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## Evaluating the Potential for Adaptive Response to Mercury in Captive-Dosed Zebra Finches

Kenton August Buck  
*College of William & Mary - Arts & Sciences*

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Evaluating the Potential for Adaptive Response to Mercury in Captive-dosed Zebra  
Finches

Kenton August Buck

Richmond, Virginia

Bachelor of Science, Christopher Newport University, 2010

A Thesis presented to the Graduate Faculty  
of the College of William and Mary in Candidacy for the Degree of  
Master of Science

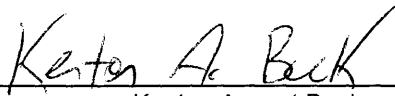
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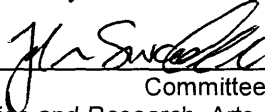
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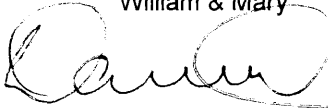
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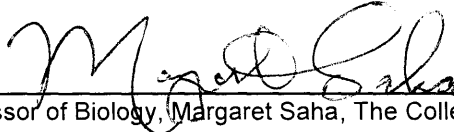
Committee Chair

Dean of Graduate Studies and Research, Arts & Sciences, John Swaddle, The College of  
William & Mary



---

Professor of Biology, Daniel Cristol, The College of William & Mary



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Chancellor Professor of Biology, Margaret Saha, The College of William & Mary

## COMPLIANCE PAGE

Research approved by

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## ABSTRACT

Mercury (Hg) is a ubiquitous heavy metal contaminant that threatens human and environmental health. Birds are sensitive bioindicators of mercury toxicity, however, current predictions of mercury accumulation and biomagnification overlook possible variation in mercury uptake/removal within species and the potential for evolution in sensitive populations. I evaluated the potential for adaptive response to mercury within a captive population of Australian Zebra Finch (*Taeniopygia guttata*) maintained on standardized diets containing 0.0, 0.3, 0.6, 1.2 or 2.4 ppm methylmercury cysteine. To control for common environmental effects, which can cause an upward bias in additive genetic variance, a small proportion of nestlings from each treatment were cross-fostered between nests. Because the genetic diversity of a study population can influence estimates of quantitative genetic variance, I measured genetic diversity in the Zebra Finch colony by genotyping the parental generation using microsatellites. Microsatellite genotyping of the Zebra Finch colony demonstrated a high level of genetic diversity, indicating that the variance estimates were not biased by a lack of genetic diversity in the colony. The close resemblance between related individuals was also not influenced by common environmental effects of nesting environment. At all dietary methylmercury treatments, Zebra Finches exhibited considerable variation in blood mercury accumulation, and this variation was highly repeatable for individuals. I observed a strong genetic influence on blood mercury accumulation, however this effect was non-linear with increasing mercury exposure; a significant heritable component for blood mercury accumulation was estimated for the 0.6 and 1.2 ppm MeHg dietary doses, but not for treatments at 0.3 and 2.4 ppm dietary MeHg. The non-linear gene by environment interactions observed could be the result of thresholds at low and high levels of exposure which limit a genetic response to mercury toxicity. If wild bird populations exhibit a heritable response to mercury accumulation, natural selection could act to produce tolerant/resistant populations. Such populations could increase total mercury biomagnification in the food web as more individuals survive to pass mercury on to the next trophic level. More research is necessary to investigate potential adaptation to mercury in wild bird populations and to understand gene expression mechanisms underlying mercury tolerance in birds.

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## **Chapter 1. Introduction**

### **1.0 Mercury**

Mercury (Hg) is a ubiquitous heavy metal contaminant that threatens human and environmental health. Anthropogenic emissions of mercury have increased by two- to three-fold over the last 200 years (Driscoll et al. 2007), chiefly as the result of coal combustion, gold mining, metal and cement production, and waste incineration (Driscoll et al. 2007). In its methylated form (MeHg), mercury readily accumulates in living tissues and biomagnifies up the food web (Eisler 2006). Methylmercury exposure in humans and wildlife is associated with decreased reproductive success and numerous behavioral and health effects (Mergler et al. 2007; Seewagen 2010).

Birds are at high risk from mercury contamination, and are sensitive to many endpoints used to measure mercury toxicity (Seewagen 2010). Many species of birds occupy high trophic positions and may have increased mercury intake from biomagnification. Birds can also be long-lived, allowing individuals to bioaccumulate higher levels of mercury throughout their lifetime (Evers 2005). Because of their sensitivity to mercury, birds are frequently used as bioindicators to evaluate environmental mercury contamination (Evers 2005).

Because mercury exposure affects survival and reproduction in birds, selection may favor individuals who are more tolerant to mercury. If variation in response to mercury exists at the population level, and if it can be attributed to heritable genetic differences, a population-level response to mercury may evolve.

## 1.1 Mercury as a Selection Pressure

Because of its numerous detrimental effects, mercury contamination may affect a population's evolutionary trajectory in a number of ways (e.g. by natural and sexual selection, gene flow, mutation, genetic drift)(Eisler 2006; Seewagen 2010). Most of the cases of evolution to environmental contaminants have been explained by natural selection mechanisms (Amiard-Triquet et al. 2011; Klerks et al. 2011). Because mercury negatively affects reproduction and survival, natural selection may favor individuals that are tolerant to its effects, presuming that the costs of developing and maintaining the mercury tolerance mechanisms do not outweigh the benefits of possessing these mechanisms in a mercury-polluted area.

### 1.1.1 Negative impacts of mercury on reproduction

Mercury exposure is associated with reduced reproductive success in several wild bird species. In Common Loons (*Gavia immer*) mercury exposure is negatively associated with fledgling production (Fimreite 1974; Meyer et al. 1998). Behavioral alterations such as a reduction in time spent incubating and foraging by parents, and a reduction in agonistic behavior may also contribute to reproductive failure in loons (Burgess and Meyer 2008; Evers et al. 2008). Common Terns (*Sterna hirundo*) have shown a 10% reduction in fledging in mercury contaminated locations compared to reference sites (Fimreite 1974). Similar to birds feeding in an aquatic environment, reproductive impairment also occurs in terrestrial birds exposed to mercury. Tree Swallows

(*Tachycineta bicolor*) breeding in uniform nest boxes in a mercury contaminated area produced fewer fledglings compared to those in nearby uncontaminated locations (Brasso and Cristol 2008).

Studies on reproduction in wild populations are influenced by variation in levels of mercury exposure as well as other environmental variables. Relatively few studies have controlled for environmental variation by studying the effects of mercury on reproduction in captivity. Mercury exposure reduced hatching success in Mallards (*Anas platyrhynchos*) and American Black Ducks (*Anas rubripes*) exposed to standardized concentrations (Finley and Stendell 1978; Heinz and Hoffman 2003). Captive dosing of White Ibises (*Eudocimus albus*) with environmentally relevant levels of mercury resulted in reproductive impairment with fledgling production decreased by 35% at a dietary exposure of 0.3 parts per million (ppm) MeHg (Frederick and Jayasena 2011). Courtship behavior among these same ibises also decreased, along with a 13% reduction in successful breeding attempts due to male-male pairing (Frederick and Jayasena 2011).

Embryotoxicity could explain decreased hatching success for birds exposed to mercury. Mercury affects numerous developmental endpoints (Eisler 2006) and is directly deposited into the eggs when the mother consumes a mercury contaminated diet (Evers et al. 2003; Eisler 2006). Maternal dietary exposure to mercury in captive Mallards has shown embryo mortality with mercury concentrations as low as 0.74 ppm MeHg (Heinz and Hoffman 2003), an

egg-mercury concentration that is lower than those reported in contaminated locations (Evers et al. 2008). Surviving mallard hatchlings experienced neurologic damage with egg mercury concentrations as low as 2.3 ppm. Embryotoxicity may partially explain declines in productivity observed in wild and captive birds exposed to mercury (Longcore et al. 2007; Tsipoura et al. 2011).

### *1.1.2 Negative impacts of mercury on survival*

Measuring survival in wild species is difficult due to the many variables that affect survival and the need for long-term data sets (Lebreton et al. 1993). Although mercury has been shown to reduce survival in more extensively studied taxa, such as fish (Scheulhammer et al. 2007), the few studies that have evaluated survival in mercury exposed birds have not demonstrated direct impacts of mercury on survival. Individual mercury concentration in Great Skuas (*Catharactskua*) and Common Loons did not show a relationship to likelihood of returning to the breeding grounds (Thompson et al. 1991; Meyer et al. 1998). A 10-year data set for Common Loons showed only a 3% difference in survival between individuals with high and low mercury levels. In long-lived species, such as loons, 3% survival differences could represent significant population declines (Mitro et al. 2008), however, these effects may be more pronounced in the survival of hatch-year loons (Scheulhammer et al. 2007). The survival rate of captive White Ibises dosed with dietary mercury in captivity and subsequently released was not impaired by mercury exposure (Frederick et al. 2011). The only

study on songbirds found a small but significant effect of mercury on survival, between 1% and 3% annually, in Tree Swallows (Hallinger et al. 2011).

Direct impacts of mercury on survival have not been demonstrated by the few studies that have been conducted with birds. Future studies should evaluate mercury impacts on survival in additional wild species and in the captive environment, where mercury's impacts on survival can be studied in isolation. Indirect and nonlethal effects of mercury could still impact survival in wild birds (Eisler 2006; Seewagen 2010). Other detrimental effects of mercury, such as those on behavior, neurology, and physiology in birds are well-documented (Seewagen 2010). Many of the neurologic effects of mercury are subtle and do have the potential to affect survival at low concentrations (Scheulhammer et al. 2007). Continuing research should prioritize these nonlethal effects in relation to survival, particularly life history events such as migration that strongly influence survival in many species (Seewagen 2010).

### *1.1.3 Among-species and within-species variation in mercury tolerance*

As described above, the effects of mercury on survival and reproduction differ among species in the wild. Some of this effect may be attributed to differences in exposure caused by mercury availability in prey items, but species are also variable in terms of sensitivity to mercury and in ability to mitigate mercury toxicity. Heinz et al. (2009) used an experimental manipulation to study among-species differences in mercury embryotoxicity, one of the most sensitive endpoints of mercury contamination. Wild-collected eggs from 23 species of birds

were injected with methylmercury in order to determine the median lethal concentration (LC<sub>50</sub>). Considerable differences in embryological sensitivity were found between species(Heinz et al. 2009b), however this experiment did not control for potential among-species variation in egg mercury deposition by females that may affect embryo sensitivity. Species also differ in their ability to mitigate mercury accumulation. Recent studies have shown species differences in rates of demethylation and metabolism in tissues (Scheuhammer et al. 2008; Eagles-Smith et al. 2009; Heinz et al. 2009b). Kim et al. (1996) demonstrated significant among-species differences in mercury accumulation in liver, muscle, kidney, and feather samples of nine species of seabirds (Kim et al. 1996).

An adaptive response to mercury would require within-species variation in mercury sensitivity and/or ability to mitigate mercury. Variation withinspecies has been demonstrated for mercury sensitivity. A captive-dosing study of Mallards found considerable among-individual variation in embryo mortality and neurological sensitivity for ducklings whose parents were fed on diets with standardized concentrations of mercury (Heinz and Hoffman 2003). Interestingly, pronounced differences in neurological sensitivity were observed between families, with some ducklings exhibiting neurological impairment at far lower mercury concentrations than ducklings of other parents (Heinz and Hoffman 2003). The variation in egg mercury deposition among females exposed at the same dietary levels was not reported. Among-individual differences in deposition by females could have resulted in lower risks of deformity and mortality in



offspring. Although among-individual differences in sensitivity were demonstrated in this study, the potential role of mitigation through decreased mercury deposition into eggs is unclear.

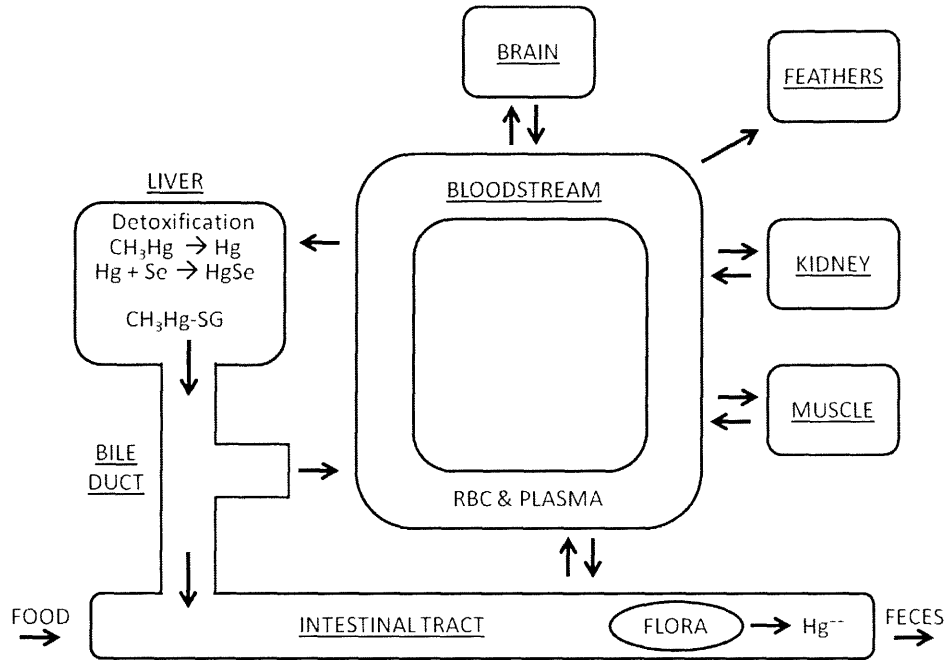
Among-individual differences in embryotoxicity suggest a difference in mercury sensitivity within species, however little attention has been paid to within-species variation in mercury mitigation. One study found high within-individual variation in feather mercury content of Arctic Terns (*Sterna paradisaea*), Common Terns, and Leach's Storm-petrels (*Oceanodromaleucorhoa*), however, variation in mercury accumulation among species, and within- and among-individuals, could be influenced by temporal differences in diet composition or molt patterns (Bond and Diamond 2008). Alternatively, among-individual variation in mercury accumulation could be driven by genetic differences in the mechanisms that control mercury balance in birds. If mercury tolerance acts to reduce mercury accumulation, the genetic differences which underlie tolerance will involve one or more of the many physiological processes that control mercury accumulation.

### *1.2 Metal Pathways in Birds*

Mercury accumulation in organisms is a balance between intake and excretion. It depends on the concentration present in food and also the physiological processes responsible for uptake, excretion, and distribution in the body. Birds uptake methylmercury from their diet and excrete it through their feces, feathers, and eggs (Ikemoto et al. 2004; Seewagen 2010).

Methylmercury will readily form a complex with cysteine (MeHgCys). This complex has a highly similar structure to the amino acid methionine, which allows mercury to enter the cell. Proposed mechanisms for MeHgCys entrance into the cell include the Large Neutral Amino Acid Transporter (Clarkson and Magos 2006), where it then preferentially enters the nucleus, mitochondria, and lysosome (Ikemoto et al. 2004). The same mechanism may also explain intestinal absorption of mercury.

After ingested mercury passes through the intestines into the bloodstream, it travels through the body and deposits within the cells of various tissues (liver, kidney, brain, muscle) (Eisler 2006). It is able to cross the blood-brain barrier as MeHgCys (Eisler 2006). Mercury can be sequestered in the liver, but also passes through the bile duct when bound to reduced glutathione (Clarkson and Magos 2006). When this complex is broken down by extracellular enzymes, MeHgCys can be reabsorbed into the blood through the gallbladder. Together, the glutathione and cysteine pathways may explain mercury mobility in the body (Figure 1) (Clarkson and Magos 2006; Eisler 2006).



**Figure 1.** Schematic diagram of hepatic recirculation of mercury in the body and its deposition into avian tissues. The liver detoxifies mercury through a process of demethylation and sequestration (see below) and also re-circulates mercury by forming a reduced-glutathione complex. Adapted from (Clarkson, 2006).

Mercury can be removed from circulation in the body through a number of different pathways. Deposition of mercury into feathers and eggs directly removes mercury from the body. Mercury can also be detoxified/sequestered in the liver where it is physiologically isolated from the rest of the body. Because these mechanisms of excretion and detoxification affect mercury balance, they could be important in the evolution of mercury tolerance, if these processes are variable/heritable.

### 1.2.1 Mercury excretion into feathers and eggs

Mercury deposition into feathers is possibly the most important route of elimination in birds. Methylmercury has a high affinity for the sulfhydryl groups found in the cysteine that contributes to the structure of the keratin in feathers (Eisler 2006). Mercury content in feathers is proportional to mercury content in the blood at the time of feather growth (Fournier et al. 2002). Measures of mercury in sequentially molting feathers show a decreased mercury content corresponding to molt pattern (Dauwe et al. 2003). Fully-grown feathers are physiologically isolated from the rest of the body (Stettenheim 2000), and mercury incorporated into feathers can be removed during regular molt.

Studies of wild birds suggest that molt has a protective influence against mercury by reducing mercury burden in the body. Early growth of down after hatching sequesters much of the systemic mercury in chicks (Fournier et al. 2002; Kenow et al. 2003; Merrill et al. 2005; Longcore et al. 2007). As an example, mercury deposition into growing feathers reduced blood mercury burden in Eastern Bluebirds (*Sialia sialis*) (Condon and Cristol 2009). During the juvenile molt period, hatch-year bluebirds had significantly lower mercury levels than adults, followed by an increase to adult levels with the completion of juvenile molt. With the onset of pre-formative, or first pre-basic, molt, blood mercury levels in the hatch-year bluebirds decreased again (Condon and Cristol 2009).

Molt represents a substantial pathway for mercury removal, and feather mercury content may represent between 70-93% of total body mercury content (Burger 1993). As many experience molt during particular seasons,

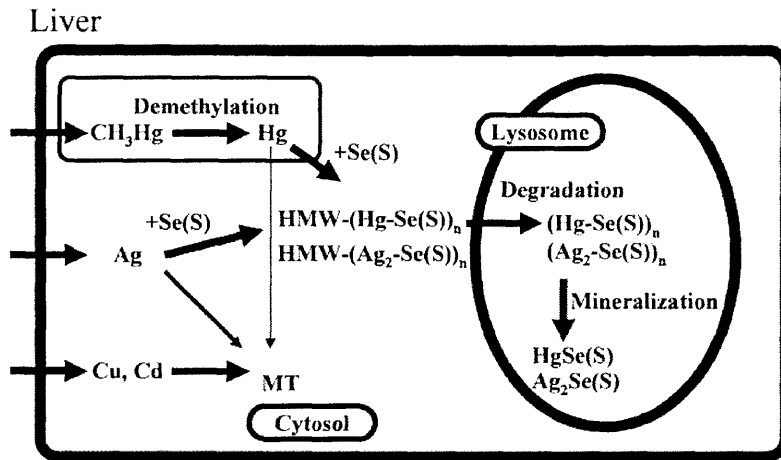
mercury elimination via feather re-growth is a transient phenomenon. A study of the toxicokinetics of mercury showed rapid excretion of mercury into feathers during the molt period, and then a much slower rate of elimination after molt was complete (Fournier et al. 2002).

The mercury body burden of females can decrease by deposition of mercury in their eggs (Eisler 2006; Seewagen 2010). Similar to feathers, mercury excretion into eggs is proportional to blood mercury at the time of egg development (Evers et al. 2003). Measurements of mercury in eggs of Herring Gulls (*Larus smithsonianus*), Common Terns, and American Oystercatchers (*Haematopus palliatus*) showed declines of mercury content (between 10%-39%) between the first and last egg laid (Becker 1992). In Tree Swallows, however, mercury content was not affected by laying order (Brasso et al. 2010). Mercury excretion into eggs may result in significant differences in mercury content between sexes. Female Laughing Gulls (*Leucophaeus atricilla*) experienced a 20% reduction in mercury body burden, compared to males, through mercury excretion into eggs (Lewis et al. 1993). Egg laying in Double-crested Cormorants (*Phalacrocorax auritus*) accounted for 20% of the mercury differences between males and females (Robinson et al. 2011), however, the between-sex differences in mercury intake as well as ability to demethylate or excrete mercury may also account for differences in mercury content between sexes.

### 1.2.2 Mercury detoxification in the liver

In addition to pathways of excretion, birds may detoxify mercury directly through a process of demethylation and formation of inert mercury complexes with selenium. The process of demethylation converts methylmercury into inorganic mercury (IoHg). IoHg is less able to move within living tissue as it does not bind to sulfur bonds like MeHg, and may be less toxic than MeHg (Eisler 2006). In birds most demethylation occurs in the liver, however, the process of demethylation may also occur in the kidneys and perhaps the brain (Scheulhammer et al. 2007). Other tissues such as skeletal muscle, feathers and eggs show no evidence of demethylation (Scheulhammer et al. 2007).

During digestion, demethylation by gut microflora occurs before mercury is absorbed into the bloodstream (Clarkson and Magos 2006), however, the liver is the primary site of demethylation after absorption. Some species of seabirds demethylate methylmercury and store it as immobilized inorganic mercury in the liver (Kim et al. 1996). Ikemoto et al. (2009) proposed a model for metal detoxification in marine mammals and seabirds (Figure 2).



**Figure 2.** Schematic diagram for a proposed process of heavy metal detoxification in high-trophic marine mammals and birds. HMW = high-molecular-weight proteins; MT = metallothionein (Ikemoto et al., 2009).

According to this model, methylmercury is taken up from the diet and demethylated by reactive oxygen species, gut microfauna, and selenium (Ikemoto et al. 2004). The resulting  $\text{Hg}$  binds to an isoform of metallothionein or forms a complex with mercuric selenide ( $\text{HgSe}$ ) and then high-molecular-weight substances (HMWS) in the liver. Glutathione (GSH) molecules attach to  $\text{HgSe}$  and form a complex that is digested in the lysosome, resulting in the formation of crystalline  $\text{HgSe}$  that can be sequestered in the liver (Ikemoto et al. 2004).

The rate of demethylation increases with mercury exposure. Comparisons among waterbirds showed a decrease in liver  $\text{MeHg}$  as the total liver mercury content ( $\text{THg}$ ) increased (Eagles-Smith et al. 2009). The increase in rate of

demethylation occurred at a threshold; a strong decline in percent MeHg occurred in waterbird livers with mercury content above  $8.51 \pm 0.93$  ppm dry weight (Eagles-Smith et al. 2009). The mechanisms involved in demethylating and sequestering mercury are likely to be energetically costly given the number of physiological pathways involved, although the extent of this cost is not well understood (Scheulhammer et al. 2007). This may partially explain the dose-response threshold for demethylation observed by Eagles-Smith et al. (2009); the cost of demethylation may exceed the benefit below toxicity thresholds. The energetic cost of demethylation could limit the evolution of tolerance if the cost of response is greater than the cost of mercury toxicity. The threshold of demethylation may also be important for populations moving in and out of contaminated environments.

Selenium has protective influence against mercury toxicity and is an important part of the detoxification pathway. Like mercury, selenium accumulates in the liver. Selenium may function during demethylation, but it mainly functions to bind to demethylated mercury in equimolar ratio (Ralston and Raymond 2010) to form mercuric selenide (HgSe), which makes the mercury toxicologically inert so long as the complex is maintained (Ikemoto et al. 2004; Eagles-Smith et al. 2009). In waterbirds, selenium concentration was correlated with loHg only above the demethylation threshold, which suggests that it may have some function in reducing secondary toxicity of loHg (Eagles-Smith et al. 2009).



Although not yet studied in birds, selenium dietary supplementation in other vertebrates reduces neurotoxicity of mercury in the brain (Ohi et al. 1976).

Mercury detoxification is variable across species. Eagles-Smith et al. (2009) observed among-species differences in the rate and threshold of demethylation in waterbird livers. American Avocets and Black-necked Stilts (*Recurvirostra americana* and *Himantopus mexicanus*, respectively) had higher rates of demethylation than terns, although the threshold at which demethylation is initiated was also higher for avocets and stilts than for terns (Eagles-Smith et al. 2009). This may reflect different strategies for mercury tolerance in different species. Avocets and stilts, who feed on invertebrates, are exposed to lower methylmercury levels than piscivorous terns. The constant replenishing of methylmercury in tern livers may favor constant demethylation at a lower threshold. To my knowledge, this is the only study to compare demethylation in birds across taxonomic groups. The threshold of demethylation has not been assessed in songbirds, although mercury levels in songbirds can exceed those reported in waterbirds (Cristol et al. 2008). Among-individual variation in mercury detoxification has not been assessed.

## **2.0 Evaluating the evolutionary response to mercury**

### *2.1 Quantifying tolerance to mercury*

The mechanisms described above may reduce mercury toxicity by increasing mercury elimination (feathers, eggs) or sequestering mercury in an inert form. Additional mechanisms that are currently undescribed could also

affect mercury accumulation in living organisms, making mercury tolerance difficult to quantify. Because many of the potential mechanisms of mercury tolerance may act directly on mercury accumulation in blood, I used mercury accumulation in blood as a proxy for traits associated with mercury excretion and detoxification.

Direct comparisons of mercury accumulation have been impossible in studies of wild birds, where individuals vary in terms of the duration and intensity of mercury exposure. Other uncontrolled variables such as environmental conditions and exposure to other pollutants prevent mercury toxicity from being studied in isolation in wild populations. This environmental variation may obscure genetic differences in tolerance. To control for these confounding factors, and to quantify mercury exposure over time, several studies have used a captive-based approach to study mercury toxicity in birds (Lewis and Furness 1991; Spalding et al. 2000; Heinz and Hoffman 2003; Heinz et al. 2009a). Captive studies may have difficulties in extrapolating to wild populations as a result of deliberate reduction in environmental variation; however, unlike studies of wild populations, the captive environment allows mercury toxicity to be studied in isolation.

We conducted a captive-dosing experiment in order to measure the variation and heritability of mercury accumulation, and thus to assess the evolutionary potential of mercury tolerance. My study species was the Australian Zebra Finch, which is a well-established model organism for studies in behavior, physiology, and quantitative genetics (Zann 1996; Tschirren and Postma 2010).

Unlike studies of environmental mercury contamination, the captive environment allowed us to standardize mercury exposure so that the resulting mercury accumulation could serve as a measure of an individual's tolerance.

There has been no assessment of the potential for adaptive response to mercury in birds. An adaptive response to mercury depends on traits associated with mercury tolerance being both variable and heritable. In the current study, mercury accumulation was treated as a phenotype representative of mercury tolerance. The variation and heritability of patterns of mercury accumulation in captive-dosed Zebra Finches was measured using quantitative genetics. A short background on quantitative genetics is necessary to understand the methodology used in this study to evaluate the evolutionary potential of mercury tolerance in captive-dosed Zebra Finches.

## *2.2 A primer on quantitative genetics*

Quantitative genetics describes how genetic influence underpins phenotypic variation in the expression of a trait at the level of the population. Provided with the relationships between individuals within a population and the ability to measure a phenotype (i.e. quantitative trait) quantitative genetics can partition phenotypic variation into genetic and environmental variation and make inferences about the inheritance and evolutionary potential of phenotypic traits (Falconer and Mackay 1996; Lynch and Walsh 1998). Most ecologically important traits are influenced by numerous genes, making them difficult to study in cases where the genetic mechanisms are poorly understood. Because

inferences in quantitative genetics are made at the level of the phenotype, specific knowledge of the genetic architecture influencing that phenotype is not required. This is useful for phenotypes such as mercury accumulation that are the result of many physiological processes.

### 2.2.1 Genetic sources of phenotypic variance

Phenotypes are the product of genes acting within an environmental context.

$$\mathbf{Phenotype (P) = Genotype (G) + Environment (E)}$$

(Equation 1)

For a single trait within a population, one can estimate the amount of phenotypic variation ( $V_P$ ) that is attributed to genetic variation ( $V_G$ ) and to environmental variation ( $V_E$ ). Genetic variation can be partitioned into several variance components including additive ( $V_A$ ), dominant ( $V_D$ ), or epistatic ( $V_I$ ) variances (Falconer and Mackay 1996; Lynch and Walsh 1998; Kruuk 2004; Wilson et al. 2010).

$$V_G = V_A + V_D + V_I \quad \text{(Equation 2)}$$

Additive genetic variance refers to the effect on phenotypic variance that is the result of the inheritance of each individual particular allele at a given locus. This is in contrast to dominance variance, which depends on the interaction of two parental alleles. Individuals only contribute one allele per locus to their offspring, and relatives will only share at most one copy of an allele that is

identical by descent (except clones and identical twins). Response to selection is correlated with additive genetic variation as each gene contributes to the expression of the phenotype. Phenotypic variance ( $V_P$ ) can be explained due to additive genetic variance ( $V_A$ ) and residual variance ( $V_R$ ), which is interpreted as environmental effect (Falconer and Mackay 1996; Lynch and Walsh 1998; Kruuk 2004; Wilson et al. 2010).

$$V_P = V_A + V_R$$

(Equation 3)

In order for adaptation to occur, selection will only have evolutionary consequences if the trait is heritable (Roff 1997). In this case, “heritability” refers to **narrow-sense heritability ( $h^2$ )**, which describes the degree of resemblance between relatives resulting from shared additive genes and is estimated as the proportion of total phenotypic variance that can be explained by additive genetic variation (Falconer and Mackay 1996).

$$h^2 = V_A/V_P$$

(Equation 4)

However, because quantitative genetics operates at the level of the phenotype, estimates of genetic variation may also capture epigenetic variance in addition to DNA sequence variation (Johannes et al. 2008). A population’s narrow-sense heritability is the best predictor of response to selection as represented in the “breeders equation”:

$$R = h^2 x S$$

(Equation 5)

where response to selection ( $R$ ) is equal to the narrow-sense heritability multiplied by the selection differential ( $S$ ). The response to selection is the change in the phenotypic mean between generations, and the selection differential is the difference in the phenotypic mean between the population as a whole and the selected parents of the next generation. When there is no resemblance of offspring to their parents ( $h^2 = 0$ ), no evolutionary change will occur for additive genes, regardless of the strength of selection (Lynch and Walsh 1998). This does not imply that traits with low heritability are not genetically determined. Genetic variance can be caused by many sources (Equation 2). In addition, traits that have become fixed in an inbred population can hypothetically have a heritability of zero as no variation exists for the trait.

While heritability is useful for predicting the absolute response to selection, it cannot be compared between populations and among traits because it can easily be influenced by the environmental variances that contribute to phenotypic variation (Houle 1992; Garcia-Gonzalez et al. 2012). Quantitative genetic studies frequently calculate dimensionless statistics in order to compare traits between populations and with other quantitative characters. Two common measures are the coefficient of additive genetic variation ( $CV_A$ ) and its square ( $I_A$ ):

$$CV_A = \frac{\sqrt{V_A}}{\bar{X}} \quad (\text{Equation 6})$$

$$I_A = \frac{\sqrt{V_A}}{\bar{X}^2} \quad (\text{Equation 7})$$

where  $\bar{X}$  is the phenotypic mean of the trait. Unlike heritability,  $CV_A$  and  $I_A$  are standardized to the trait mean and not phenotypic variance and are not influenced by other sources of variance (Garcia-Gonzalez et al. 2012). Mean-scaled estimates of other variance components can be used to make similar comparisons across studies and are calculated as the square of the respective variance component divided by the trait phenotype mean.

### *2.2.2 Environmental sources of phenotypic variance*

In addition to resemblance between individuals that results from genetic effects, individuals may resemble each other as a result of environmental effects. Similar to genetic variance, environmental variance ( $V_E$ ) can be partitioned into various subcategories including: general environmental variance ( $V_{EG}$ ), specific environmental variance ( $V_{ES}$ ), and gene by environment interaction (Byers 2008).

$$V_E = V_{EG} + V_{ES} + V_{GxE}$$

General environmental variance refers to non-genetic sources of variance that are experienced by multiple individuals in a population. This is the largest source of environmental variance, and can increase residual variance if additional fixed or random effects of the environment are not modeled (Byers 2008; Wilson et al. 2010). In addition to statistical methods to account for

environmental variance, captive studies reduce general environmental variance by creating a more homogenous environment. The deliberate reduction in environmental variance can inflate estimates of heritability, as heritability increases as phenotypic variance decreases (Equation 4). This sometimes makes quantitative genetic parameters obtained in captive populations difficult to relate to wild populations. The captive approach allows quantitative traits to be studied in isolation, although it should be supplemented by studies with wild populations when possible (Kruuk 2004; Wilson et al. 2010).

One form of general environmental variance, the common environmental effect, is especially frequent in captive settings and can cause an upward bias of genetic influence if not modeled. Quantitative genetic methodologies regularly rely on comparisons between relatives, such as parents and offspring and full siblings. Relatives are often more closely grouped in space and time compared to non-relatives, and therefore tend to share more environmental effects. These common environmental effects ( $V_{CE}$ ) are associated with the pedigree structure and the resulting autocorrelation between relatives can cause an upward bias of genetic variance. For example, siblings who share the same nest may be more similar due to common environmental effects, such as parental behaviors. Bias introduced by common environment can be reduced with the inclusion of additional random/fixed effects to separate genetic effects from common environmental effects (Kruuk and Hadfield 2007; Wilson et al. 2010)

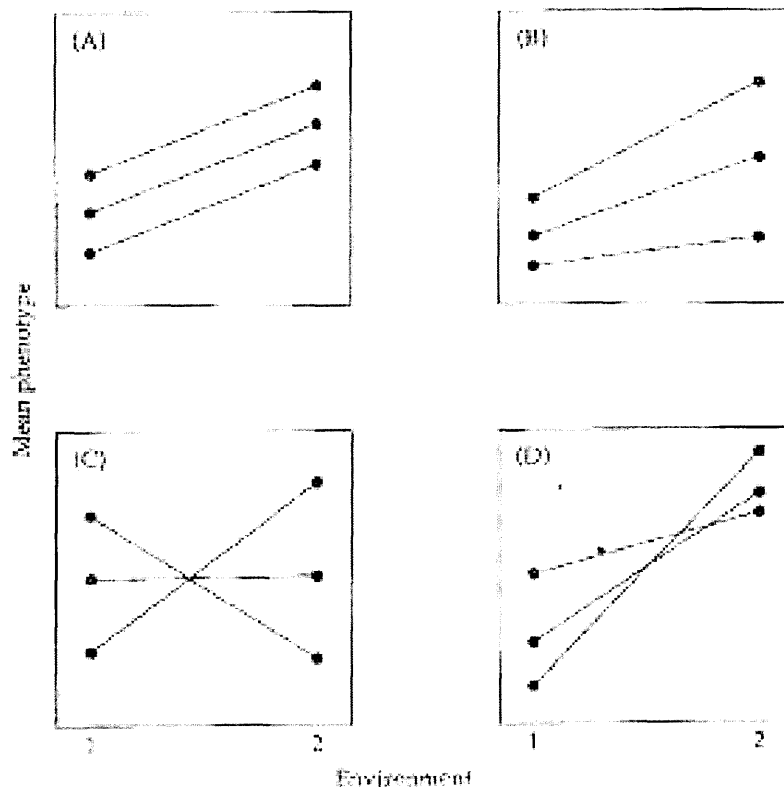


Cross-fostering is a common approach to assess common environmental effects in which offspring are switched between age- and size-matched clutches or broods (Falconer and Mackay 1996; Kruuk and Hadfield 2007; Tschirren and Postma 2010). Quantitative genetic analysis then partitions resemblance due to nest environment from phenotypic variance, thus reducing any potential bias of additive genetic variation. There is no agreed method/sample size for cross-fostering in quantitative genetic studies, and many studies risk upward bias of genetic influence by not incorporating cross-fostering into breeding designs. Cross-fostering can only separate common environmental effects after the cross-fostering has taken place. Maternal effects and environmental effects that take place early in development may still confound genetic effects (Kruuk and Hadfield 2007).

Specific environmental variance ( $V_{ES}$ ) are deviations from the mean phenotype that result from environmental conditions experienced by individuals (Lynch and Walsh 1998). This includes microenvironmental variation and effects of the permanent environment. Just as phenotypic variance can be influenced by non-genetic effects for individuals who share a common environment, each individual permanently shares a common environment with itself causing a permanent-environmental effect on phenotype (Kruuk 2004; Wilson et al. 2010). For example, the environment of early development may influence phenotype through the rest of an individual's life (Kruuk 2004). Similar effects may also occur as a result of an individual's home range and territory. The inclusion of

multiple measurements per individual allows Permanent-environmental variance ( $V_{PE}$ ) to be partitioned from the residual variance. Failure to model  $V_{PE}$  in studies with repeated measurements can result in an upward bias of  $V_A$ .

The third subcategory of environmental variance, gene by environment interactions (abbreviated GEI, the variance of which is expressed as  $V_{G \times E}$ ) describes how genotypes respond differently to the general environment variation. Gene by environment interactions can be visualized using the reaction norm, and describe how genotypes react to an environment gradient to produce a range of phenotypes (Figure 3).



**Figure 3.** Reaction norms for three genotypes in response to two environments. (A) No gene by environment interaction. (B) Gene by environment interaction due to a change in scale. (C) Gene by environment interaction due to a change in ranking. (D) Gene by environment interaction as a result of both change of scale and ranking. Figure reproduced from (Lynch and Walsh 1998).

The figure above illustrates two forms of gene by environment interaction: 1) a change in scale, when genotypes respond more or less strongly to a change in environment; and 2) a change in phenotype rank between environments (Lynch and Walsh 1998). These two types of GEI are not mutually exclusive (Figure 3D). The reaction norm also describes the degree of phenotypic plasticity present in the genotype, which is represented by the slope of each reaction norm. Gene by environment interactions can predict how genotypes can adapt to heterogeneous environments. GEI could be important to the adaptation of mercury tolerance as populations move in and out of contaminated locations. The plasticity of traits associated with mercury tolerance could allow individuals to avoid the energetic costs associated with mechanisms of mercury tolerance when in uncontaminated locations.

The mechanisms by which gene by environment interactions occur are still unclear, however, epigenetic processes such as DNA methylation may cause gene by environment interactions (Feil and Fraga 2012). Mercury affects DNA methylation, but this has only been demonstrated in mammals (Pilsner et al. 2010). Epigenetic effects could alter the individual phenotypic measurements in quantitative genetic studies, however the effects over multiple generations are unknown (Vandegheuchte and Janssen 2011).

Estimates of genetic and environmental variance are generated by comparing phenotypic resemblance between individuals. The genetic variance which underlies phenotypic variation is determined by comparing individuals of known relatedness. Traditional methods in quantitative genetics rely on one-level of relatedness (e.g. parent-offspring), where  $h^2$  is calculated as the slope of the regression of mid-offspring phenotype on mid-parent phenotype (Falconer and Mackay 1996). Methods such as this are limited because they compare individuals at only one level of relatedness (Lynch and Walsh 1998; Visscher et al. 2008), however, advances in the use of linear-mixed models, often called “Animal Models”, allow for comparisons at all levels of relatedness described in a population pedigree.

### *2.2.3 The Animal Model*

Both genetic and environmental variances can be estimated through a method of mixed modeling known as the Animal Model. Unlike traditional quantitative genetics methodology, the Animal Model can incorporate the entire pedigree structure to generate more robust estimates of variance components and heritability. This quality also makes the Animal Model less sensitive to unbalanced data sets by utilizing all available data. The Animal Model is a type of mixed modeling where terms include both fixed and random effects. Fixed effects are present within all levels of a data set (e.g. sex, sampling date), and are often constants that affect sample variation and can obscure underlying patterns of variance (Kruuk 2004). Random effects have multiple levels and are samples

from a larger population of potential values (Kruuk 2004). In the Animal Model, an individual's breeding value, or genetic merit, is included as a random effect in order to explain variation in a phenotype. The breeding value measures the additive effect of an individual's genotype on phenotype expression relative to the phenotypic mean in the population. In the simple case where treatment mean is a fixed effect and breeding value is a random effect, any resemblance among individuals must be the result of shared genes. In the most basic form of the Animal Model, the phenotype of a single trait ( $y$ ) for individual ( $i$ ) is given as:

$$y = \mu + a_i + e_i \quad (\text{Equation 6})$$

where  $\mu$  is the population mean,  $a_i$  is the breeding value (the additive genetic effect of  $i$ 's genotype relative to  $\mu$ ) and  $e_i$  is the residual term. Variance components are estimated directly by fitting random effects into a general mixed model (Kruuk 2004), where  $a_i$  will have a variance equal to additive genetic variance ( $V_A$ ), and  $e_i$  will have a variance equal to residual variance ( $V_R$ ). The form of the general mixed model is:

$$y = X\beta + Zu + e \quad (\text{Equation 7})$$

where  $y$  is a vector of observations on all individuals,  $X$  is a design matrix that relates fixed effects to individuals,  $\beta$  is a vector of fixed effects,  $Z$  is a design matrix that relates random effects to individuals,  $u$  is a vector of random effects, and  $e$  is a vector of residual error. Based on this framework, the Animal Model given in equation 6 can be re-written as:

$$\mathbf{y} = \boldsymbol{\mu} + \mathbf{u} + \mathbf{e} \quad (\text{Equation 8})$$

where  $\mathbf{X}$  is a vector of 1s,  $\boldsymbol{\beta}$  is equal to  $\mu$ ,  $\mathbf{Z}$  is the identity matrix, and  $\mathbf{u}$  is the vector of additive genetic effects. The covariance of random effects associated with vector  $\mathbf{u}$  (in this case breeding values) can be described by a matrix  $\mathbf{G}$ , which is calculated based on expectations of additive covariance between relatives. Because individuals share genes, the population pedigree allows for a prediction of how breeding values should vary between individuals. More closely related individuals share more additive genes underlying a phenotype and should exhibit stronger covariance compared to nonrelatives. The additive genetic covariance matrix  $\mathbf{G}$  is calculated as  $\mathbf{G} = 2\boldsymbol{\theta}_{ij} \times \mathbf{V}_A$ , where  $\boldsymbol{\theta}_{ij}$  is the coefficient of coancestry, or probability that an allele from individual  $i$  is identical by descent to an allele from individual  $j$ . A matrix corresponding to matrix  $\mathbf{G}$  is calculated for residual error as  $\mathbf{R} = \mathbf{I}\mathbf{V}_R$ , where  $\mathbf{I}$  is the identity matrix.

The estimation of variance components based on the Animal Model involves the use of maximum likelihood. Maximum likelihood estimates parameters in a model by selecting values for those parameters that have the highest probability (of likelihood) of supporting the actual data. For example, if parameters with low likelihood were true, there would be a low probability of observing the actual data. In applying maximum likelihood to equation 8, the vector  $\mathbf{y}$  has a mean of  $\mathbf{XB}$  and a variance ( $\mathbf{V}$ ) determined by additive genetic effects ( $\mathbf{G}$ ) and residuals ( $\mathbf{R}$ ). The likelihood of the model in equation 8 is then

calculated to determine maximum likelihood estimates for  $G$  and  $R$ , from which  $V_A$  and  $V_R$  can be calculated. Maximum likelihood estimated can become biased by underestimating residual variance (Kruuk 2004). For application to the Animal Model the maximum likelihood is restricted (restricted maximum likelihood, or REML) by transforming vector  $y$  to remove potential bias of residual variance.

The simplest REML-based Animal Models can generate estimates of  $V_A$  and  $V_R$ . In reality, other sources of variance may contribute to the resemblance among individuals. The inclusion of additional random effects can be used to calculate other variances, including common environmental, permanent environment, maternal effects, and dominance effects, depending on the data available and the statistical significance of the effect (Kruuk 2004; Wilson et al. 2010). Based on these variance components, total phenotypic variance ( $V_P$ ) is calculated as the sum of all the variance components included in the model. Heritability and coefficient of additive genetic variation can be calculated as in Equations 4 and 6-7.

Multiple measurements per individual can be accommodated by the Animal Model framework. **Repeatability** ( $r$ ) of a trait describes how strongly measurements in the same group (i.e. same individual) resemble each other. In application to the quantitative genetics of mercury accumulation, a high repeatability would indicate that individuals tend to accumulate mercury consistently. Traits that are highly repeatable are more stable under selection (Lynch and Walsh 1998). Repeatability also sets the upper limit to

heritability in the case where all phenotypic variance can be attributed to additive genetic variance (Falconer and Mackay 1996), although there are circumstances where this is not the case (Dohm 2002). In order to measure repeatability, the Animal Model partitions phenotypic variance into within- and among-individual components by including individual identity ( $V_{Ind}$ ) as a random effect (Wilson et al. 2010). Repeatability is calculated as the proportion of individual variance to phenotypic variance.

$$r = V_{Ind}/V_P \quad (\text{Equation 9})$$

Repeated measures per individual increase the likelihood of autocorrelation between measurements as a result of permanent environmental effects. The Animal Model can control for this by fitting an individual's identity twice: once in association with the pedigree to partition  $V_A$ , and secondly as a standard random effect which includes fixed non-genetic differences between individuals to partition out  $V_{PE}$  (Kruuk 2004; Wilson et al. 2010).

Formal hypothesis testing in REML-based models can be accomplished using the log-ratio test (LRT). The LRT compares the log-likelihood of the model to a reduced model from which the effect of interest has been dropped. The test statistic is twice the difference between log-likelihoods with an assumption that this follows a  $\chi^2$  distribution with degrees of freedom equal to the difference in variance components estimated (Lynch and Walsh 1998; Kruuk 2004; Wilson et al. 2010).



$$\chi^2_{1DF} = 2 \times \frac{\text{Log}L_{\text{complete}}}{\text{Log}L_{\text{reduced}}} \quad (\text{Equation 7})$$

The LRT can be used to determine the statistical significance of random effects. Because variance components are usually constrained to be positive, the LRT may be overly conservative (Gilmore et al. 2009). Some authors adjust to the conservative LRT by halving the p-value obtained from the LRT (Stram and Lee 1994), however, this may result in an anticonservative test (Wilson et al. 2010). There is no agreement by statisticians on the use of the LRT, however, quantitative genetic studies should report methods for calculating the LRT.

#### *2.2.4 Summary*

In summary, phenotypic variation among individuals can be attributed to both genetic and environmental sources, and both sources of variation must be understood in order to assess the potential for a population to evolve. The Animal Model uses restricted maximum likelihood to estimate variance components associated with quantitative genetic studies. Most importantly, the variance components estimated by the Animal Model can be used to assess the variability and heritability of quantitative traits, such as mercury accumulation, and evaluate their evolutionary potential.

#### *2.3 Heritability across populations*

Because heritability is a population parameter, estimations can vary between populations. Heritability estimates for the same trait can differ more than twofold between captive Zebra Finch populations (Tschirren and Postma 2010).

Heritabilities for body-mass were estimated at  $h^2 = 0.32$  (Airey et al. 2000),  $h^2 = 0.68$  (Birkhead et al. 2006), and  $h^2 = 0.34$  (Ronning et al. 2007) in three different captive Zebra Finch populations. Similarly, differences in heritability estimates among populations have occurred for tarsus length,  $h^2 = 0.32$  (Ronning et al. 2007) and  $h^2 = 0.78$  (Birkhead et al. 2006); PHA response,  $h^2 = 0.76$  (Gleeson et al. 2005) and  $h^2 = 0.22$  (Birkhead et al. 2006), and bill color,  $h^2 = 0.45$  ♂/0.48 ♀ (Price and Burley 1994) and  $h^2 = 0.42$  ♂/0.41 ♀ (Price 1996). These differences can be minimized by a standardized measure of genetic variation that is more suitable for comparison, such as  $CV_A$ , however, quantitative genetics studies should document potentially confounding sources of environmental and genetic variation.

Some of the variation described above may be explained by sampling differences or differences in environment (See: Environmental sources of variance), however, the genetic history of study populations may also influence trait heritabilities between populations (Tschirren and Postma 2010). As exports of the Australian Zebra Finch stopped in 1960, the number of founders for current laboratory populations may be small (Zann 1996). Moreover, relatively little interbreeding among captive populations may have facilitated drift among populations, leading to different trait expressions (Forstmeier et al. 2007a; Tschirren and Postma 2010).

Microsatellites have been used to assess the influence of founder effects and genetic drift among captive populations of Zebra Finch (Forstmeier et al.

2007a). Microsatellites, or short-tandem repeats (STRs), are short, repeating sequences of non-coding DNA. They are highly polymorphic and are frequently used to assess kinship and genetic diversity in populations (Ball et al. 2010). Several sets of microsatellite primers have been developed for the Zebra Finch (Dawson et al. 2005; Forstmeier et al. 2007a; Ball et al. 2010). Genetic variation differs significantly between captive populations of Zebra Finch, as well as between captive and wild populations (Forstmeier et al. 2007a). Captive Zebra Finch populations have also shown lower genetic diversity than wild populations (Forstmeier et al. 2007a).

There are no formal tests for comparing genetic variation among study populations, even though this information is essential for the interpretation of variation in quantitative traits. The range of variation in quantitative traits across study populations is also unclear (Tschirren and Postma 2010). Neutral markers, such as microsatellites, may provide a method for measuring genetic diversity, although there is disagreement about the ability of neutral markers to evaluate quantitative genetic variation among populations (Leinonen et al. 2008). The use of neutral markers assumes that variation at neutral loci accurately represents variation at quantitative loci (Tschirren and Postma 2010). Future research should examine this assumption by comparing diversity in neutral markers with quantitative genetic variation across many traits and study populations.

### **3.0 Adaptive response to mercury and consequences for biomagnification**

Quantitative genetics has been applied to study the evolution of resistance to ecotoxins (Klerks et al. 2011), but there has been limited application to the evolution of mercury tolerance. Most studies related to mercury tolerance have investigated the mechanisms underlying tolerance directly without consideration of genetic variation in tolerance (Barkay et al. 2003) or have measured the response to selection directly in laboratory or wild settings (Berk et al. 1978).

Evolutionary responses to mercury have been demonstrated across a wide variety of taxa. Mercury resistance in bacteria is widespread among both Gram-positive and Gram-negative bacteria (Barkay et al. 2003). The majority of studies in animals have measured tolerance in invertebrates (Berk et al. 1978; Roesijadi et al. 1982; Kraus et al. 1988; Benton and Guttman 1992; Capolino et al. 1997; Vidal and Horne 2003; Tsui and Wang 2005; Mahapatra et al. 2010). Mercury tolerance has been evaluated in few vertebrates; to my knowledge, mercury tolerance in vertebrates has only been described in fish (Blanc et al. 2003; Burnett et al. 2007). Because environmental mercury is primarily methylated in aquatic systems, investigations of mercury tolerance have naturally focused aquatic organisms. As terrestrial species can accumulate mercury to levels as high as aquatic consumers (Cristol et al. 2008), experiments, such as the current study, should broaden their focus to include terrestrial species.

The evolution of mercury tolerance could have significant consequences for biomagnification and conservation. Mercury tolerance could take the form of:

1) increased mitigation of mercury (limited uptake, increased elimination through feathers and eggs, detoxification/deposition pathway), or 2) decreased sensitivity to the detrimental effects of mercury. Both limited uptake and increased elimination of mercury in birds would result in decreased biomagnification of mercury as populations adapt to mercury contamination. However, if tolerance to mercury involves sequestering mercury in the liver or other tissues, individuals that survive mercury toxicity and eventually become prey items themselves may accumulate far more mercury than predicted by current estimates of biomagnification. A better understanding of mercury transfer through food webs will allow for better predictions of mercury availability and biomagnification.

Mercury tolerance could imply decreased risks for populations experiencing environmental mercury contamination. Although mercury-tolerant populations would be at a decreased risk from mercury toxicity, the evolution of tolerance may itself pose a risk to populations if the mechanisms of tolerance are costly. This may be especially relevant to migratory bird populations that move in and out of mercury contaminated environments.

#### **4.0 Research questions**

In order to measure the potential for adaptive response to mercury in songbirds, I measured the phenotypic variation and heritability of mercury accumulation in captive-dosed Zebra Finches. This captive-dosing experiment allowed mercury exposure to be standardized within treatments and reduced the environmental variance associated with studies of wild populations. Although

terrestrial songbirds can accumulate mercury to levels comparable to aquatic foragers (Cristol et al. 2008), the effects of mercury on songbirds remains understudied (Seewagen 2010). This experiment represents one of the first efforts to study mercury toxicity in songbirds in a controlled setting and numerous endpoints for physiology, reproduction, development, and behavior were measured by other experiments.

I evaluated the potential for adaptive response to mercury in a Zebra Finch population by measuring phenotypic variation and heritability of blood mercury accumulation. These parameters were estimated using a mixed-effects Animal Model. I hypothesized that blood mercury accumulation would be variable in Zebra Finches exposed at the same concentration of dietary methylmercury, and that individuals would be highly consistent (or repeatable) in their levels of mercury accumulation. I predicted variation in accumulation to increase linearly with dietary exposure, as among-individual differences in mercury intake/excretion/detoxification would become more pronounced at higher concentrations of mercury exposure. With regard to genetic influence on variation in mercury accumulation, I hypothesized that blood mercury accumulation would be highly heritable. As among-individual differences were predicted to increase with exposure, I expected estimates of heritability to increase with dietary mercury exposure. Assuming mercury accumulation to be a physiological trait, I predicted estimates of genetic influence to be comparable to other physiological traits in Zebra Finches (see Tschirren and Postma 2010).

To control for common environmental effects, which can cause an upward bias of additive genetic variance (Kruuk and Hadfield 2007), a small proportion of nestlings from each treatment were cross-fostered between nests. I also measured genetic diversity in the Zebra Finch colony by genotyping the parental generation using microsatellites. Because genetic diversity of the study population can influence estimates of quantitative genetic variance, microsatellite genotyping was used to compare genetic diversity in the William and Mary Zebra Finch colony with other captive populations.

## **Chapter 2. Materials and Methods**

### **1.0 Captive-dosing study**

#### *1.1 Study population*

This study was conducted with a population of 353 Australian Zebra Finches maintained in indoor and outdoor aviaries located at The College of William & Mary. The colony pedigree extended over four generations, with original founders obtained from multiple aviaries. The parental generation for this study consisted of 180 individuals paired at random, and pairs were bred continuously for one year. All breeding pairs were maintained in standardized cages with appropriate mercury or control food, ad libitum water, perches, nesting material and nest box, and on a long day (14:10 L:D) photoperiod. Because common environmental and maternal effects inflate estimates of genetic influence within broods (Kruuk and Hadfield 2007), age- and size-matched broods were cross-fostered as nestlings when available (nestlings cross fostered:  $N_{0.3} = 15$ ;  $N_{0.6} = 15$ ;  $N_{1.2} = 7$ ;  $N_{2.4} = 0$ ). Offspring were removed to same-sex group cages on appropriate diets after reaching fledged independence (approximately 50 days).

#### *1.2 Dietary mercury dosing*

Zebra Finch pairs (18 per treatment) were randomly assigned to one of five dietary concentrations (0.0, 0.3, 0.6, 1.2, 2.4 ppm) of methylmercury cysteine (MeHgCys). Mercury-dosed foods were prepared by homogenizing stock concentrations of chemically pure MeHgCys into a pelletized ZuPreem finch food



(contents: 14.0% min. protein, 4% min. crude fat, and 3.5% max fiber). Selenium concentrations in the prepared finch food were negligible. Each diet was sampled 10 times for mercury content in every batch and average mercury content was  $0.00425 \pm 0.00165$  ppm in the control diet. Mercury dosed diets contained between 99.27-102.13% of desired values with a mean concentration of 100.79% of the calculated mercury concentration. All mercury measurements in food are reported as wet weight (ww), however the moisture content in food was only 10% max.

### *1.3 Mercury quantification*

Blood mercury content is a common measurement to assess overall mercury exposure (Seewagen 2010). Blood samples of approximately 20-50  $\mu\text{L}$  were collected monthly using a 30-gauge needle to puncture the cutaneous ulnar vein and then collecting blood from the surface droplet in 70 $\mu\text{L}$  heparinized capillary tubes. Each capillary tube was sealed with Crito-Caps, stored in an individually labeled 10cc BD Vacutainer, and frozen at  $-20^{\circ}\text{C}$  until analysis. All samples were analyzed for total mercury content (THg) using a Direct Mercury Analyzer DMA-80 (Milestone, Monroe, CT, USA). Mercury quantification occurred through an automated sequence of heating and decomposition, catalysis, amalgamation, and atomic absorption spectroscopy.

Quality assurance measures were maintained using two certified reference materials: dogfish muscle tissue and dogfish liver (DORM-3 and

DOLT-4, National Research Council of Canada, Ottawa, ON, Canada). DORM-3 and DOLT-4 were also used to calibrate the mercury analyzer. Each batch of samples was preceded and followed by the following sequence of system/method blanks and reference materials: System Blank, System Blank, Method Blank, DORM-3, DOLT-4, H<sub>2</sub>O, System Blank, System Blank, System Blank. Recoveries for certified reference materials averaged 103.48% ± 0.43 (n = 1489) for DORM-3 and 100.32% ± 0.22 (n = 1461) for DOLT-4. Matrix spikes were performed regularly, and recoveries averaged 101.15% ± 3.56 (n = 62).

## **2.0 Quantitative genetics**

We measured the phenotypic variation and heritability of blood mercury accumulation within dietary mercury treatments using a repeated-measures Animal Model. The Animal Model is a method of mixed modeling that partitions phenotypic variation for a quantitative trait into separate genetic and environmental variance components and includes an individual's breeding value, or individual genetic merit, as a random effect (Lynch and Walsh 1998). Mercury levels in offspring included in the model were those obtained after full maturity was reached (approximately 100 days). All analyses were conducted using ASReml version 3 (Gilmore et al. 2009).

We ran Animal Models for each dietary mercury treatment separately with independent variance components partitioned for each. Additionally, I ran a model combining all four mercury dose treatments which included treatment as a fixed effect in order to normalize blood mercury levels. The initial models included

sampling date, age, and sex as fixed effects, and additive genetic effect, permanent environment, and foster nest environment as random effects. Because estimates of genetic influence are prone to upward bias for repeated-measures traits (Kruuk and Hadfield 2007), individual identity was included twice in the model: once in association with the pedigree to partition additive genetic variance ( $V_A$ ) and secondly as a standard random effect which includes fixed non-genetic differences between individuals to partition out permanent environmental variance ( $V_{PE}$ ) (Kruuk 2004; Wilson et al. 2010).

The model partitioned variance components for each random effect. Variation of blood mercury accumulation within treatments was measured by total phenotypic variance ( $V_P$ ), which was calculated as the sum of all variance components for each random effect plus the residual error ( $V_P = V_A + V_{PE} + V_F + V_R$ ). Between-individual variance ( $V_{IND}$ ) was calculated as the sum of additive genetic variance and permanent environmental variance ( $V_{IND} = V_A + V_{PE}$ ). Repeatability ( $r^2 = V_{IND}/V_P$ ), narrow-sense heritability ( $h^2 = V_A/V_P$ ), and permanent environmental effect ( $pe^2 = V_{PE}/V_P$ ) within each treatment were calculated as the proportion of the related variance component to total phenotypic variance. Comparisons between treatments, including a comparison of relative phenotypic variation, were made using mean-scaled coefficients of variation for total phenotypic variance ( $CV_P$ ), permanent environmental ( $CV_{PE}$ ), foster environmental ( $CV_F$ ), and residual variance ( $CV_R$ ). All coefficients of variance were calculated as the square root of the respective variance component divided

by the treatment mean of blood mercury. Two mean-scaled measures of evolvability (i.e., additive genetic variance or potential to evolve given the right circumstances), coefficient of additive genetic variation and  $I_A$ , were calculated for all mercury dose treatments as  $CV_A = \sqrt{V_A}/\bar{X}$  and  $I_A = V_A/\bar{X}^2$ , where  $\bar{X}$  is the blood mercury treatment mean. Mean-scaled coefficients of variation could not be calculated for the normalized blood mercury model, as these statistics are inappropriate for data transformed in this way.

Significance values for fixed effects were estimated using conditional Wald  $F$  statistics (Gilmore et al. 2009); non-significant effects ( $p > 0.05$ ) were removed from the model, leaving only the main effect and other significant interactions. The significance of random effects was tested using the likelihood ratio test (LTR). The significance of variance ratios ( $r^2$ ,  $h^2$ ,  $pe^2$ ) was calculated using one-sample  $t$ -tests with the standard errors reported by ASReml.

### **3.0 Microsatellite genotyping**

The variance components estimated by quantitative genetics methodology can be influenced by the genetic history of the study population (Tschirren and Postma 2010). In order to measure genetic diversity of the William & Mary colony in relation to other captive Zebra Finch populations, I genotyped the breeding pairs within each treatment. The analysis used previously developed microsatellites ZF02-129, ZF01-025, ZEST09-018, ZF01-190 (Primers and fluorescent dyes listed in Table 1) (Ball et al. 2010). PCR amplifications were performed using a QIAGEN Multiplex PCR kit (QIAGEN). Each multiplex PCR

contained 4 $\mu$ L QIAGEN Multiplex PCR Master Mix, 10ng DNA, and 2 $\mu$ L of the primer mix. The cycling conditions were: an initial denaturation at 95°C for 5-min, 28 cycles of 30-s denaturation at 95°C, 30-s annealing at 57°C, and 90-s extension at 72°C. Thermocycling was followed by a 30-min final extension at 57°C. PCR products were separated and visualized using an ABI 3130 Genetic Analyzer (Applied Biosystems) calibrated to DS-33 and the LIZ size standard. Data were analyzed using GENEMAPPER 3.7 and GENEPOP online to record genotypes at microsatellite loci, calculate expected ( $H_E$ ) and observed ( $H_O$ ) heterozygosity, and test for deviation from Hardy-Weinberg Equilibrium. Inbreeding coefficient ( $F_{IS}$ ) was calculated as  $F_{IS} = (H_E - H_O)/H_E$ .

## **Chapter 3. Results**

### **1.0 Quantitative genetics of mercury accumulation**

Blood mercury accumulation showed considerable variation at all dietary mercury treatments. Mean-standardized estimates of variation, represented by coefficients of total phenotypic variation ( $CV_P$ ), were equivalent across all levels of dietary exposure and ranged from 0.239 to 0.283 (Table 2). Repeatability ( $r^2$ ) of blood mercury ranged from 0.1996 to 0.4577 and was highly significant for all mercury treatments and in the normalized blood mercury model (Table 1).

The contribution of additive genetic variation on blood mercury accumulation was non-linear with increasing Hg exposure. Significant heritabilities were calculated for the 0.6 and 1.2 mercury treatments (0.4577 and 0.341, respectively); a significant heritability of 0.444 was calculated for the normalized blood mercury model (Table 1). Similarly, the mean-scaled measures of evolvability, coefficient of additive genetic variation ( $CV_A$ ) and  $I_A$ , were highest for Zebra Finches dosed at 0.6 ppm MeHg ( $CV_A = 0.192 \pm 0.069$ ;  $I_A = 0.037 \pm 0.017$ ;  $p < 0.05$ ) and were marginally non-significant ( $CV_A = 0.139 \pm 0.052$ ;  $I_A = 0.019 \pm 0.010$ ;  $p = 0.09$ ) for the 1.2 ppm MeHg treatment (Table 1). High  $CV_A$  and  $I_A$  values indicate a high degree of genetic influence on mercury accumulation and a greater evolutionary potential. Zebra Finches dosed at 0.3 and 2.4 ppm MeHg showed a low contribution of additive genetic variance ( $p = 0.913$  and  $0.806$ , respectively) on blood mercury (Table 1), although the partitioning of among-individual variance into significant permanent environmental effects may

have prevented an upward bias of additive genetic variation in these treatments (Table 1). Common environmental effects, measured as foster environment ( $CV_F$ ), had a negligible effect in all treatments except in the 1.2 ppm dietary mercury dose (Table 1).  $CV_F$  was not estimated for the 2.4 treatment due to reduced nestling survival.

All variance estimates were conditioned by sampling date, age, and sex with the inclusion of fixed effects for these terms. Sampling date significantly affected blood mercury across all treatment levels ( $p < 0.01$ ; Appendix 1). Age at time of sampling affected blood mercury accumulation in the 0.3 ( $p = 0.002$ ), 0.6 ( $p = 0.030$ ) and the 1.2 ( $p = 0.034$ ) ppm dietary mercury treatments and in the normalized blood mercury model ( $p < 0.001$ ; Appendix 1). Females had lower levels of mercury accumulation than males in the 0.3 ( $p < 0.001$ ) and the 1.2 ( $p < 0.001$ ) ppm dietary mercury treatments and in the normalized blood mercury model ( $p = 0.002$ ; Appendix 1).

## **2.0 Microsatellite genotyping**

Microsatellite genotyping of the breeding pairs within each treatment demonstrated a high level of genetic diversity in the captive Zebra Finch colony. All microsatellite loci were highly polymorphic, with an average of  $17.25 \pm 4.25$  alleles. There was no significant deviation from Hardy–Weinberg equilibrium ( $p > 0.05$ ), and the inbreeding coefficient was low ( $F_{IS} = -0.00562$ ; Table 2).

## **Chapter 4. Discussion**

We exposed breeding pairs of Zebra Finches to one of four standardized levels of dietary methylmercury and measured the resulting mercury accumulation in the blood of both parents and offspring. I used a repeated-measures Animal Model to investigate the phenotypic variation of blood mercury accumulation within dietary treatments and the influence of genetics on patterns of mercury accumulation. At all dietary methylmercury treatments, Zebra Finches exhibited considerable variation in blood mercury accumulation, and this variation was highly repeatable for individuals. I observed a strong genetic influence on blood mercury accumulation, however this effect was non-linear with increasing mercury exposure; only a negligible genetic contribution to blood mercury accumulation was detected in the lowest and highest dietary mercury treatments. Microsatellite genotyping of the Zebra Finch colony demonstrated a high level of genetic diversity, indicating that the variance estimates were not biased by a lack of genetic diversity in the colony. The close resemblance between individuals was also not influenced by common environmental effects of nesting environment.

### **1.0 Quantitative genetics of mercury accumulation in captive-dosed Zebra Finches**

#### *1.1 Variation and repeatability of blood mercury accumulation*

Blood mercury accumulation varied substantially within all dietary mercury treatments and in the normalized blood mercury model. This confirms my initial



hypothesis that a population exposed at the same concentration of dietary methylmercury would exhibit variation in their levels of blood mercury accumulation. The relative phenotypic variation ( $CV_P$ ) of blood mercury accumulation was equivalent between treatments; my initial prediction hypothesized that relative variation would increase with dietary exposure. Phenotypic variation of blood mercury accumulation was comparable to variation in physiological traits reported in a recent review of quantitative genetics in the Zebra Finch and exceeded  $CV_P$  values for morphologic, physiological, and ornamental traits combined (Appendix 3, Figure 4).

Blood mercury levels were highly repeatable for individuals. Repeatabilities are frequently reported with quantitative genetic estimates and may serve two functions: indication of measurement error for static traits and as a measure of the consistency of a dynamic trait measured over time (Falconer and Mackay 1996). Blood mercury accumulation represents the latter type of repeatability, although repeatabilities of mercury levels may also include measurement error. Repeatability estimates of blood mercury accumulation exceed the average (0.32) reported in a recent review of repeatabilities in quantitative characters (Wolak et al. 2012) in all treatments except the 0.3 ppm MeHg dietary dose. The repeatability in this treatment was reduced from a previous model with the inclusion of fixed effects for sex and age, which reduced permanent environmental variance.

### *1.2 Heritability of mercury accumulation*

In contrast to my prediction, genetic influence on mercury accumulation was non-linear with increasing dietary mercury exposure, and I observed significant gene by environment interactions. A high genetic influence on blood mercury accumulation was observed at dietary mercury concentrations of 0.6 and 1.2 ppm MeHg ( $h^2 = 0.458$  and  $0.341$  respectively); high genetic influence was also detected when mercury accumulation was normalized by treatment ( $h^2 = 0.444$ ). Birds exposed to diets containing 0.3 and 2.4 ppm MeHg did not show significant genetic contribution to mercury accumulation. Mean-scaled measures of additive genetic variation ( $CV_A$ ) for the 0.6 and 1.2 ppm MeHg dietary treatments exceeded  $CV_A$  values reported for physiological, ornamental, and morphological traits in a recent review of quantitative genetics in the Zebra Finch (Tschirren and Postma 2010) (Appendix 3, Figure 5). Birds exposed to diets containing 0.3 and 2.4 ppm MeHg did not show significant additive genetic variance, although significant effects of permanent environment were observed. When blood mercury was modeled for these treatments without inclusion of permanent environment, estimates of genetic contribution to blood mercury increased, indicating the potential for upward bias of additive genetic effects without the inclusion of permanent environment.

A lack of genetic influence on mercury accumulation in both the lowest and highest dietary mercury treatments may be the result of thresholds which limit tolerance to mercury. Below the threshold where mercury toxicity negatively affects individual health, the energetic cost of a response to mercury may

outweigh the benefits of tolerance. This theory is consistent with research on mercury detoxification in wild birds; Eagles-Smith et al. (2009) reported a threshold for mercury demethylation in waterbird livers where demethylation occurred only when liver mercury concentrations increased above  $8.51 \pm 0.93$  ppm. Thresholds of demethylation have not yet been demonstrated in the Zebra Finch (or any songbird), and it is unclear if this effect is responsible for low genetic contribution to blood mercury accumulation in the 0.3 ppm treatment group. Future captive-dosing studies should investigate the potential for demethylation thresholds in Zebra Finches and the probable co-variation between liver detoxification of mercury and blood mercury accumulation.

For Zebra Finches exposed to dietary mercury levels at 2.4 ppm, an upward threshold may have prevented a genetic influence on blood mercury accumulation. Blood mercury levels in the range represented by the 2.4 ppm MeHg treatment are associated with reproductive and health costs (Eisler 2006; Seewagen 2010). The effects of high mercury levels on protective mechanisms that may be responsible for mercury mitigation in birds are unknown, although it is possible that high mercury levels in this treatment may have exhausted the capacity of individuals to affect mercury accumulation through genetic means.

### *1.3 Mechanisms of genetic influence on mercury accumulation*

The current study cannot describe the mechanisms that underlie the quantitative genetic variation described because inferences of genetic influence were made at the level of the phenotype. As described more fully in the

introduction to this thesis, numerous processes affect the balance of mercury absorption and excretion in vertebrates including deposition into eggs and feathers (Seewagen 2010) and detoxification in the liver (Ikemoto et al. 2004). The variation in these processes and their potential for evolutionary change in response to mercury remains unclear, and future research should examine (co)variation among these mechanisms in order to develop a more accurate understanding of mercury tolerance.

Additional mechanisms beyond those described may contribute to mercury tolerance, and a number of techniques are available to identify candidate gene sets. Quantitative trait loci (QTL) methodology combines quantitative genetics with molecular markers such as microsatellites and single-nucleotide polymorphisms (SNP) in order to locate regions of a chromosome responsible for trait differences (Falconer and Mackay 1996). Application of QTL methodology to toxicology has been limited, although QTL were used to identify candidate genes for cold tolerance in *Drosophila melanogaster* (Poynton and Vulpe 2009). The ability to profile gene expression using microarray and transcriptome technologies offers an alternative to QTL methods. The development of these technologies makes it possible to compare patterns of expression across the entire genome for individuals at contaminated and non-contaminated locations (Poynton and Vulpe 2009). This approach, specifically microarray and quantitative real-time PCR, was recently utilized to identify candidate genes for tolerance to mercury in *Drosophila* (Mahapatra et al. 2010).

The immune-response gene Turandot A (TotA) showed strong up-regulation with mercury exposure in *Drosophila* (Mahapatra et al. 2010), however, this gene has no homologue in vertebrates. Additionally, significant up-regulation was observed in cytochrome P450 (CYP) (Mahapatra et al. 2010), which has high activity in liver and kidney tissues in vertebrates (Suda and Hirayama 1992).

Both QTL and genomic techniques could be used to expand knowledge of mercury tolerance beyond *Drosophila*. Microsatellite and SNP markers are available for the Zebra Finch for QTL analysis (Ball et al. 2010).

#### *1.4 Forecasting evolution in response to mercury*

In addition to partitioning phenotypic variation into sources of genetic and environmental variation, the prediction of the evolutionary potential of quantitative traits is a main goal of quantitative genetics (Lynch and Walsh 1998). In order for a trait to evolve under selection it must be both variable and heritable. The current study has demonstrated substantial variation in blood mercury accumulation within dietary mercury treatments that could be acted on by selection. The repeatability of blood mercury accumulation in individuals may make this more stable as a trait for selection. Variation in mercury accumulation was highly heritable for the 0.6 and 1.2 ppm MeHg treatments. Taken together, the variation and heritability of mercury accumulation suggest that adaptation in response to mercury would be possible in the current captive Zebra Finch population. Because quantitative genetics estimates population parameters, difficulties arise when attempting to extrapolate across populations/species.

Variance components for mercury accumulation may differ between populations, with variable levels of exposure, and between captive and wild birds. In addition, caution must be employed when attempting to forecast evolutionary change from the prediction of breeding values. Estimates of heritability can vary over time and under different environmental conditions (Postma and Charmantier 2007).

Likewise, selection pressures can vary, making long-term prediction of micro-evolutionary change highly uncertain.

Estimates of heritability for one particular trait do not provide insight into how other traits evolve. Characters may not evolve independently if they genetically correlate with other traits. For example, if mercury contamination results in a selection pressure for increased tolerance to mercury, and mercury tolerance negatively correlate with a separate trait affecting fitness, then the response to selection will be constrained by the genetic correlation. Given the number of physiological processes potentially involved in metal pathways in vertebrates (see Introduction), mercury accumulation is likely to be correlated with many traits. The selection pressure induced by mercury contamination may also act to drive correlated traits in different directions. Understanding the covariation of traits related to mercury tolerance is essential to predicting an evolutionary response.

The degree to which traits are genetically variable and genetically correlated can be related using a G-matrix (Steppan et al. 2002). The G-matrix is a matrix that displays additive genetic variances and co-variances. Correlated

traits do not evolve independently, and strong genetic correlation can constrain evolutionary trajectory. G-matrix methodology can be used to describe the evolutionary potential of a population with respect to quantitative characters and the direction in which selection may drive a population (Steppan et al. 2002). Although used throughout other evolutionary contexts, the G-matrix approach has not been applied to the evolution of contaminant tolerance (Klerks et al. 2011). Future research should examine co-variation among genetic mechanisms for tolerance to mercury, as well as possible constraints on adaptation to mercury.

## **2.0 Genetic and environmental variables**

The estimation of quantitative genetic variances can be influenced by both the genetic history of the study population and environmental covariates (Falconer and Mackay 1996; Roff 1997). In order to assess the genetic diversity of the Zebra Finches used in this study, I typed the breeding pairs used in this study at four microsatellite loci (Ball et al. 2010). Common environmental effects were included in models for the 0.3, 0.6, and 1.2 dietary MeHg treatments by cross-fostering a portion of nestlings within each treatment. The addition of fixed effects for date, age, and sex were included in the models to condition variance estimates based on these effects.

### *2.1 Genetic diversity in the Zebra Finch colony*

Microsatellite genotyping of breeding pairs showed the parental generation to be highly diverse at all loci typed, and to have genotypic

frequencies within Hardy–Weinberg equilibrium. High genetic diversity was observed in the William & Mary colony; I observed a higher number of alleles per locus in this colony than in the population from which the microsatellites were developed (Table 3). The levels of inbreeding observed in the breeding pairs used for this study are comparable to those calculated from published heterozygosity values (Table 3).

**Table 3.** Genetic diversity and level of inbreeding in the parental generation typed at four microsatellite loci in comparison to a captive population maintained at the University of Sheffield, Sheffield, UK (Ball et al. 2010).  $N_A$ , average number of alleles;  $H_E$ , expected heterozygosity;  $H_O$ , observed heterozygosity;  $F_{IS}$ , inbreeding coefficient.

Population	$N_A$	$H_E$	$H_O$	$F_{IS}$
<b>This study</b>	17.25	0.889	0.894	-0.00562
<b>Sheffield</b>	10.5	0.785	0.8025	-0.02229

The Zebra Finch population used in the present study had higher genetic diversity than was reported in the colony at the University of Sheffield, which has been used for numerous quantitative genetics studies (Tschirren and Postma 2010) and was the same pedigree used to construct a linkage map of the Zebra Finch genome (Stapley et al. 2008). Deliberate outbreeding during the creation of the William & Mary colony may be responsible for the high genetic diversity observed. Assuming that high variation in neutral markers indicates similar variation at quantitative loci, estimates of genetic influence on mercury accumulation made by this study are unlikely to be biased by inbreeding.



Few studies have attempted to measure colony genetic diversity in conjunction with quantitative genetic estimates in Zebra Finches (Tschirren and Postma 2010), although the genetic diversity of captive Zebra Finches varies considerably among laboratory populations, and also between captive and wild populations (Forstmeier et al. 2007b). Understanding the differences in genetic diversity among study populations is essential to comparing relative variation among quantitative characters. There are currently no formal tests for assessing genetic diversity in association with quantitative genetic methodology, however, neutral markers, such as microsatellites, may provide a method for measuring genetic diversity. Considering the increasing number of studies that use microsatellites in the search for quantitative trait loci (more below) (Lynch and Walsh 1998; Ball et al. 2010), calculation of genetic diversity and inbreeding could easily be incorporated into experimental designs.

## *2.2 Common environmental effects*

In general, nest environment did not influence blood mercury accumulation, although inclusion of foster nest identity significantly improved the model for the 1.2 ppm mercury treatment. The magnitude of common environmental effects ( $0.024 \pm 0.119$ ) in the 1.2 ppm mercury treatment was small in comparison to the coefficients of phenotypic variance ( $0.239 \pm 0.066$ ) and additive genetic variance ( $0.139 \pm 0.052$ ). These results indicate that common environmental effects were negligible and also that the resemblance between relatives was not inflated by environmentally-induced autocorrelation.

Failure to include cross-foster data could have allowed an upward bias of additive genetic variance if common environmental effects were present (Wilson et al. 2010). Maternal and common environmental effects that occurred before nestlings were cross-fostered may still contribute to the uncertainty of variance component estimates (Kruuk and Hadfield 2007). In particular, maternal deposition of mercury into eggs has numerous effects on embryo development (Heinz 1975; Heinz and Hoffman 2003; Heinz et al. 2006). Lifelong patterns of mercury accumulation could be influenced by the developmental stress induced by maternal deposition of mercury into eggs, although this effect could be resolved by the current study.

### *2.3 Fixed effects of date, age, and sex on blood mercury accumulation*

Additional fixed effects are often fitted in Animal Models in order to separate effects of the environment from additive genetic effects (Wilson et al. 2010). The inclusion of fixed effects may thus provide better estimates of variance components in quantitative genetics studies. In the current study, fixed effects for sampling date, sex, and age were included into models of blood mercury accumulation.

Both sampling date and age were included as fixed effects in order to condition variance estimates on the basis of temporal effects. The inclusion of fixed effects for sample date significantly improved all models of blood mercury accumulation, and age had a significant influence in all models except the 2.4 ppm MeHg treatment. Estimates of additive genetic variance increased in all

models after conditioning for the effect of sampling date; the further inclusion of age did not raise estimates of additive genetic variation beyond condition based on sampling date. This suggests that both date and age reflect temporal differences in mercury accumulation over the course of this study, however, the factors responsible for differences in mercury accumulation with respect to date and age could not be determined. Variation among batches of mercury-dosed food is unlikely to explain differences with respect to sampling date, as measures of quality assurance for food preparation indicate high consistency of mercury concentrations (99.27-102.13% of desired concentrations) between batches. Changes in environmental conditions experienced by offspring, who were removed to outdoor cages after fledged independence, may account for variation by both sampling date and age. In this case, the inclusion of date as a fixed effect may have conditioned for variation in temperature and other environmental changes experienced by offspring housed outside.

Sex had significant effects on blood mercury accumulation for all treatments except at the 2.4 ppm MeHg dose. Females had lower mercury accumulation than males. Mercury excretion into eggs may explain lower accumulation in females; the effect was larger when offspring (which are too young to lay eggs) were excluded from the model. This result is consistent with other studies, which have reported lower mercury concentrations in females as a result of egg-laying (Lewis et al. 1993; Robinson et al. 2011). The lack of effect in

the 2.4 ppm MeHg treatment may be the result of a reduced number of clutches produced by females in this treatment.

In summary, I did not find evidence that quantitative genetic estimates in this study were influenced by a low genetic diversity of the captive Zebra Finch population or by common environmental effects of nest environment. The inclusion of fixed effects conditioned variance components on the basis of effects of sex and temporal variation resulting from sampling date and age.

### **3.0 Implications for environmental mercury contamination**

This research has demonstrated heritable variation in patterns of mercury accumulation for captive-dosed Zebra Finches, which suggests a strong potential for evolution in this captive population. A primary goal of studying contamination in a captive setting is the ability to make predictions for wild populations. Extrapolation to wild populations based on this or other captive studies must be made with caution, as numerous differences in environmental conditions may lead to differences in responses between captive and wild populations. Evolution of tolerance to mercury, either in the form of decreased sensitivity to its detrimental effects or through mechanisms of increased excretion and/or detoxification, could have serious implications for conservation and our understanding of mercury biomagnification.

#### *3.1 Mercury exposure in captive and wild settings*

The dietary mercury treatments used in this study at 0.3, 0.6, and 1.2 ppm MeHg span the range of dietary levels of exposure for songbirds living at contaminated sites (Cristol et al. 2008); dietary exposure at 2.4 ppm MeHg exceeds levels of exposure reported in songbird prey items. Prey items collected from Carolina Wrens (*Thryothorus ludovicianus*), Eastern Bluebirds, and House Wrens (*Troglodytes aedon*) foraging near the mercury-contaminated South River had mercury concentrations of  $1.24 \pm 1.47$  ppm (Spiders),  $0.38 \pm 2.08$  ppm (Lepidopterans), and  $0.31 \pm 1.22$  ppm (Orthopterans) (Cristol et al. 2008); these food sources accounted for >80% of food delivered to nestlings (Cristol et al. 2008). These dietary levels of mercury exposure at the South River produced adult blood mercury concentrations of  $4.49 \pm 2.27$  (Carolina Wrens),  $1.39 \pm 0.95$  (Eastern Bluebirds), and  $2.38 \pm 1.14$  (House Wrens) (Cristol et al. 2008).

Although the dietary levels of mercury used in this study accurately reflect the mercury content in highly contaminated prey items reported at the South River, they produced blood mercury concentrations that were much higher than those reported by studies of wild songbirds (Cristol et al. 2008; Jackson et al. 2011). The amount of biologically available methylmercury in relation to total mercury may explain these observed differences in accumulation. Prey items on the South River contained lower percentages of methylmercury relative to total mercury compared to this study, and wild diets were also much less uniform in mercury content. The methylmercury content of prey items on the South River ranged between  $49 \pm 21\%$  (Spiders),  $38 \pm 24\%$  (Orthoperans), and  $24 \pm 20\%$

(Lepidopterans) (Cristol et al. 2008). In contrast, this study used chemically pure methylmercury. As inorganic mercury may not be absorbed at the same rate as methylmercury (Clarkson and Magos 2006), higher levels of mercury accumulation by birds in this study may be explained by an increased percentage of dietary methylmercury.

Conditions of the captive environment likely contributed to an increased availability of mercury and may also help to explain differences in mercury accumulation between captive and wild songbirds. Zebra Finches in this experiment had access to mercury-dosed food ad libitum, with virtually no cost associated with foraging. Levels of dietary exposure were consistent throughout the course of the experiment, whereas exposure levels in wild birds are likely to fluctuate with prey availability and with variation in contamination between foraging patches. Because many songbirds are migratory, mercury exposure varies as individuals move in and out of contaminated locations.

More methylmercury was available to birds in this study than under conditions of environmental mercury contamination, both through an increased ratio of methylmercury to total mercury and through increased food availability. However, the presence of environmental stressors absent in the captive environment may increase the cost of mercury toxicity in wild songbirds. Compared to wild songbirds, Zebra Finches in captivity live in a more uniform and much less challenging environment. Birds in a captive environment lack risks such as predators and parasites and are less susceptible to fluctuations in

climatic conditions, and these environmental effects may increase the effects of mercury toxicity. For example, reduction in fledgling production in Tree Swallows on the South River was associated with increased temperature during early nestling growth in mercury contaminated locations, but not in uncontaminated reference sites (Hallinger and Cristol 2011). Environmental conditions were standardized in this study in order to create a uniform environment. If the presence of environmental stressors absent in the captive environment increases the effects of mercury toxicity, the threshold at which genetic influence becomes important may be lower than observed in this study.

In summary, comparisons of mercury accumulation between natural and captive conditions are somewhat limited because captive settings cannot approximate environmental conditions. The dietary mercury concentrations used in this study have a higher proportion of methylmercury than prey items at contaminated sites, and this may explain higher levels of accumulation in this study. Numerous environmental variables that were not included in this study have the potential to affect mercury accumulation in wild birds. The use of domestic Zebra Finches in a captive setting to study mercury toxicity suffers from the same limitations common to all captive model systems. Captive populations cannot answer some of the questions that may be addressed using wild populations, however, the environmental variation present in wild populations prevents mercury accumulation from being studied in isolation. The incorporation of controls lacking in natural habitats has allowed this study to estimate the level

of genetic influence on mercury accumulation in a population of Zebra Finches. The use of captive-dosed Zebra Finches is relevant to mercury contamination in wild songbirds as the same mechanisms of mercury tolerance would likely be affected. Continued research using captive Zebra Finches may lead to a greater understanding of the mechanisms underlying mercury tolerance. The potential for evolution of mercury tolerance demonstrated in this study may eventually be tested in wild populations.

### *3.2 Adaptive response in wild populations*

As stated above, the extrapolation of the genetic influence on mercury accumulation found in this study to wild populations of songbirds experiencing environmental mercury contamination must be done with extreme caution. Quantitative genetics partitions sources of variance at the population level, and the parameters derived for one population are not necessarily shared by other populations (Tschirren and Postma 2010). While this study cannot be used to make direct inferences about the evolutionary potential of mercury tolerance in wild birds, the methodology used in this study may in time be applied to environmental mercury contamination.

The application of quantitative genetic methodology to wild populations is increasingly common (Kruuk 2004; Postma and Charmantier 2007; Wilson et al. 2010). The Animal Model is particularly suitable for use in natural populations because it estimates variance components using all the relationships shown in the pedigree structure, and can therefore accommodate the complex and often



incomplete pedigrees common in studies with wild populations (Postma and Charmantier 2007). The construction of more accurate pedigrees in wild populations has improved with advances in the use of molecular markers to assign paternity. Except in cases where the nesting environment can be closely monitored, such as with species that will use nest boxes, measures of genetic variation in wild populations may become biased by common environmental effects (Kruuk 2004). As described above, dietary mercury availability is highly variable in natural environments, and variations in dietary intake will influence mercury levels among individuals. For this reason, a direct measure of mercury accumulation cannot be used as a quantitative trait in wild populations unless levels are measured alongside a mechanism of tolerance shown to strongly co-vary with mercury accumulation.

In addition to quantitative genetics approaches, evidence for adaptation of tolerance to ecotoxins can be provided by comparing the sensitivity of populations exposed to a contaminant with uncontaminated reference sites. Genetic differences between populations can be demonstrated by rearing individuals obtained from different environments (contaminated vs. uncontaminated) in a common environment. This approach was successfully used to document cadmium resistance among European populations of *Daphnia magna*, where wild-obtained clones were subjected to lethal levels of cadmium (Barata et al. 2002). The common environment removes covariance as the result

of environmental variation, but it may also eliminate gene by environment effects unless the experimental design includes a gradient of environmental exposure.

Adaptation in response to mercury will be influenced by the heritable variation in mercury tolerance and the intensity of the selection pressure caused by mercury in individual populations. Demonstration of tolerance to mercury in wild populations, either through estimating its potential with quantitative genetics or documenting differences between contaminated and reference sites, could have important implications for conservation and estimates of mercury biomagnification.

### *3.3 Consequences of mercury tolerance on conservation and biomagnification*

Adaptation in response to mercury, either in the form of increased mitigation or decreased sensitivity could have serious consequences for conservation and biomagnification. Current models for biomagnification are based on mercury concentration regressed to trophic position. Trophic position is quantified using stable isotope analysis (most often the ratio of  $^{14}\text{N}$  to  $^{15}\text{N}$ ); light isotopes are eliminated faster than heavy isotopes, and organisms at a higher trophic position will have a higher percentage of  $^{15}\text{N}$  (Newman and Unger 2002). Mercury biomagnification in flood plains near the South River, expressed as food web magnification factor (FWMF) or fold increase per trophic level, was 9.3 and 25.1 at distances of 11.8 and 22.4 miles from the site of point-source contamination (Newman et al. 2011). This biomagnification was higher than

modeled values for river biomagnification at the same distances from the point source contamination (Tom et al. 2010).

Models of the kind proposed for the South River are used to predict trophic biomagnification and are frequently used to make recommendations for land management and human health advisories (Eisler 2006; Tom et al. 2010). Current models for mercury biomagnification do not consider the potential for adaptive response to mercury. Because models of biomagnification do not measure changes in biomagnification over time, it is possible that evolutionary changes could influence patterns of biomagnification in unpredicted ways unless models are updated constantly. Consequently, forecasting future food web magnification factors and the spread of contamination based current predictions of mercury biomagnification could be inaccurate if adaptation in response to mercury occurs in wild populations.

Adaptation in response to mercury could take the form of: 1) increased mitigation of mercury (limited uptake, increased elimination, detoxification/deposition pathway), or 2) decreased sensitivity to the detrimental effects of mercury. Under the first scenario, individuals could reduce systemic mercury levels through increased deposition into feathers or eggs. In addition or alternatively to these mechanisms, detoxification pathways could sequester biologically inert mercury in the liver; mercury stored in the liver in the form of mercuric selenide would be less readily bioaccumulated by predators (Ikemoto et al. 2004). With a reduction in mercury bioaccumulation, less mercury would be

available to biomagnify up the food chain. While mercury-tolerant populations would be at a decreased risk from mercury toxicity, the evolution of tolerance may itself pose a risk to populations if the mechanisms of tolerance are costly. For example, increased mercury deposition into eggs may increase embryonic mortality (Heinz and Hoffman 2003), although the deposition of mercury into eggs could simply be a byproduct of egg-laying rather than a mechanism of tolerance. Mercury tolerance may also impose an energetic cost if the mechanisms associated with tolerance are energetically expensive.

The evolution of mercury tolerance may alternatively result in a decreased sensitivity to the numerous detrimental effects of mercury. Decreased sensitivity to mercury implies that risks may be lower than predicted for some populations, but also that after selection for generations, individuals that survive mercury toxicity and eventually become prey items themselves may accumulate far more mercury than predicted by current models. This could result in increased biomagnification of mercury through surrounding food webs. Increased mercury biomagnification may lead to greater mercury toxicity in predators, including the possibility of higher risks for hunters who consume wild-caught waterfowl, many of which accumulate high levels of mercury at contaminated locations (Cristol et al. 2012). As many bird species are migratory, individuals who accumulate higher levels of mercury as a result of decreased sensitivity could transport more mercury out of contaminated environments. Migration has been linked to the transport of mercury and mercury tolerant individuals could intensify the

movement of mercury out of contaminated areas. Seabird-mediated mercury transport into high arctic ponds accounted for a 25-fold increase in mercury concentration compared to locations unused by seabirds (Blais et al. 2005). Similarly, waterfowl exposed to mercury on the South River have been collected by hunters as far away as 1,054 km (Cristol et al. 2012). Some of mercury concentrations reported in these waterfowl exceeded health advisory guidelines for consumption of seafood (Cristol et al. 2012).

It is also possible that populations would not adapt in response to mercury. Wild birds may not show a similar pattern of genetic influence on mercury accumulation as observed in this captive population. Lack of an adaptive response could imply persistent detrimental effects of mercury toxicity as populations do not acquire tolerance. Alternatively, the cost of mercury tolerance could be too high for tolerance to evolve, or variation in exposure as populations move between contaminated and non-contaminated areas could induce a selective pressure that is insufficient to cause adaptive change. In this scenario, current models of mercury biomagnification could still accurately predict the movement of mercury up trophic chains.

Concerns about the potential for bias in models for mercury biomagnification should be closely moderated by evidence of evolution in response to mercury in wild populations. As future research examines the potential for mercury tolerance in wild populations, the potential effects of

mercury tolerance on conservation and mercury biomagnification should be closely monitored.

#### **4.0 Conclusion**

The potential for adaptive response to mercury exists within the experimental population of Zebra Finches used in this study. I observed substantial variation in blood mercury accumulation within all dietary mercury treatments, and this variation was highly repeatable for individuals. A significant heritable component for blood mercury accumulation was estimated for the 0.6 and 1.2 ppm MeHg dietary doses and in the normalized blood mercury model, but not for treatments at 0.3 and 2.4 ppm dietary MeHg. The non-linear gene by environment interactions observed could be the result of thresholds at low and high levels of exposure which limit a genetic response to mercury toxicity. To my knowledge, this is the first study to evaluate the potential for adaptive response to mercury in birds, and the first to employ Animal Model methodology for tolerance to an ecotoxin. Many new lines of research can be generated from this study, including investigation into the mechanisms underlying genetic influence on mercury accumulation, identification of tolerance genes, and the covariance among tolerance mechanisms (and other quantitative traits). Extrapolating the results of the current study must be done with caution as variance estimates are population specific and because numerous differences in mercury exposure and environmental conditions may produce differences in mercury accumulation patterns and in relative sensitivity to mercury between wild and captive

songbirds. The results of this study should direct future research to investigate the potential for adaptation of mercury tolerance in wild birds. As global mercury pollution increases and bird populations decline, mercury tolerance in the wild could have significant effects on biomagnification and wildlife conservation in mercury-contaminated areas.

## Appendix

**Table 1.** Coefficients of variation and variance ratios for blood mercury accumulation in Zebra Finches exposed to dietary methylmercury: coefficient of total phenotypic variation ( $CV_P$ ), coefficient of additive genetic variation ( $CV_A$ ), coefficient of permanent environmental variation ( $CV_{PE}$ ), coefficient of foster environmental variation ( $CV_F$ ), coefficient of environmental variation ( $CV_R$ ), permanent environmental effects ( $pe^2$ ), repeatability ( $r^2$ ), heritability ( $h^2$ ),  $I_A$ . Variance components for the normalized blood mercury model presented without mean-scaling.

Treatment	N	Mean	$CV_P$ (SE)	$CV_A$ (SE)	$CV_{PE}$ (SE)	$CV_F$ (SE)	$CV_R$ (SE)	$pe^2$ (SE)	$r^2$ (SE)	$h^2$ (SE)	$I_A$
0.3	741	4.22 (1.196)	0.268 (0.076)	0.022 (0.085) $p = 0.913$	<b>0.118</b> <b>(0.040)</b> $p = 0.002$	0	0.239 (0.068)	<b>0.193</b> <b>(0.066)</b> $p = 0.002$	<b>0.200</b> <b>(0.043)</b> $p < 0.001$	0.007 (0.053) $p = 0.449$	0.0005 (0.032)
0.6	807	8.526 (2.482)	0.283 (0.087)	<b>0.192</b> <b>(0.069)</b> $p < 0.001$	0	0	0.208 (0.063)	0	<b>0.458</b> <b>(0.116)</b> $p < 0.001$	<b>0.458</b> <b>(0.116)</b> $p < 0.001$	0.037 (0.017)
1.2	582	16.5 (4.343)	0.239 (0.066)	0.139 (0.052) $p = 0.09$	0	<b>0.024</b> <b>0.119</b> $p < 0.001$	0.192 (0.053)	0	<b>0.341</b> <b>(0.141)</b> $p < 0.001$	<b>0.341</b> <b>(0.141)</b> $p < 0.001$	0.019 (0.010)
2.4	511	30.81 (7.989)	0.259 (0.068)	0.042 (0.084) $p = 0.806$	<b>0.146</b> <b>(0.048)</b> $p = 0.002$	0	0.210 (0.055)	<b>0.318</b> <b>(0.113)</b> $p = 0.003$	<b>0.344</b> <b>(0.059)</b> $p < 0.001$	0.026 (0.102) $p = 0.401$	0.0017 (0.007)
Normalized Blood Hg	2641	-	$V_P$ (SE) 7.327 (0.673)	$V_A$ (SE) <b>3.254</b> <b>(1.28)</b> $p < 0.001$	$V_{PE}$ (SE) 0.087 (0.11) $p = 0.887$	$V_F$ (SE) 0	$V_R$ (SE) 3.99 (0.234)	$pe^2$ (SE) 0	$r^2$ (SE) <b>0.456</b> <b>(0.052)</b> $p < 0.001$	$h^2$ (SE) <b>0.444</b> <b>(0.142)</b> $p < 0.001$	-

Values reported with standard error (SE); values in bold have statistically significant variance components ( $p < 0.05$ ). Dashes (-) denote parameters that could not be estimated. Original variance components reported in Appendix 2.



**Table 2.** Summary of genotyping results for the parental generation. Primer sequence includes the fluorescent label used.  $N_A$ , number of alleles;  $H_o$ , observed heterozygosity;  $H_E$ , expected heterozygosity;  $F_{IS}$ , inbreeding coefficient. Averages presented with standard error (SE).

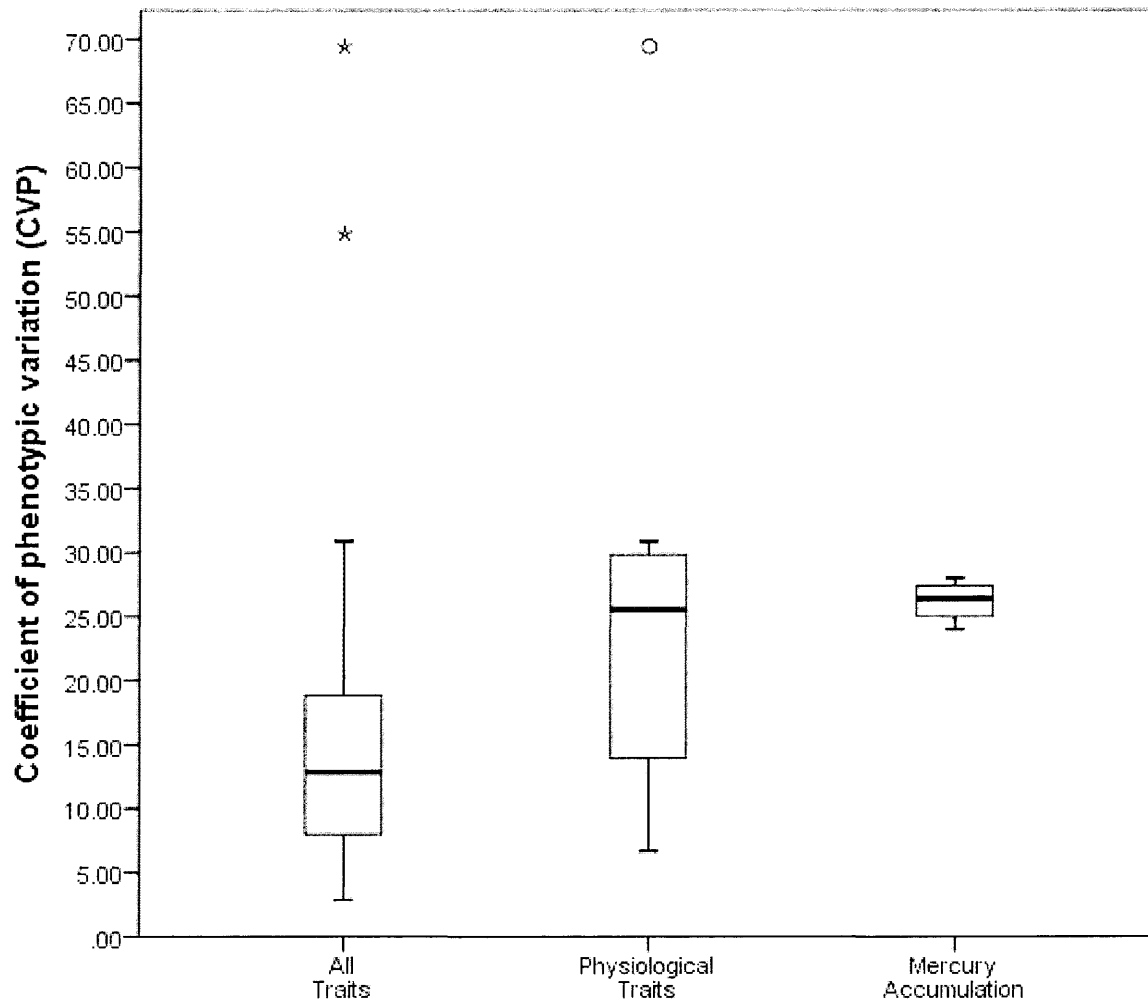
Locus	Primer sequences (5'-3')	Size range (bp)	$N_A$	$H_o$	$H_E$	$H_E-H_o$
<b>ZF02-129</b>	F: [PET]-TGCAAAATATACATTGCCACAAAC R: ATCACAGAGAGGCTCAACTGAC	125-172	22	0.936	0.935	-0.001
<b>ZF01-025</b>	F: [6-FAM]-AAACAGGTTCTTAGGTTCTATTCAAAG R: TGGTATCACAGATGGTGTGG	185-217	16	0.858	0.881	0.023
<b>ZEST09-018</b>	F: [VIC]-TGTCTTGATTTGTACTACCATATCACTG R: ATCCTGCAGTGCTTCTC	277-291	12	0.859	0.863	0.003
<b>ZF01-190</b>	F: [NED]-AGCACCTGGGATGAAGACAC R: CTGCAGCAAAATCAGGAAGC	238-263	19	0.923	0.875	-0.048
		<b>Average (SE)</b>	17.25 (4.25)	0.894 (0.041)	0.889 (0.032)	-0.005 (0.03)
						<b><math>F_{IS}</math></b> -0.00562

**Table 4.** Fixed or random effects that were significant additions to models of blood mercury. Fixed effects were eliminated when  $p > 0.05$ . The significance value for each fixed effect is based on conditional Wald F statistics, and the significance of random effects was calculated using likelihood ratio tests. Levels of significance are as follows:  $p < 0.05$  (\*);  $0.001 < p < 0.01$  (\*\*);  $p < 0.001$  (\*\*\*) ; a dash (-) represents a non-significant effect.

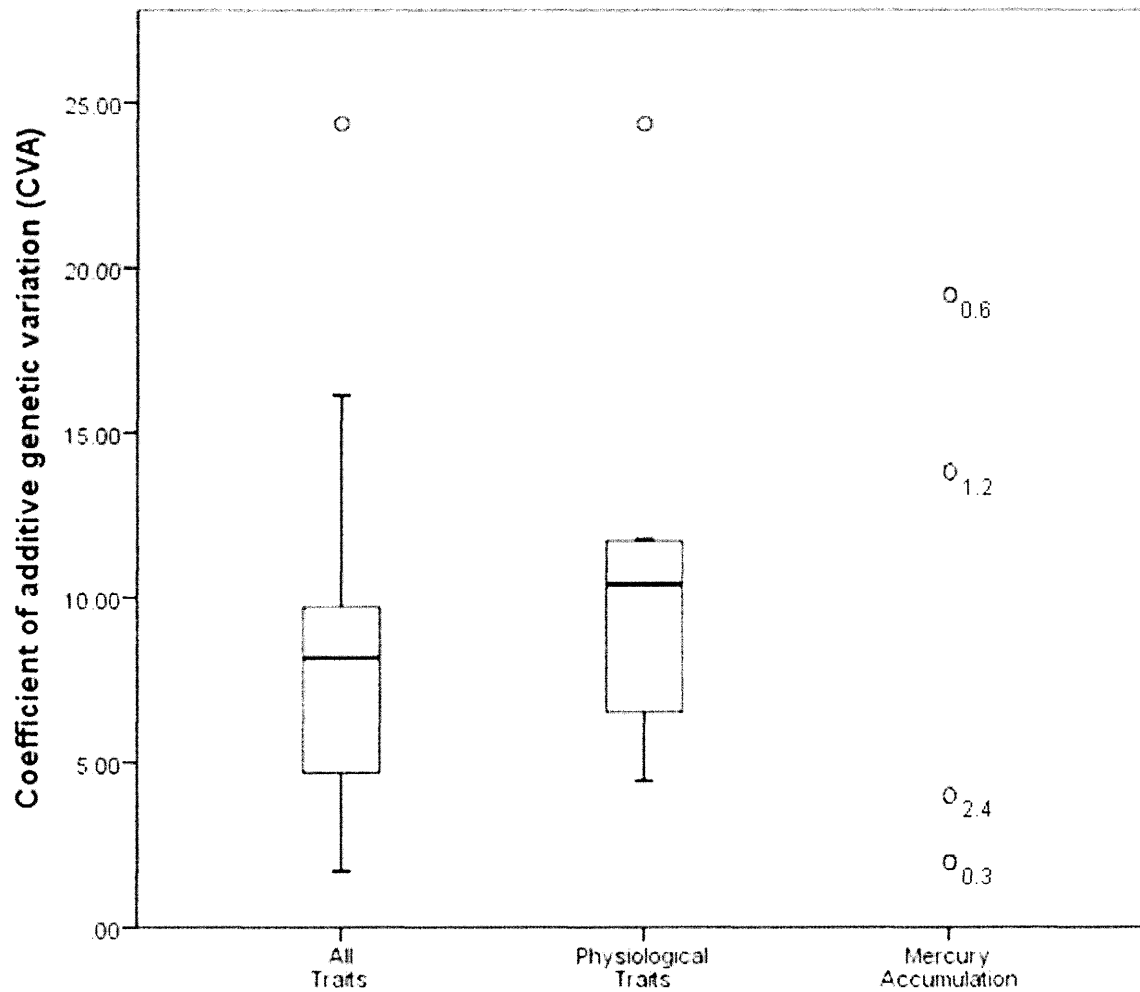
Trait	Fixed Effects				Random Effects	
	DOSE	DATE	SEX	AGE	PE	FOSTER
0.3	NA	$p < 0.001$	$p < 0.001$	$p = 0.002$	$p = 0.002$	$p = 0.731$
0.6	NA	$p = 0.008$	$p = 0.871$	$p = 0.030$	$p = 1$	$p = 1$
1.2	NA	$p = 0.003$	$p < 0.001$	$p = 0.034$	$p = 0.065$	$p < 0.001$
2.4	NA	$p < 0.001$	$p = 0.656$	$p = 0.093$	$p = 0.002$	NA
Normalized Blood Hg	$P < 0.001$	$p < 0.001$	$p = 0.002$	$p < 0.001$	0.1	$p < 0.001$

**Table 5.** Un-scaled variance components for blood mercury accumulation in Zebra Finches exposed to dietary methylmercury: Total phenotypic variance ( $V_P$ ), between-individual variance ( $V_{IND}$ ), additive genetic variance ( $V_A$ ), permanent environmental variance ( $V_{PE}$ ), foster environmental effects ( $V_F$ ), residual variance ( $V_R$ ). These models include significant fixed effects for blood mercury (see Appendix 1). Values reported with standard error (SE); dash (-) denotes a parameter that could not be estimated.

Parameter Estimates							
Treatment	N	$V_P$ (SE)	$V_{IND}$	$V_A$ (SE)	$V_{PE}$ (SE)	$V_F$	$V_R$ (SE)
0.3	741	1.275 (0.08)	0.2544 (0.0642)	0.0087 (0.0666)	0.246 (0.09)	0	1.02 (0.059)
				p = 0.913	p = 0.002		
0.6	807	5.827 (1.188)	2.667 (1.136)	2.667 (1.13)	0	0	3.16 (0.489)
				p < 0.001			
1.2	582	15.538 (2.675)	5.297 (2.74)	5.297 (2.744)	0	0.153 (1.53)	10.088 (1.46)
				p = 0.09		p < 0.001	
2.4	511	63.845 (5.792)	21.964 (5.39)	1.637 (6.55)	20.326 (7.97)	-	41.882 (2.82)
				p = 0.806	p = 0.002		
Normalized Blood Hg	2641	7.327 (0.673)	3.341 (0.655)	3.254 (1.28)	0.087 (0.11)	0	3.99 (0.234)
				p < 0.001	p = 0.887		



**Figure 4.** Phenotypic variation of quantitative characters in the Zebra Finch compared to phenotypic variation in blood mercury accumulation. Characters included morphological, physiological, and ornamental traits. Physiological traits separated to show direct comparison with mercury accumulation. Coefficients of variation presented as percents. Source information: (Tschirren and Postma 2010).



**Figure 5.** Additive genetic variation of quantitative characters in the Zebra Finch compared to phenotypic variation in blood mercury accumulation. Characters included morphological, physiological, and ornamental traits. Physiological traits separated to show direct comparison with mercury accumulation. Coefficients of variation presented as percents. Source information: (Tschirren and Postma 2010).

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