Lethal and non-lethal effects of exposure to methylmercury in the Zebra Finch (*Taeniopygia guttata*) during development

by

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Abstract

Methylmercury is an environmental contaminant that bioaccumulates, and has multiple toxic modes of action. Aquatic species have traditionally been the focus of wildlife toxicological research on mercury, but terrestrial receptors, including passerines, may also be exposed to similarly elevated levels of methylmercury. In this study we exposed a model passerine, the Zebra Finch (*Taeniopygia guttata*), to methylmercury in-ovo (embryonic exposure, pre-hatching), only as a chick (post-hatching exposure), and with a combined in-ovo chick treatment (embryonic and post-hatching exposure). Exposure to methylmercury in-ovo resulted in a significant reduction in hatching success, but there was no significant difference in behavioural or reproductive outcomes for the individuals that survived to maturity. Birds dosed both in-ovo and as chicks had reduced numbers of females surviving to maturity and altered male courtship behaviours. Birds dosed only as chicks had reduced survival rates. No long-term effects were seen on male courtship in the birds dosed only as chicks. Continuous exposure of chicks during embryogenesis and chick development had a deleterious effect on bird survival and fertility. Passerines may be able to withstand exposure to elevated levels of methylmercury during development at the nestling stage, but chronic exposure may reduce survival and fertility.

Keywords: Methylmercury; Avian; Growth; Behaviour; Reproduction; Zebra Finch

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List of Acronyms

ACF	Animal Care Facility
GnRH	Gonadotropin-releasing hormone
MeHg	Methylmercury (CAS No: 22967-96-6)
MeHgCl	Methylmercury chloride (CAS No: 115-09-3
SFU	Simon Fraser University
T3	Thyroxine
T4	Triiodothyronine

Chapter 1.

1.1. Introduction

Mercury is a naturally occurring element that is found most commonly in the earth's crust as cinnabar (HgS). When mercury enters terrestrial and aquatic systems it can become toxic, persistent and bioaccumulative (Bank, 2012). Natural sources of mercury emissions include forest fires and volcanic eruptions (Gochfeld, 2003). While there will inevitably be some degree of cycling associated with a naturally occurring element, anthropogenic activities have contributed to an increase in the levels of mercury measured in surface water, sediment, terrestrial systems and the atmosphere globally (Bank, 2012). Artisanal small-scale gold mining and coal burning are the greatest contributors to anthropogenic emissions, followed by the production of ferrous and non-ferrous metals and cement production (UNEP, 2013). As a result of both increased emissions and the recirculation of available mercury, the levels of mercury detected in surface waters, terrestrial systems and the atmosphere are now two to three times higher than before the industrial revolution (Gobeil et al.,1999; Lamborg et al., 2014).

A United Nations review of global mercury levels that was produced as a part of the Minimata Convention on Mercury found that the total global emission of mercury from anthropogenic sources is estimated to be between 1,875,000 and 1,960,000 kg per year (UNEP, 2013). North America emits approximately 60,000 kg of mercury annually; 92% of this is emitted by the United States with the remaining 8% emitted by Canada. One of the greatest issues associated with mercury is that atmospheric emissions are subject to long-range transport (Bank, 2012; Driscoll et al., 2013; Selin, 2009). Mobilisation of mercury far from the source has resulted in elevated levels of mercury in remote environments with negligible mercury emissions, including mountainous forests, in Antarctica, and north of the Arctic Circle (Guigueno et al., 2012). For example, Rimmer et al. (2010) found elevated levels of mercury in food webs in remote mountainous Montane forests. Elevated levels of methylmercury (MeHg) in Antarctica have been linked to long-range atmospheric transport, deposition onto the snowpack (Gionfriddo et al., 2016). Likewise, elevated levels of MeHg in Arctic ecosystems have been attributed to anthropogenic emissions, long-range atmospheric transport and subsequent deposition (Hammerschmidt & Fitzgerald, 2006). As a result of this long-range transport, organisms in some of the most remote parts

of the planet are now exposed to diffuse levels of mercury for the full extent of their life, from conception until death. The capacity for mercury to bioaccumulate has resulted in elevated levels of this contaminant in organisms that live in some of the most remote environments and contributes to the high levels of MeHg seen in higher trophic level organisms (Bank, 2012; Guigueno et al. 2012; Elliott and Elliott 2016).

Mercury can be present in the environment in three forms: as elemental (metallic), inorganic and organic mercury. Mercury is also capable of existing in three oxidation states: Hg(0), Hg(1) and Hg (II); each of these valence states can be found in the solid, liquid and gaseous phases, resulting in a wide range of possible forms and states (Gochfeld, 2003). Mercury transforms between these forms and valence states via a range of biotic and biogeochemical pathways (Hammerschmidt & Fitzgerald, 2006; Selin, 2009). The global cycle of mercury involves atmospheric emissions that are the result of natural geological and geothermal activity and forest fires (Gochfeld, 2003), or anthropogenic emissions, including coal-fired power plants and artisanal gold mining (UNEP, 2013). Mercury (Hg0 and HgII) is released into the atmosphere, where it may undergo long-range transport (Driscoll et al., 2013). Over time, atmospheric mercury is oxidized and dissolved in water contained in the atmosphere (Selin, 2009), or deposited as particulate matter (HgP) where it enters both terrestrial and aquatic systems (Hammerschmidt & Fitzgerald, 2006). Over 90% of Mercury that is deposited in soil is associated with organic matter, binding to reduced sulphur functional groups (Selin, 2009). When deposited in surface water bodies, inorganic mercury may be transformed by sulphate-reducing bacteria to methylmercury (MeHg) (King et al., 2000). Methylmercury is highly bioavailable, and moves through the food web, bioaccumulating and biomagnifying in aquatic systems and, in some instances, entering terrestrial food webs (King et al., 2000; Selin, 2009).

1.2. Mercury toxicity

Because mercury is capable of a wide range of valence states, it is able to bind to sulfhydryl, carboxyl, amide, amine and phosphoryl groups, resulting in multiple potential toxicokinetic pathways (Hulla, 2014). The form of mercury encountered by an organism will determine the major exposure pathways and physiological outcomes, the most toxic and most bioavailable of which is methylmercury (MeHg) (Clarkson, 1993; Bank, 2012; Hulla, 2014). Ingestion of MeHg-contaminated food is the most important exposure route for humans and wildlife. Between 80-95 percent of ingested MeHg is absorbed in the

gastrointestinal tract, after which it is transported in red blood cells and binds to thiol-containing biomolecules such as cysteine, glutathione and homocysteine (Clarkson et al. 2003; Rutchik & Ramachandran, 2014). This binding enables mercury to cross the blood-brain barrier, resulting in disproportionately high concentrations of mercury in the Central Nervous System (CNS) (Choi et al., 2008). Movement of MeHg across biological membranes appear to be associated with its affinity for –SH functional groups and the L-transport system where MeHg-Cys complexes effectively emulate methionine, which is a substrate for amino acid carriers (Choi et al., 2008). Because MeHg readily binds to –SH groups, it has an affinity for these groups in keratin; as a result, feathers or hair can contain MeHg and feather/hair growth is a potential excretion pathway (Lewis & Furness, 1991; Rutkiewicz et al., 2013; Whitney & Cristol, 2017).

As a consequence, the developing brain is especially sensitive to MeHg. Prolonged exposure at moderate doses can result in widespread neuronal damage (Bertossi et al., 2004). The neurotoxic mode of action of MeHg has been attributed to its irreversible inhibition of selenium (Se)-dependent enzymes (selenoenzymes). These selenoenzymes, including selenomethionine and selenocysteine, are present in the brain and neuroendocrine tissues, where they prevent and reverse oxidative damage (Bertossi et al., 2004 Choi et al., 2008). This irreversible binding contributes to the fetotoxicity of methylmercury. Fetal tissues require an ongoing source of selenium for neurodevelopment, and Se reserves are rapidly depleted during development. When MeHg binds irreversibly to the selenoenzymes, the fetal brain is no longer protected from the by-products of respiration. Adult tissues have Se reserves, so damage does not occur until these reserves have been exhausted (Rutchik & Ramachandran, 2014). Mammals exposed to MeHg during development show clear signs of neurotoxicity. The fishing village in Minimata, Japan was subject to one of the worst mercury poisoning events known (Uchino et al., 2001). From the late 1930s until 1968 a chemical company discharged mercury-laden wastewater into the ocean, resulting in heavy contamination of the surface water and sediment. Epidemiological studies of villagers who resided in Minimata during the 1960s showed that the population had a higher rate of cerebral palsy in the offspring of the exposed population (Myers, 2005). A series of neurological tests were also undertaken on children born between 1955 and 1958 in Minimata. Children in this population were significantly more likely to experience sensory disturbances, poor muscle control (dysarthia), and mental retardation when compared to children in neighbouring villages (Uchino et al., 2001).

The neurotoxic effects of exposure to elevated levels of MeHg have been replicated in other mammalian toxicological studies. For example, rats exposed to methylmercury during gestation had disturbed behavioural functions that are linked to the frontal cortex and dopamine neurotransmitters (Reed et al., 2008). The same rats also showed lifelong disrupted behavioural plasticity, diminished GABA-A sensitivity and an overall disturbance in reward processing (Reed et al., 2008). Specific developmental stages may be more sensitive to MeHg exposure; Sakamoto et al. (1993) dosed rats on different days during development and found that the severity of motor coordination and growth impairment depended on the point at which the rats were exposed. The sensitivity of specific developmental stages to MeHg exposure is, however, still poorly understood.

To date, toxicological research has predominantly focused on the neurotoxic effects of MeHg exposure. There is, however, increasing recognition that MeHg may be preferentially retained in endocrine organs, and that subsequent changes to endocrine function can adversely affect the behaviour, metabolism and fertility of an organism (Tan et al., 2009). Lobsters (*Homarus americanus*), were injected with or fed a diet containing MeHg and after one month of excretion, the largest remaining proportion of MeHg was found in the egg masses of females and the gonads of males (Guarino et al., 1976). Mercury has been shown to accumulate in and affect the function of the thyroid, adrenal glands, the ovaries and testes across a range of mammalian species (Lamperti & Printz, 1974; Møller-Madsen & Thorlacius-Ussing,1 986; Zhu et al., 2000). There is also evidence that mercury has deleterious effects on the endocrine systems of other vertebrate species. Western Pond Turtles (*Emys marmorata*) are a long-lived reptile that is in decline throughout California and the Pacific Northwest. Western Pond Turtles exposed to elevated levels of mercury had a concurrent increase in the rate of thyroxine (T4) deiodination, a mechanism of toxicity that may cause excess T4 levels and depressed concentrations of triiodothyronine (T3) (Meyer et al., 2014).

Methylmercury has been implicated in the global pattern of decreasing mammalian fertility (National Research Council, 2000; Wirth & Mijal, 2010). In Minimata during the 1960s, the exposed population had a higher rate of abnormal pregnancies when compared to surrounding Japanese villages. In the late 1950s, the increased male stillbirth rate resulted in a skewed sex ratio, indicating a higher susceptibility of male foetuses to the toxicant (Sakamoto et al., 2001). Studies have also linked exposure to elevated levels of MeHg to altered endocrine function and lowered fecundity in other vertebrates

(Hammerschmidt et al, 2002; Drevnick & Sandheinrich, 2003). Drevnick and Sandheinrich (2003) provided dietary MeHg to fathead minnows (*Pimephales promelas*). Treated Male and female fish had significantly lower levels of testosterone and estradiol (E2) respectively when compared to control fish. Hammerschmidt et al. (2002) also exposed fathead minnows to dietary MeHg, and found that exposure to environmentally relevant concentrations of MeHg resulted in a decreased adult fecundity. While the exact toxicokinetic mechanism for altered endocrine function and decreased fertility was not elucidated in these studies, endocrine disruption along the hypothalamic-pituitary-gonadal axis, which is common to all vertebrate taxa, has been implicated.

1.3. Mercury Toxicity: Avian species

Reproductive and developmental effects

Reproductive success is a critical toxicological endpoint (Wolfe et al. 1998) but measuring the effect of MeHg exposure on reproduction is complicated. This is because the term 'reproduction' encompasses multiple life-stages, biological processes and behaviours, including courtship (e.g. song performance, song complexity and mate selection), spermatogenesis, oogenesis, fertility and fertilization, embryogenesis, embryonic development, hatching success, and paternal and maternal care of eggs and, with the exception of superprecocial species, chicks. Each of these endpoints has the potential to affect the fitness of an individual, and this is further complicated by the likelihood that MeHg has multiple toxic pathways and that different life stages may have differing sensitivity to MeHg exposure. The following section provides an overview of current knowledge of the effects of MeHg exposure on reproduction and development in avifauna.

Courtship effects

Several studies have shown that elevated levels of blood MeHg can alter courtship behavior and mate selection in non-passerines. American White Ibises (*Eudocimus albus*) captured as nestlings and then exposed to environmentally relevant concentrations of MeHg for 3.5 years showed significantly altered courtship behaviours when compared to control birds (Frederick & Jayasena, 2010). The MeHg-treated males had significantly lower rates of head bobbing and pair bowing and reduced female approaches. Overall,

MeHg-dosed American White Ibis were also more likely to engage in same-sex pairings - a phenomenon unknown in wild populations of this species with no exposure to the pollutant (Frederick & Jayasena, 2010). In a follow-on study, MeHg-dosed male American White Ibis had decreased testosterone during nest building and increased testosterone during nesting, when compared to the male White Ibis engaging in heterosexual pairings (Jayasena et al., 2011). In these studies the authors propose that MeHg acts as an endocrine disruptor (Jayasena et al., 2011).

In passerines, song is a critical part of reproduction and is used as an honest means of assessing mate quality and health (Nowicki et al., 1998; Nowicki et al., 2002; Spencer et al., 2003; DeVoogd, 2004; Hoogesteijn et al. 2008). Changes to song production, whether it be by pitch, frequency or complexity may potentially disrupt courtship and pair selection (Hoogesteijn et al. 2008). The exact mechanism for the alteration of song in poor-quality individuals has been attributed to reduced musculature because of a nutritional deficiency (Brumm et al., 2009), and to developmental neuroanatomical changes (Nowicki et al., 1998; DeVoogd, 2004; Hoogesteijn et al., 2008). In a study on free-living Nelson's sparrows (*Ammodramus nelson*), individuals with higher higher blood Hg had faster songs and sang at higher frequencies than sparrows with lower blood Hg (McKay & Maher, 2012). McKay and Maher (2012) suggest that MeHg could be acting as a developmental stressor or acting as an endocrine disruptor, but do not delve into how these respective modes of action could result in alteration of song pitch and frequency. Developmental stress and endocrine disruption are not mutually exclusive, and there are likely to be additional toxicokinetic pathways that should be considered when investigating the mechanisms behind the apparent alteration of song as a result of MeHg exposure. A comparison of the song complexity of passerines living on contaminated and uncontaminated sites found that Carolina Wrens, House Wrens and Song Sparrows had lower song complexity and sang at a lower frequency than those same species living at the reference sites (Hallinger et al., 2010). This pattern of altered song was not, however observed across all species; there was no significant difference in any of the measured song parameters for Eastern Phoebes living on the reference or contaminated sites (Hallinger et al., 2010). These differences have been attributed to the fact that Eastern Phoebes don't learn songs from conspecifics, but instead have an innate suboscine song, which is independent from learning (Hallinger et al., 2010; Liu et al., 2013). This 'innate versus learned' pattern of MeHg sensitivity wasn't supported by the findings of Yu et al.,

(2017) or Morran et al., (2015). Yu et al., (2017) found no evidence that exposure to environmentally relevant concentrations of MeHg in-ovo had an effect on song quality in the Zebra Finch (*Taeniopygia guttata*), an oscine passerine. In a follow-on study, Morran et al. (2015) exposed zebra finches to low but environmentally relevant levels of MeHg after chicks had hatched, and also found no evidence that continued dosing of low but environmentally relevant levels of MeHg altered male song performance. That leads to the possibility that cumulative effects associated with ongoing exposure at multiple life stages may be required to impact on male song performance. An alternative explanation may be that the birds in Morran et al. (2015) and Yu et al.'s (2017) studies were not dosed with sufficient amounts of MeHg to create noticeable effects; in both studies, the treated birds had low levels of THg in their blood when they reached sexual maturity. Their birds were dosed during periods of critical feather growth, which is a recognised excretion pathway for Hg in birds (Whitney & Cristol, 2017). As a result, both the in-ovo and chick dosed birds in the studies by Morran et al. (2015) and Yu et al. (2017) ended up with low blood THg, relative to the blood THg seen in passerines found in contaminated sites with noted song changes.

Fertility and Hatching Success

Maternal transfer of MeHg to the developing egg is a known exposure pathway Methylmercury dissolved in water is thought to mimic the effects of maternal transfer of mercury into the egg, as water-bound MeHg distributes itself uniformly through the albumen and yolk (Heinz et al., 2009, 2009b). Exposure to MeHg reduces the number of eggs that successfully hatch in a wide range of bird species in both field and laboratory studies (Finley & Stendell, 1978; Heinz & Hoffman, 1988; Heinz et al., 2009; Heinz et al., 2009b; Yu et al., 2016). In an early study on the effects of maternal exposure to MeHg, Finley and Stendell (1978) fed black ducks (Anas rubipres) MeHg over two breeding seasons. They found that a significant number of eggs of exposed mothers failed to hatch. Behavioural effects may also contribute to a reduction in egg hatching success; Heinz and Locke (1976) investigated the developmental effects of MeHg ingestion. Mallards (Anas platyrhynchos) fed mercury-laced food had smaller clutch sizes than the control ducks, and some eggs were laid outside the nest suggesting that, in some instances, altered parental behaviour may contribute to egg failure. Egg sensitivity to MeHg does not appear to follow a simple dose-response relationship. Heinz and Hoffman (2003) found that egg hatching rates in mallards were influenced both by dose and also by parental lineage, and

that the embryotoxic effects of MeHg differed by more than an order of magnitude between parents. There are also issues associated with the partitioning of MeHg between the yolk and albumen, as the embryo depends on albumen during earlier development and the yolk during later developmental stages (Heinz et al., 2009). Studies across a range of species have found that MeHg preferentially partitions to the albumen (Brasso et al., 2012; Heinz et al., 2009). As a result, the use of 'whole egg' MeHg concentrations does not address relative exposure at different time points during embryogenesis. The mechanism of delivery of MeHg also appears to influence overarching toxicity; in a comparative study of the toxicity of maternally transferred MeHg vesus injected MeHg, the LC_{50}s of maternally transferred MeHg were found to be higher than the LC_{50}s of injected MeHg. The toxicokinetic mechanism associated with the difference in toxicity between the two delivery methods is not currently known (G. Heinz, *pers. comm*.). Finally, in field studies, the presence of other environmental stressors complicates the relationship between MeHg toxicity and egg hatching success. For example, selenium has both antagonistic and synergistic effects on MeHg embryotoxicity, and this is entirely dependent on the timing of exposure (Heinz & Hoffman, 1998).

Survival of Nestlings

Chicks are particularly vulnerable to the effects of MeHg (Finley & Stendell, 1978; Taylor & Cristol, 2015), but this pattern is not universal (Sepúlveda et al., 1999). It is also complicated by the possibility of multiple mechanisms of failure; notably parental behavior versus a physiological impact on chick development. In non-passerines, laboratory studies support the notion that increased exposure to MeHg results in higher levels of nestling mortality. In their experiment with Black Ducks (*Anas rubipres*), Finley and Stendell (1978) found that reduced hatchability and poor duckling survival were the most notable effects of maternal exposure to MeHg. The brains of ducklings that died after hatching contained between 3.25 and 6.98 ppm of Total Mercury (THg), and pathology results revealed lesions which are considered indicative of MeHg exposure. Wild-living Common Loons (*Gavia immer*) with higher blood THg levels had lower numbers of chicks survive to fledge when compared to Common Loons with lower blood THg (Meyer et al., 1998). Sepúlveda et al. (1999) did not, however, see any increase in nestling mortality in juvenile free-ranging Great Egrets (*Ardea albus*) exposed to dietary MeHg. They concluded that

excretion into new feathers was acting as a protective mechanism for the growing chicks (Sepúlveda et al., 1999).

Passerines also appear to have a wide range of species-specific sensitivities to MeHg. Taylor and Cristol (2015) identified a threshold of 5–10 µg/g feather of THg with increased nestling mortality in the Tree Swallow (*Tachycineta bicolor*), that was lower than the THg concentrations associated with mortality for other life stages, suggesting that nestlings are the most sensitive life stage in this species. However Varian-Ramos et al. (2014) found that exposing Zebra Finch chicks to dietary MeHg up to 2.4 ug.g-1 had no effect on chick survival. Likewise, Yu et al. (2016) found no increase in nestling mortality in Zebra Finch chicks dosed in-ovo, and Morran et al. (2015) also found no treatment-based effects on nestling survival in her study on chick-dosed Zebra Finches.

Developmental Effects

Birds have been used as a model organism to study developmental neurotoxicity; this is because exposure of the yolk to a contaminant mimics long-term pre-natal exposure during in-ovo development (Carvalho et al., 2008). These studies have also proved useful when investigating potential toxic effects on wildlife. There is evidence that exposure to MeHg can have a negative impact on chick development. Heinz and Locke (1976) investigated the effects of MeHg ingestion on Mallard Ducks. Mallard ducklings with elevated blood THg had a reduced response to the call of their mothers, and maternal transfer of methylmercury appeared to be responsible for the development of lesions in the brains of the ducklings (Heinz and Locke 1976; Heinz, 1979). Evers et al. (2002) found a correlation between a decrease in egg volume and an increase in the body burden of MeHg. In addition, chicks treated with methylmercury had damaged cerebellar granules and Purkinje neurons; that damage was not seen in rat studies, suggesting that there is inter-species variability (Carvalho et al., 2008).

Endocrine Effects

There is now increasing recognition that MeHg acts as an endocrine disruptor, but to date few studies have looked at this effect in birds (Jayasena et al., 2011; Wada et al., 2009). In their study on White Ibises (*Eudocimus albus*), Jayasena et al. (2011) dosed a

captive population with up to 0.3 ppm ww of MeHg in their diet. At the highest MeHg levels, adult ibis of both sexes had altered levels of estradiol and testosterone, and altered breeding behaviours. A correlational study found that Black-legged Kittiwakes (*Rissa tridactyla*) with higher blood THg were more likely to skip a breeding season (Tartu et al., 2013) and also found GnRH-induced LH levels increased with increasing mercury concentration in both sexes, suggesting that MeHg may have been reducing the ability of Kittiwakes to respond to suitable breeding conditions. The authors of that study suggest that future studies could investigate whether MeHg disrupts GnRH input to the pituitary. In their study on wild-living Tree Swallows, Wada et al. (2009) found that the Tree Swallows living at the Hg-impacted sites had adrenocortical responses (*i.e*. the 'fight or flight' response) that changed depending on the age of the bird, with adrenocortical responses enhanced earlier nestling stages, and suppressed as the birds were about to fledge. The responses differed significantly from those seen in the birds living at the reference sites (Wada et al., 2009). In addition the swallow population living at the Hg-impacted sites also had lowered plasma T4 and T3 concentrations when compared to the Tree Swallow populations living at the reference sites (Wada et al., 2009). While the mechanisms behind the apparent endocrine-disrupting effects of MeHg are still poorly understood, endocrine disruption is seen across all vertebrates and is likely to be a mode of action that should be considered in both laboratory and field studies when assessing the impacts of MeHg on avifauna.

Hormesis

While many studies emphasise the toxicity of higher MeHg levels/doses, there is there also evidence that at lower levels MeHg may exert an hormetic effect. Mallards fed 0.5 µg/g MeHg were significantly more fertile, with a higher hatching success and higher mean duckling weight than untreated mallards (Heinz, 2010). Likewise, Herring et al. (2009) found a positive correlation between wild-living Great Egret (*Ardea alba*) and White Ibis (*Eudocimus albus*) nestlings and levels of MeHg in feathers. In both instances, the authors suggest that at lower doses MeHg may kill internal parasites (Herring, 2009; Heinz, 2010). A reduction in the number of parasites being carried may outweigh the toxic effects of mercury, providing birds with a net physiological benefit, at least during periods of growth. Varian-Ramos et al. (2014) also found hormetic effects. In their study, the

offspring of Zebra Finches dosed with 0.3 ppm of MeHg had a slightly higher hatching success than the control birds. The offspring of the 2.4 ppm MeHg treatment group had much lower hatching success. The study was run on laboratory birds that are less likely to be burdened with high parasite loads, so it is possible that there may be other mechanistic processes at work that are contributing to this hormetic effect. Varian-Ramos et al. (2014) posit that the reproductive success at high doses may be the result of strong selection pressures at higher MeHg doses, suggesting evolutionary selection for mercury tolerance. Heinz (2010) surmises that "…one cannot rule out the possibility that low concentrations of Hg in eggs may be beneficial, and this possibility should be considered when setting regulatory thresholds for methylmercury."

1.4. Justification for this study

Mammalian studies have found specific developmental stages that are particularly sensitive to MeHg exposure (Sakamoto et al.,1993). Few studies have, however, investigated the sensitivity of passerines to MeHg at key developmental stages. In previous studies from our laboratory, Yu et al., (2017) found that exposure to MeHg in-ovo reduced hatching success, but after hatching there was no evidence that exposure to environmentally relevant concentrations of MeHg had effects on breeding behavior or fertility in zebra finches. In a follow-on study, Morran et al. (2015) exposed zebra finch chicks to low but environmentally relevant levels of MeHg for 21 days post-hatch and also found no evidence that continued dosing of low but environmentally relevant levels of MeHg altered affected finch breeding behavior or fertility. These studies contradict many field and laboratory studies which show that birds exposed to MeHg have reduced fitness and fertility (Finley & Stendell, 1978; Meyer et al., 1998; Tartu et al., 2013; Varian-Ramos et al., 2014; Taylor & Cristol, 2015), but are supported by other field and laboratory studies which have found little to no effect, or even some degree of hormesis in MeHg-exposed bird populations (see Herring et al. 2009 and Heinz, 2010). Mercury excretion during feather growth, combined with dilution as a result of growth of chicks may have reduced the toxicity of MeHg in both Yu et al., (2017) and Morran's (2015) studies (Whitney & Cristol, 2017). An alternative explanation may be that the birds in Morran et al. (2015) and

Yu et al.'s (2017) studies were not dosed with sufficient amounts of MeHg to create noticeable effects; as discussed previously, in both studies their treated birds had low levels of THg in their blood when they reached sexual maturity, relative to the blood THg seen in passerines found in contaminated sites with behavioural changes. In order to address these gaps, we undertook a final study, exposing a model terrestrial passerine species, the Zebra Finch (*Taeniopygia guttata*) to MeHg in-ovo, only as a chick, and as a combined in-ovo and chick treatment. By dosing at different life-stages we hope to establish if: 1) terrestrial passerines have a specific life stage that is more sensitive to MeHg exposure; 2) to assess potential cumulative effects associated with exposure during more than one early life stage; and 3) to investigate the effect of different exposure scenarios on breeding success, which is a non-lethal but environmentally relevant endpoint. The mercury dosing methods involved: 1) injecting eggs with MeHg in a water vehicle to simulate maternal transfer and embryonic exposure to MeHg; and 2) dosing of chicks orally from hatching until fledging to simulate provision of food by parents until chicks are ready to disperse from the nest.

1.5. References

Ackerman, J.T., Takekawa, J.Y., Eagles-Smith, C.A. and Iverson, S.A., 2008. Mercury contamination and effects on survival of American avocet and black-necked stilt chicks in San Francisco Bay. Ecotoxicology, 17(2), pp.103-116.

Bank, M.S., 2012. Mercury in the environment: pattern and process. Univ. of California Press.

Bertossi, M., Girolamo, F., Errede, M., Virgintino, D., Elia, G., Ambrosi, L. and Roncali, L., 2004. Effects of methylmercury on the microvasculature of the developing brain. Neurotoxicology, 25(5), pp.849-857.

Brasso, R.L., Abel, S. and Polito, M.J., 2012. Pattern of mercury allocation into egg components is independent of dietary exposure in Gentoo penguins. Archives of environmental contamination and toxicology, 62(3), pp.494-501.

Brumm, H., Zollinger, S.A. and Slater, P.J., 2009. Developmental stress affects song learning but not song complexity and vocal amplitude in zebra finches. Behavioral ecology and sociobiology, 63(9), pp.1387-1395.

Choi, A.L., Budtz-Jørgensen, E., Jørgensen, P.J., Steuerwald, U., Debes, F., Weihe, P. and Grandjean, P., 2008. Selenium as a potential protective factor against mercury developmental neurotoxicity. Environmental research, 107(1), pp.45-52.

Clarkson, T.W., 1993. Mercury: major issues in environmental health. Environmental Health Perspectives, 100, p.31.

Clarkson, T.W., Magos, L. and Myers, G.J., 2003. The toxicology of mercury - current exposures and clinical manifestations. New England journal of Medcine, 2003 (349), pp.1731-1737.

DeVoogd, T.J., 2004. Neural constraints on the complexity of avian song. Brain, Behavior and Evolution, 63(4), pp.221-232.

Driscoll, C.T., Mason, R.P., Chan, H.M., Jacob, D.J. and Pirrone, N., 2013. Mercury as a global pollutant: sources, pathways, and effects. *Environmental science & technology*, *47*(10), pp.4967-4983.

Finley, M.T. and Stendell, R.C., 1978. Survival and reproductive success of black ducks fed methyl mercury. Environmental Pollution (1970), 16(1), pp.51-64.

Frederick, P. and Jayasena, N., 2010. Altered pairing behaviour and reproductive success in white ibises exposed to environmentally relevant concentrations of methylmercury. Proceedings of the Royal Society of London B: Biological Sciences, p.rspb20102189.

Gionfriddo, C.M., Tate, M.T., Wick, R.R., Schultz, M.B., Zemla, A., Thelen, M.P., Schofield, R., Krabbenhoft, D.P., Holt, K.E. and Moreau, J.W., 2016. Microbial mercury methylation in Antarctic sea ice. Nature Microbiology, 1, p.16127.

Gobeil, C., Macdonald, R.W. and Smith, J.N., 1999. Mercury profiles in sediments of the Arctic Ocean basins. Environmental Science & Technology, 33(23), pp.4194-4198.

Gochfeld, M., 2003. Cases of mercury exposure, bioavailability, and absorption. Ecotoxicology and Environmental Safety, 56(1), pp.174-179.

Guigueno, M.F., Elliott, K.H., Levac, J., Wayland, M. and Elliott, J.E., 2012. Differential exposure of alpine ospreys to mercury: Melting glaciers, hydrology or deposition patterns?. Environment international, 40, pp.24-32.

Hallinger, K.K., Zabransky, D.J., Kazmer, K.A. and Cristol, D.A., 2010. Birdsong differs between mercury-polluted and reference sites. The Auk, 127(1), pp.156-161.

Hammerschmidt, C.R., Sandheinrich, M.B., Wiener, J.G. and Rada, R.G., 2002. Effects of dietary methylmercury on reproduction of fathead minnows. Environmental Science & Technology, 36(5), pp.877-883.

Hammerschmidt, C.R. and Fitzgerald, W.F., 2006. Methylmercury in freshwater fish linked to atmospheric mercury deposition. Environmental science & technology, 40(24), pp.7764-7770.

Heinz, G.H. and Hoffman, D.J., 1998. Methylmercury chloride and selenomethionine interactions on health and reproduction in mallards. Environmental Toxicology and Chemistry, 17(2), pp.139-145.

Heinz, G.H. and Hoffman, D.J., 2003. Embryotoxic thresholds of mercury: estimates from individual mallard eggs. Archives of Environmental Contamination and Toxicology, 44(2), pp.0257-0264.

Heinz, G.H., Hoffman, D.J., Klimstra, J.D. and Stebbins, K.R., 2009. Rapid increases in mercury concentrations in the eggs of mallards fed methylmercury. Environmental Toxicology and Chemistry, 28(9), pp.1979-1981.

Heinz, G.H., Hoffman, D.J., Klimstra, J.D., Stebbins, K.R., Kondrad, S.L. and Erwin, C.A., 2009b. Species differences in the sensitivity of avian embryos to methylmercury. Archives of Environmental Contamination and Toxicology, 56(1), pp.129-138.

Heinz, G.H., Hoffman, D.J., Klimstra, J.D. and Stebbins, K.R., 2010. Enhanced reproduction in mallards fed a low level of methylmercury: an apparent case of hormesis. Environmental toxicology and chemistry, 29(3), pp.650-653.

Herring, G., Gawlik, D.E. and Rumbold, D.G., 2009. Feather mercury concentrations and physiological condition of great egret and white ibis nestlings in the Florida Everglades. Science of the Total Environment, 407(8), pp.2641-2649.

Hoogesteijn, A.L., Kollias, G.V., Quimby, F.W., De Caprio, A.P., Winkler, D.W. and DeVoogd, T.J., 2008. Development of a brain nucleus involved in song production in zebra finches (*Taeniopygia guttata*) is disrupted by Aroclor 1248. Environmental toxicology and chemistry, 27(10), pp.2071-2075.

Hulla, J. Chapter 17. Metals. In Hayes, A.W. and Kruger, C.L. (Eds, 2014). Hayes' principles and methods of toxicology. CRC Press.

Jayasena, N., Frederick, P.C. and Larkin, I.L., 2011. Endocrine disruption in white ibises (*Eudocimus albus*) caused by exposure to environmentally relevant levels of methylmercury. Aquatic Toxicology, 105(3), pp.321-327.

King, J.K., Kostka, J.E., Frischer, M.E. and Saunders, F.M., 2000. Sulfate-reducing bacteria methylate mercury at variable rates in pure culture and in marine sediments. Applied and Environmental Microbiology, 66(6), pp.2430-2437.

Lamborg, C.H., Hammerschmidt, C.R., Bowman, K.L., Swarr, G.J., Munson, K.M., Ohnemus, D.C., Lam, P.J., Heimbürger, L.E., Rijkenberg, M.J. and Saito, M.A., 2014. A global ocean inventory of anthropogenic mercury based on water column measurements. Nature, 512(7512), pp.65-68.

Lamperti, A.A. and Printz, R.H., 1974. Localization, Accumulation, and Toxic Effects of Mercuric Chloride on the Reproductive Axis of the Female Hamster. Biology of Reproduction, 11(2), pp.180-186.

Lewis, S.A. and Furness, R.W., 1991. Mercury accumulation and excretion in laboratory reared black-headed gullLarus ridibundus chicks. Archives of Environmental Contamination and Toxicology, 21(2), pp.316-320.

Liu, W.C., Wada, K., Jarvis, E.D. and Nottebohm, F., 2013. Rudimentary substrates for vocal learning in a suboscine. Nature Communications, 4, p.2082.

McKay, J.L. and Maher, C.R., 2012. Relationship between blood mercury levels and components of male song in Nelson's sparrows (*Ammodramus nelsoni*). Ecotoxicology, 21(8), pp.2391-2397.

Meyer, E., Eagles-Smith, C.A., Sparling, D. and Blumenshine, S., 2014. Mercury exposure associated with altered plasma thyroid hormones in the declining Western Pond Turtle (Emys marmorata) from California mountain streams. Environmental Science & Technology, 48(5), pp.2989-2996.

Meyer, M.W., Evers, D.C., Hartigan, J.J. and Rasmussen, P.S., 1998. Patterns of common loon (*Gavia immer*) mercury exposure, reproduction, and survival in Wisconsin, USA. Environmental Toxicology and Chemistry, 17(2), pp.184-190.

Møller-Madsen, B. and Thorlacius-Ussing, O., 1986. Accumulation of mercury in the anterior pituitary of rats following oral or intraperitoneal administration of methyl mercury. Virchows Archiv B Cell Pathology Zell-pathologie, 51(1), pp.303-311.

Morran, S.A., 2016. Investigating potential growth, behavioural, and reproductive effects of nestling exposure to methylmercury in Zebra Finches (Taeniopygia guttata) (Masters of Environmental Toxicology Thesis). Retrieved from Simon Fraser University (Identifier: etd9737)

Nowicki, S., Peters, S. and Podos, J., 1998. Song learning, early nutrition and sexual selection in songbirds. American Zoologist, 38(1), pp.179-190.

Nowicki, S., Searcy, W.A. and Peters, S., 2002. Brain development, song learning and mate choice in birds: a review and experimental test of the" nutritional stress hypothesis". Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology, 188(11), pp.1003-1014.

Rimmer, C.C., McFarland, K.P., Evers, D.C., Miller, E.K., Aubry, Y., Busby, D. and Taylor, R.J., 2005. Mercury concentrations in Bicknell's thrush and other insectivorous passerines in montane forests of northeastern North America. Ecotoxicology, 14(1), pp.223-240.

Rutchik, J.S., Ramachandran, T. and Centers for Disease Control and Prevention (CDC), 2014. Toxic neuropathy. Emedicine, Neurology.

Rutkiewicz, J., Bradley, M., Mittal, K. and Basu, N., 2013. Methylmercury egg injections: Part 2—Pathology, neurochemistry, and behavior in the avian embryo and hatchling. Ecotoxicology and Environmental Safety, 93, pp.77-86.

Selin, N.E., 2009. Global biogeochemical cycling of mercury: a review. Annual Review of Environment and Resources, 34.

Sepúlveda, M.S., Williams Jr, G.E., Frederick, P.C. and Spalding, M.G., 1999. Effects of mercury on health and first-year survival of free-ranging great egrets (Ardea albus) from southern Florida. Archives of Environmental Contamination and Toxicology, 37(3), pp.369-376.

Spencer, K.A., Buchanan, K.L., Goldsmith, A.R. and Catchpole, C.K., 2003. Song as an honest signal of developmental stress in the zebra finch (*Taeniopygia guttata*). Hormones and Behavior, 44(2), pp.132-139.

Tan, S.W., Meiller, J.C. and Mahaffey, K.R., 2009. The endocrine effects of mercury in humans and wildlife. Critical reviews in toxicology, 39(3), pp.228-269.

Tartu, S., Goutte, A., Bustamante, P., Angelier, F., Moe, B., Clément-Chastel, C., Bech, C., Gabrielsen, G.W., Bustnes, J.O. and Chastel, O., 2013. To breed or not to breed: endocrine response to mercury contamination by an Arctic seabird. Biology letters, 9(4), p.20130317.

Taylor, C.E. and Cristol, D.A., 2015. Tissue mercury concentrations and survival of tree swallow embryos, nestlings and young adult females on a contaminated site. Bulletin of environmental contamination and toxicology, 95(4), pp.459-464.

Thompson, D.R., Hamer, K.C. and Furness, R.W., 1991. Mercury accumulation in great skuas Catharacta skua of known age and sex, and its effects upon breeding and survival. Journal of applied ecology, pp.672-684.

UNEP, 2013. Global Mercury Assessment 2013: Sources, Emissions, Releases and Environmental Transport. UNEP Chemicals Branch, Geneva, Switzerland

Varian-Ramos, C.W., Swaddle, J.P. and Cristol, D.A., 2014. Mercury reduces avian reproductive success and imposes selection: an experimental study with adult-or lifetime-exposure in zebra finch. PLoS One, 9(4), p.e95674.

Wada, H., Cristol, D.A., McNabb, F.A. and Hopkins, W.A., 2009. Suppressed adrenocortical responses and thyroid hormone levels in birds near a mercury-contaminated river. Environmental science & technology, 43(15), pp.6031-6038.

Whitney, M. and Cristol, D., 2017. Rapid depuration of mercury in songbirds accelerated by feather molt. Environmental Toxicology and Chemistry.

Wirth, J.J. and Mijal, R.S., 2010. Adverse effects of low level heavy metal exposure on male reproductive function. Systems biology in reproductive medicine, 56(2), pp.147-167.

Wolfe, M.F., Schwarzbach, S. and Sulaiman, R.A., 1998. Effects of mercury on wildlife: a comprehensive review. Environmental toxicology and chemistry, 17(2), pp.146-160.

Yu, M.S., Eng, M.L., Williams, T.D., Basu, N. and Elliott, J.E., 2016. Acute embryotoxic effects but no long-term reproductive effects of in ovo methylmercury exposure in zebra finches (*Taeniopygia guttata*). Environmental toxicology and chemistry, 35(6), pp.1534-1540.

Yu, M.S., Eng, M.L., Williams, T.D., Guigueno, M.F. and Elliott, J.E., 2017. Assessment of neuroanatomical and behavioural effects of in ovo methylmercury exposure in zebra finches (*Taeniopygia guttata*). Neurotoxicology, 59, pp.33-39.

Zhu, X., Kusaka, Y., Sato, K. and Zhang, Q., 2000. The endocrine disruptive effects of mercury. Environmental health and preventive medicine, 4(4), pp.174-183.

Chapter 2.

Ongoing anthropogenic release of mercury into the biosphere has resulted in a concurrent increase in the levels of mercury detected in the environment and in organisms (Bank, 2012). Since the onset of the Anthropocene, both diffuse and point-source emissions have contributed to a two to three-fold increase in the levels of mercury detected in the atmosphere, soil and water (Gobeil et al., 1999; Lamborg et al., 2014). While mercury can exist in many valence states, natural biological processes result in the methylation of mercury into its most toxic form, methylmercury (MeHg). Methylmercury is persistent, has multiple toxicokinetic pathways, and is capable of bioaccumulation and biomagnification (Gochfeld, 2003; Wiener et al., 2003). Methylmercury is also a potent neurotoxin, and avifauna exposed to sub-lethal but environmentally relevant levels have shown physiological and behavioural changes that may affect reproduction and survival (Heinz et al, 1976; Heinz et al., 1979; Scheuhammer et al., 2007). In controlled feeding experiments juvenile Great Egrets (*Ardea alba*) exposed to environmentally relevant levels of MeHg had impaired neurological function, reducing their ability to forage effectively (Bouton et al, 1999), and American White Ibis (*Eudocimus albus*) exposed to environmentally relevant concentrations of MeHg were more likely to engage in same-sex pairings — a phenomenon unknown in wild populations of this species with no exposure to the pollutant (Frederick & Jayasena, 2010).

Because MeHg is known to biomagnify in aquatic food webs, the majority of MeHg wildlife research has focused on piscivorous aquatic species, but new research has shown that terrestrial organisms, including passerines, may potentially be exposed to equivalently elevated concentrations of MeHg (Heinz & Locke, 1976; Heinz, 1979; Yu et al, 2015). There is a growing body of evidence linking environmentally relevant levels of MeHg to negative effects on terrestrial passerines; female Tree Swallows (*Tachycineta bicolor*) living in close proximity to sites contaminated with mercury had smaller eggs and a lower number of chicks fledging when compared to the same species living at reference sites (Brasso & Cristol, 2008). In a laboratory study, Zebra Finches (*Taeniopygia guttata*) fed environmentally relevant levels of MeHg showed significant reproductive impairment (Varian-Ramos et al., 2014). There is also evidence that some life stages are more sensitive to the toxic effects of MeHg than others. Zebra Finches exposed to MeHg in-ovo had significantly reduced hatching success but there was no apparent change in chick

growth or survival post-hatch between treated and untreated chicks (Yu et al, 2015). A parallel study dosed zebra finch chicks daily for 21 days post hatch and found no significant difference in the breeding behaviour or reproductive success between treated or control finches (Morran et al., 2016). Growing chicks may be protected from the effects of elevated mercury exposure by sequestering MeHg into their feathers; in both of these studies the blood mercury levels during periods of rapid growth decreased rapidy, even when the chicks were dosed daily post-hatch. Several studies have identified feather growth as a key excretion mechanism in post-hatch nestlings (Rutkiewicz et al., 2013; Whitney & Cristol, 2017). A study of the MeHg body burden of wild Forster's Tern (*Sterna forsteri*), Black-necked Stilt (*Himantopus mexicanus*), and American Avocet (*Recurvirostra americana*) chicks suggest that immediately post-hatch and just prior to fledging are the two periods when chicks are most vulnerable to elevated MeHg body burdens (Ackerman et al., 2011). All three species had a U-shaped pattern in blood MeHg levels. Methylmercury body burdens were highest in chicks immediately after hatching, and then declined rapidly as chicks aged and 'diluted' their own mercury body burden through growth in size and mercury excretion into growing feathers. Chicks then showed an increase in blood mercury concentrations, presumably a direct result of continued dietary Hg intake, when they fledged and feather growth slowed, reducing these excretion pathways.

To date most studies on effects of MeHg on passerines have focused on single life-stages (Ackerman et al., 2011; Morran et al., 2016; Yu et al, 2015) or chronic, life-long-exposure (Heinz, et al., 2009; Hester et al., 1978; Heinz, et al., 2009; Fimreite & Karstard, 1971; Kenow et al., 2003; Frederick et al., 2011), but few studies have investigated the potential cumulative effects associated with ongoing exposure to MeHg at different life-stages. In this thesis we measure the effect of exposure to MeHg in-ovo (embryonic exposure, pre-hatching), only as a chick (post-hatching exposure), and with a combined in-ovo chick treatment (embryonic and post-natal exposure). The aim of this work is to identify specific life-stages in birds that are most sensitive to MeHg exposure, assess potential cumulative effects associated with chronic exposure or exposure at key developmental stages, and also to investigate the effect of different exposure scenarios on non-lethal but environmentally relevant endpoints. Specifically, we investigate the

combined effects of in-ovo and post-natal MeHg dosing, a situation that might represent the most realistic pattern of exposure for numerous, free-living birds species.

2.1. Materials and Methods

In January 2016 a pilot study was run with 12 pairs of stock zebra finches. The offspring of these 12 pairs were used to test MeHg dosing methods that are described in more detail in this section. All methods used in the pilot study were identical to those described in this main study, but blood THg was centrifuged after collection and only the plasma was analysed; as a result, the THg levels measured in the pilot study aren't comparable to the main study and are not discussed further. Feathers were, however, taken from the pilot study for analysis of MeHg sequestration, the results of which are discussed further below.

2.1.1. General Zebra Finch Husbandry

This project was conducted at the Animal Care Facility at Simon Fraser University in Burnaby, British Columbia, Canada. Non-breeding birds were housed in single-sex cages (100 x 39 x 43 cm), with a maximum of 10 birds per cage. Non-breeding birds were provided with mixed seeds (panicium and white millet 1:2; 11.7% protein, 0.6% lipid and 84.3% carbohydrate by dry mass), water, grit and cuttlefish bone *ad libitum*. Food and water were changed daily. Once every seven days, a multivitamin supplement was added to the drinking water. The animal rooms were regulated to maintain a consistent temperature (19 - 25 degrees Celsius) and humidity (35-55%) with a constant photoperiod of 14L:10D. All experimental work was conducted by trained individuals under a Simon Fraser University Animal Committee Permit (1070B-08) according to the guidelines of the Canadian Committee on Animal Care.

2.1.2. Zebra Finch Breeding Protocols

Male and female zebra finches were taken from stock cages and were paired at random in May 2016. Birds that had successfully reared chicks in the past were given priority over birds that were inexperienced or had previously failed to breed. Because there were limited successful breeding pairs, some inexperienced birds were also used. Experienced and inexperienced birds were equally distributed between the four treatment groups. Breeding pairs were housed in single cages (51 x 39 x 43 cm). A cardboard nesting box was attached to the outside of each breeding cage. Each nesting box was filled with fresh hay for nesting material. All birds were given access to 'Just for Birds ™' seed mix, water, grit and cuttlefish bone ad libitum. Once every seven days, Nekton-S multivitamin supplement was added to the drinking water at a ratio of 1 gram of supplement per 250 ml of water. Breeding pairs were also supplemented with egg food (a mash of hard-boiled chicken eggs including shells, breadcrumbs and cornmeal; approximately 20% protein, 7% lipid) for the duration of the breeding process, from pairing until chicks were aged 30 days for the first generation. The females and chicks in the F2 breeding trial were not provisioned with egg food. Pairs that did not successfully lay eggs within 15 days of pairing were separated, and females were re-paired with a new male. Nest boxes were monitored daily for egg laying. Within 24 hours of laying, all new eggs were weighed and numbered with a fine-tipped marker for recognition. Nest boxes were checked twice daily (morning and afternoon) starting at 12 days post first egg to monitor for hatching.

Chicks were weighed (± 0.01g) daily, starting on the day of hatching. Plastic weigh boats were used to weigh younger chicks, and older chicks capable of flying were weighed in a felt bag. Tarsus length was taken on day 30. Chicks were banded with an aluminum or split plastic band on approximately day 10, enabling recognition of individual chicks after the loss of the initial down feathers. Once chicks reached 30 days of age they were considered 'independent' and were removed from the breeding cage. Chicks were then placed in regular non-breeding cages (100 x 39 x 43 cm), and small dishes with extra seed were placed at the bottom of the cages to ensure that those chicks that had not learned to use the feeder had access to seeds. Once chicks reached 60 days post-hatch, sexual dimorphism became apparent and chicks could be segregated and placed into single-sex cages. While chicks were segregated by sex, they were randomly assigned to cages so that all treatments were found in each cage. At a minimum of 90 days post-hatch,

reproduction and courtship experiments were run on females and males respectively to investigate adult phenotypic quality.

2.1.3. MeHg solution preparation

For solution preparation, methylmercury (II) chloride (MeHg chloride PESTANAL® analytical standard from Sigma-Aldrich; CAS: 115-09-3) was dissolved in double deionised water. Solutions were prepared and stored in new glassware that had been autoclaved, rinsed three times with acetone and hexane and then washed in nitric acid using the following procedure: clean lab ware was fully submerged in a dilute nitric acid tray containing 1.5 % HNO3 (Sigma-Adlrich; CAS: 7697-37-2) for at least 8 hours. Glassware was then manually triple rinsed four times with Reverse Osmosis (RO) water, and was then air-dried in a still-air hood. When completely dry, glassware was covered with Parafilm™. Two stock solutions were prepared; one for egg injection and one for chick dosing via pipette. The final analysed concentration of the egg stock solution was 1.6 µg Hg per µl (2 µg MeHg per µl), and the final analysed concentration of the chick stock solution was 0.96 µg Hg per µl (1.20 µg MeHg per µl). These concentrations were confirmed as total mercury concentrations (THg) at the laboratory of Dr Nil Basu in the Faculty of Agricultural and Environmental Sciences at McGill University in Montreal, QC, Canada, using EPA Method 7473 (Limit of Reporting = 0.0534 µg Hg/L). All samples were analysed on a Nippon Instruments MA-300 in accordance with the U.S. Environmental Protection Agency Method 7473, as detailed in Basu et al., (2014). A second stock solution was shipped to Dr Basu's facilities at the end of the project to check for potential degradation of the stock at the end of the project, but the vial containing the stock solution broke in transit, so it was not possible to obtain these results.

2.1.4. Egg dosing

Eggs in each clutch were randomly assigned to either: 1) control (un-injected); 2) vehicle injected, or 3) MeHg injected treatments. Before dosing, eggs were weighed (± 0.0001g). Egg MeHg doses were based on the previous study of zebra finch embryotoxicity by Yu et al. (2015) [7]. The egg stock solution contained 1.6 µg/µl of

methylmercury chloride (MeHgCl) PESTANAL® analytical standard from Sigma-Aldrich. At the first signs of fertility, 2-3 eggs were injected with a single dose of 2 µl of stock solution per gram of egg, resulting in a nominal dose of 3.2 µg/g egg of MeHg. Eggs were injected using 10 µl Hamilton syringes (Gastight 1700 Series) and sterile 26-gauge beveled needles. The needle was pushed through the side of the shell and the dose was injected into the albumen. The methods follow those used by Yu et al. (2017) and as described in Winter et al. (2013). The injection hole was sealed with cyanoacrylate glue (Loctite Gel Control), and once the glue was dry the egg was returned to the nest. Remaining eggs in the clutch were be injected with the vehicle (water) using the same method, or assigned as 'control' (un-injected) eggs. After returning eggs the nest, each egg was monitored to record hatching success, teratogenicity (i.e., deformities) and, for those chicks that successfully hatched, body mass daily for 30 days.

2.1.5. Chick dosing

Chick dosing methods follow Morran et al. (2016). Clean stock birds were paired, and their nests were monitored closely at hatch to identify which chicks hatched from which eggs (in-ovo treated versus un-injected). At hatching, each chick was marked by plucking down feathers on different areas of the body for individual identification within the nest. Chicks were assigned to one of four treatment groups: 1) MeHg dosed in-ovo (embryonic exposure, pre-hatching); 2) MeHg dosed only as a chick (post-hatching exposure); 3) MeHg dosed both in-ovo and as a chick (embryonic and post-natal exposure); or 4) control (no dose), assigned randomly, but contingent on the egg treatment. Chicks from the embryonic and postnatal exposure treatment (the 'both' treatment) and the post-natal exposure group (the 'chick' treatment) were dosed with MeHg from day 1 (24 hours after hatching) to day 30. The target MeHg doses for the chicks in this study used previous dietary MeHg doses developed Morran et al. (2016), which were based on previous laboratory and field studies of environmentally relevant dietary MeHg doses (Heinz et al., 2009; Varian-Ramos et al., 2013). Stock was created using methylmercury chloride (MeHgCl) PESTANAL® analytical standard from Sigma-Aldrich and double deionised water. Our targeted dose for chicks was based on the originally intended targeted "high" dose (0.27 µg/g BW) in the study by Morran et al. (2016), which was based on a seed-dosing study by Varian-Ramos et al. (2014). However,

in Morran et al.'s (2016) study the measured concentrations of the dosing solution (0.15 µg/µl MeHg) were only 28% of the intended concentration. Chicks were given 0.5 ul of this "high" solution per g BW per day, so chicks received an actual dose of 0.075 µg MeHg/g BW. In our study, the measured concentration of our stock solution was 0.96 µg/µl, we diluted this 1:3 to give a dosing solution of 0.24 µg/µl, and chicks received 1 ul/g BW per day, pipetted directly into their gape. Our chick dose was 0.24 µg/g MeHg BW per day, which was 3.2 x Morran et al,'s (2016) dose. Chicks were dosed early (7.30 – 10.30 am) in the day prior to provision of egg food. Chicks were weighed every day to determine the precise dose and volume of water vehicle for dosing (± 0.01g), and also to monitor effects of MeHg on growth.

2.1.6. Blood and Feather Mercury Analysis

Blood was collected from all chicks 30 days post-hatch from the brachial vein of the wing using heparinized capillary tubes. A maximum of 2 capillary tubes of blood was taken from each bird. The collected blood was then placed into heparinized vials, which were immediately frozen and were stored at -20 degrees Celsius until they were shipped on dry ice to McGill University for analysis. In January of 2017 a pilot study was run to test MeHg dosing methods. A single P2 feather was taken from seven birds at 30 days when blood was sampled, and these were placed in individual labelled envelopes and shipped with the blood samples to McGill University for analysis. Blood analyses from these pilot birds could not be compared to the current study as blood was centrifuged after extraction and later analysis revealed that the vehicle in the pilot test was contaminated with MeHg so blood THg levels between the two studies are not comparable. Additional feathers were not analysed by McGill researchers because of time constraints. All samples were analysed on a Nippon Instruments MA-300 in accordance with the U.S. Environmental Protection Agency Method 7473, as detailed in Basu et al., (2014). Standard Reference Materials (SRMs) were measured each day of analysis to determine validity of the calibration curves. The SRMs for this study were Dorm-4 (Fish Protein; National Research Council of Canada) and Human Hair 13 (National Institute for Environmental Studies, Japan). One SRM and an empty quartz boat were run at least every nine samples, and one replicate sample was included at least every nine samples. Precision (reproducibility) was measured by comparing within-day and between-day replicate analysis of SRMs.

2.1.7. Assessment of adult phenotype: Male Courtship Trials

Male courtship behaviour was assessed following the methods outlined by Yu et al. (2015), and Morran et al. (2016) and described in Zann (1996). During the male courtship trials, the males were housed in a separate room, isolated from the main colony. Cages of clean, stock females that had not been dosed with MeHg were also kept in this room. Each female was only used once per day, but some females were re-used for courtship trials on subsequent days. Before each courtship trial, records were checked to ensure that male chicks were not paired with their mothers and that a different female was used for repeat trials. Courtship trials were conducted in single cages (51 x 39 x 43 cm), containing water and grit ad libitum. A female was chosen at random and introduced to the trial cage where she was allowed to acclimate for 5 minutes. After the acclimation period, a randomly selected experimental male was introduced to the cage and the behaviour of both birds was observed approximately 1.5 m from the cage for 10 minutes. During this time, the following courtship behaviours of the male were recorded: a) number of bill wipes (male wiping his bill on the perch); b) number of follows (male follows when the female moves between perches or between the bottom of the cage and a perch); c) number of unsuccessful mounts; d) number of successful mounts (male is able to make cloacal contact); e) whether the male sang (yes or no); and f) if the male attempted to court the female by showing any of the aforementioned behaviours (yes or no). The female's response to the male was recorded on a scale of 1-5 with 1 meaning she did not acknowledge the male and 5 meaning she allowed him to copulate. A microphone (Sennheiser ME62) was placed through the top bars of the cage to record the male song. The microphone was connected to a small laptop and the songs were recorded on Syrinx-PC software (version 2.6h, J. Burt, Seattle WA). Once all the males were used in mating trials, each was used for a second mating trial. At the time of submission of this thesis, the song analysis was still being analyzed. The song performance results are therefore not presented in the results section.

2.1.8. Assessment of adult phenotype: Female Breeding Trials

At 90+ days of age, females were paired with a random clean experienced male that was not her father under the same conditions as described above for breeding pairs. If a female did not lay any eggs within 15 days of pairing, she was un-paired and labeled as a “non-breeder”. All eggs were checked for signs of fertility and infertile eggs or eggs that showed signs of fertility but failed were noted. For the females that laid eggs within 15 days of pairing, the laying interval (number of days between pairing and first egg), clutch size, mean egg mass, brood size at hatch, brood size at 21 days, and brood size at 30 days were recorded. For those that successfully raised chicks, the resulting chicks were weighed (± 0.01 g) tarsus measurements (± 0.01 mm) were taken 30 days post-hatch.

2.2. Statistical Analysis

All statistical analyses were conducted in R (Version 3.4.1, packages used: nlme, lsmeans, lme4, lmerTest, multcomp, plyr, ggplot2). Data were tested for normality and heteroscedasticity. Post-hoc tests for differences between means were adjusted for multiple comparisons using the Tukey-Kramer method. All values are presented as mean ± standard error of the mean (SEM), and statistical significance for all tests was set at $p < 0.05$. The mean blood mercury concentration of each treatment group was compared using a single-factor completely random design ANOVA. Egg hatching success was modeled with generalized linear mixed models using a binomial distribution and a logit link with the pair (nest) as a random factor. Mean chick mass was compared between treatments using linear mixed-effects models, correcting for egg mass (covariate) and blocking by nest (random factor). The proportion of chicks surviving to maturity was compared among the treatments using a Pearson Chi-Square analysis.

Treatment effects on latency to breed and the fertility of females was tested using linear mixed-effects models, correcting for egg mass (covariate) and blocking by nest (random factor). The proportion of females that laid eggs was compared among the treatments using a Pearson Chi-Square analysis except in instances where there were insufficient sample sizes, when Fisher’s Exact Test was used. Offspring growth was

compared between treatments using linear mixed-effects models, correcting for egg mass (covariate) and blocking by nest (random factor). Female birds that laid eggs that failed to hatch were included in fertility assessments, but birds that failed to nest and lay eggs were excluded from these analyses For the male mating trials, attempts to court were analysed using Fisher's Exact Test. For analysis of male courting behaviours, only those that invited the female to court (i.e. performed a courtship behaviour) were used in the analysis. Courtship behaviours that were normally distributed were analysed using a Pearson's Chi2 test, and behaviours that were non-normally distributed were analysed using a Kruskal-Wallace test.

2.3. Results

2.3.1. Total blood and feather mercury levels

There was a significant effect of in ovo and/or chick MeHg treatment on total blood mercury (THg) in chicks sampled at 30 days post-hatching ($F_{3,87} = 342$, $p < 0.0001$, DF = 3, Figure 1). There was no difference in mean THg of chicks exposed to MeHg in-ovo compared to control chicks ($p = 0.74$). Similarly, there was no difference in THg for chicks exposed to MeHg post-hatching only compared with those exposed in-ovo and as a chick ($p = 0.17$). Blood Thg was significantly higher in both chick-dosed groups compared to chicks exposed to MeHg in-ovo only and to controls ($p < 0.001$ in all cases; Table 1). The THg concentrations in the vehicle-dosed P2 feathers for the control birds from the pilot study were between 9-29 ppb (n=4; mean = 16.5; SE ± 4.3) and the mean Thg in the feathers of birds dosed in-ovo and as chicks were 538 and 715 ppb (n=2; mean = 626.5; SE ± 88.4) (Table 2).

2.3.2. Effects of MeHg exposure on egg hatching success

A total of 154 eggs were laid by 58 pairs of F1 generation zebra finches in this experiment. Of these, 17 were infertile (17/155; 11%), 7 were broken during handling or injection (7/155; 4.5%), and 130 showed signs of fertility (131/155; 84.5%). Therefore, a

total of n=27 fertile eggs were not injected, n=74 fertile eggs were injected with methylmercury, and n=29 fertile eggs were injected with the vehicle used to dissolve the methylmercury chloride. There was a significant effect of egg treatment on hatching success ($\chi 2 = 11.77$, d.f. = 2, $p = 0.003$; egg mass was controlled for, though this term was not significant in the model, $p = 0.56$). Hatching success was lower in eggs treated in ovo with MeHg (64%), compared with control (non-injected) eggs (92%, $z = 2.47$, $p = 0.013$), but there was no difference in hatching success of control eggs and sham-injected eggs (89%, $z = 0.398$, $p = 0.69$; Table 3).

2.3.3. Effects of MeHg exposure on Chick Growth

There was no treatment*age interaction for chick mass ($F_{12,425} = 0.72$, $p = 0.7363$) although there was (not surprisingly) a highly significant effect of age on chick mass ($F_{4,425} = 1094$, $p < 0.0001$; controlling for egg mass). Also, there was no overall main effect of treatment ($F_{3,425} = 0.03$, $p = 0.99$). Post-hoc multiple comparisons confirmed there were no difference in chick mass at any age (0, 5, 10, 21, 30 days) among different treatments (Tukey test, $p > 0.05$ in all cases).

2.3.4. Effects Treatment on Survival

Similar numbers of chicks hatched across each of the four treatments (Table 3). There was no significant treatment effect on the proportion of hatched chicks that survived to fledging (21 days post-hatch) ($\chi 2 = 6.37$, d.f. = 3, $p = 0.095$; Table 3), and there was no significant treatment effect on post-fledging survival between days 30 and days 90 ($\chi 2 = 1.51$, d.f. = 3, $p = 0.69$). However, chick survival was generally lower in the ‘both’ (69%) treatment group when compared with the ‘control’ (88%), in-ovo (76%) and ‘chick-only’ (84%) treatment groups. There were no treatment-based effects on the sex ratio of the chicks surviving to 90 days ($\chi 2 = 2.81$, d.f. = 3, $p = 0.42$). There were no treatment-based effects on the sex ratio of the chicks surviving to 90 days ($\chi 2 = 2.81$, d.f. = 3, $p = 0.42$) (Table 4).

2.3.5. Effects of MeHg Exposure on the Breeding Success of Females

A total of 136 eggs were laid by 34 pairs of the F2 generation of zebra finches. Of these, 56 were fertile (56/136; 41%), 73 were infertile (73/136; 53%), 3 showed signs of fertility, but failed to hatch (3/136; 2%), and 4 were broken during handling (4/136; 3%). Table 6 summarises the percent hatching success for each treatment. There was no significant treatment effect on the interval between pairing of the females and the time taken to lay the first egg ($F_{3,26} = 0.35$, $p = 0.79$). There was no significant treatment effect on clutch size ($F_{3,26} = 0.22$ $p = 0.88$); latency was controlled for but was not significant in the model ($p = 0.41$).

There was no significant effect of maternal treatment on egg hatching success; egg mass was controlled for, but mean egg mass effects were not significant in the model ($F_{3,26} = 0.38$, $p = 0.44$). There was a significant treatment effect on the brood size at hatching ($F_{3,25} = 0.35$, $p = 0.05$). Post-hoc multiple comparisons confirmed there was a significant difference in the number of chicks that survived the chick-only treatment when compared to the chicks exposed to MeHg both in-ovo and as chicks (Tukey test, $p < 0.05$). There were no significant differences between any of the other treatments or the control. Because low numbers of females in the 'both' group survived to sexual maturity, and then had low levels of egg productivity and subsequent productivity, females were pooled into two groups for analysis: 1) females with 'low' blood MeHg levels (the 'control' and 'in-ovo' treatment groups, n = 37), and 2) females with 'high' blood MeHg levels (the 'chick' and the combined 'in-ovo and chick' treatment groups n = 15). Females in the 'low' or 'high' blood MeHg groups had no significant difference in hatching success ($\chi 2 = 1.03$, d.f. = 1, $p = 0.31$) or fledging success (Fisher's Exact Test, $p = 0.29$).

There was no significant treatment effect on the proportion of hatched chicks that survived to fledging (21 days post-hatch) ($p = 0.086$; Fisher's Exact Test for Count Data; Table 4). There was no treatment*age interaction for chick mass up to 30 days ($F_{12,225} = 1.54$, $p = 0.11$) although again there was a highly significant effect of age on chick mass ($F_{4,225} = 37.72$, $p < 0.0001$; controlling for egg mass). Also, there was no overall main effect of treatment ($F_{3,17} = 0.36$, $p = 0.78$). Post-hoc multiple comparisons confirmed there were

no difference in chick mass at any age (0, 5, 10, 21, 30 days) among different treatments (Tukey test, $p > 0.05$ in all cases).

2.3.6. Male Courtship Trials

Forty-four males were tested in mating trials across four treatments; n = 9 for the "control", n = 10 for "in-ovo", n = 8 for the "chick" and n = 9 for the "both" treatments (Table 7). A total of n = 26 (59%) of males showed positive attempts to court during their first introduction to the females, and an additional n = 8 males attempted their first courtship during a second breeding trial, resulting in a total of 34 (77%) successful pooled trials. Ten males did not engage in any courtship behaviour in any trial; n = 6 were from the "chick", n = 3 from "both" and n = 1 from the "control" treatments. There was a significant treatment effect on the number of males engaging in courtship behaviour (Fisher's Exact Test, $p = 0.0014$). The data for the males that engaged in courtship in the second trial was pooled with the successful first trials for subsequent analysis to maximise the data set available for analysis (n = 34 males). There was no overall treatment effect on the frequency of 'follow' behaviours ($F_{3,30} = 1.03$, $p = 0.39$), 'bill wipes' ($F_{3,30} = 0.16$, $p = 0.93$), the number of mount attempts ($\chi 2 = 3.95$, d.f. = 3, $p = 2.67$), successful mount attempts ($\chi 2 = 4.80$, d.f. = 3, $p = 0.19$) or the time taken to mount females ($\chi 2 = 0.93$, d.f. = 3, $p = 0.81$) by the male birds. There was also no significant treatment effect on the response of the females to the male birds ($\chi 2 = 0.11$, d.f. = 3, $p = 0.99$).

2.4. Discussion

In this study we exposed a model terrestrial passerine species, the zebra finch (*Taeniopygia guttata*) to methylmercury (MeHg) in-ovo, only as a chick, and as a combined in-ovo and chick treatment. The dosing at different life-stages was to determine if: 1) terrestrial passerines have a specific life stage that is more sensitive to MeHg exposure; 2) to assess potential cumulative effects associated with exposure during more than one early life stage; and 3) to investigate the effect of different exposure scenarios on breeding success, which is a non-lethal but environmentally relevant endpoint. The mercury dosing methods involved injecting eggs with MeHg in a water vehicle to simulate maternal transfer and embryonic exposure to MeHg, and dosing of chicks orally from hatching until fledging

to simulate provision of food by parents until chicks are ready to disperse from the nest. Both dosing concentrations were based on environmentally relevant concentrations, and while the use of blood THg is not a precise measure of mercury body burden, it is a good indicator of MeHg exposure and doesn't involve destructive sampling. The blood THg results obtained reflected the range of blood Hg concentrations found in wild birds, so the outcomes of this study may be indicative of mercury toxicity in wild birds. Exposure to MeHg in-ovo had a significant effect on the hatching success of birds; eggs injected with MeHg had lower hatching success when compared to both control and vehicle-injected eggs. Exposure to MeHg had no significant effects on the growth rate or the weight of birds at maturity. There may, however, have been a treatment-based effect on the survival of birds to sexual maturity (90 days), as the chicks in the two treatment groups that were dosed with MeHg orally each day for 30 days ("chick" and "both") had lower overall survival rates compared to the "control" and "in-ovo" chicks. The sex ratio of the birds reaching sexual maturity appeared to be skewed in favour of males in the "chick" and "both" treatment groups when compared to the sex ratios of both the "control" and "in-ovo" chicks, but this skew was not significant. Female birds exposed to the higher levels of MeHg (the "chick" and "both" treatment groups) exhibited a large reduction in reproductive success. This was a function of both the number of MeHg-dosed females dying before reaching sexual maturity and the failure of the surviving females to lay fertile eggs. There were, however, no significant treatment effects on the growth of this second generation of chicks, or on their weight at 30 days post-hatch. Exposure to higher levels of MeHg also appeared to have an effect on male breeding behaviour, with males exposed to MeHg both in-ovo and as chicks showing a lower inclination to engage in courtship behaviour when compared to the other treatment groups.

Our dosing methods resulted in mean blood THg concentrations of 0.03 ppm for the control birds, 0.3 ppm for the birds dosed in-ovo, and 6 ppm for the birds dosed as only chicks and the birds dosed both in-ovo and as chicks. These blood THg concentrations were within the target range for each of the treatments and reflect environmentally relevant concentrations. The blood THg concentrations seen in these in-ovo studies are in line with levels seen in Yu et al. (2015), who had blood THg levels of 0.066 ± 0.015 μg g-1 in the highest dosed zebra finch cohort at 30 days post-hatch. A wide range of passerine species from across the north-east of North America had mean blood THg between 0.044 - 1.060

ppm (Jackson et al., 2015), which are within the range of the blood THg levels seen in the control and in-ovo dosed chicks in this study. The lack of in-ovo dosed treatment effects in this study after hatching mirror the findings of previous lab-based research that found negligible treatment effects on chicks post-hatch (Morran et al., 2016; Yu et al., 2015). The levels of THg seen in the feathers of the 'both' dosed birds (XX) were within the range of THg seen in birds in the wild. For example, a range of gull species sampled on the Southern Baltic coast in Poland had feather mercury levels between 79.0 – 9186 ppb (Szumiło-Pilarska et al., 2017), and Tree Swallows living at mercury impacted sites had feather THg levels of 13,550 ± 6940 ppb (Brasso & Cristol, 2008). Methylmercury appears to be excreted in feathers, and this strategy is likely to act as a protective mechanism for growing passerines, reducing the total body burden. When combined with the dilution effect that occurs during growth, MeHg toxicity appears to be low if birds survive exposure in-ovo. The selection pressure associated with surviving an elevated dose of MeHg in-ovo may also skew the selection of chicks in favour of those that are better able to manage a residual MeHg burden, increasing survival and reducing overt signs of toxicity.

The zebra finches in this study dosed daily as chicks (the 'chick' treatment group), and dosed both in-ovo and as chicks (the 'both' treatment group) had mean blood THg levels of 6 ppm. These blood THg concentrations are an order of magnitude higher than those seen in the preceding study by Morran et al. (2016). The initial chick target dose of Morran et al. (2016) was 0.27 µg g-1 bw.day-1, but loss of MeHg in the stock solution resulted in an actual chick dose of 0.075 µg g-1 bw.day-1. In this study we dosed chicks with 0.24 µg MeHg g-1 bw.day-1, which was 94% of the target dose of Morran et al. (2016), and was 3.3 times higher than the final 'high' chick dose achieved in their study. In this study we also dosed chicks for an extra 9 days post-hatch, during which time feather growth and, as a result, a critical source of MeHg excretion, slowed down. The estimated dose received by chicks in the high-dose treatment in Morran et al. (2016) at 21 days post-hatch was 14.6 µg MeHg, whereas in this study it was estimated to be 48.6 µg MeHg (3.3 x higher). Because chicks were dosed to 30 days post-hatch in the present study, chicks received an additional 29.1 µg MeHg, resulting in a final dose of 77.7 µg MeHg. That explains the elevated blood THg concentrations seen at 30 days in our current study, which are in-line with the higher range of blood THg levels seen in wild birds at heavily contaminated sites. Tree swallows at contaminated sites in the North-eastern United

States had blood mercury in the range of 3.56 +/- 2.41 ppm [42], and free-living Black-footed Albatross (*Phoebastria nigripes*) had blood THg of up to 6.4 ppm (Finkelstein et al., 2007). Those concentrations are also associated with sub-lethal but sensitive endpoints like fertility and courtship behaviour in free-living avifauna (Schoch et al., 2014; Fuchsman et al., 2017; Evers et al., 2008). The concentrations seen in the current study were in the lower range of total blood THg seen in a chronic Zebra Finch dietary exposure study where birds had blood THg levels between 4 and 34 ppm (Varian-Ramos et al., 2014). That is likely due to cessation of dosing at 30 days post-hatch in the current study. Nevertheless, we found a trend of increased mortality and overall reduced female fertility at mean THg blood levels of 6 ppm. In field studies, birds with elevated blood THg may experience a significant reduction in fertility. Wild female tree swallows near contaminated sites had mean blood THg levels of 3.56 ppm and had reduced hatching success, wild Carolina Wrens had blood THg levels between 1.96 to 3.38 ppm and a concurrent reduction in fertility at higher blood THg levels, and wild Common Loons had impaired reproductive success with a mean blood THg of 3.0 ppm (Evers et al., 2008; Jackson et al., 2015). In our study, elevated but environmentally relevant blood THg levels were associated with a reduction in fecundity in female Zebra Finches.

We provide evidence that MeHg is excreted via feathers in the Zebra Finch during growth and fledging. Birds in the pilot study that were dosed in-ovo and also as chicks had up to 715 ppb THg in P2 feathers, while vehicle-dosed birds had between 9 and 29 ppb THg in their feathers. This supports previous work on the toxicity of MeHg to zebra finches exposed in-ovo or as chicks by Yu et al. (2015), and Morran et al. (2016) respectively. Excretion of MeHg is likely providing some degree of protection from the toxic effects of MeHg during this critical development phase in passerines (Whitney & Cristol., 2017).

The results of the current study support the findings of Yu et al. (2015) that exposure to 4.0 µg/g egg^{-1} MeHg (3.2 µg/g egg^{-1} Hg) in-ovo reduces hatching success in zebra finches. Interestingly, while there were too few surviving birds dosed both in-ovo and as chicks to enable a robust analysis of final female fertility in this treatment group, there was no significant treatment effect on the fertility of chick-dosed birds. Heinz et al. (2009) also found that that direct in-ovo injection of MeHg is more ‘toxic’ than maternally transferred MeHg. Some studies have also shown that at low levels exposure to MeHg may result in an hormotic response, with mallards (*Anas platyrhynchos*) exposed to 0.5 µg/g bw^{-1} of MeHg producing significantly larger clutches containing heavier chicks than

those in the control group (Heinz et al., 2010). There is, however, an extensive body of evidence linking increased exposure to MeHg in-ovo with reduced hatching success in avifauna (Heinz et al., 2009; Kenow et al., 2011; Rutkiewicz et al., 2013; Yu et al., 2015). Heinz et al. found that both Mallard and Chicken (*Gallus gallus*) egg hatching rates were affected at MeHg concentrations of 1 µg/g ww, and that survival dropped significantly at 1.6 µg/g ww (Hester et al., 1978; Heinz, 1979). Common Loons showed a reduced hatching rate when eggs were injected with 1.3 µg/g ww of MeHg (Kenow et al, 2011). In a comparative study, Heinz et al. (2009) also found a wide range of interspecies in-ovo sensitivity to MeHg exposure, with some species demonstrating a 'high' tolerance, defined as an LD_{50} of > 1 µg/g ww, 'moderate' tolerance of an LD_{50} greater than 0.25 µg/g mercury but less than 1 µg/g), and low tolerance as < than 0.25 µg/g mercury. Methylmercury is a neuroteratogen in mammals, with foetal exposure causing changes to neuronal structure, gross brain structure and an overall reduction in brain weight (Hulla, 2014). In birds, egg failure may also be attributed to the teratogenic effects of MeHg (Heinz et al, 2011). To date, no studies have determined the mechanism by which maternal transfer reduces embryotoxicity (G. Heinz, *pers. comm*.). The findings that egg injections enhance MeHg toxicity when compared to maternally transferred MeHg should be taken into consideration when extrapolating from the results of this study to the potential impacts of MeHg egg exposure on wild bird populations (Heinz, 2009; Heinz et al., 2011).

We found no growth-related effects for any of the treatment groups, supporting previous studies on zebra finch sensitivity to MeHg exposure (Morran et al, 2016; Yu et al., 2015). The mechanism of action for MeHg growth suppression in birds has been linked to behaviour; organisms exposed to elevated levels of MeHg may experience appetite suppression (Frederick et al., 2011). Exposure to MeHg during critical development phases can affect bird growth, but this does not appear to be consistent across species. Chickens provisioned with water containing 500 mg/L of MeHg had reduced growth (Hester et al., 1978), and Red-tailed Hawks (*Buteo jamaicensis*) had significant reductions in chick growth when provisioned with food containing 10 mg/g of MeHg (Fimreite & Karstad, 1971). In both instances, growth reduction appeared to be related to a reduced appetite. Some studies found that there was no change to growth when birds were exposed to environmentally relevant levels of MeHg; Common Loons fed an environmentally relevant dose of 1.5mg MeHg g^{-1} of wet fish (Kenow et al., 2003) had no significant difference in mean body weight when compared to the control group, and no

treatment-related growth effects were observed when Zebra Finches were dosed with 1.2 µg/g bw^{-1} of MeHg daily for 21 days post-hatch (Morran et al., 2016), or when they were dosed in-ovo with 3.2 µg Hg g^{-1} egg (Yu et al., 2015).

In this study, survival rates of zebra finches exposed to MeHg 'both' in-ovo and as chicks (69%) and just 'in-ovo' (68%) were much lower than the 'control' (83%) and 'chick-only' (76%) treatment groups. While not statistically significant, this may have pertinent population-level effects on wild birds, especially when coupled with the observed lower successful breeding rates for MeHg-exposed birds. There are limited studies assessing the long-term impacts of MeHg exposure to bird survival in the wild, mainly because of challenges in determining exposure and effects in wild birds. The studies that have been done do not strongly support the hypothesis that exposure to MeHg has significant effects on bird survival rates. Forster's Terns (*Sterna forsteri*) had no apparent relationship between blood THg and survival for the first 35 days post-hatch (Ackerman et al, 2008a), and there was only weak evidence of a relationship between Hg exposure and the survival of fledgling American Avocets (*Recurvirostra americana*) and Black-necked Stilts (*Himantopus mexicanus*) (Ackerman et al, 2008b). Yu et al (2015) found that exposure to MeHg decreased survival of Zebra Finch eggs, but found no long-term effects on the growth of birds exposed in-ovo. Likewise, Morran et al. (2016) exposed zebra finch chicks with up to 0.15 µg/g bw^{-1} MeHg for 21 days post-hatch, and found no significant effects on survival between treatment groups. Varian-Ramnos et al. (2014) reported that patterns of MeHg affected reproductive success in Zebra Finches, and suggested there may be population-level artificial selection for MeHg tolerance, which could explain the low correlation between MeHg body burdens and survival in wild bird populations. Frederick et al. (2011) fed White Ibises (*Eudocimus albus*) up to 0.3 MeHg µg/g ww in their diet and found that survival was significantly lower in the control and high-dosed birds when compared to the low or medium-dosed birds, suggesting a possible hormetic effect. Wild Common Loon populations experienced a 50% drop in productivity when fish Hg levels were 0.21 ug/g ww, and failed completely when fish Hg concentrations were 0.41 ug/g ww (Kenow et al., 2003), but that study assessed productivity rather than survival. The dearth of comprehensive long term survival studies suggest that more research is required to understand the effects of MeHg on survival of wild birds.

In the current study, of the 26 birds that were dosed with MeHg both in-ovo and as chicks, 12 males and 5 females survived to breeding age. This was an unexpected result, especially in light of the additional excretion route available to female birds to mitigate their total MeHg body burden. The result suggests that birds may have gender-based differences to MeHg sensitivity. Few environmental toxicology studies have tested for gender differences of MeHg toxicity, despite evidence across multiple species that males and females have differing sensitivities to heavy metals, including MeHg (Haber & Jennings, 1964; Hirayama & Yasutake, 1986; Vahter et al, 2007). Androgens have been implicated in the higher levels of MeHg seen in the urine of male mice when compared to female mice (Hirayama & Yasutake, 1986), and gender-related differences in the patterns of nephrotoxicity have been observed in rats exposed to MeHg (Haber & Jennings, 1964). Robinson et al. (2012) undertook a meta-analysis of THg concentrations in birds of both genders and found that female birds of all species had lower overall THg body burdens when compared to males. This difference has been attributed to maternal transfer of some of her THg body burden into eggs

The present study found treatment-based effects on the brood size at hatching; however, the analysis was hampered by loss of the females with the highest exposure and the failure of these females to lay any fertile eggs. Only five females dosed both in-ovo and as chicks survived to 90 days, n = 4 of these birds laid eggs, and only n =1 egg was fertile and developed into a chick. There was also a reduction in the number of females dosed only as chicks, with n = 7 surviving to breed. The chick-only dosed females had a higher success rate with breeding, but again, it is difficult to make any strong assertions with respect to overarching effects because of the reduced number of mercury-dosed females reaching sexual maturity. Elevated environmental levels of MeHg have been implicated in the decline of a range of species, and animal and human studies have shown that MeHg has a deleterious effect on the endocrine system and on reproductive success (Zhu et al, 2000; Tartu et al, 2013; Varian-Ramos et al., 2014). There is some evidence that THg may supress the ability of the pituitary gland to release lutenising hormone (LH) and follicle stimulating hormone (FSH) (Tartu et al., 2013). Gonadotropin-releasing hormone (GnRH) in the hypothalamus may also be suppressed in the presence

of THg (Tartu et al, 2013). Both of these mechanisms are likely to impact reproductive performance of birds. Other mechanisms that may impact productivity include behavioural changes; for example, White Ibises (*Eudocimus albus*) had lower fecundity, which was attributed to an increasing predisposition towards homosexual pairing in birds with higher MeHg body burdens (Frederick & Jayasena, 2010).

In the present study there was a treatment-based effect on the number of males initiating courtship behaviour. Exposure to MeHg may adversely impact male pairing efforts (Heinz, 1979; Frederick & Jayasena, 2010; Heinz et al, 2009). White Ibis exposed to environmentally relevant levels of MeHg had an increased number of homosexual pairings and reduced clutch success (Frederick & Jayasena, 2010), and songbirds living in mercury-impacted areas had a reduced song complexity, potentially reducing their perceived fitness by mates (Hallinger et al., 2010). There was, however, no evidence of a change in male courtship behaviour in zebra finches exposed to MeHg either in-ovo or as chicks (Morran et al., 2016; Yu et al., 2015).

2.5. Conclusions

This study has shown that sub-lethal and lethal endpoints of MeHg are contingent on the dose, timing and duration of exposure in Zebra Finches. There are two life stages that are particularly sensitive to MeHg exposure: 1) the developing embryo, as MeHg-injected eggs had a significantly lower hatching success than vehicle-injected or control eggs, and 2) the fledged chicks. While exposure to MeHg in-ovo resulted in a significant reduction in hatching success, there was no significant difference in behavioural or reproductive outcomes for the individuals that survived to maturity. Birds dosed both in-ovo and as chicks had reduced numbers of females surviving to maturity and altered male courtship behaviours. Birds dosed only as chicks had reduced survival rates. No long-term effects were seen on male courtship in the birds dosed only as chicks. Continuous exposure of chicks during embryogenesis and chick development had a deleterious effect on bird survival and fertility. Passerines may be able to withstand exposure to elevated levels of methylmercury during development at the nestling stage, but chronic exposure may reduce survival and fertility. Because the Zebra Finch is used as a model organism for toxicity tests, its ability to tolerate large doses of MeHg, especially during the growth phase, should be considered when investigating passerine MeHg toxicity.

2.6. References

Ackerman, J.T., C.A. Eagles-Smith, J.Y. Takekawa, and S.A. Iverson. 2008a. Survival of postfledging Forster's terns in relation to mercury exposure in San Francisco Bay. Ecotoxicology 17:789–801

Ackerman, J.T., Takekawa, J.Y., Eagles-Smith, C.A. and Iverson, S.A., 2008b. Mercury contamination and effects on survival of American avocet and black-necked stilt chicks in San Francisco Bay. Ecotoxicology, 17(2), pp.103-116.

Ackerman, J.T., Eagles-Smith, C.A. and Herzog, M.P., 2011. Bird mercury concentrations change rapidly as chicks age: toxicological risk is highest at hatching and fledging. Environmental Science & Technology, 45(12), pp.5418-5425.

Bank, M.S., 2012. Mercury in the environment: pattern and process. Univ. of California Press.

Basu, N., Tutino, R., Zhang, Z., Cantonwine, D.E., Goodrich, J.M., Somers, E.C., Rodriguez, L., Schnaas, L., Solano, M., Mercado, A. and Peterson, K., 2014. Mercury levels in pregnant women, children, and seafood from Mexico City. Environmental research, 135, pp.63-69.

Bouton, S.N., Frederick, P.C., Spalding, M.G. and McGill, H., 1999. Effects of chronic, low concentrations of dietary methylmercury on the behavior of juvenile great egrets. Environmental Toxicology and Chemistry, 18(9), pp.1934-1939.

Brasso, R.L. and Cristol, D.A., 2008. Effects of mercury exposure on the reproductive success of tree swallows (*Tachycineta bicolor*). Ecotoxicology, 17(2), pp.133-141.

Evers DC, Savoy LJ, DeSorbo CR, Yates DE, Hanson W., 2008. Adverse effects from environmental mercury loads on breeding common loons. Ecotoxicology 17: 69–81.

Fimreite, N. and Karstad, L., 1971. Effects of dietary methyl mercury on red-tailed hawks. The Journal of Wildlife Management, pp.293-300.

Finkelstein, M.E., Grasman, K.A., Croll, D.A., Tershy, B.R., Keitt, B.S., Jarman, W.M. and Smith, D.R., 2007. Contaminant-associated alteration of immune function in black-footed albatross (*Phoebastria nigripes*), a North Pacific predator. Environmental Toxicology and Chemistry, 26(9), pp.1896-1903.

Frederick, P. and Jayasena, N., 2010. Altered pairing behaviour and reproductive success in white ibises exposed to environmentally relevant concentrations of methylmercury. Proceedings of the Royal Society of London B: Biological Sciences, pp.2010-2189.

Frederick, P., Campbell, A., Jayasena, N. and Borkhataria, R., 2011. Survival of White Ibises (*Eudocimus albus*) in response to chronic experimental methylmercury exposure. Ecotoxicology, 20(2), pp.358-364.

Fuchsman, P.C., Brown, L.E., Henning, M.H., Bock, M.J. and Magar, V.S., 2017. Toxicity reference values for methylmercury effects on avian reproduction: Critical review and analysis. Environmental toxicology and chemistry, 36(2), pp.294-319.

Gobeil, C., Macdonald, R.W. and Smith, J.N., 1999. Mercury profiles in sediments of the Arctic Ocean basins. Environmental Science & Technology, 33(23), pp.4194-4198.

Gochfeld, M., 2003. Cases of mercury exposure, bioavailability, and absorption. Ecotoxicology and Environmental Safety, 56(1), pp.174-179.

Haber, M.H. and Jennings, R.B., 1964. Sex differences in renal toxicity of mercury in the rat. Nature, 201, p.1235.

Hallinger KK, Zabransky DJ, Kazmer KA, Cristol DA (2010) Song differs between birds on mercury-polluted and reference sites. Auk 127: 156–161.

Heinz, G.H. and Locke, L.N., 1976. Brain lesions in mallard ducklings from parents fed methylmercury. Avian Diseases, pp.9-17.

Heinz, G.H., 1979. Methylmercury: reproductive and behavioral effects on three generations of mallard ducks. The Journal of Wildlife Management, pp.394-401.

Heinz GH, Hoffman DJ, Klimstra JD, Stebbins KR, Kondrad SL, Erwin CA. 2009. Species differences in the sensitivity of avian embryos to methylmercury. Archives of Environmental Contaminant Toxicology 56:129–138.

Heinz, G.H., Hoffman, D.J., Klimstra, J.D., Stebbins, K.R., Kondrad, S.L. and Erwin, C.A., 2011. Teratogenic effects of injected methylmercury on avian embryos. Environmental Toxicology and Chemistry, 30(7), pp.1593-1598.

Hester, P.Y., Brake, J., Sikes, C.V., Thaxton, P. and Pardue, S.L., 1978. The excretory system of young chickens experiencing mercury toxicity—effects on kidney development, morphology, and function. Archives of environmental contamination and toxicology, 7(1), pp.257-271.

Hirayama, K. and Yasutake, A., 1986. Sex and age differences in mercury distribution and excretion in methylmercury administered mice. Journal of Toxicology and Environmental Health, Part A Current Issues, 18(1), pp.49-56

Hulla, J. Chapter 17. Metals. In Hayes, A.W. and Kruger, C.L. (Eds, 2014). Hayes' principles and methods of toxicology. CRC Press.

Jackson, A.K., Evers, D.C., Adams, E.M., Cristol, D.A., Eagles-Smith, C., Edmonds, S.T., Gray, C.E., Hoskins, B., Lane, O.P., Sauer, A. and Tear, T., 2015. Songbirds as sentinels of mercury in terrestrial habitats of eastern North America. Ecotoxicology, 24(2), pp.453-467.

Kenow, K.P., Gutreuter, S., Hines, R.K., Meyer, M.W., Fournier, F. and Karasov, W.H., 2003. Effects of methyl mercury exposure on the growth of juvenile common loons. Ecotoxicology, 12(1), pp.171-181.

Kenow, K.P., Meyer, M.W., Rossmann, R., Gendron-Fitzpatrick, A. and Gray, B.R., 2011. Effects of injected methylmercury on the hatching of common loon (Gavia immer) eggs. Ecotoxicology, 20(7), pp.1684-1693.

Lamborg, C.H., Hammerschmidt, C.R., Bowman, K.L., Swarr, G.J., Munson, K.M., Ohnemus, D.C., Lam, P.J., Heimbürger, L.E., Rijkenberg, M.J. and Saito, M.A., 2014. A global ocean inventory of anthropogenic mercury based on water column measurements. Nature, 512(7512), pp.65-68.

Morran, S.A., 2016. Investigating potential growth, behavioural, and reproductive effects of nestling exposure to methylmercury in Zebra Finches (Taeniopygia guttata) (Masters of Environmental Toxicology Thesis). Retrieved from Simon Fraser University (Identifier: etd9737

Robinson, S.A., Lajeunesse, M.J. and Forbes, M.R., 2012. Sex differences in mercury contamination of birds: testing multiple hypotheses with meta-analysis. Environmental science & technology, 46(13), pp.7094-7101.

Rutkiewicz, J., Bradley, M., Mittal, K. and Basu, N., 2013. Methylmercury egg injections: Part 2—Pathology, neurochemistry, and behavior in the avian embryo and hatchling. Ecotoxicology and Environmental Safety, 93, pp.77-86.

Scheuhammer, A.M., Meyer, M.W., Sandheinrich, M.B. and Murray, M.W., 2007. Effects of environmental methylmercury on the health of wild birds, mammals, and fish. AMBIO: a Journal of the Human Environment, 36(1), pp.12-19.

Schoch, N., Glennon, M.J., Evers, D.C., Duron, M., Jackson, A.K., Driscoll, C.T., Ozard, J.W. and Sauer, A.K., 2014. The impact of mercury exposure on the Common Loon (Gavia immer) population in the Adirondack Park, New York, USA. Waterbirds, 37(sp1), pp.133-146.

Szumiło-Pilarska, E., Falkowska, L., Grajewska, A. and Meissner, W., 2017. Mercury in feathers and blood of gulls from the Southern Baltic coast, Poland. Water, Air, & Soil Pollution, 228(4), p.138.

Tartu, S., Goutte, A., Bustamante, P., Angelier, F., Moe, B., Clément-Chastel, C., Bech, C., Gabrielsen, G.

Varian-Ramos, C.W., Swaddle, J.P. and Cristol, D.A., 2014. Mercury reduces avian reproductive success and imposes selection: an experimental study with adult-or lifetime-exposure in zebra finch. PLoS One, 9(4), p.e95674.

Vahter, M., Åkesson, A., Lidén, C., Ceccatelli, S. and Berglund, M., 2007. Gender differences in the disposition and toxicity of metals. Environmental research, 104(1), pp.85-95.

Whitney, M. and Cristol, D., 2017. . Environmental Toxicology and Chemistry.

Wiener, J.G., Krabbenhoft, D.P., Heinz, G.H. and Scheuhammer, A.M., 2003. Ecotoxicology of mercury. Handbook of ecotoxicology, 2, pp.409-463.

Winter, V., Elliott, J.E., Letcher, R.J. and Williams, T.D., 2013. Validation of an egg-injection method for embryotoxicity studies in a small, model songbird, the zebra finch (Taeniopygia guttata). Chemosphere, 90(1), pp.125-131Yu, M.S., Eng, M.L., Williams, T.D., Basu, N. and Elliott, J.E., 2015. Acute embryotoxic effects but no long term reproductive effects of in ovo methylmercury exposure in zebra finches (Taeniopygia guttata). Environmental Toxicology and Chemistry.

Zann, R.A., 1996. The zebra finch: a synthesis of field and laboratory studies (Vol. 5). Oxford University Press.

Chapter 3.

3.1. Conclusions

In this thesis we exposed a model passerine, the Zebra Finch, to MeHg in-ovo (embryonic exposure, pre-hatching), only as a chick (post-hatching exposure), and with a combined in-ovo chick treatment (embryonic and post-natal exposure). The aim of this work was to answer the following questions about the effects of MeHg on passerines: 1) are any life-stages in passerines particularly sensitive to MeHg exposure; 2) does exposure to MeHg over several critical developmental stages have a cumulative effect; and 3) how do these different exposure scenarios affect reproduction, a non-lethal but environmentally relevant endpoint. Our study found that there are two life stages that are particularly sensitive to MeHg exposure. The first was the developing embryo; MeHg-injected eggs had a significantly lower hatching success than vehicle-injected or control eggs. That supports the findings of a previous study by Yu et al. (2015), who also found that injection of MeHg resulted in elevated levels of egg failure. Heinz et al. (2011) have attributed MeHg's teratogenic effects to the likely increase in embryo mortality in birds, but the exact mechanisms of toxicity have not yet been established. This study supports the findings that MeHg decreases egg survival but the heightened toxicity of egg injections when compared to maternally transferred MeHg should be taken into consideration when extrapolating from the results of this study to the potential impacts of MeHg egg exposure on wild bird populations (Heinz, 2009; Heinz et al., 2011). The second sensitive life stage is the fledgling chick after it has completed the majority of its feather production and growth. Our chicks were exposed to MeHg for 30 days post-hatch, and feather growth, which is a known excretion mechanism, and that slows down after 20 days post-hatch (T. Williams, *pers. comm*.). Morran et al. (2016) exposed chicks to MeHg for 21 days post-hatch, and found no significant behavioural or reproductive changes between dosed birds and control birds. The chicks in the study by Morran et al. (2016) were thought to be reducing their MeHg body burdens via dilution (i.e. growth) and excretion into the keratin of the feathers. Because we dosed our chicks for a further 9 days, the chicks were provided with additional 9 doses of MeHg, during which their excretory pathways were markedly reduced.

There was an apparent reduction of female fertility for birds exposed both in-ovo and also as chicks, but a large reduction in the number chick-dosed females surviving to sexual maturity reduced the statistical power of this sample size and the results were not significant. Overall, our study supports the findings of previous studies that growing songbirds have a means for reducing MeHg toxicity during the sensitive growth stage, but after this stage is completed, passerines are more susceptible to MeHg exposure. The loss of all but five of the in-ovo and chick-dosed (i.e. the 'both' treatment) females hampered analysis of possible synergisms between the timing and duration of MeHg exposure and the resulting toxicity. Of the five females dosed both in-ovo and as chicks that survived to 90 days, four laid eggs and only one egg was fertile. The female birds dosed only as chicks also had a low survival rate, with only seven females surviving to maturity. These seven females had a higher fertility rate than those exposed both in-ovo and as chicks, with 13 eggs hatching. The survival and fertility rates for the in-ovo dosed chicks were higher still, but their fertility rates were in line with the chick-only dosed females, with 11 eggs hatching. These trends point to increased sensitivity when passerines are exposed both in-ovo and as chicks, with ongoing exposure during growth promoting increased failure to reach maturity, and exposure at both in-ovo and during growth as the most 'toxic' pattern of exposure. Overall it is difficult to make any strong assertions with respect to treatment-based effects because of the reduced number of mercury-dosed females reaching sexual maturity, but this pattern of fertility drop-off appears to be related to a constant exposure from conception and past the stage at which birds are able to excrete MeHg.

There was evidence that being exposed to MeHg both in-ovo and also as a chick affected male courtship behaviour, with a significant number of males in this treatment group showing changes in courtship initiation. It would be interesting to see if male fertility in these groups had also been affected, as multiple inputs (e.g. increased female die-off, reduced female fertility and reduced propensity of males to initiate courtship) would be likely to have possible population-level effects. Animal and human studies have shown that elevated body burdens of MeHg can have deleterious effect on reproductive success, and elevated levels of MeHg in the environment have been implicated in the decline of a range of species (Zhu et al, 2000; Tartu et al, 2013; Varian-Ramos et al., 2014). The present study supports these notions, but at the same time these birds were exposed to

levels of MeHg that are at the upper limit of those found in heavily contaminated environments (Varian-Ramos et al., 2014), suggesting that Zebra Finches may have a high tolerance for MeHg exposure. It is also possible that in wild populations the presence of MeHg may also act to select individuals who can tolerate elevated MeHg burdens, and these confounding factors should be considered if incorporating these data into Toxicity Reference Values for wildlife.

This project is the last of three studies into the effects of MeHg exposure on passerine survival, development and behavior done in the Williams Laboratory at Simon Fraser University (Morran et al., 2015; Yu et al., 2015). This study demonstrates that the injection of 3.2 $\mu g.g^{-1}$ of MeHg per gram of egg results in a statistically significant reduction in egg hatching rates and also demonstrated that the production of feathers may protect the developing chick from some of the more acute effects of MeHg toxicity. Because this study continued to dose chicks after the majority of feather growth had been completed, we were also able to provide the birds with a higher MeHg body burden, and as expected, we saw both non-lethal and lethal signs of MeHg toxicity. Future studies into MeHg will need to take this excretion mechanism into account when designing experiments, as moulting patterns and excretion mechanisms are species-specific, with some birds losing many feathers in a short period of time, providing a large sink for MeHg excretion. The lethal signs of toxicity were related to egg failure and also survival to maturity, with only a small number of high-dosed females surviving to sexual maturity. Because of the high rate of mortality, we were unable to analyse the fertility of the remaining females. So, while this survival pattern was not statistically significant, it warrants further study both in the laboratory and also, more importantly, in the field. The endocrine-disrupting properties of MeHg are also of interest and may prove to be a fertile field for future research. Testing endocrine levels would be a non-destructive addition to future laboratory and field studies into MeHg toxicity in passerines, and would help to answer some of the questions that have emerged as a result of our MeHg studies.

Our studies have shown that the Zebra Finch does show signs of both sub-lethal and lethal toxicity, and that these signs are contingent on the dose, timing and duration of exposure. Because the Zebra Finch is used as a model organism for toxicity tests, its ability to tolerate large doses of MeHg, especially during the growth phase, should be considered when investigating passerine MeHg toxicity. The doses used in this study may provide useful for future range-finding studies.

3.2. References

Bank, M.S., 2012. Mercury in the environment: pattern and process. Univ. of California Press.

Finkelstein, M.E., Grasman, K.A., Croll, D.A., Tershy, B.R., Keitt, B.S., Jarman, W.M. and Smith, D.R., 2007. Contaminant-associated alteration of immune function in black-footed albatross (*Phoebastria nigripes*), a North Pacific predator. Environmental Toxicology and Chemistry, 26(9), pp.1896-1903.

Heinz, G.H., Hoffman, D.J., Klimstra, J.D. and Stebbins, K.R., 2009. Rapid increases in mercury concentrations in the eggs of mallards fed methylmercury. Environmental Toxicology and Chemistry, 28(9), pp.1979-1981.

Jackson, A.K., Evers, D.C., Adams, E.M., Cristol, D.A., Eagles-Smith, C., Edmonds, S.T., Gray, C.E., Hoskins, B., Lane, O.P., Sauer, A. and Tear, T., 2015. Songbirds as sentinels of mercury in terrestrial habitats of eastern North America. Ecotoxicology, 24(2), pp.453-467.

Meyer, M.W., Evers, D.C., Hartigan, J.J. and Rasmussen, P.S., 1998. Patterns of common loon (Gavia immer) mercury exposure, reproduction, and survival in Wisconsin, USA. Environmental Toxicology and Chemistry, 17(2), pp.184-190.

Morran, S.A., 2016. Investigating potential growth, behavioural, and reproductive effects of nestling exposure to methylmercury in Zebra Finches (Taeniopygia guttata) (Masters of Environmental Toxicology Thesis). Retrieved from Simon Fraser University (Identifier: etd9737)

Tan, S.W., Meiller, J.C. and Mahaffey, K.R., 2009. The endocrine effects of mercury in humans and wildlife. Critical reviews in toxicology, 39(3), pp.228-269.

Taylor, C.E. and Cristol, D.A., 2015. Tissue mercury concentrations and survival of tree swallow embryos, nestlings and young adult females on a contaminated site. Bulletin of environmental contamination and toxicology, 95(4), pp.459-464.

Wada, H., Cristol, D.A., McNabb, F.A. and Hopkins, W.A., 2009. Suppressed adrenocortical responses and thyroid hormone levels in birds near a mercury-contaminated river. Environmental science & technology, 43(15), pp.6031-6038.

Whitney, M. and Cristol, D., 2017. Rapid depuration of mercury in songbirds accelerated by feather molt. Environmental Toxicology and Chemistry.

Wolfe, M.F., Schwarzbach, S. and Sulaiman, R.A., 1998. Effects of mercury on wildlife: a comprehensive review. Environmental toxicology and chemistry, 17(2), pp.146-160.

Yu, M.S., Eng, M.L., Williams, T.D., Basu, N. and Elliott, J.E., 2015. Acute embryotoxic effects but no long term reproductive effects of in ovo methylmercury exposure in zebra finches (Taeniopygia guttata). Environmental Toxicology and Chemistry.

Appendix A.

Tables and Figures

Table A1. Summary Statistics for total blood mercury (THg) in parts per million for chicks 30 days post-hatch.

Treatment	Number of Chicks	Mean THg (ppm)	Std Dev	Std Err Mean	Lower 95%	Upper 95%
Both	24	6.03	1.07	0.12	5.58	6.48
Chick	23	6.58	1.42	0.3	5.97	7.19
In-ovo	20	0.31	0.34	0.08	0.15	0.4
Control	24	0.03	0.02	0.08	0.02	0.03

Table A2. Summary of THg results from P2 feather analysis in the pilot study. Note: the vehicle was contaminated with THg in the pilot study. Additional feathers were not analysed because THg levels in the birds dosed in-ovo and as chicks were elevated.

Treatment	THg (ppb)
In-ovo and chick ('both')	538
In-ovo and chick ('both')	715
Vehicle	14
Vehicle	13
Vehicle	9.8
Vehicle	29

Table A3. Summary data showing survival across the four treatment groups. A total of n=101 chicks hatched across the four treatment groups. This table shows survival at 21 days, 30 days, 90 days, and 90-120 days (females) and 120-140 days (males). There was no significant treatment effect on survival of chicks.

	Number and % of Chicks per Treatment			
	Control (uninjected)	In ovo (MeHg injection)	Chick dosed (sham injected)	Both (in-ovo and chick dosed)
Number hatched	24	25	26	26
Number survived (21 days)	23 (96%)	20 (80%)	23 (88%)	24 (92%)
Number survived (30 days)	22 (91%)	20 (80%)	23 (88%)	23 (88%)
Number survived (90 days)	20 (83%)	17 (68%)	20 (76%)	18 (69%)
Number survived to 90 days (male / female)	8 / 12	9 / 8	13 / 7	12 / 6
Number of females surviving to breed (90 – 120 days)	12	8	7	5
Number of males surviving to breed (120 - 140 days)	8	9	13	12

Table A4. **Summary data showing all fertile eggs assigned a treatment in this experiment. A total of 154 eggs were laid by n=57 F1 finch pairs. Of these, 7 eggs were broken during handling, and 17 eggs were infertile. A total of 130 fertile eggs were treated with: 1) no injection ('control'); 2) an injection of a vehicle ('sham') or 3) an injection of methylmercury (MeHg). There was a significant effect of egg treatment on hatching success ($\chi 2 = 11.77$, d.f. = 2, p = 0.003; egg mass was controlled for, though this term was not significant in the model, p = 0.56).**

	Number of eggs per treatment			% eggs per treatment		
	Control (uninjected)	Sham (vehicle injection)	MeHg injected	Control (uninjected)	Sham (vehicle injection)	MeHg injected
Fertile eggs used	27	29	74	20.6	22.1	57.3
Number (%) hatched	24	26	49	88.9	89.7	64
Fertile, but failed to develop	1	3	25 **	3.7	10.3	33.3 **

** Indicates a significant treatment effect

Table A5 **Summary data showing survival across the four treatment groups in the F2 generation. A total of n=101 chicks hatched across four treatment groups. There was no significant treatment effect on the proportion of hatched chicks that survived to fledging (21 days post-hatch) ($\chi 2 = 6.37$, d.f. = 3, $p = 0.095$; Table 3), and there was no significant treatment effect on post-fledging survival between days 30 and days 90 ($\chi 2 = 1.51$, d.f. = 3, $p = 0.69$). However, chick survival was generally lower in the 'both' (69%) treatment group when compared with the 'control' (88%), in-ovo (76%) and 'chick-only' (84%) treatment groups. There were no treatment-based effects on the sex ratio of the chicks surviving to 90 days ($\chi 2 = 2.81$, d.f. = 3, $p = 0.42$).**

	Number and % of Chicks per Treatment			
	Control (uninjected)	In ovo (MeHg injection)	Chick dosed (sham injected)	Both (in-ovo and chick dosed)
Number hatched	24	25	26	26
Number survived (21 days)	23 (96%)	20 (80%)	23 (88%)	24 (92%)
Number survived (30 days)	22 (91%)	20 (80%)	23 (88%)	23 (88%)
Number survived (90 days)	21 (88%)	19 (76%)	22 (84%)	18 (69%)
Number survived to 90 days (male / female)	8 / 13	9 / 10	13 / 9	12 / 6
Number of females surviving to breed (90 – 120 days)	13	10	9	5
Number of males surviving to breed (120 - 140 days)	8	9	13	12

Table A6. Summary data showing female breeding success across the four treatment groups. A total of n=34 female chicks survived until day 90. Mean clutch size did not differ significantly between treatment groups, but analysis of overall fecundity was hampered by reduced female survival in the 'both' treatment group. This table also compares the relative fecundity of all females ('Total') and the females who showed nesting behavior ('Laying') – the females that failed to nest were excluded from the 'Laying' group.

	Female breeding success by Treatment			
	Control (uninjected)	In ovo (MeHg injection)	Chick dosed (sham injected)	Both (in-ovo and chick dosed)
Number of females (Total)	12	10	7	5
Number of females (Laying)	11	9	6	3
Number of eggs laid	51	37	28	15
Number of fertile eggs laid	25	13	19	1
Number of infertile eggs laid	26	24	9	14
Mean clutch size (± SE)	4.2 (± 0.5)	3.9 (± 0.7)	3.6 (± 0.9)	3.2 (± 1.5)
Mean brood size at hatching (Total) (± SE) *	1.85 (± 0.43)	1.33 (± 0.42)	2.1 (± 0.7)	0.2 (± 0.2)
Mean brood size at hatching (Laying) (± SE) *	2.0 (± 0.44)	1.33 (± 0.41)	2.37 (± 0.84)	0.33 (± 0.3)
Mean brood size at fledging (Total) (± SE)	1.84 (± 0.44)	1.31 (± 0.42)	1.45 (± 0.78)	0.2 (± 0.2)
Mean brood size at fledging (Laying) (± SE)	2.0 (± 0.44)	1.33 (± 0.41)	2.16 (± 0.98)	0.33 (± 0.3)
Number of offspring surviving to day 30	22	11	13	1

Table A7 Summary statistics for the multiple measures of male courtship behaviour and the response of females to this behaviour

	Control (n = 9)	In-ovo (n = 10)	Chick dosed (n = 8)	Both In-ovo and chick dosed (n = 9)
Total Bill Wipes during 10 minute trial	10.56 ± 8.74	12.70 ± 7.00	12.75 ± 8.32	13.00 ± 9.38
Male following female during 10 minute trial	18.00 ± 15.23	20.7 ± 16.70	29.50 ± 18.20	33.44 ± 23.79
Female response (0 = no response, 5 = female presenting)	2.33 ± 1.50	2.70 ± 1.49	2.75 ± 1.58	2.56 ± 1.94
Time to first mount (seconds)	107.56 ± 198.105	56.20 ± 83.67	83.63 ± 134.40	35.56 ± 53.40
Number of unsuccessful mounts	1.23 ± 2.04	3.10 ± 3.57	3.38 ± 3.70	5.56 ± 4.56
Number of successful mounts	0.23 ± 0.67	0.10 ± 0.32	1.37 ± 2.07	1.76 ± 0.59

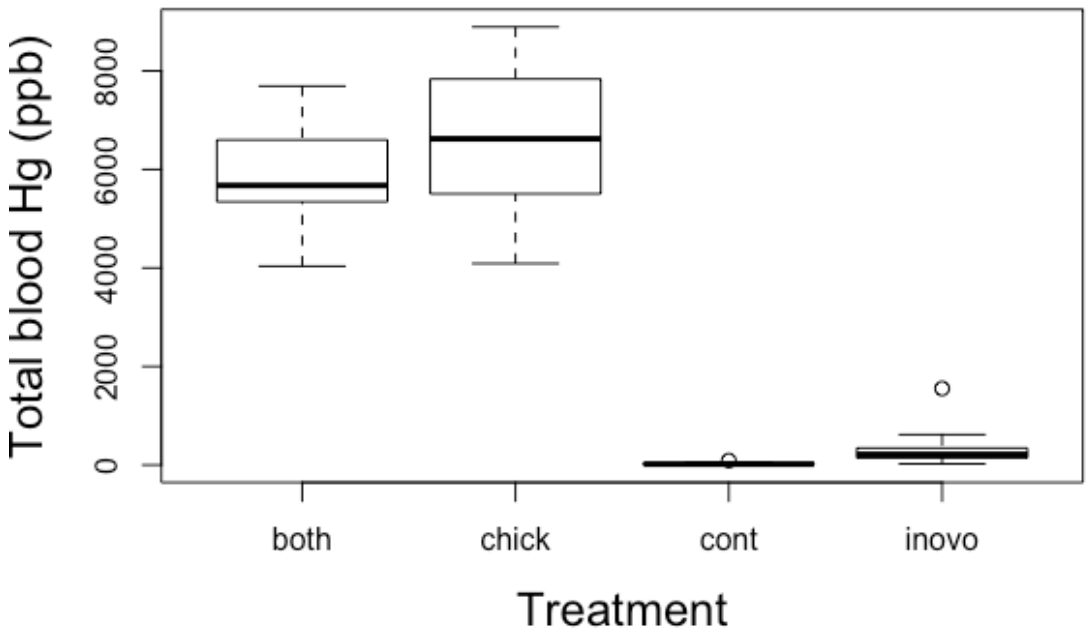

Figure A1. **Boxplots showing blood Hg concentrations at 30 days post-hatch for the four treatment groups. The thick line represents the median blood Hg concentration, and the outer squares of the boxplot represent the and 1st and 3rd quartiles.**