Investigating potential growth, behavioural, and reproductive effects of nestling exposure to methylmercury in Zebra Finches (*Taeniopygia guttata*)

by

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Ethics Statement

The author, whose name appears on the title page of this work, has obtained, for the research described in this work, either:

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Abstract

Methylmercury is a widespread contaminant that has been shown in multiple studies to cause behavioural and reproductive effects on piscivorous birds. Previously, it was thought that non-aquatic birds (such as passerines) were not at risk for methylmercury toxicity. However, in recent years high blood mercury levels have been found in free-living passerines. In the current study, zebra finch (*Taeniopygia* guttata) chicks were treated with methylmercury during the nestling stage of early development to simulate exposure from food provisioning by the parents. Despite a dose response relationship shown in the blood mercury analyses, no effects of dose were found for growth, development, or behaviour of the chicks. No long-term effects were seen on male courtship and song or female reproductive success. The lack of treatment effects in these experiments indicates that the nesting stage may be less sensitive in passerines, possibly due the sequestration of mercury into growing feathers.

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

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List of Acronyms

bw	Body Weight
ddH_2O	Double Deionized Water
Hg	Mercury
Hg[0]	Elemental Mercury
Hg[II]	Oxidized Elemental Mercury
Hg[P]	Particulate Elemental Mercury
MeHg	Methylmercury
ppm	Parts Per Million
µg	Microgram
µl	Microlitre
ww	Wet Weight

Chapter 1. Introduction

Mercury is a naturally occurring element found below the Earth's surface that can enter the atmosphere naturally through both volcanic and geological activity [1]. However, this natural emission of mercury is now relatively minor compared to anthropogenic sources such as fossil fuel combustion, cement production, metal production, and waste incineration [1,2]. For example, it was estimated in 2010 that 2300 tonnes of mercury are emitted into the atmosphere globally each year from anthropogenic sources, which can be compared to 90 tonnes from volcanoes and geological activity [3]. In terms of anthropogenic emissions, the biggest contributors are power plants driven by fossil fuels which contribute approximately 810 tonnes per year, small scale artisanal gold mining with 400 tonnes per year and manufacturing of non- ferrous metals with 310 tonnes per year [3]. The production of cement and caustic soda along with waste incineration are also major contributors by releasing 236, 187, and 163 tonnes of mercury respectively each year [3]. Though there has been movement towards mercury emission reduction (the 2013 Minamata Convention on Mercury for example), mercury emissions apparently continue to rise [4].

Mercury has multiple chemical forms. Elemental mercury (Hg[0]) is the form released by both natural sources and anthropogenic sources [1]. Its oxidized form (Hg[II]), and particulate matter associated elemental mercury (Hg[P]) are emitted by anthropogenic sources only [1]. These three forms of elemental mercury are the most abundant species found in the Earth's atmosphere [1]. These mercury species are persistent in the atmosphere and this persistence allows them to travel worldwide via atmospheric routes and be deposited in areas far away from point source emissions and contaminated sites [5]. Elemental and oxidated mercury along with particulate mercury in the atmosphere can then be deposited into soil or water through precipitation or dry deposition [1]. Oxidized (Hg[II]) and particulate associated (Hg[P]) are more likely to be deposited from the atmosphere due to their higher water solubility compared to elemental mercury (Hg[0]) [1].

Methylation of oxidized mercury to form methylmercury (MeHg) then occurs in anoxic environments mediated by sulphate reducing bacteria in soil and sediments [1,5,6]. Methylmercury is known to bioaccumulate and biomagnify in food chains. That means that methylmercury can accumulate in organisms and its concentration can increase as trophic level increases [6,7].

Neurotoxic and Developmental Effects of Methylmercury in Non-Avian Species

Methylmercury is the most toxic and bioavailable form of mercury found in the environment and targets the central nervous system [1,6–9]. It moves throughout the body as a complex with cysteine which is similar in structure to methionine and therefore gains easy access into cells via the large amino acid carrier [6]. This cell access allows methylmercury to cross the blood-brain barrier [9,10]. In adults, methylmercury toxicity is fairly selective in that only certain parts of the brain show damage [11]. These areas include the visual cortex and the cerebellum [11]. Damage to these areas causes symptoms such as visual impairment (most commonly constriction of the visual field), impaired senses in the hands and feet, muscle weakness, hearing loss, and tremors [11]

In young organisms, infiltration of the brain by methylmercury can be of particular concern due to the increased sensitivity of the brain to neurotoxins during development [12]. Methylmercury exposure of the developing organism can cause widespread effects on many regions of the brain, unlike the adult brain which shows more specified damage [11]. Exposure to methylmercury during foetal development has been shown to cause declines in motor and sensory abilities (especially vision impairment) as well as altered behaviours [9,11]. This is likely due to brain lesions, degradation of the spinal cord, and other dysfunctions in the central nervous system [9] In mammals, it has been shown that this early methylmercury exposure can negatively impact the developing hippocampus (associated with learning and memory) and these effects may not be seen until later in life [12]. This is supported by the finding that in rats exposed to methylmercury during gestation, the hippocampus showed the highest mercury content (when corrected for tissue weight) compared to the cerebrum [13].

Studies in Non-Passerine Species

The methylation of mercury to give methylmercury occurs at high rates in wetlands and lake sediments where particular sulphate and iron reducing bacteria are present [1]. Due to this high rate of methylation in aquatic ecosystems, it has been long thought that animals in those areas were at highest risk for methylmercury poisoning. That increased risk extended to birds inhabiting aquatic systems, particularly birds that eat fish (piscivorous birds) due to methylmercury's high ability to biomagnify. These high risks of methylmercury poisoning have previously driven the avian methylmercury research towards a focus on piscivorous birds. Death is a common endpoint of high methylmercury exposure while at sublethal levels, methylmercury can cause decreased reproductive success due to embryonic death, eggshell thinning, reduced clutch size, and abnormal chick behaviour [9].

Multiple correlational studies have found potential links between mercury tissue concentrations and detrimental effects. For example, great egret (*Ardea alba*) deaths due to chronic diseases in southern Florida have been correlated with high liver mercury levels (2.9-59.4 ppm) [14]. That study showed that liver mercury levels in the egrets who were found to have suffered acute death (trauma, poisoning) were much lower (0.6-4.0 ppm) compared to those dying from chronic diseases [14]. It is important to note that in that study only birds that died were studied and therefore the results cannot be extrapolated to the sublethal level [14]. Also in Florida (the Everglades more specifically), wading bird populations have been decreasing due to habitat loss, but high mercury contamination in the area may also be to blame [15]. That study also found that liver mercury burdens are higher in birds living in more contaminated areas, those that have more fish in their diet, and that adults and post-moulting chicks had higher mercury levels than chicks which had not yet moulted [15].

One study conducted in the New York Bight showed that egg mercury levels in snowy egrets (*Egretta thula*), black skimmers (*Rynchops niger*), common terns (*Sterna hirundo*), Forster's terns (*Sterna forsteri*), roseate terns (*Sterna dougallii*), and herring gulls (*Larus* argentatus) in addition to feather mercury levels in great egrets, snowy egrets, black skimmers, common terns, and herring gulls exceeded laboratory derived values at the time (1997) associated with detrimental effects (0.5 ppm in eggs, 5 ppm in feathers)

[16]. The authors speculated that declines in reproductive success of birds in the area may be due to this association [16].

Common loons (*Gavia* immer) breeding in areas with high mercury contamination have shown evidence of reduced reproductive success [17]. These reductions in reproductive success in the loons with high mercury loads were due to decreased time spent on the nest and lethargy (therefore, reduced ability to feed chicks) of the parents, which resulted in fewer chicks fledged when compared to those with lower mercury loads [17]. Reduced reproductive success may also be attributed to feather asymmetry [17]. Birds with abnormal feather growth will likely use more energy during migration and therefore may have difficulty maintaining territory and rearing young during the breeding season [17]. A long term study of common loons living in Adirondack Park (New York), which have shown decreased productivity, indicated that 21% of males and 8% of females had blood mercury concentrations that correlate with high risk of behavioural and reproductive detriments [18]. In males, the average blood mercury concentration was 2.16 µg per g while females had an average of 1.72 µg per g [18]. The authors determined that the reason for the differences in blood mercury concentrations between sexes is due to the ability for females to depurate the mercury into eggs as they are formed [18].

A number of lab studies of potential effects of methylmercury exposure in piscivorous birds have been conducted. For example, in great egrets, dietary methylmercury exposure from days 12 to 105 post hatch has been shown to impact general activities and maintenance behaviours [19]. That included a significant decrease in standing and perching activity in high dose group birds (5 mg methylmercury chloride per kg of food) compared to those in the low (0.5 mg per kg of food) and control (no methylmercury) groups [19]. Those in the high dose group also spend less time on feather maintenance and tended to keep their heads down or under their wings [19] It was speculated that this may be due to loss of coordination and motor control in the high dose group birds [19]. In another study, it was shown that great egret chicks fed fish containing methylmercury (0.5 or 5 mg MeHgCl per kg fish weight) had decreased appetite and subsequently suffered weight reduction [20]. A multigenerational study in mallards (*Anas platyrhynchos*) showed that females fed methylmercury (0.5 ppm in food) tended to lay a higher proportion of their eggs outside of the nest box compared to controls not fed

methylmercury [21]. Those females also had lower hatching success and their eggs had thinner shells than those produced by control females [21]. That study also showed that ducklings from parents fed 3 ppm methylmercury in food tended to have an increased avoidance response to frightening stimuli [22]. Similar reproductive results were shown in black ducks treated with 3 ppm of methylmercury in their food [23]. It was found that females in the methylmercury treatment group laid fewer eggs and incubated less eggs [23]. There was also reduced hatching success in eggs from treated mothers and lower duckling survivability compared to the control group [23].

Raptors can be exposed to methylmercury by consuming fish, but raptors that do not consume fish may also be at risk. Methylmercury poisoning in raptors can occur from catching prey items in fields with mercury-treated seeds [24]. In American kestrels (*Falco sparverius*), diets high in methylmercury (3 ppm, 6 ppm, or 12 ppm oil solution mixed with meat 1% ww) have been shown to cause poor balance, incoordination, and death [24]. It has also been shown that American kestrels fed a diet supplemented with mercury (0.6, 1.7, 2.8, 3.9, or 5 mg per kg food) had impaired reproduction including reduced clutch size, decreased incubation, reduced hatching success, and a lower percentage of chicks which survived to fledging [25]

New Realizations: Methylmercury Exposure Passerines

Methylmercury has previously been primarily associated with aquatic systems and therefore much research has been done on effects of methylmercury exposure in aquatic and piscivorous birds [26–28]. However, more recent studies have found high concentrations of methylmercury (similar levels to piscivorous in contaminated sites) in passerines, including those not associated with aquatic systems [29,30]. For example, blood mercury concentrations in Bicknell's Thrushes (*Catharus bicknelli*) living in north-eastern North America showed mean blood mercury concentrations ranging from 0.03 to 0.42 µg per g (ww) [31]. Spiders [26,32], dragonflies [33], black flies [34] can be a source of methylmercury in passerine diets, especially in areas of with high mercury contamination Studies have also found evidence for methylmercury bioaccumulation within a Montane forest ecosystem, which is not considered an aquatic system [35].

Correlational field studies are currently limited due to the recent scientific interest in the consequences of methylmercury exposure in passerines. Wild female tree swallows (*Tachycineta bicolor*) living in mercury contaminated sites in Virginia showed average blood mercury levels of 3 ppm while those in reference sites showed only 0.17 ppm average blood mercury levels [36]. Those elevated blood mercury levels were associated with smaller eggs and a decreased number of fledglings but clutch size, date to first lay, and egg hatchability were not different between contaminated and reference females [36]. Similar results have been shown in Carolina Wrens (*Thryothorus ludovicianus*) also found in contaminated sites in Virginia. Mean blood mercury concentrations ranged from 1.96-3.38 µg per g (ww) in females and 1.07-3.27 µg per g (ww) in males found in contaminated sites compared to reference sites which showed mean mercury concentrations ranging from 0.21-0.48 µg per g (ww) in females and 0.18-0.34 µg per g (ww) in males [27]. Those differences in mean blood mercury concentrations were associated with a 34% decrease in nest success for wrens breeding in contaminated sites compared to reference sites [27].

In oscine passerines (those who learn their song), apparent differences in male songs between birds living in mercury contaminated sites when compared to those living in reference sites has been reported [8,37]. More specifically, one study in wild Carolina wrens, house wrens, and song sparrows showed that those living in the contaminated sites had lower diversity of notes and lower tonal frequency [8]. The researchers speculated that may be due to hearing impairment, which has been shown in lab mammal studies, but direct evidence for this is lacking [8]. Those results are contrary to the findings of a study in Nelson's sparrows (*Ammodramus nelsoni*), which showed that males with a higher blood mercury concentration tended to have higher tonal frequency songs and also sang faster [37]. It was speculated that differences in song may occur due to learning detriments [37], unlike the previously mentioned study which attributed it to potential hearing impairments [8]. These differing results indicate that the effects of methylmercury on male song are complex and may differ between species. Regardless of the type of effects and their mechanisms, effects on male song can be detrimental to a bird's reproductive success because song is the main mode of communication used to attract females and defend territories [37,38].

Due to the recent realization that passerines are highly exposed to methylmercury, there are few lab studies currently published on the subject. Most studies focus on non-behavioural endpoints. For example, a study in zebra finches (*Taeniopygia guttata*) looking at immune response showed that birds exposed to methylmercury had decreased immune function [39]. In another adult zebra finch study, lifetime methylmercury exposure was associated with decreased corticosterone stress response [40]. Decreased flight performance has been shown in European Starlings exposed to methylmercury [41]. Reproductive success in captive zebra finches exposed to methylmercury has been studied previously. In adult and lifetime studies, methylmercury exposure was shown to reduce reproductive success [42]. That included a reduction in number of chicks which reached independence, decreased hatching and fledging success, and a longer latency to re-nest [42]. That study did not show a relationship between methylmercury exposure and clutch size [42]. It was also shown that reproductive success was reduced to a greater extent in birds treated during their entire lifetime compared to those treated during adulthood only [42]. That finding reinforces the notion that the birds are most sensitive to methylmercury during development [42].

Zebra Finches (*Taeniopygia guttata*) as an Avian Model for Toxicological Studies

Zebra finches (*Taeniopygia guttata*) are a passerine species used very commonly for lab studies in birds, mainly due to many factors related to their life history and general behaviour. For example, zebra finches will breed continuously once they reach sexual maturity (90 days of age) as long as ample resources are present. That allows researchers to conduct reproductive studies year round as well as breed multiple generations in a relatively short time [42]. These birds are also fairly tolerant of handling which can reduce the impact of experimental manipulation. Zebra finches are highly gregarious which allows for efficient use of space in zebra finch colonies because multiple birds of the same sex can be housed in one cage without incident.

Goals of the Current Research Project

The goal of the Williams Lab is to determine what effects in passerines (with zebra finches as a model) result from methylmercury exposure at different life stages. A previous

study was conducted using egg injections of methylmercury to simulate maternal transfer in zebra finches as a model passerine. The results of that study showed decreased hatching success of eggs injected with methylmercury compared to vehicle control injected eggs [43]. However, those chicks that did survive showed no long term effects on growth or reproduction [43]. These long term results were somewhat surprising considering reproductive effects have been shown in lifetime zebra finch studies [42]. This has lead to the current research project to look at effects at other life stages. This thesis will discuss the present Master of Environmental Toxicology research project that investigates potential effects of methylmercury exposure during the nestling stage (from hatching to 21 days post-hatching. Dosing at this stage simulates exposure to chicks due to food provisioning from the parents. Chicks were weighed and standard measurements were made (bill, tarsus, wing) throughout the nesting period to determine if the methylmercury exposure has any effects on nestling growth. Fledge date and, age of self-feeding, and fledgling feeding behaviour were also investigated to determine if there are effects on chick maturation and learning. Once the chicks reached sexual maturity, reproductive trials were done with the females to investigate potential long-term reproductive effects. Males were used in mating trials and their songs were analyzed to determine whether there have been long-term effects on courtship behaviour or song.

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Chapter 2. Data Chapter

Introduction

Mercury is a common and widespread pollutant released into the atmosphere in large amounts worldwide from anthropogenic activities [1,2]. Atmospheric mercury can be deposited into aquatic systems where bacteria in the sediments transform it into methylmercury, the most toxic form [1,3,4]. Methylmercury is highly bioaccumulative and also biomagnifies in food chains [4,5]. Effects of methylmercury exposure in aquatic and piscivorous birds have been studied to a great extent [6,7]. Many effects of methylmercury exposure have been observed including neurotoxicity (loss of balance, loss of coordination, etc.), behavioural changes, and reproductive impairment [8–19]. It was previously thought that land dwelling birds such as passerines (songbirds) were not a risk for methylmercury toxicity. However, high concentrations of mercury have been found in the blood of passerines in more recent studies [20,21]. For example, mean blood mercury measurements in Bicknell's Thrushes living in north-eastern North America have been shown to range from 0.03 to 0.42 µg per g (ww) [22]. This relatively recent realization that passerines may be at risk for toxicity due to methylmercury contamination has opened up an entirely new area of avian mercury research. However, this also means that the number of studies published on the subject is significantly lower compared to the plethora of methylmercury research in aquatic and piscivorous birds.

Field studies have reported correlations between high female blood mercury concentration and reduced reproductive success in tree swallows [23]. A similar result was also shown in Carolina wrens, with elevated mean blood mercury concentrations being associated with a 34% decrease in nest success when comparing birds breeding in contaminated sites to those in reference sites [7]. It has also been shown in multiple species (Carolina wrens, house wrens, song sparrows, and Nelson's sparrows) that male song differs between those living in contaminated sites compared to those in reference

sites [24,25]. These differences in contaminated males included lower diversity of notes and lower tonal frequency in the Carolina wrens, house wrens, and song sparrows while the Nelson's sparrows showed higher tonal frequency and also sang faster [24,25]. It was speculated that those differences may be due to hearing impairment [24] or learning detriments [25].

In terms of lab studies, most of the previously published research in passerines has focused on lifetime exposure studies or adult only exposure studies. In zebra finch studies, both adult and lifetime exposure to methylmercury has been shown to decrease reproductive success [26]. There is a significant lack of lab studies focusing on early exposure to methylmercury in passerines. This is somewhat surprising because there is evidence that developmental mercury exposure has been shown to cause multiple effects such as impaired behaviour, motor and sensory abilities [27,28]. For example, in mallards it has been shown that ducklings whose parents were fed mercury in their food showed an increase in their avoidance response to frightening stimuli compared to ducklings from clean parents [29]. In mammals, there is a significant amount of evidence that early exposure to methylmercury targets the hippocampus, which is responsible for learning and memory [30–33]. It is thought that the hippocampus in birds serves a similar function [34–37]. In zebra finches, studies have shown that the hippocampus may also be involved in song learning [38]. It has also been shown that imprinting (recognition of parents) [39], and sexual imprinting (i.e. learning what to look for in an ideal mate) may be associated with the hippocampus [34].

A study conducted previously in the same lab focused on early developmental effects by focusing on embryonic exposure to methylmercury using egg injections as a proxy for maternal transfer [40]. The results of that study showed decreased hatching success with *in ovo* methylmercury exposure [40]. However, for those chicks that did hatch, no long term effects on growth and reproduction were found [40]. Based on the literature review, no studies involving post-hatching early exposure to methylmercury in passerines have been conduced. However, this type of study has been conducted previously with zebra finch chicks being orally treated with a brominated flame retardant, BDE-99 [41]. In that study, chicks were treated with BDE-99 orally with a micropipette from 1-21 days post hatching (nestling stage) [41].

The goal of the present research project was to investigate the impacts of oral methylmercury exposure to the chicks from days 1-21 post-hatching (nestling stage). More specifically, growth, development, and behaviour were measured for the chicks until 30 days of age (independence). Once chicks reached sexual maturity (90 days post-hatching), courtship trials and song analyses were conducted with the males while reproductive success was measured in the females. It was expected that differences would be observed in at least one of these parameters (growth, development, behaviour, courtship, song, and reproduction) when comparing birds treated with methylmercury to control birds.

Materials and Methods

Zebra Finch Husbandry

This research project was conducted in the Animal Care Facility at Simon Fraser University in Burnaby, British Columbia where a well-established breeding zebra finch colony has been housed since 1994. Non-breeding birds were held in single-sex double sized cages (100 x 39 x 43 cm) and provided with mixed seeds (panicum 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*. A multivitamin supplement was added to the regular drinking water once per week. Animal housing rooms are regulated to maintain a consistent temperature (19-23°C) and humidity (35-55%) with a constant photoperiod of 14L:10D. All experimental work was conducted under a Simon Fraser University Animal Committee Permit (1070B-08) according to the guidelines of the Canadian Committee on Animal Care.

Zebra Finch Breeding Protocols

Male and female zebra finches were paired at random in May 2015. Preference was given to experienced birds (those that had successfully reared chicks in the past). However, there was a shortage of experienced birds during this breeding period and therefore inexperienced birds were also used. Breeding pairs were housed in single cages (51 x 39 x 43 cm) with a cardboard nest box (14 x 14.5 x 20 cm) attached to the outside of the cage. Nest boxes were filled with hay for nest building materials. Birds were given

access to seeds (as above), water, grit, and cuttlebone *ad libitum* and vitamin supplemented water was also provided once per week. During breeding, zebra finch pairs were supplemented with egg food (mash of hard boiled chicken eggs including the shells, breadcrumbs, and cornmeal; 20.3% protein, 6.6% lipid) during the entire breeding process (pairing until chicks were 30 days of age). Pairs that did not successfully lay and incubate eggs in the nest within 15 days of pairing were separated and females re-paired with a new male.

Nest boxes were monitored daily for egg laying, and upon laying all new eggs were weighed (± 0.01 g) and numbered with a fine tip marker for easy recognition. Nest boxes were checked twice daily (morning and afternoon) starting at 12 days post first egg to monitor for hatching. Parent birds were weighed at pairing, the day the first egg was laid, at clutch completion (fourth consecutive day without a new egg), the day the first chick hatched, day 21 post-hatching and day 30 post-hatching in order to monitor mass change.

MeHg Solution Preparation

The target doses for this experiment were based on those used in a lifetime lab zebra finch study in which the methylmercury was administered in the seeds [26]. In that study, four treatment groups were used [26]. Low to moderate doses of 3.0 and 6.0 ppm were used to simulate the amount of mercury found in prey insects, a high dose of 1.2 ppm was used to simulate prey items in a “worst-case-scenario” contaminated site, and a 2.4 ppm dose was used as a very high dose, comparative to exposure that some wild aquatic birds would experience [26]. Since chicks in the present study were treated directly, only three treatments total (two mercury treatments and one control) were used in order to have better assurance that each nest would have at least one chick from each treatment group to control for between nest variation. The 6.0 and 1.2 ppm treatments from the aforementioned study were chosen to be replicated. The highest dose (2.4 ppm) was not used because it is not comparable to the exposure expected in free-living passerines. The doses used in the lifetime exposure study were mixed into the seeds and therefore required conversion to a daily amount using the seed consumption rate for zebra finches. The amount of seeds consumed per day was calculated as 3.31 grams per day by taking an average of the food consumption values reported in multiple published papers [42–44]. In order to determine the seed consumption as a rate per gram of body mass, an

average zebra finch body mass of 14.59 grams was used based on measurements of zebra finches in the colony at Simon Fraser University. Together, these values (seed consumption rate/body mass or 3.31g/14.59g) gave an approximate daily seed consumption rate of 0.23 grams of seed per gram of body weight per day. This could then be multiplied by the seed concentration used in the lifetime zebra finch study to give an approximate daily dose of mercury. Next, the target solution concentrations needed to be obtained. The high dose (corresponding to 1.2 ppm in seeds) was calculated as 0.54 µg Hg per µl and the low dose (corresponding to 6.0 ppm in seeds) was calculated as 0.27 µg Hg per µl. For solution preparation, methylmercury (II) chloride (MeHg chloride, PESTANAL ®, analytical standard from Sigma-Aldrich) was dissolved in double deionized water. The actual mercury concentrations in the solutions were 0.063 and 0.15 µg mercury per µl, confirmed as total mercury concentrations (THg) at the lab of Dr. Nil Basu in the Faculty of Agricultural and Environmental Sciences at McGill University in Montreal QC, Canada using EPA Method 7473 (practical mercury detection limit = 0.0534 µg Hg per µl). These concentrations were lower than expected (0.27 and 0.54 µg mercury per µl for low and high respectively). This represents 28% of the expected mercury concentration for the high dose solution and 23% of the expected mercury concentraiton for the low dose solution. Solutions were prepared and stored in autoclaved glassware that had been chemically cleaned (washed with acetone and hexane three times each) prior to use. During storage, glass vials were covered with aluminum foil (also autoclaved and chemically cleaned) in order to prevent photodegradation of the solutions.

MeHg Dosing

Chicks were randomly assigned either to a) control: double deionized water, b) low MeHg: 0.126 µg Hg per gram bw per day, or c) high MeHg: 0.30 µg Hg per gram bw per day) within each nest using a random number generator (The Random Number Generator App for iPhone 5S, Nicholas Dean). Each nest contained at least one chick per treatment before treatments were repeated within a nest, and treatments were randomized among hatch order (i.e. roughly equal numbers of each treatment for first hatched, second hatched, etc). At hatching, each chick was marked by plucking down feathers on different areas of the body for individual identification within each nest. Chicks were removed from the nest box and weighed daily (± 0.01 g) and the amount of original dosing solution

administered was calculated as 0.5 µl per gram body weight per day. Dosing solutions or vehicle were administered by pipetting the solution directly into the chick's mouth and chicks were dosed from days 1 to 21 post-hatching. Chicks were then returned to the home nest box once dosing was complete. Dosing methods have been used previously in a toxicological study with zebra finches [41].

Chick Growth Measurements

Chicks were weighed (± 0.01 g) daily starting on the day of hatching. Initially plastic weigh boats were used to weigh young chicks but once chicks were fully feathered and able to fly (therefore would just fly out of the weigh boat), they were weighed in a felt bag. Tarsus and bill lengths were measured on days 0, 5, 10, 15, 21, and 30 post-hatch using digital callipers (± 0.01 mm) and wing measurements were conducted on the same days. Prior to feather growth, wing measurements were done from the "elbow" to the end of the phalanges. Once feathers had grown in, standard wing measurements ("shoulder" to tip of longest primary feather) were used. The ninth primary (P9) was measured on days 15, 21, and 30 if the feather was present. Chicks were banded with an aluminum band with a unique identification number on the right leg at approximately 10 days of age and at the same time chicks were also banded with two colour bands on the right leg for easy identification during behavioural observations. Colour band combinations were unique within the nest but due to limited colours, combinations were repeated within the experimental colony.

Timing of Fledging, Development of Feeding Behaviour and Neophobia

Once chicks reached 13 days post-hatching, breeding cages were observed daily to check for fledglings. Fledge date, defined as the first time a chick left the nest box, was recorded which allowed for fledge age to be determined. If a chick was seen out of the nest box one day but back in the nest the next day, this was recorded. The final fledge date was recorded as the date after which the chick was not seen back in the nest box on subsequent days and this was the date used to determine the fledge age. There were times when chicks "force fledged" during removal from the nest for dosing, meaning the chicks flew out of the nest box before they were truly ready due to being disturbed. These "force fledge" events were not recorded as the fledge date. Chicks that "force fledged"

were returned to the nest box and the opening was covered for a short period of time in order to let them recover and avoid flying out again. Extra seeds were added to the cage in small dishes on the floor to ensure chicks that were initially unable to use the regular seed feeder had access to seeds.

After the first chick from each nest had fledged, the chicks were monitored to determine development of feeding behaviour, defined as the date each chick was self-feeding. The chicks were observed for 5 minutes right after the egg food was replenished in the cage each day and the date that each chick was first observed self-feeding (eating from the egg food on their own) was recorded. This allowed for an age of self-feeding to be determined.

In general, when all chicks in the nest had been seen self-feeding, further feeding behavioural observations began. The only exception to this occurred when older chicks in the nest were approaching 30 days of age (independence) but the younger chicks were not self feeding yet. Feeding behavioural observations were done prior to all chicks self feeding in order to obtain feeding behaviour data for the older chicks. Feeding behavioural observations occurred on at least two consecutive days. On the first day, the latency to feed of each chick was recorded: egg food was placed in the cage, chicks were then observed for 5 minutes and the time taken for each chick to begin feeding was recorded (stopwatch on iPhone 5S used, Apple Inc). Chicks were identified in each cage using their coloured leg bands. On the second day of observations, the effect of neophobia (fear of novel objects) on feeding behaviour was measured. In these trials, a novel object (either a balloon or a plastic toy beetle) was attached to the egg food dish. The chicks were again observed for 5 minutes after the food dish was added to the cage. The original plan was to record how long each chick took to approach the food when the novel object was attached. However, no chicks (or adults for that matter) approached the food dish while the novel object was attached. Instead, the time for each chick to approach the food dish after the novel object was removed was recorded. If the chicks in each nest were all self-feeding early enough, these paired trials (day 1 of latency to feed and day 2 of latency to feed post removal of a novel object) were repeated until the oldest chicks reached independence (30 days post-hatching).

Blood Sampling and Blood Mercury Analysis

Blood samples were collected at 30 days of age from the brachial vein of the wing into heparinized capillary tubes. A maximum volume of 1% of the body weight was collected from each bird (approximately 2 capillary tubes full). Blood was transferred to heparinized centrifuge tubes and promptly frozen at -20°C until analysis. Whole blood samples were analysed for total mercury (THg) by Marie Perkins in the lab of Dr. Nil Basu in the Faculty of Agricultural and Environmental Sciences at McGill University in Montreal QC, Canada. Using EPA Method 7473 (practical mercury detection limit = 0.1867 ppm). Total blood mercury values for each bird can be found in Appendix A along with total mercury dose and an estimate of the total body mercury. Estimated body mercury was obtained by multiplying the bird's blood mercury measurement by its 30 day mass (i.e. mass at time of blood collection). Summary statistics of these values by treatment can be found in Table 2.1. This is an approximation based on an assumption that that blood mercury and body mercury are similar [45]. However, it is important to note that this is a very rough estimate and these calculations are used to confirm that the results of the dosing solution and blood analyses make sense rather than to accurately determine mercury body burden [46].

From Independence to Sexual Maturity

Once chicks reached 30 days of age, they were considered "independent" and removed from the breeding cage, 30 day measurements were taken (see chick growth measurements for details) and blood samples were collected (see blood sampling section). Chicks were then placed in regular double cages (100 x 39 x 43 cm) with an adult male tutor to enhance song learning. Small dishes with extra seeds were added to the bottom of the cages to ensure those that had not learned to use the regular seed feeder had access to seeds. At this time, chicks cannot be definitively sexed and therefore the cages were mixed sex. Once all chicks had reached approximately 60 days of age (approximately when they show their full adult plumage), they were sorted into single sex cages. At this point, only the male chicks required an adult tutor. It was assumed that by this point all chicks had learned to use the regular seed feeders and therefore did not need extra seeds in the bottom of the cage. Chicks were weighed (± 0.01 g) and their tarsus, bill, wing, and P9 measurements recorded (± 0.01 mm) again at 90 days of age, i.e. at

sexual maturation, and subsequently reproduction experiments to assess adult phenotypic quality were conducted: mating or courtship trials in males and breeding in females (see below).

Male Courtship Behaviour

During courtship trials, the males were housed in a room separate from the main colony in the room adjoining to the trial room together with 14 clean (not dosed with methylmercury) experienced females that were used for the male courtship trials. Each female was only used once per day but the same female was used in trials on subsequent days. It was ensured that males were not paired with their mother and that a different female was used when the same male was used for multiple trials, and courtship trials were conducted blind to treatment. Courtship trials were conducted in a single half cage (50 x 39 x 43 cm) with a single water fountain and grit *ad libitum*. A female was chosen at random and introduced into 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 pair's behaviour was observed from a distance (approximately 5 feet away) for 10 minutes. During this time, courtship behaviour of the male was 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), and e) whether the male sang (yes or no). Whether the male invited the female to court was recorded simply as whether he did any of the previously mentioned behaviours or not. 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.

Song Analysis

For each mating trial in which the male sang, the sonogram recordings were analyzed using Syrinx-PC software (version 2.6h, J. Burt, Seattle WA) based on methods used by Airey and DeVooged [47]. A laboratory assistant worked on this portion of the analysis and was blind to each bird's treatment prior to statistical analysis. Ten song phrases were chosen from each recording and the song phrase duration, number of syllables per phrase, and number of unique syllables per phrase were measured for each. The average value of each measurement was taken from the 10 song phrases to give a final measurement. The number of song phrases was counted in each 10 minute recording then this was multiplied by 6 to find the number of song phrases per hour (song rate).

Female Breeding Trials

At 90+ days of age, females were paired with a random clean experienced male (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". For the remaining females (those that laid eggs within 15 days), 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) and their bill, tarsus, wing, and P9 lengths (± 0.01 mm) were recorded at 21 and 30 days post-hatching (average age for chicks in the nest). Chicks were sexed between days 30 and 60 post-hatching in order to determine sex ratio of each nest.

Statistical Analysis

The mean blood mercury concentration of each treatment group was compared using a single-factor completely random design ANOVA.

Mean chick mass at days 0, 5, 10, 15, 21, 30, and 90 post-hatching were compared for each treatment using linear mixed-effects models, correcting for tarsus (covariate) and blocking by nest (random factor). Fledge age, age of self feeding, latency to feed, and latency to feed after removal of a novel object were all non-normally distributed and

therefore a Kruskal-Wallace (nonparametric) test was used to compare these measurements between treatments.

For the male mating trials, only those that invited the female to court (i.e. performed a courtship behaviour) were used in the analysis. As with the chick behaviour, none of the variables measured (number of bill wipes, number of follows, time to first mount, number of unsuccessful mounts, number of successful mounts, female response) were normally distributed and therefore a Kruskal-Wallace test was also used to compare these measurements between treatments.

Single-factor completely randomized design ANOVAs were used to compare phrase duration, number of syllables, number of unique syllables, and song rate (phrases per hour) between the males of each treatment group. Correlations were also done to determine if these measures of song quality correlate with one another as well as with the male courtship behaviours mentioned previously.

For the female breeding trials, the proportion of females that laid eggs was compared among the treatments using a Pearson Chi-Square analysis. Further analyses were then conducted including all females except those that did not lay eggs (non-breeders). Single-factor completely random design ANOVAS were used to compare the lay interval, clutch size, average egg mass, brood size at hatch, and brood size at fledge between treatments. These analyses were then repeated with a subset of the females that successfully fledged chicks. For analyses of mean chick mass at days 21 and 30 post-hatching, as well as sex ratio, only those females that successfully raised chicks were included. Mean chick masses were compared between treatments using linear mixed-effects models, correcting for mean tarsus length (covariate). To determine if the sex ratio of the chicks from mothers of each treatment group differed, the proportion of female chicks in each nest was also compared using the same method. These proportions were then compared to a null hypothesis of a proportion of 0.5 (i.e. 50/50 sex ratio) using a Student's t-test to determine if the sex ratio was skewed for any of the treatment groups.

All statistical analyses were conducted in R (Version 3.2.1, packages used: nlme, lsmeans, lme4, lmerTest, multcomp, plyr, ggplot2, multcompView, MASS, Rmisc, lattice, pbkrtest).

Results

Blood Mercury

There was a significant effect of treatment on blood mercury in chicks at 30-days post-hatching ($F_2 = 333.9$, $p < 0.001$). Mean blood mercury (total mercury, THg) was 0.006 ± 0.006 ppm for control birds, 0.297 ± 0.067 ppm for low MeHg treatment group birds, and 0.734 ± 0.163 ppm for high MeHg treatment group birds (

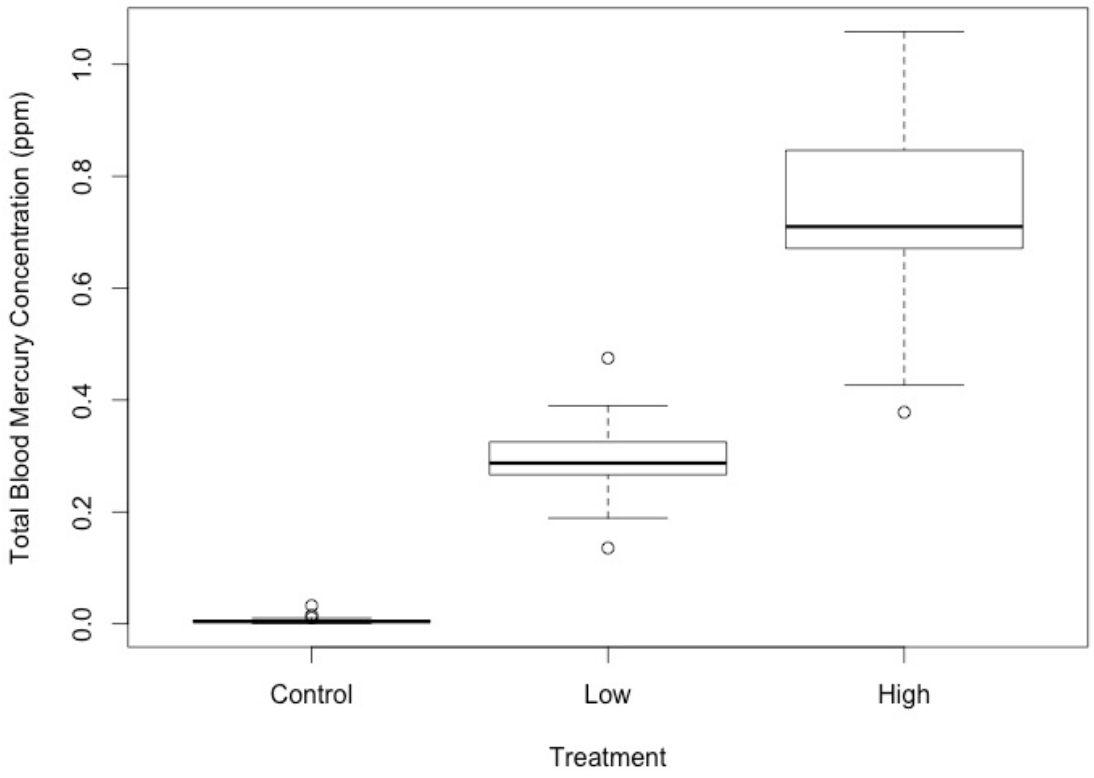

Figure 2.1). Chick Growth, Fledging, and Development of Self-feeding Behaviour There was no significant difference in average chick mass between treatments for any chick age ($P > 0.10$ in all cases; Table 2.2).

Chick Growth, Fledging, and Development of Self-Feeding Behaviour

There was no significant difference in average chick mass between treatments for any chick age ($p > 0.10$ in all cases; Figure 2.2). Figure 2.1 shows growth of each treatment group from days 0 to 90 post-hatching. There was no significant difference in

mean fledge age between treatments ($X^2 = 0.27$, D.F. =2, p = 0.88; Table 2.3) or the mean age of self-feeding between treatments ($X^2 = 0.71$, D.F. = 2, p = 0.70; Table 2.3). There was also no evidence for a difference in either latency to feed or latency to feed post removal of a novel object among treatment groups ($X^2_2 = 0.09$, D.F. = 2, p = 0.96 and $X^2 = 0.19$, D.F. = 2, p = 0.91, respectively; Table 2.3).

Male Courtship Trials and Song

There was no evidence for a difference in any of the male courtship behaviours (bill wipes, number of follows, female response, time to first mount, number of unsuccessful mounts, number of successful mounts) between treatments (Table 2.4).

There was no evidence for a difference in the phrase duration, number of syllables, number of unique syllables, or song rate in males among the three treatment groups (Table 2.5). There was a strong correlation (0.958) between the number of unique syllables and the number of syllables. There were also moderately strong correlations between phrase duration and number of syllables (0.771) and number of unique syllables (0.717). The correlations between song rate and phrase duration (0.035), number of syllables (-0.016), and number of unique syllables (-0.066) were all considered weak. Correlations between each song quality measure and all of the male courtship behaviours were also considered weak (Table 2.6).

Female Breeding Experiments

The proportion of females that laid eggs did not show evidence of differing between treatments ($X^2 = 0.04$, DF = 2, p = 0.98). Raw counts and the proportion of females in each treatment that laid eggs can be found in Table 2.7. There was no evidence for a difference between the treatment groups for mean lay interval, mean clutch size, mean egg mass, mean brood size at hatch, and mean brood size at fledge when comparing all females which laid eggs (Table 2.8). There was also no evidence for a difference among treatments in any of these measurements for females that successfully produced chicks (Table 2.9). Mean chick mass at day 21 post-hatching and mean chick mass at day 30

post-hatching did not show evidence for a difference among treatments for those females that successfully produced fledged chicks (Table 2.9). There was no evidence for a difference in the proportion of female chicks in these nests between treatments ($F_2 = 0.35$, $p = 0.71$). There was also no evidence that the proportion of female chicks in the nest was different than 0.5 (i.e. 50/50 sex ratio) for control ($t_{15} = -0.49$, $p = 0.63$), low ($t_{15} = -1.53$, $p = 0.15$), or high ($t_{15} = -0.160$, $p = 0.88$) treatment group females.

Discussion

In this research project, zebra finch chicks were treated with methylmercury during the nestling period in order to investigate potential short-term effects on growth, chick behaviour, and development, along with long-term effects on courtship behaviour, song, and female reproductive success. No evidence for treatment effects were found for any of the short-term or long-term endpoints measured. This is despite a clear dose-response effect in the total blood mercury levels of the birds and these blood mercury levels being comparable to those found in free-living juvenile birds.

Research involving methylmercury exposure in passerines is limited compared to the more extensive body of research available for aquatic and piscivorous birds. It was previously thought that land-dwelling birds, such as passerines, were not at risk for methylmercury poisoning because their diet is not dependant on aquatic systems. However, recent studies have found high amounts of blood mercury in wild passerines [20–22]. Previous lab studies dealing with methylmercury in zebra finches have focused on lifetime or adult only exposures [26,48–50]. The Williams Lab has chosen to focus on early methylmercury exposure in order to elucidate potential effects during critical developmental periods. Previously, egg injections were used to simulate maternal transfer of methylmercury. This previous research looked at embryotoxicity and potential post-hatching growth effects along with long-term effects on behaviour and reproduction [51]. Results from that research project showed evidence for reduced hatching success for eggs injected with 3.2 µg mercury per gram of egg compared to controls. However, no treatment effects were observed for chick hatching mass and growth. There were also no detectable long-term effects on courtship behaviour and female reproductive success [40,51]. This lack of detectable effects for any post-hatching endpoints from *in ovo*

exposure led to the decision to focus on the nestling stage. Treatment during this stage simulates methylmercury exposure due to contaminated food provisioned to the chicks by their parents. A treatment period of days 1 to 21 post-hatching was chosen because 21 days represents average fledge age [52]. The chicks were treated directly with the methylmercury solution via pipette in order to control the dose administered and to allow for dose randomization within each nest. Based on the literature review, this is the first time a direct-dosing method has been used to administer methylmercury to passerine chicks.

The measured mercury concentrations in the dosing solutions were much lower than the target mercury concentration. Solutions had a target concentration of 0.54 µg Hg per µl for the high dose and 0.27 µg Hg per µl. However, chemical analysis of the solutions revealed the actual concentrations as 0.15 µg Hg per µl and 0.063 µg Hg per µl for high and low doses, respectively. That corresponds to 28% of the expected concentration in the high dose and a 23% of the expected concentration in the low dose. Previous studies have reported difficulties in obtaining highly accurate analysis of organic mercury solutions is difficult due to solution instability [53,54]. It is thought that mercury will tend to sorb to solution containers and can also be degraded by exposure to light [53,54].

Though the mercury recovery in the dosing solutions was lower than expected, the total blood mercury measurements obtained from the chicks in this experiment are reasonable. There is a clear dose response, with the comparison of the total blood mercury levels between the treatments being statistically significant. The measured values are also within the range of total blood mercury concentrations found in some free-living juvenile birds in mercury contaminated environments. For example, samples from juvenile common loons collected in various North American locations had blood mercury concentrations ranging from 0.3 ppm to 0.78 ppm [55]. The treated chicks in the present study had blood mercury concentrations ranging from 0.136 ppm to 1.059 ppm, meaning that the values found in the juvenile loons were encompassed. This is significant because common loons are aquatic birds feeding at a reasonably high trophic level, meaning that they are expected on average to have a higher mercury burden than a land-dwelling passerine. As a passerine example, nestling red-winged blackbirds living in areas with historical urban, industrial, and agricultural pollution showed blood mercury concentrations

ranging from 0.009 ppm to 0.284 ppm [56]. The total blood mercury values obtained for the low treatment chicks alone ranged from 0.136 ppm to 0.475 ppm, which is on the high end of the range found in the wild red-winged blackbird chicks. Interestingly, the red-winged blackbird chicks also did not show evidence of having growth impairment due to their blood mercury concentrations [56]. The results of the present lab study may help reinforce the results of the red-winged blackbird field study. It is possible that passerine chicks may not be particularity sensitive to mercury exposure. In birds, it is thought that a majority of the methylmercury excretion occurs in growing feathers [14,18,45,57]. This is particularly important in juveniles because methylmercury they are exposed to during feather growth is deposited into the feathers [13] and is therefore not as readily available to cause toxicity to the developing brain. However, this would only be true up to time of fledging when feather growth decreases [13,57]. Field measurements of tree swallows in Virginia have supported this by showing the nestlings have blood mercury concentrations an order of magnitude lower than adults in the same area [23]. Based on this known excretion route, it is suggested that the chicks in the present study did not show detectable short or long-term effect because the mercury was being diverted from the bloodstream into the feathers.

In this study, 30 day blood mercury levels achieved were an order of magnitude higher than those obtained in the previous egg injection study (0.297 ppm and 0.734 ppm in the present study, 0.0081 ppm and 0.066 ppm in the *in ovo* study for low and high doses, respectively) [40,51]. That suggests a greater likelihood of long-term effects due to the higher mean blood mercury levels achieved. Mean blood mercury levels ranged from 0.044 ppm to 1.060 ppm in a variety of terrestrial songbird species collected in diverse habitats in eastern North America [20]. However, those songbirds were said to be living in non-contaminated sites [20]. In terms of the long-term results from the present study (male courtship behaviour, song, and female reproductive success), the results are somewhat surprising because both field and lab studies have reported treatment effects of mercury exposure on those endpoints [7,23,25,26]. However, when comparing the blood mercury results from studies in adults, these results become less surprising. In the study comparing male song components between male Nelson's sparrows living in contaminated sites and reference sites, the blood mercury levels measured in the males from the contaminated sites were much higher than measured values in the present study [25]. The lowest blood

mercury values in the Nelson's sparrows were greater than 1.0 ppm, while the highest 30 day blood mercury levels were around 1.0 ppm in the present study [25]. Similar comparisons can be made for the field reproductive success studies with a mean blood mercury levels in female tree swallows of 3.56 ppm and mean blood mercury levels in female Carolina wrens ranging from 1.96 to 3.38 ppm being associated with reduced reproductive success [20,23]. In a study using both lifetime and adult-only exposures in zebra finches, reduced hatching success, fledging success, and total number of chicks fledged were seen in birds with blood mercury levels at least an order of magnitude higher than the present blood mercury levels (averages ranging from 4 to 34 ppm blood mercury) [26]. However all of these comparisons are between adult blood mercury levels in the published papers and the 30 day blood mercury levels in the present study may not be applicable because chicks were no longer being exposed. It is expected that the adult blood levels in the present study would be comparable or lower than the 30 blood mercury measurements. These comparisons indicate that the shortened treatment period used in the current study may not have allowed for enough mercury accumulation to occur in order to detect long-term effects on song or reproduction.

In summary, the lack of effects observed in any of the measured endpoints is likely attributed to the relatively short exposure period, which also corresponded to a time of rapid feather growth. The blood mercury levels observed in the present study were higher than those in a juvenile red-winged blackbird field study which also showed a lack of any growth effects despite elevated blood mercury [56]. Such complementary results indicate that young passerines may be relatively unaffected by mercury exposure, likely due to the sequestration of mercury into their growing feathers [14,18,45,57]. The measured blood mercury levels in the present study are much lower than those in which long-term effects on song and reproduction were seen [7,23,25,26]. This indicates that the comparatively short exposure period used in this experiment is not sufficient enough to elevate blood mercury levels to those needed to cause long-term effects. However, the present study has tested an important question of whether exposure to mercury at reasonably elevated levels during the nestling period, which is a critical time of growth and development, had any short or long-term effects. Multiple endpoints were investigated including growth, maturation, feeding behaviour, female reproductive success, male courtship and song.

Based on the literature review knowledge, the nestling developmental period has not been the focus of any previous mercury research.

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Table 2.1 **Mean and standard deviation for total blood mercury values, total mercury dose, and an estimate of mean total body mercury for each treatment. Estimated body mercury was obtained by multiplying the bird's blood mercury measurement by its 30 day mass (i.e. mass at time of blood collection). These values are all approximations based on an assumption that that blood mercury and body mercury are similar and should be considered very rough estimates.**

	Mean ± Standard Deviation		
	Control (n = 26)	Low (n = 29)	High (N = 27)
Measured Blood Mercury Concentration (ppm)	0.006 ± 0.006	0.297 ± 0.070	0.734 ± 0.163
30 Day Mass (g)	13.62 ± 0.75	13.49 ± 1.03	13.71 ± 0.88
Estimated Mercury Body Burden (ppm/g bw)	0.089 ± 0.037	3.994 ± 0.896	10.073 ± 2.351
Total Mercury Administered (µg)	0.036 ± 0.034	5.362 ± 0.452	12.798 ± 0.973

Table 2.2. Mean and standard deviation of zebra finch chick mass for each measurement day (post-hatching) for control (double deionized water), low (0.063 µg mercury per 0.5 g body weight per day), and high (0.15 µg mercury per 0.5 g body weight per day) treated nestlings. Corresponding F statistics and p-values are also given. There was no evidence for a difference when comparing mean chick mass between treatments for any of the chick ages.

	Chick Mass (grams) ± Standard Deviation (n)				
	Control	Low	High	F_2 Statistic	P-Value
Day 0	0.80 ± 1.31 (31)	0.80 ± 0.13 (33)	0.82 ± 0.11 (31)	0.36	0.70
Day 5	3.97 ± 0.71 (26)	3.92 ± 0.97 (26)	3.98 ± 0.90 (26)	0.02	0.98
Day 10	9.23 ± 1.10 (28)	9.25 ± 1.39 (29)	9.41 ± 1.18 (28)	0.14	0.87
Day 15	10.80 ± 0.71 (28)	10.86 ± 0.68 (29)	10.76 ± 0.71 (27)	0.62	0.54
Day 21	11.77 ± 0.80 (28)	11.88 ± 0.78 (29)	11.95 ± 0.85 (28)	0.55	0.58
Day 30	13.62 ± 0.75 (26)	13.49 ± 1.03 (29)	13.71 ± 0.88 (28)	1.13	0.14
Day 90	14.77 ± 1.15 (24)	14.62 ± 0.80 (28)	14.80 ± 1.17 (28)	0.33	0.87

Table 2.3. Mean and standard deviation of zebra finch fledge age, age of self-feeding, latency to feed, and latency to feed post removal of a novel object for fledglings in control (double deionized water), low (0.063 µg mercury per 0.5 g body weight per day), or high (0.15 µg mercury per 0.5 g body weight per day) treatment groups.

	Mean ± Standard Deviation				
	Control (n = 28)	Low (n = 28)	High (N = 29)	X^2_2 Statistic	P-Value
Fledge Age (Days)	19.2 ± 2.0	19.8 ± 2.4	19.6 ± 2.5	0.27	0.88
Age of Self-Feeding (Days)	24.4 ± 2.1	25.0 ± 2.5	24.4 ± 1.6	0.71	0.70
Latency to Feed (Seconds)	57.67 ± 47.74	68.13 ± 69.40	70.13 ± 69.32	0.09	0.96
Latency to Feed Post Removal of Novel Object (Seconds)	53.67 ± 48.94	56.21 ± 57.30	54.36 ± 65.92	0.19	0.91

Table 2.4. Mean and standard deviation of multiple measures of male courtship behaviour for zebra finches treated with control (double deionized water), low (0.063 µg mercury per 0.5 g body weight per day), or high (0.15 µg mercury per 0.5 g body weight per day) mercury treatment during their nestling stage (days 0-21 post-hatching).

	Mean ± Standard Deviation				
	Control (n = 15)	Low (n = 27)	High (n = 17)	X^2_2 Value	P-Value
Bill Wipes (total during 10 minute trial period)	20.6 ± 15.1	18.2 ± 20.8	27.5 ± 18.1	3.48	0.18
Follow (total during 10 minute trial period)	6.2 ± 8.3	7.9 ± 15.8	17.7 ± 20.9	6.09	0.05
Female Response (scale of 1 to 5)	2.9 ± 0.8	3.1 ± 1.1	3.4 ± 0.8	2.60	0.27
Time to First Mount (seconds)	241.6 ± 156.3	43.4 ± 42.2	139.1 ± 170.5	5.01	0.08
Number of Unsuccessful Mounts	2.2 ± 4	1.6 ± 2.4	2.7 ± 4.8	0.55	0.76
Number of Successful Mounts	0.40 ± 0.6	0.5 ± 1.5	1.0 ± 1.8	1.96	0.38

Table 2.5. Mean and standard deviation of multiple measures of male song quality for zebra finches treated with control (double deionized water), low (0.063 µg mercury per 0.5 g body weight per day), or high (0.15 µg mercury per 0.5 g body weight per day) mercury treatment during their nestling stage (days 0-21 post-hatching).

	Mean ± Standard Deviation			F_2 Statistic	P-Value
	Control (n = 12)	Low (n = 13)	High (n = 28)		
Phrase Duration (Seconds)	0.41 ± 0.09	0.38 ± 0.16	0.38 ± 0.15	0.136	0.87
Number of Syllables	10.73 ± 3.14	9.51 ± 4.56	9.50 ± 3.27	0.55	0.58
Number of Unique Syllables	11.25 ± 3.57	9.46 ± 4.07	10.32 ± 3.58	0.73	0.49
Song Rate (Phrases per Hour)	465.50 ± 228.79	367.38 ± 375.13	438.44 ± 342.54	0.31	0.73

Table 2.6. Correlation values between measures of song quality and courtship behaviours of zebra finches that were treated with mercury during the nestling stage.

	Phrase Duration	Number of Syllables	Number of Unique Syllables	Song Rate
Bill Wipes	0.092	0.074	0.102	-0.201
Follows	-0.162	-0.187	-0.148	0.152
Female Response	0.250	0.160	0.196	0.105
Time to First Mount	-0.204	-0.030	-0.119	-0.115
Number of Unsuccessful Mounts	0.319	0.179	0.172	0.220
Number of Successful Mounts	0.297	0.201	0.216	0.099

Table 2.7. Counts of females which laid eggs and did not lay eggs for zebra finches treated with control (double deionized water), low (0.063 µg mercury per 0.5 g body weight per day), or high (0.15 µg mercury per 0.5 g body weight per day) mercury treatment during their nestling stage (days 0-21 post-hatching). Proportions of females in each treatment which laid eggs are also shown.

Treatment	Laid	Did not Lay	Total	Proportion
Control	8	3	11	0.73
Low	11	4	15	0.73
High	5	1	6	0.83

Table 2.8. Mean and standard deviation of multiple measures of female breeding success for zebra finches treated with control (double deionized water), low (0.063 µg mercury per 0.5 g body weight per day), or high (0.15 µg mercury per 0.5 g body weight per day) mercury treatment during their nestling stage (days 0-21 post-hatching).

	Mean ± Standard Deviation			F_2 Statistic	P-Value
	Control (n = 8)	Low (n = 11)	High (n = 5)		
Lay Interval (Days)	7.6 ± 1.9	4.9 ± 2.3	6.8 ± 3.0	3.36	0.05
Clutch Size	5.8 ± 1.0	6.1 ± 1.1	5.2 ± 0.8	1.24	0.31
Mean Egg Mass (g)	1.06 ± 0.07	1.11 ± 0.06	1.07 ± 0.07	1.07	0.37
Brood Size at Hatch	3.5 ± 2.4	3.6 ± 2.0	4.2 ± 0.8	0.27	0.80
Brood Size at Fledge	3.0 ± 2.6	2.9 ± 2.1	3.4 ± 2.1	0.04	0.96

Table 2.9. **Mean and standard deviation for multiple measures of female breeding success of the female zebra finches which successfully produced chicks. The birds treated with control (double deionized water), low (0.063 µg mercury per 0.5 g body weight per day), or high (0.15 µg mercury per 0.5 g body weight per day) mercury treatment during their nestling stage (days 0-21 post-hatching).**

	Mean ± Standard Deviation			F_2 Statistic	P-Value
	Control (n = 7)	Low (n = 8)	High (n = 4)		
Lay Interval (Days)	7.6 ± 1.9	5.0 ± 2.4	6.2 ± 3.1	2.58,	0.10
Clutch Size	6.1 ± 0.8	6.3 ± 1.2	5.0 ± 0.8	2.41	0.12
Mean Egg Mass (g)	1.06 ± 0.70	1.12 ± 0.05	1.06 ± 0.06	2.47	0.11
Brood Size at Hatch	4.7 ± 1.2	4.3 ± 1.2	4.3 ± 1.0	0.27	0.77
Brood Size at Fledge	4.0 ± 2.1	4.0 ± 1.2	4.25 ± 1.0	0.04	0.96
Mean Chick Mass at Day 21 Post-hatching (g)	12.21 ± 0.57	12.74 ± 0.28	12.17 ± 0.65	3.00	0.08
Mean Chick Mass at Day 30 Post-hatching (g)	13.41 ± 0.43	13.78 ± 0.30	13.64 ± 0.40	1.87	0.18

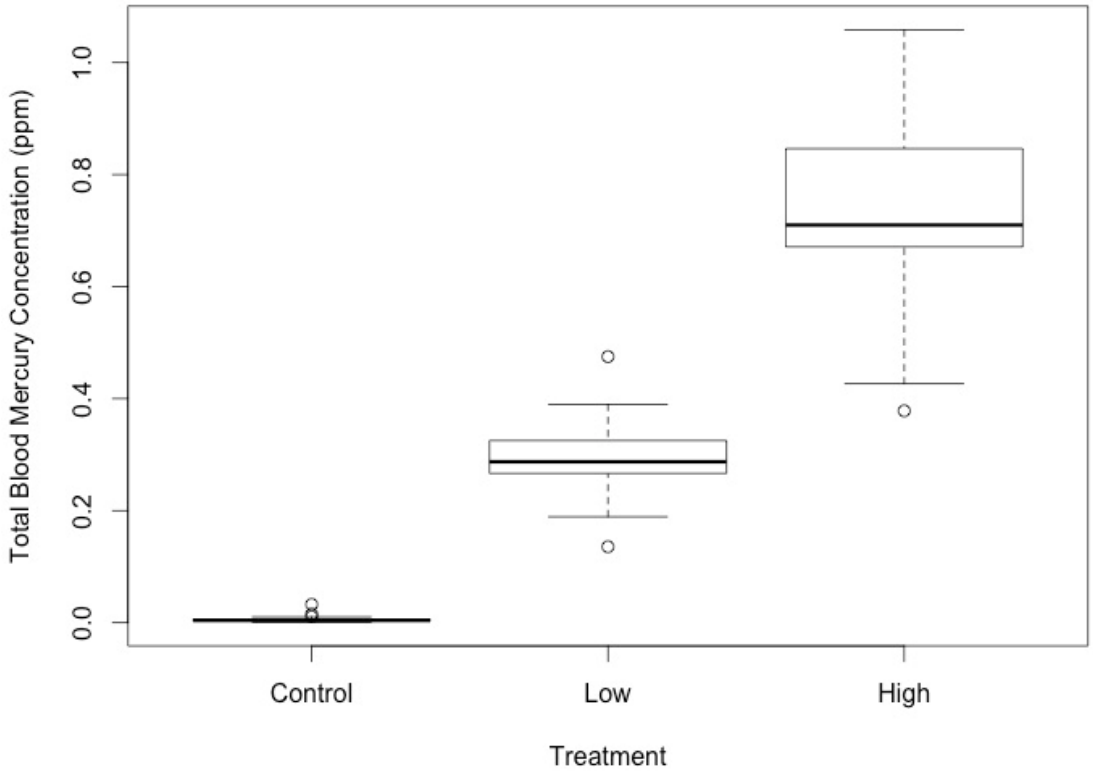

Figure 2.1. Average total mercury concentration in the zebra finch blood for control (double deionized water), low (0.063 µg mercury per 0.5 g body weight per day) and high (0.15 µg mercury per 0.5 g body weight per day)

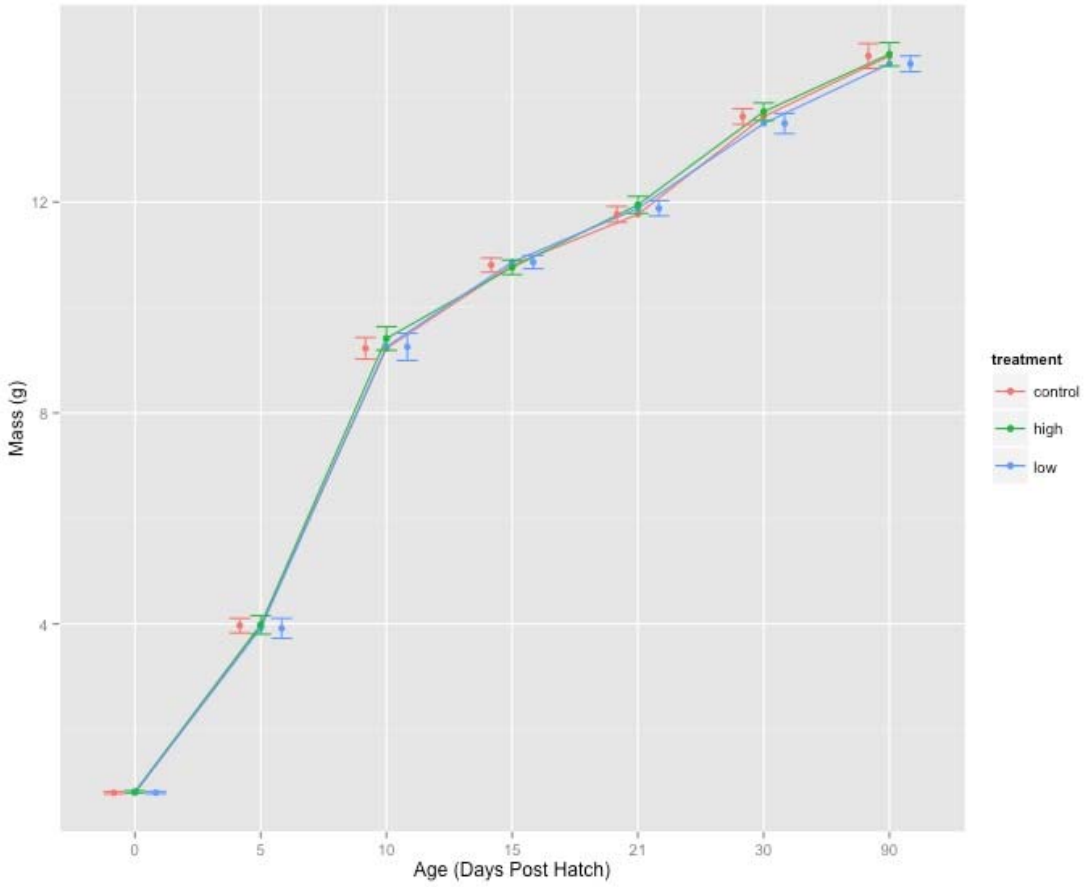

Figure 2.2 **Growth of zebra finch nestlings in control (double deionized water), low (0.063 µg mercury per 0.5 g body weight per day), or high (0.15 µg mercury per 0.5 g body weight per day) treatment groups. Error bars represent standard error and are dodged for clarity.**

Chapter 3. Conclusions

General Conclusions

In the present study, zebra finch nestling were treated with methylmercury solutions orally for a period of 21 days post hatching in order to simulate exposure from contaminated food provisioned by the parents. Three treatment doses (low, high, control) were used and randomized within each nest. The low and high dose solutions were confirmed to have concentrations of 0.063 µg Hg per µl and 0.15 µg Hg per µl respectively, and double deionized water was used for the control. Blood samples were collected at 30 days post hatching. Mean blood mercury levels for control, low, and high treatment groups were 0.006 ppm, 0.297 ppm, and 0.734 ppm, respectively while the range of blood mercury levels for the treated birds was 0.136 ppm to 1.059 ppm. Free-living juvenile loons living in North America have been shown to have blood mercury levels ranging from 0.3 ppm and 0.78 ppm [1]. Nestling red-winged blackbirds, a passerine species, living in contaminated sites were shown to have blood mercury levels ranging from 0.009 ppm to 0.284 ppm [2]. Both of these species may be expected to have high mercury loads compared to others due to their association with aquatic systems and, in the case of the red-winged blackbirds, because they are living in a contaminated area. In the present study, blood mercury levels were achieved within the same range or higher than both of the previously mentioned species, indicating the mercury load is ecologically relevant for juveniles.

Chick mass, tarsus length, bill length, wing length, and P9 length were recorded periodically from days 0-30 in order to determine if the methylmercury treatment had an impact on chick growth. Fledge age, age of self-feeding, and response to novel stimuli were observed in the chicks were compared among the treatment to determine if there are effects on maturity and early learning. Once the chicks reached sexual maturity at day 90 post-hatching, the males participated in courtship trials and their songs were also analyzed. The mature females were paired with a novel male to determine their breeding

propensity and success. Statistical analyses showed no evidence of a treatment effect on any of the endpoints measured, despite blood mercury levels showing a clear dose-response. It is speculated that the relatively short dosing period may not be sufficient enough to elicit observable effects on the chicks. This is in addition to speculation that the blood mercury may have been sequestered into the feathers because this is a strong excretion route in birds [3–6].

Success of Oral Exposure Methods

The method of pipetting the mercury solution directly into each chick's mouth daily has been used previously to study effects of PBDE exposure in zebra finches [7]. However, this dosing method has not been used previously for methylmercury studies. Pipetting the solution directly into the chick's mouth is advantageous because the exact dose each chick receives daily can be determined. This also removes potential pseudoreplication associated with mixing the methylmercury into the food shared by all birds in one cage. Between nest variation was eliminated by randomizing treatments within the nest, which could not be done by treating the communal food with mercury. The blood mercury analyses showed a clear dose-response and there was a statistical difference between the blood mercury concentrations among the treatment groups. This indicates that the pipette method was indeed successful for administering individual doses.

Lower than Expected Mercury Concentration in Dosing Solutions

The measured concentration of mercury in the dosing solutions was much lower than the target mercury concentrations. The solutions had target concentrations of 0.54 µg Hg per µl for the high dose and 0.27 µg Hg per µl for the low dose. These concentrations were based on those used in a lifetime exposure study which had the mercury mixed into the seeds [8]. Results of the conformational analysis showed the dosing solution concentrations to be 0.15 µg Hg per µl and 0.063 µg Hg per µl for high and low doses, respectively. These values correspond to 28% of the expected concentration for the high dose and a 23% of the expected concentration for the low dose. It is unclear whether this large discrepancy is due to error when creating the solutions or mercury degradation and sorption to the glass vials between the time the solutions were

made and the time the analysis was completed (approximately 8 months). One way to determine which solution concentration (expected or measured) the birds received would be to compare the body burden of mercury to the total amount of mercury the birds were given. However, only have blood mercury measurements were obtained and therefore this comparison cannot be done without mercury measurements from other body compartments such as feathers, liver, or brain. In the future, mercury solutions should be analyzed closer to the time they are made to be sure the measurements are accurate.

Future Directions

Currently, a project is already underway which extends off of the current project as well as the previous egg-injection project. The new project combines both *in ovo* and nestling treatments with methylmercury. Since in previous *in ovo* study and my current study both did not show evidence for long-term effects, combining the two treatment methods was the obvious next step. This longer exposure may be sufficient enough to yield detectable long-term effects. The current researcher is working hard to resolve some of the shortfalls from the current project. For example, the dosing solutions are being analyzed shortly after they are prepared rather than months later. There is also a push for mercury levels in the feathers to be measured. This is particularly important because it can only be speculated that the mercury was sequestered into the feathers during the present experiment but without the feather mercury concentrations measured this cannot be confirmed.

Due to the success of the direct dosing methods used in this current project, long-term studies could be conducted using this method. Pipette dosing could be used in adult zebra finches fairly easily. This would allow researchers to eliminate pseudoreplication by replacing mercury treated communal food with individual dosing. The exact dose the birds received would also be controlled and therefore an expected body burden could be found to compare with mercury concentrations measured in blood, feathers, liver, brain, and other organs and tissues. It is important to note that individual dosing is a time consuming process. In order for a long-term study to be conducted successfully, only a manageable amount of birds can be treated at once. This means that these studies would need to be

smaller scale unless there were multiple researchers or techs available to dose the birds daily.

In future studies, it is strongly recommend that future researchers measure the mercury levels in feathers along with taking blood samples. It is well known that feathers are a major excretion route for mercury in birds [3–6,9]. With blood samples alone, it can be difficult to determine to true body burden of mercury that was achieved in the study. However, having feather measurement to go along with the blood sample measurements would allow for a more accurate estimate of body burden. This is especially important when studying juvenile birds because they are going through rapid feather growth. This may explain why they appear to be relatively unaffected by mercury exposure, but this cannot be confirmed without the measurements of mercury in the feathers. Feather measurements are also advantageous because they can be taken with little to no harm being done to the bird. This is in contrast with other tissues such as the brain, kidney, and liver, which also contain some of the mercury body burden [5].

Currently, the body of research for effects of methylmercury in passerines is small. This is mostly because mercury contamination was not thought to be a major issue in terrestrial ecosystems until fairly recently [10–12]. There is a large amount of research that could potentially be done in this field. For example, studies in aquatic and piscivorous birds can be repeated in passerines in order to determine if similar effects are seen. This is particularly important for behavioural studies because the current mercury passerine research has very few behavioural studies. More field observations and measurements of passerines living in contaminated areas can also be conducted. Further blood mercury level measurements should be taken in order to determine which birds are at risk for detrimental effects due to mercury accumulation. There is also little research on the cycling of mercury in these terrestrial ecosystems. New research on how the passerines are being exposed to mercury in the wild would be valuable for conservation purposes.

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Appendix A.

Individual measurements of total blood mercury, 30 day mass, and total mercury administered for each zebra finch chick that survived to day 30.

A rough estimate of total body mercury was calculated by multiplying the blood mercury value by the 30 day mass.

Chick ID	Treatment	Total Blood Mercury (µg Hg/mL)	30 Day Mass (g)	Estimated Total Body Hg (µg Hg) Based on 30 Day Mass	Total Mercury (µg Hg) Administered
M15D153	Control	0.01036	15.27	0.1581972	0.0322
M15D154	Control	0.00477	14.61	0.0696897	0.03122
M15D155	Control	0.00845	13.81	0.1166945	0.032025
M15D161	Control	0.00608	14.02	0.0852416	0.03178
M15D164	Control	0.00475	12.51	0.0594225	0.028035
M15D168	Control	0.00599	13.88	0.0831412	0.029435
M15D172	Control	0.03264	13.99	0.4566336	0.028525
M15D174	Control	0.00415	12.81	0.0531615	0.027965
M15D186	Control	0.00779	13.86	0.1079694	0.03045
M15D190	Control	0.01076	13.66	0.1469816	0.028875
M15D196	Control	0.00326	13.66	0.0445316	0.03066
M15D197	Control	0.00319	12.94	0.0412786	0.026285
M15D199	Control	0.00316	13.37	0.0422492	0.02884
M15D200	Control	0.00294	14.1	0.041454	0.029085
M15D208	Control	0.01536	13.43	0.2062848	0.030975
M15D210	Control	0.00314	13.2	0.041448	0.029645
M15D212	Control	0.0033	13.22	0.043626	0.028735

M15D215	Control	0.00378	13.52	0.0511056	0.02835
M15D218	Control	0.00398	14.09	0.0560782	0.030975
M15D219	Control	0.00421	13.07	0.0550247	0.027895
M15D220	Control	0.00402	15.3	0.061506	0.03395
M15D221	Control	0.00232	11.93	0.0276776	0.024605
M15D227	Control	0.00076	13.33	0.0101308	0.204645
M15D294	Control	0.00246	13.14	0.0323244	0.03052
M15D298	Control	0.00204	13.24	0.0270096	0.031255
M15D299	Control	0.00207	14.16	0.0293112	0.034825
M15D157	Low	0.18914	13.11	2.4796254	4.8818341
M15D160	Low	0.30531	14.56	4.4453136	5.242068
M15D162	Low	0.25618	13.54	3.4686772	5.109117
M15D166	Low	0.30789	13.18	4.0579902	5.007821
M15D167	Low	0.25553	12.95	3.3091135	4.355728
M15D170	Low	0.3872	14.2	5.49824	5.545956
M15D176	Low	0.39003	13.71	5.3473113	5.659914
M15D177	Low	0.325235	13.52	4.3971772	4.919187
M15D179	Low	0.31629	15.36	4.8582144	5.989126
M15D180	Low	0.36751	14.04	5.1598404	5.653583
M15D183	Low	0.21287	13.33	2.8375571	5.12811
M15D184	Low	0.36372	14.44	5.2521168	6.20438
M15D185	Low	0.27154	13.74	3.7309596	5.216744
M15D188	Low	0.2865	14.2	4.0683	5.4206022
M15D189	Low	0.26499	12.86	3.4077714	5.368688
M15D194	Low	0.30055	13.96	4.195678	5.413005
M15D195	Low	0.3347	14.18	4.746046	6.071429

M15D201	Low	0.27738	14.86	4.1218668	5.691569
M15D204	Low	0.32087	14.34	4.6012758	5.672576
M15D207	Low	0.26648	13.01	3.4669048	5.394012
M15D209	Low	0.27725	12.73	3.5293925	5.450991
M15D211	Low	0.475	9.84	4.674	5.615597
M15D214	Low	0.28082	13.08	3.6731256	5.900492
M15D216	Low	0.28733	12.59	3.6174847	5.5124017
M15D222	Low	0.20934	13.59	2.8449306	5.0192168
M15D224	Low	0.36439	13.43	4.8937577	5.330702
M15D225	Low	0.29355	13.73	4.0304415	5.4364297
M15D292	Low	0.1356	13.37	1.812972	4.173799681
M15D296	Low	0.282865	11.7	3.3095205	5.102786
M15D151	High	0.64369	13.24	8.5224556	12.675
M15D152	High	0.76678	13.11	10.0524858	12.09
M15D158	High	0.42649	13.84	5.9026216	12.36
M15D159	High	0.47451	14.23	6.7522773	12.495
M15D163	High	0.96519	13.54	13.0686726	12.87
M15D165	High	0.879985	13.85	12.18779225	13.05
M15D169	High	0.69815	14.34	10.011471	13.8
M15D171	High	0.83801	14.81	12.4109281	13.275
M15D173	High	0.85447	15.71	13.4237237	14.43
M15D175	High	0.82465	12.54	10.341111	11.1
M15D178	High	0.88928	14.05	12.494384	13.74
M15D181	High	0.68428	13.08	8.9503824	12.315
M15D182	High	0.96397	12.13	11.6929561	12.045
M15D187	High	0.71229	13.37	9.5233173	12.45

M15D191	High	0.70032	14.51	10.1616432	12.72
M15D192	High	0.81128	13.74	11.1469872	13.485
M15D193	High	1.0585	14.6	15.4541	13.365
M15D198	High	0.572015	12.38	7.0815457	12.18
M15D202	High	0.66477	13.68	9.0940536	12.465
M15D203	High	0.70989	12.16	8.6322624	11.685
M15D205	High	0.77004	14.35	11.050074	13.425
M15D206	High	0.73502	14.17	10.4152334	13.02
M15D213	High	0.69112	13.48	9.3162976	12.615
M15D217	High	0.68852	13.83	9.5222316	13.755
M15D223	High	0.37816	12.97	4.9047352	9.81
M15D228	High	0.67738	14.06	9.5239628	13.665
M15D297	High	0.5456	15.26	8.325856	14.355
M15D300	High	0.93826	12.89	12.0941714	13.11