Results

4.3.1. Selenium concentrations

All Se concentrations in diets, water samples, maternal zebrafish (whole-body), and eggs are provided in Table 4.1. Dietary Se-Met closely matched the nominal Se concentrations of 3, 10, and 30 $\mu\text{g/g}$ dw, with the control diet containing only 0.9 $\mu\text{g/g}$ dw. Maternal concentrations followed this dose-dependent pattern ($F_{3,8} = 65.08$, $p < 0.001$), with significantly more accumulation after dietary treatment with 12.8 and 34.1 $\mu\text{g/g}$ in comparison to the control (both $p \leq 0.002$). Egg concentrations also differed ($F_{3,1.70} = 290.2$, $p = 0.007$), with significant accumulation resulting from all maternal Se-Met diets (all $p < 0.05$). Selenium concentrations in

the exposure water did not vary among the treatments ($F_{3,12} = 0.67$, $p = 0.59$), and the bloodworm portion of the diets contained only $0.1 \pm 0.05 \mu\text{g/g}$ Se-Met wet weight.

Table 4.1. Selenium concentrations in adult females (whole-body), their eggs ($\mu\text{g/g}$ wet weight), and water ($\mu\text{g/l}$).

<i>Dietary Se ($\mu\text{g/g}$ dry weight)</i>	<i>Adult Se concentrations ($\mu\text{g/g}$ wet weight)</i>	<i>Egg Se concentrations ($\mu\text{g/g}$ wet weight)</i>	<i>Water Se ($\mu\text{g/l}$)</i>
0.90 ± 0.02 (control)	0.58 ± 0.03	0.21 ± 0.03	0.35 ± 0.01
3.64 ± 0.11	1.00 ± 0.23	1.24 ± 0.01 *	0.34 ± 0.02
12.80 ± 0.42 *	2.27 ± 0.22 *	1.90 ± 0.20 *	0.37 ± 0.02
34.10 ± 1.10 *	4.38 ± 0.33 *	3.94 ± 0.30 *	0.38 ± 0.03

Asterisks represent significant differences from control at $\alpha = 0.05$.

4.3.2. Experiment 1: Antipredator behaviour

The vertical position of maternally exposed offspring in response to alarm cues differed significantly across treatments (time \times Se-Met treatment: $F_{3,36} = 3.58$, $p = 0.023$; Fig. 4.1). Before the injection of alarm cues, fish that were maternally exposed to the high concentration showed lower vertical position in comparison to the control group ($F_{3,36} = 4.67$, $p = 0.007$; control vs. 34.1: $p = 0.006$; other comparisons: $p > 0.05$; Fig. 4.1). However, the fish from all treatment groups showed a similar level of reduced vertical position after the introduction of alarm cues ($F_{3,14,11} = 1.16$, $p = 0.36$).

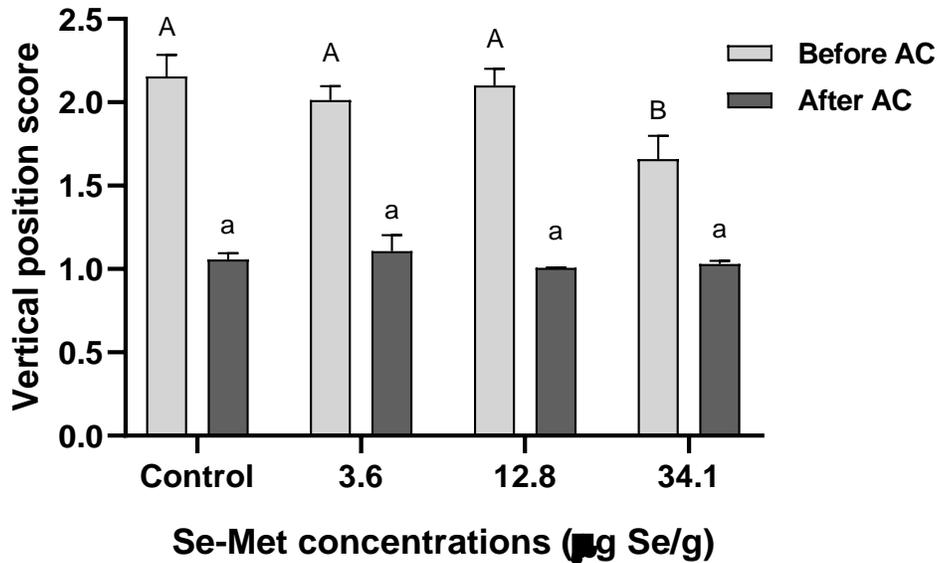


Fig. 4.1. Mean (\pm SE) vertical position scores before and after the exposure of alarm cue to zebrafish maternally treated to different concentrations of Se-Met. Bars with different letters are significantly different from each other at $\alpha < 0.05$.

4.3.3. Experiment 2: General individual activity

General individual activity differed significantly among treatment groups ($F_{3,41.6} = 4.91$, $p = 0.005$; Fig. 4.2), with the two higher Se exposures causing higher activity compared to the lowest exposure (both $p < 0.05$). However, none of the maternally exposed groups differed significantly from the control group (all $p > 0.3$; Fig. 4.2).

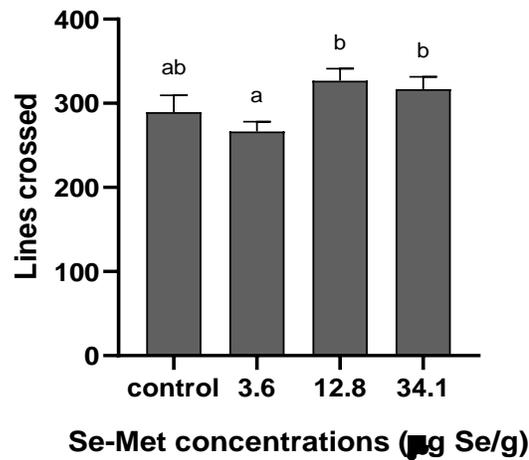


Fig. 4.2. Mean (\pm SE) the number of lines crossed by zebrafish exposed maternally to different concentrations of Se-Met. Bars with different letters are significantly different from each other at $\alpha < 0.05$.

4.3.4. Experiment 3: Group preference

Maternal exposure to Se-Met significantly affected attraction to the conspecific shoals ($F_{3,84} = 4.13$, $p = 0.009$; Fig. 4.3A), with the high dose causing zebrafish to spend less time near shoals compared to the lowest dose and the control ($p = 0.007$ and 0.044 respectively; other comparisons: $p > 0.05$). We also found that the preference for the larger shoal varied significantly among treatments ($F_{3,46.2} = 3.46$, $p = 0.02$; Fig. 4.3B). Maternal exposure to the lowest dose resulted in marginally more time spent in the conspecific zone near the larger shoal compared exposure to the highest dose ($p = 0.05$), but no treatment groups differed significantly from the control (all $p > 0.14$; Fig. 4.3B).

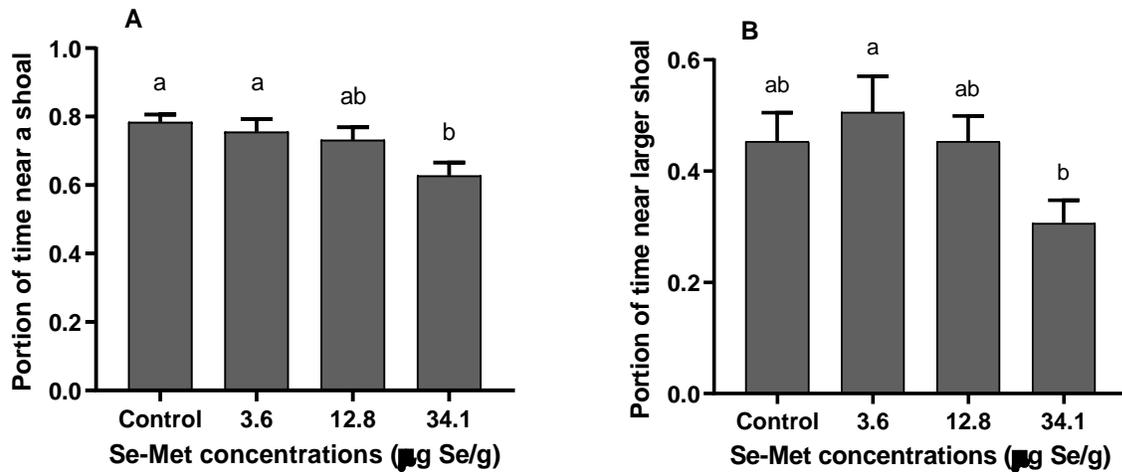


Fig. 4.3. Mean (\pm SE) proportion of time spent in a conspecific zone (A) and in the zone near the larger shoal (B) for zebrafish maternally exposed to different concentrations of Se. Bars with different letters are significantly different from each other at $\alpha < 0.05$.

4.3.5. Experiment 4: Social learning

The escape latency of observers was significantly influenced by an interaction between exposure treatment and the type of training (i.e., trained or sham demonstrators) ($F_{3,74} = 2.84$, $p = 0.04$; Fig. 4.4A). For observers paired with trained demonstrators, escape latency differed among the exposure treatments ($F_{3,24.02} = 6.91$, $p = 0.002$), with control fish escaping almost three times faster than those maternally exposed to the high concentration ($p = 0.017$; Fig. 4.4A). Fish maternally exposed to the lowest concentration also escaped significantly faster than those from the medium and high treatments ($p = 0.045$ and 0.008 , respectively; other comparisons: $p > 0.05$). For observers conditioned with sham demonstrators, there was no significant difference in escape latency across Se-Met treatments ($F_{3,14.37} = 1.18$, $p = 0.35$).

For correct route preference, individuals with the highest maternal exposure failed to locate the correct escape route about twice as often as control fish. This decreased accuracy was not statistically significant, however (exposure treatment \times training type: $F_{3,74} = 0.43$, $p = 0.73$; Fig. 4.4B). Instead, we found only a significant main effect of the demonstrator training, where fish trained with knowledgeable demonstrators chose the correct route significantly more than those trained with sham demonstrators ($F_{1,74} = 34.7$, $p < 0.001$).

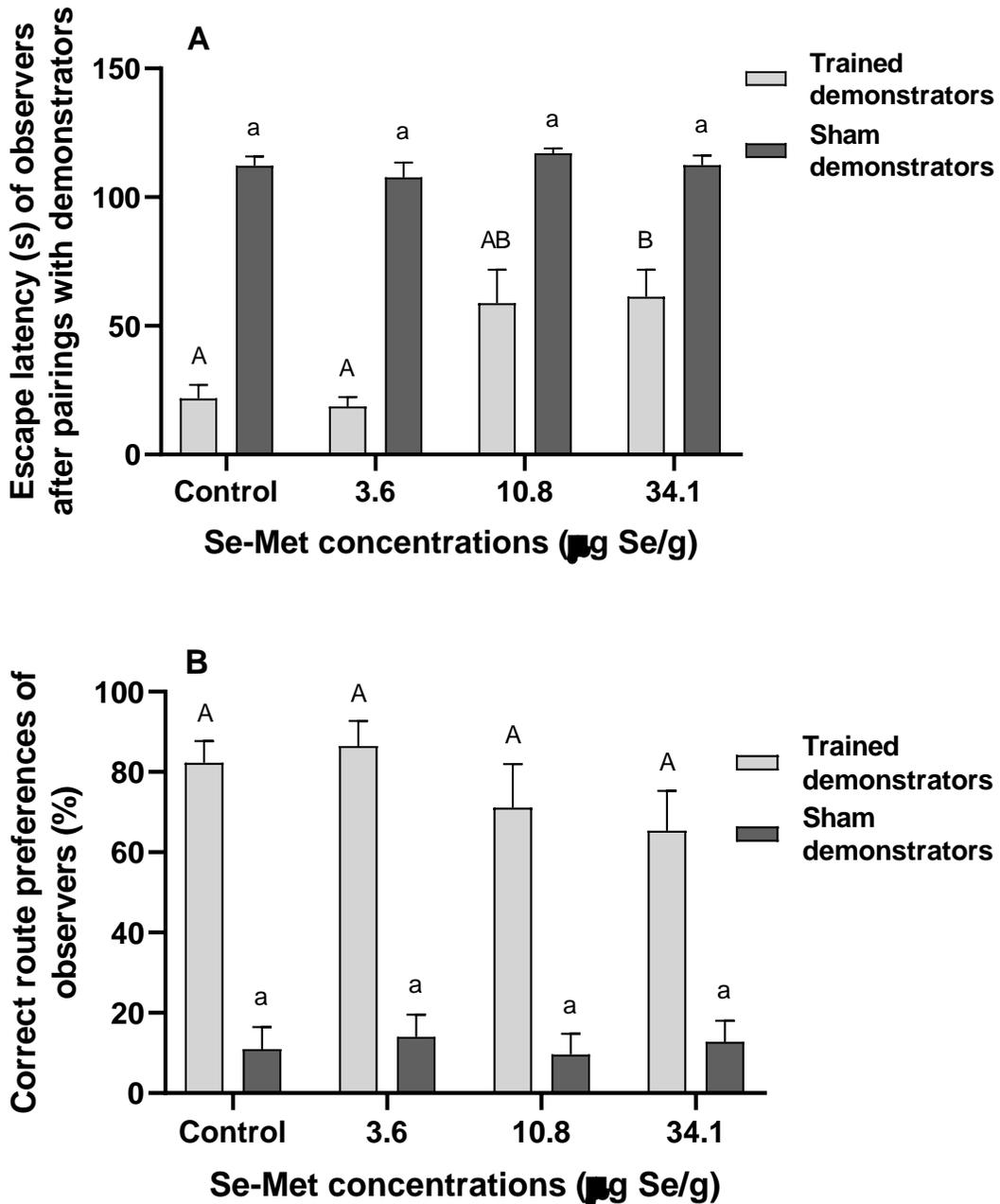


Fig. 4.4. Mean (\pm SE) escape latency (A), and the percentage of correct route preference (B) of zebrafish maternally exposed to different Se-Met concentrations after training with either trained demonstrators or sham demonstrators. Bars with different letters are significantly different from each other at $\alpha < 0.05$.

4.3.6. Serotonergic cell markers in the zebrafish brain

We found maternal exposure to Se-Met significantly affected the expression of 5-HT receptors [*htr1aa* ($F_{3,11} = 16.43$, $p < 0.001$; Fig. 4.5C), *htr1ab* ($F_{3,5.55} = 8.70$, $p = 0.016$; Fig. 4.5D), *htr1b* ($F_{3,11} = 16.73$, $p < 0.001$; Fig. 4.5E), and *htr1d* ($F_{3,11} = 21.27$, $p < 0.001$; Fig. 4.5F)], the 5-HT transporter, *slc6a4a* ($F_{3,11} = 5.38$, $p = 0.016$; Fig. 4.5A) and the transcription of *mao* ($F_{3,11} = 9.52$, $p = 0.002$; Fig. 4.5B) in the brain of F1-generation adult zebrafish. In comparison to the control, maternal exposure to medium and high Se-Met concentrations caused significant upregulation (~2-4 fold) in transcription of *hr1aa* (12.8: $p < 0.001$, 34.1: $p = 0.42$), *htr1b* (12.8: $p < 0.001$, 34.1: $p = 0.008$), *slc6a4a* (12.8: $p = 0.48$, 34.1: $p = 0.027$), and *mao* (12.8: $p = 0.004$, 34.1: $p = 0.036$). Moreover, the maternal exposure to the highest concentration of dietary Se-Met significantly elevated the relative expression of *htr1ab* by ~1.5 fold ($p = 0.013$). However, maternal exposure to Se-Met (all treatment groups) caused a marked down regulation in the expression of *htr1d* compared to control fish (all $p \leq 0.006$). No other genes analysed in this study showed significant variation across treatment groups (all $p > 0.05$).

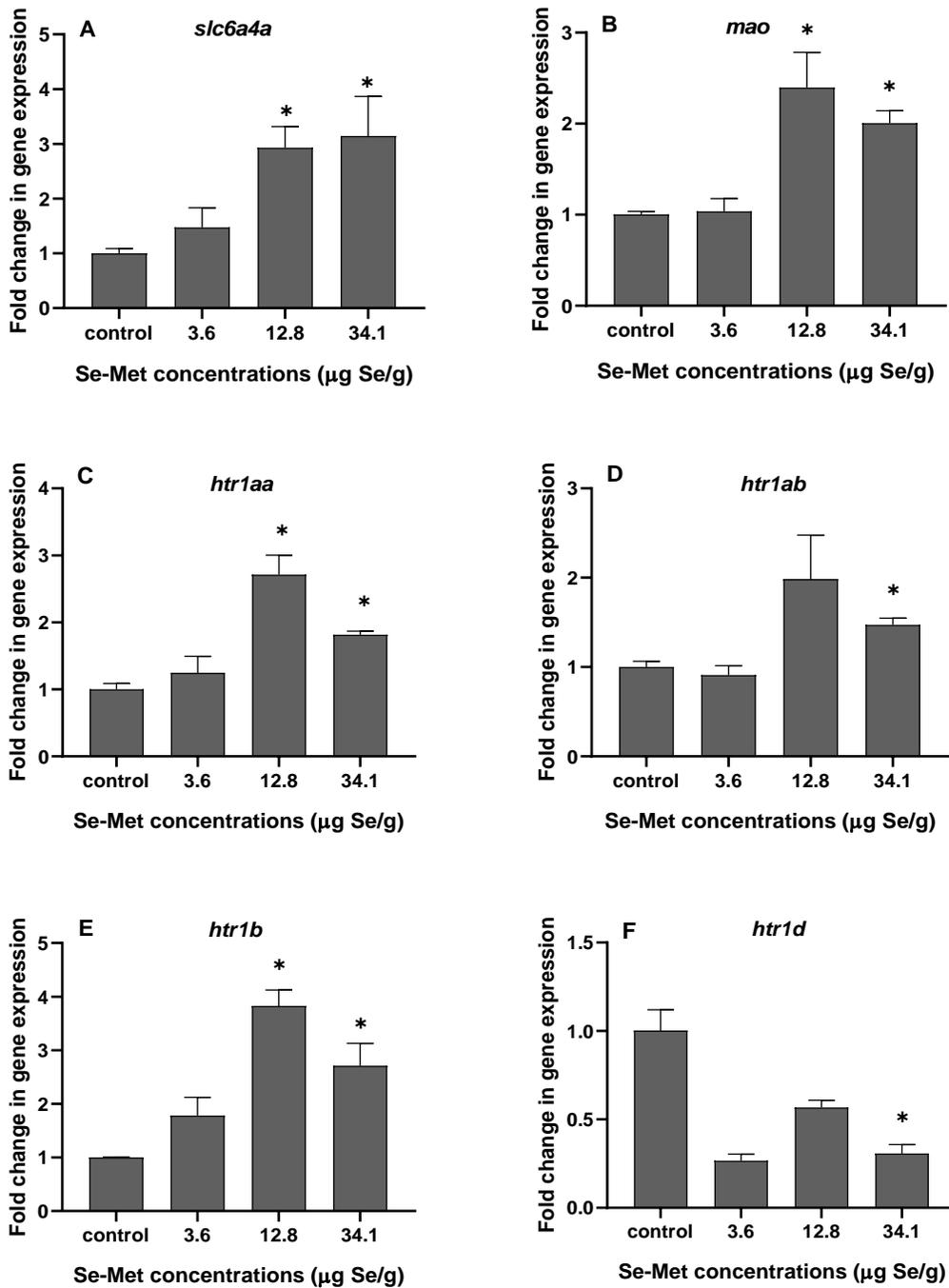


Fig. 4.5. Mean (\pm SE) fold change in the genes involved in 5-HT reuptake (*slc6a4a*; A), and metabolism (*mao*; B), as well as 5-HT receptors *htr1aa* (C), *htr1ab* (D), *htr1b* (E), and *htr1d* (F) in the brain of zebrafish that were treated with different concentrations of dietary Se-Met. Asterisks represent significant differences from control at $\alpha < 0.05$.

4.4. Discussion

This study is the first to demonstrate that maternal exposure to dietary Se-Met can impact space use, social preferences, social learning, and the transcription levels of important genes in the serotonergic system. The measured concentrations of Se in zebrafish eggs (1.2–3.9 $\mu\text{g Se/g}$ wet weight) after maternal exposure to a broad range of environmentally relevant Se-Met (3.6 – 34.1 $\mu\text{g Se/g dw}$) were comparable to the levels reported in previous laboratory studies, as well as field-collections from Se-impacted aquatic environments (Lemly, 2014, 2018; May et al., 2008; Muscatello et al., 2006; Muscatello and Janz, 2009; Naderi et al., 2018a; Phibbs et al., 2011; Raine et al., 2016; Rudolph et al., 2008; Thomas and Janz, 2015).

Sudden changes in space use and increased bottom-dwelling are innate escape responses of zebrafish to predation risk (Kalueff et al., 2013). Our antipredator behaviour test (experiment 1) demonstrated that fish from all exposure treatments (controls, low, medium, and high) displayed this appropriate response (sinking to the bottom of the tank) to chemical alarm cues. However, individuals maternally exposed to the high Se-Met concentration swam lower in the water prior to any predation risk. Zebrafish normally prefer to spend their time close to the surface of the water, often searching for food, so a baseline reduction in their vertical position may indicate a higher overall level of anxiety and stress.

Being part of a group is critically important for many social species such as zebrafish. Our group preference test (experiment 3) demonstrated that zebrafish treated maternally with the high Se concentration spent less time (~16%) near conspecifics. However, we found only a marginal effect on the amount of time spent near the larger shoal. This finding contrasts with our previous study where zebrafish spent significantly less time near the larger shoal after direct Se-Met exposure, rather than maternal. Thus, both maternal and direct exposure appear to weaken the desire to shoal, but direct exposure appears to have a larger effect on group size discrimination.

In our social learning test (experiment 4), maternal exposure to Se-Met impaired learning outcomes. Although all exposure groups escaped threat faster after being paired with knowledgeable demonstrators, maternal exposure to the high Se concentration resulted in escape times that were $\sim 3\times$ slower than control individuals. Such learning deficiency is consistent with reports for other learning modalities (spatial learning and latent learning) in previous studies

(Naderi et al., 2018a; Smith et al., 2010). Poor cognitive performance in our study did not appear to result from physical impairment, as we saw no sign of such effects in our study. However, poor performance could also result from higher stress levels, as was indicated by our space use data from experiment 1.

Our gene expression analyses revealed that maternal exposure to Se-Met altered the transcription of 5-HT receptors, including 5-HT1A, 5-HT1B, and 5-HT1D subtypes, which can play direct and indirect roles in the regulation of social behaviour, anxiety and stress, and learning and memory (Barnes and Sharp, 1999; Cavallaro, 2008; Katsurabayashi et al., 2003). The medium and high maternal exposures caused upregulation of *htr1aa* and *htr1b*, whereas *htr1ab* was significantly upregulated only in the high exposure group. These results are consistent with our previous research involving direct Se-Met exposures to adult zebrafish (Attaran et al., 2019; Attaran et al., 2020). However, we observed downregulation in the transcription of *htr1d* after maternal exposures to dietary Se-Met, contrasting with our previous study involving direct exposures (Attaran et al., 2020). Possible explanations for this disparity may derive from differences in exposure methods (direct vs. maternal) and/or age (12 vs. 6 month old).

We also found that the high and medium exposures increased the expression of the genes associated with serotonin transportation (*slc6a4a*) and metabolism (*mao*). The serotonin transporter plays a major role in regulating serotonin signalling via reuptake of this neurotransmitter from the extracellular space. Upregulation in this gene may result in changes in transporter functioning. For example, 5-HT transporter knockout mice have been reported to show anxiety-like behaviours, reduced aggression, and higher stress responses (Holmes et al., 2003; Wellman et al., 2007). Likewise, *mao* is an important mitochondrial enzyme that catalyses the oxidative deamination of monoamine neurotransmitters, including 5-HT, dopamine, and norepinephrine, producing hydrogen peroxide as a by-product (Meiser et al., 2013). Naderi et al. (2018a) found that upregulated expression of *mao* was accompanied by induced oxidative stress in the zebrafish brain. Several lines of evidence show that high oxidative stress can impair learning and memory (Ruhl et al., 2016), presumably leading to neurodevelopmental and psychiatric disorders that are characterised by abnormal social behaviour and elevated anxiety (e.g., autism and attention deficit hyperactivity disorder) (Ng et al., 2008; Rossignol and Frye, 2014; Salim, 2014). Hence, the elevated level of stress and impaired social behaviour and learning found in this

study might be, at least partially, due to upregulation in the expression of *mao* and induction of oxidative stress in the brain of zebrafish offspring. This possibility warrants further investigation in future studies.

In conclusion, our findings are the first to reveal that maternal exposure to high, but environmentally relevant, concentrations of Se-Met can impair normal space use, group preference, and social learning in F1-generation adult zebrafish. We also found that such exposure affected the serotonergic system in the brain, which is likely one of the main underlying mechanisms for these behavioural and cognitive impairments. Being part of a social group and learning indirectly from social information is critical for the fitness activities of many species. However, our results show that living in Se-impacted ecosystems can compromise these abilities in resident fish.

Chapter 5: General discussion

5.1. Introduction

The rate of deposition of natural elements like minerals and metals due to anthropogenic activities has exceeded and reached toxic levels in many aquatic ecosystems around the world. For example, Se, which is an essential metalloid in low doses, has reached toxic concentrations in different areas (Janz et al., 2014; Lemly, 2004; Muscatello and Janz, 2009). In this thesis, I explored the impacts of direct and transgenerational dietary exposure to Se-Met on different aspects of social behaviour, antipredator behaviour, and social learning in zebrafish as the model organism with a focus on a serotonergic neurotransmitter in the brain. In the first two experiments (chapter 2), I evaluated the impacts of Se-Met exposure on social and antipredator behaviours. I also quantified the expression levels of important genes in the serotonergic pathway, known as a modulator of social behaviour, and stress, after chorionic exposure to dietary Se-Met. Exposure to Se can impair different aspects of cognition; therefore, I investigated the impacts of chronic exposure to dietary Se-Met on social learning in zebrafish (chapter 3). The serotonergic system also plays a key role in cognition and learning and memory, so I evaluated the levels of this neurotransmitter and the expression of its different receptors in the brain of zebrafish (chapter 3). Due to the fact that maternal transfer is the primary exposure pathway of Se to embryonic fish, in chapter 4, I measured the impacts of maternal exposure to Se-Met on group preference, antipredator response, and social learning in adult F1-generation of zebrafish. Since 5-HT plays a fundamental role in the development of brain in larval fish, I investigated the effects of maternal exposure to dietary Se-Met on the serotonergic system in the next generation.

5.2. Effects of chronic exposure to dietary selenomethionine on social behaviour, antipredator response, and social learning as well as the serotonergic system in zebrafish

A notable finding of this research was that environmental contaminant selenium could influence different aspects of social behaviours and learning and memory in animal model zebrafish. In the first experiment (chapter 1), I found exposure to elevated concentration of dietary Se-Met (31.5 µg Se/g) can impact zebrafish antipredator responses by influencing their shoaling cohesion and vertical position before and after exposure to alarm cues. Zebrafish are social animals, and one mechanism that they use to protect themselves from predators is increasing shoaling cohesion;

however, we did not find any significant change in shoaling behaviour of exposed zebrafish to the highest concentration of Se-Met after injection of alarm cues compare to before that. Another response to predator threats is increasing bottom-dwelling time as it can help fish to hide in vegetation and muddy water. One potential reason we did not see any significant difference in these behaviours might be that zebrafish exposed to the highest concentrations of Se-Met were already swimming in a tighter shoal and closer to the bottom of the tanks even before alarm cue injection. As zebrafish naturally swim freely in upper levels of the water column for foraging (Gerlai et al., 2000; Saverino and Gerlai, 2008), swimming in lower levels can be a sign of anxiety and stress (Engeszer et al., 2007b). Previous studies demonstrate zebrafish exposed to a high concentration of Se (26.6 $\mu\text{g Se/g}$) had elevated cortisol level, which is an indicator of stress in various organisms (Thomas and Janz, 2011a).

In the second behavioural experiment (chapter 2), we found exposure to high concentrations of Se-Met (31.5 $\mu\text{g Se/g}$) can impact the interest of zebrafish to join a group of conspecifics as they spend less time near shoals of 3 or 4 zebrafish. However, zebrafish fed with control diets not only spent most of their time near groups of zebrafish, but they also displayed some tendency to join the bigger shoal. Fish in larger shoals are expected to face foraging benefits and a lowered predation risk due to factors such as increased predation confusion (Landeau and Terborgh, 1986), collective vigilance, and transfer of social information (Mathis et al., 1996). However, it has also been shown that the overall activity of alternative shoals influences zebrafish group preference (Pritchard et al., 2001). Zebrafish preferred to associate with more active shoals even though there was a lower number of fish because probably zebrafish using shoal activity to gauge shoal size (Pritchard et al., 2001).

Social learning is of critical importance for social species. This is particularly evident within the context of predator-prey interactions. After confirming that zebrafish can learn about an escape route from knowledgeable demonstrators (3rd experiment; chapter 3), in the fourth behavioural experiment (chapter 3), I found that zebrafish exposed to the highest concentration of Se-Met (31.4 $\mu\text{g Se/g}$) displayed significantly slower escape responses toward an oncoming trawl compared to fish in control and lower levels of Se exposure (3.6 and 12.8 $\mu\text{g Se/g}$). In addition, the number of the correct route preference of observers exposed to the highest concentration of Se-Met was significantly less than control groups (~ 55 vs. 82%). It has been demonstrated that exposure to

elevated concentrations of Se disrupted different aspects of learning and memory in zebrafish (Naderi et al., 2018b; Naderi et al., 2017) and other organisms like honey bees (Burden et al., 2016). Therefore, Se at lower doses can have essential roles in learning and memory functions while inducing neurotoxicity and cognition impairment at higher concentrations.

In chapter 3, increased oxidative stress biomarkers, including LPO level and transcriptional levels of antioxidant enzymes (*gpx1*, *cat*, *Cu/Znsod*, and *Mn-sod*) in zebrafish brains treated with the highest Se-Met exposure concentration (31.4 µg Se/g) indicated excess Se can generate ROS. The brain is highly susceptible to oxidative stress due to its limited antioxidant capacity, higher energy requirement, and higher amounts of lipid and iron (Juurlink and Paterson, 1998). The brain has a limited capacity to detoxify ROS because of the lack of glutathione-producing capacity by neurons (Chauhan and Chauhan, 2006). In the presence of oxidative stress, the lipid-rich constitution of the brain favours lipid peroxidation that results in a decrease in membrane fluidity and damage in membrane proteins inactivating receptors, enzymes, and ion channels (Bouayed et al., 2009). Therefore, oxidative stress can impact neurotransmission, neuronal function and overall brain activity. Oxidative damage in the brain causes nervous system impairment, and it has been implicated in different neurological diseases, including depression, anxiety disorders, and Autism (Chauhan and Chauhan, 2006; Patki et al., 2013). This induced oxidative stress also lead to learning impairment through different pathways, including dopamine and serotonin (Chauhan and Chauhan, 2006; Naderi et al., 2018b; Naderi et al., 2017; Praticò et al., 2002). The endogenous enzyme MAO that catalyses the oxidation of amine-containing neurotransmitter 5-HT in the cell produces H₂O₂ as a byproduct, and the overproduction of ROS can lead to neuronal apoptosis and mitochondrial dysfunction (Lopresti et al., 2013; Scapagnini et al., 2012; Vaváková et al., 2015). Here, we found dysregulations in MAO gene (*mao*) expression levels (down-regulation in the second chapter and upregulation in the third chapter) in the zebrafish brain that were exposed to the highest concentrations of Se-Met. This discrepancy may stem from different exposure regimes and behavioural paradigms used in these experiments.

The results of these experiments demonstrated that the dysfunction of the serotonergic system is one of the main pathological characteristics of chronic exposure to Se-Met. Changes in *mao* were associated with alterations in mRNA expression of 5-HT synthesis, 5-HT receptors, the transporter of 5-HT. The results were quite similar for social behaviours and social learning experiments. An

upregulation of *tph2*, which generates 5-HT in the brain, was observed in the zebrafish that were exposed to the highest concentrations of Se-Met. However, we did not find a statistically elevated 5-HT level in the brain of Se-Met exposed zebrafish (chapter 3). Tph2 dysregulation has been associated with anxiety-like behaviours, fear learning and memory, and depression-like behaviours in human and other animal models (Lesch et al., 2012). The upregulated expression of the 5-HT transporter gene, *slc6a4a*, was the common manifestation of serotonergic dysfunction in Se-treated zebrafish exhibiting impaired social behaviours and social learning performances (chapter 2 and 3). An increase in the transcript levels of the genes associated with 5-HT_{1A} (*htr1aa*) and 5-HT_{1B} (*htr1b*) receptor was detected in fish treated with different Se-Met concentrations in both experiments. We also found significantly higher mRNA expression of genes coding 5-HT_{1D}, 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors in zebrafish brains following exposure to dietary Se-Met in chapter 3. Nonetheless, an upregulation in these 5-HT receptor subtypes' expressions was mainly found in the zebrafish brain with social behaviour and social learning impairments. These receptors have essential roles in the regulation of learning and memory, anxiety, and different aspects of social behaviour. Overall, the results of experiments in chapters 2 and 3 revealed that chronic exposure to dietary Se-Met impairs different social behaviours, including group preference and antipredator response and social learning in zebrafish. Induction of oxidative stress and dysfunction of the serotonergic neurotransmitter are two mechanisms by which Se exerts its neurotoxic effects. However, this toxic trace element's effects on other neurotransmitters systems like dopamine cannot be ruled out.

5.3. Transgenerational effects of dietary exposure to Se-Met on social behaviour and social learning in zebrafish offspring

Exposure to environmental contaminants during development have both acute consequences to the developing embryos, leading to congenital anomalies and poor birth outcomes, and long-term health consequences during the life of individuals. Maternal transfer is the main pathway of Se exposure to fish embryos, which is generally demonstrated as a suit of developmental toxicities in the early life stages of F1-generation fishes (Janz et al., 2010). It has also been documented that both maternal and early life (from 2 to 24 hpf) exposure to Se-Met causes enduring learning impairments in adult zebrafish (Naderi et al., 2018a; Smith et al., 2010). It also has been demonstrated that maternal exposure to Se-Met induces oxidative stress and dopaminergic

dysfunction in adult F1-generation zebrafish (Naderi et al., 2018a). The brain is highly vulnerable to oxidative stress, particularly during the early part of development that may result in neurodevelopmental disorders. 5-HT and its corresponding receptors appear early during brain development (Côté et al., 2007; Lillesaar, 2011). Alterations in 5-HT and its receptors' expression during early development may lead to improper development of the CNS and impair normal neurological functionality that produces long-lasting changes in 5-HT-related neurological functions. Chapter 4 assessed whether maternal exposure to dietary Se-Met via serotonergic signalling changes would result in social behaviours and social learning impairment in F1-generation adult zebrafish. The results of behavioural experiments demonstrated adult zebrafish that were treated maternally to the highest concentration of Se-Met (34.1 µg Se /g) performed differently in tasks including vertical position, group preference, and social learning compared to control fish. Increased bottom-dwelling prior to exposure to alarm cues could be a sign of having higher levels of anxiety or stress in the fish that were maternally exposed to the highest concentration of Se-Met, as we did not see any sign of activity impairments in these exposed groups (Fig. 4.2). In the group preference experiment, we found that F1-generation adult zebrafish treated maternally with the highest Se-Met spent significantly less time in conspecific zones. Maternal exposure to the highest level of Se-Met also impaired social learning in F1-generation by increasing their escape latency after training with demonstrators.

The behavioural impairments were associated with 5-HT dysregulation. In contrast with the findings of chapters 2 and 3, where an upregulation in the expression of the *tph2* gene was observed in fish exposed to the highest concentrations of Se-Met, we did not find any changes in zebrafish maternally treated to Se-Met. The reason for these discrepancies between these studies may reside in differences in exposure regimes (adult vs. maternal exposure) and age of experimental zebrafish (12 months vs. 6 months). An upregulation in both *mao* and *slc6a4a* expression levels were found in the two highest exposed groups. We also found similar results in zebrafish that were treated to Se directly. Higher expression of *slc6a4a* may cause an increase in the activity of the 5-HT transporter; therefore, more 5-HT might be transferring back to the neurons where higher MAO was needed to metabolise extra 5-HT. MAO is also involved in the sequestration and metabolism of other neurotransmitters, such as dopamine, which has also been impacted by maternal exposure to Se-Met in F1-generation adult zebrafish (Meiser et al., 2013; Naderi et al., 2018a). An upregulation in the expression of different 5-HT receptor subtypes, including 5-HT_{1A}, 5-HT_{1B} was

found in the high treatment groups. However, in contrast with our previous finding (chapter3), we found a downregulation in mRNA expression of the gene encoding 5-HT_{1D} receptor in all treated groups. As previously mentioned, these receptors are involved in a broad repertoire of physiological effects, including modulation of learning and memory, anxiety and stress, and other aspects of social behaviours. Depending on the localization of these receptors, they may act as autoreceptors, regulating serotonin activity, and as heteroreceptors on nonserotonergic neurons like dopamine, GABA, and glutamate. Therefore, impairment in these behaviours could also result from the impacts of 5-HT on other neurotransmitters. Higher activity of 5-HT_{1A} and 5-HT_{1B} autoreceptors can inhibit the release of 5-HT. It has been demonstrated that maternal exposure to Se-Met leads to elevated levels of oxidative stress in F1 generation adult zebrafish (Naderi et al., 2018a). Therefore, Se-induced oxidative stress during early life stages may contribute to permanent neurobehavioral abnormalities in zebrafish via dysregulation of signal transduction and macromolecular damage like lipids, proteins, and DNA. Further studies are necessary to explain the mechanisms underlying transgenerational and the neurodevelopmental effects of exposure to Se.

5.4. Conclusion

Overall, my thesis suggests that environmentally relevant dietary Se-Met exposure can cause social behaviours and social learning impairments in adult zebrafish and their offspring. Moreover, we found that Se-Met exposure can alter the serotonergic system and induce oxidative stress in the zebrafish brain. The preference to associate with their conspecific is an innate behaviour in zebrafish, and they benefit from living in social groups by enhancing foraging success, increasing mate choice, and reducing predator risk. They also can collect potentially life-saving information about different circumstances like potential predators by learning from social information. Therefore, Se-contaminated ecosystems may cause alteration in these behaviours, and a wide range of environmental stimuli and consequently compromise fish fitness and survival. Because impaired social learning and social behaviours, dysregulation in the serotonergic neurotransmitter, and enhanced oxidative stress are characteristics of different neuropsychiatric and neurodevelopmental disorders, my research confirms the potential role of zebrafish as an animal model organism that can be used to explore the possible role of environmental contaminants in the development of brain disorders.

5.5. Future studies

Selenium is a primary concern in many environmental contaminant ecosystems because of its bioaccumulative nature in food webs. My research provided novel and important information on the effects of selenium on cognition and social behaviour in zebrafish as an animal model organism. However, fish living in contaminated ecosystems sometimes are simultaneously facing multiple contaminants like selenium, arsenic, and cadmium. Therefore, understanding their interactive effects on animals' health and development is essential and should be considered in future research.

In my thesis, we evaluated the impacts of exposure to dietary Se-Met on some aspects of social behaviours, including group preference and antipredator response in zebrafish. I found that zebrafish treated to the highest concentration of Se-Met increased their bottom-dwelling even before exposure to alarm cues (chapter 2, and 4). This reduction in vertical position can be considered as a sign of anxiety and stress. Previously, it also has been shown that Se exposure induces some physiological stress responses, including increasing the whole-body cortisol levels and oxygen consumption in zebrafish. Despite the fitness benefits of normal anxiety and stress responses, excessive or abnormal stress responses reduce individuals' ability to cope with other environmental challenges. Enhanced anxiety and stress responses, even in a safe environment and toward safe stimuli, decrease the time and energy commitment to other fitness-enhancing behaviours such as foraging and mate choice (Sih et al., 2003). Anxiety and stress also can interfere with social behaviour and learning and memory. Therefore, more behavioural studies, including novel tank tests, are needed to evaluate the impact of Se-Met exposure on anxiety and stress.

Moreover, based on the results presented in chapters 2 and 4, exposure to high concentrations of Se-Met can impact group preference and neurotransmitter 5-HT in zebrafish. Within group-living animals, individuals sometimes need to compete over limited resources such as food and mates, resulting in dominance hierarchies. Such hierarchies are essential for enhancing reproductive success because dominant males mate more frequently with females than do subordinate males, and dominant females produce more eggs when mating with a dominant male in comparison to a subordinate one (Paull et al., 2010). 5-HT also has been linked to the regulation of social hierarchies (Best et al., 2020). Therefore, it will be interesting to examine the effects of dietary

exposure to Se-Met on dominance hierarchies and reproductive behaviours. In my research, to evaluate the social learning of escape routes in zebrafish and F1-generation, we used both positive and negative reinforcement (a group of conspecifics and a trawl net, respectively). However, we do not know about the impacts of exposure to Se-Met on social learning when there are other types of reinforcement (e.g., food) or only one reinforcement.

Behavioural impairments in treated zebrafish with high Se-Met concentrations could be mainly due to dysregulation of 5-HT and induction of oxidative stress (chapter 3) in the zebrafish brain. 5-HT heteroreceptors also mediate the modulation of other neurotransmitters, including dopaminergic, cholinergic, glutamatergic, and GABAergic neurotransmitter systems (Fink and Göthert, 2007), which are involved in critical physiological processes such as learning and memory, social behaviours, and stress (Horzmann and Freeman, 2016). Future investigations can further explore the effects of exposure to Se-Met on these neurotransmitter pathways relevant to social behaviour and social learning.

Finally, although in all of these experiments we found exposure to Se-Met affects the serotonergic system by impacting the expression levels of its receptors, transporter, and enzymes essential in its synthesis and degradation, the mechanisms underlining these effects remain to be clarified. In addition to transcriptional levels, further studies will be helpful on translational and post-translational levels of enzymes involved in the 5-HT synthesis (MAO) and reuptake (TPH).

In chapter 4, I established that maternal exposure to dietary Se-Met impairs the serotonergic system and subsequently led to social behaviour and social learning impairments in the F1-generation of zebrafish. It will be interesting to investigate how 5-HT intervention and activation of its receptors influence the development of brain structures and neural circuits involved in social learning and behaviours. While dysregulation in transcription levels of genes in the serotonergic pathway has been demonstrated here, we could not measure the 5-HT level in the brain of the F1-generation zebrafish, and thus the mechanisms underlying the transgenerational behavioural effects of Se remain to be fully understood. Therefore, additional experiments will be helpful to measure 5-HT in zebrafish offspring and evaluate factors that might be responsible for neurodevelopmental consequences of Se-Met.

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