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The Effects of Methylmercury on Corticosterone and Thyroid Hormones in a Breeding Songbird Model Organism

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ABSTRACT PAGE

Mercury is a ubiquitous environmental contaminant associated with a host of adverse effects in wild birds. Although songbirds accumulate mercury at rates equivalent to more well-studied bird species, the effects of mercury bioaccumulation in songbirds remain understudied. Songbirds are an important part of many ecosystems and a growing number of species are experiencing precipitous declines. Little is known about mercury's effect on endocrine physiology, yet mounting evidence indicates that mercury may disrupt the function of the Hypothalamic-Pituitary-Adrenal (HPA) and Hypothalamic-Pituitary-Thyroid (HPT) axes. The HPA and HPT axes play fundamental physiological roles in stress responses, metabolic activity, development, and reproduction. Hence, proper functioning of these endocrine systems is imperative in maximizing individual fitness. Field-based correlations suggest that mercury exposure may alter levels of the primary avian stress hormone, corticosterone, as well as thyroid hormones T4 and T3. Thus, mercury exposure may compromise fitness through deregulation of the HPA and/or HPT axes. Adult zebra finches, a model system songbird, dosed with 0, 0.5, and 1.0 ppm dietary methylmercury (environmentally relevant levels) were sampled for corticosterone 3 days after pairing at week 10 of treatment. Birds were re-sampled approximately 25 weeks later while caring for late-stage fledglings. Upon fledging of young from the third clutch, adult pairs were sampled for T4 and T3. Circulating levels of corticosterone, T4, and T3 were not statistically significantly affected by mercury exposure. We detected a statistically significant effect of sex on T3 concentrations in breeding adults, however implications of this finding are obscured by a lack of information regarding thyroid function in passerine birds. Results indicate a discrepancy between results obtained from mercury exposed passerines in field-based versus captive studies; an inconsistency which may result from the significant physiological challenges encountered by wild birds. Findings from the present study do not provide evidence that mercury disrupts function of the HPA or HPT axes, however additional research in both the field and in captive settings is needed to determine if mercury has endocrine-disrupting properties in passerine birds.

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Section 1: Mercury in the Environment

Mercury: Introduction

Mercury is a global, naturally occurring element. In the Periodic Table of Elements, mercury is found in Group IIB of the transition metals, along with zinc and cadmium (Schroeder & Munthe 1998). Unlike zinc and cadmium, which are relatively electropositive, mercury is an inert metal (Schroeder & Munthe 1998). In its elemental state, mercury is a silver-colored metal that is liquid at standard temperature and pressure (Schroeder & Munthe 1998). Additional properties include high surface tension, high specific gravity, low electrical resistance, and a constant volume of expansion over its entire temperature range as a liquid (Schroeder & Munthe 1998).

Mercury is released into the environment by natural processes as well as anthropogenic activity. Despite the significant contribution of natural sources to global distribution, anthropogenic deposition has rendered mercury contamination a severe threat to human health, ecological integrity, and the persistence of wildlife populations. Within the past two centuries, human activity has caused a two to three-fold increase in deposition (Driscoll et al. 2007) and mercury emissions continue as we move into the 21st century. Despite substantial accumulation in water, air, and soil, little is known about the effects of mercury on ecosystems and associated wildlife. One of the most significant research gaps exists on the effects of environmental mercury exposure on insectivorous songbirds, also known as passerines.

Natural Sources

Release or re-emission of mercury into the environment occurs via numerous natural processes including volcanoes, geothermal activity, wildfires, and the outgassing of earth's mantle/crustal material (Schroeder & Munthe 1998). Mercury has a crustal abundance of approximately 0.5 ppm ($\mu g\ g^{-1}$), and over 25 mercury-containing minerals are present in earth's mantle (Schroeder & Munthe 1998). Thus, geothermic and volcanic activity results in a significant source of mercury emissions to the atmosphere (Gustin et al. 2008). Mercury gas flux of geothermally active areas exhibits significant spatiotemporal variability, as it is influenced by the age and type of geothermal system, heat flow, and geologic host rocks (Gustin et al. 2008). Processes associated with emissions include volatile loss resulting from heat flow, gas release from hot springs and fumaroles, and release from enriched substrate as a result of geothermal processes (Gustin et al. 2008). Despite considerable effort to quantify the geothermal flux of mercury emissions, estimates range from 1.4 to 60 tons per year, indicating the difficulty in quantifying natural emissions and a clear need for additional research (Nriagu & Becker 2003).

Volcanoes are the only direct source of mercury emissions to the free troposphere and stratosphere (Pyle & Mather 2003). Mercury is released from both eruptions and degassing from non-eruptive volcanoes (Nriagu & Becker 2003). In a time-averaged inventory of mercury emissions from volcanic sources from 1980-2000, worldwide flux of mercury from volcanoes was estimated to be 57 tons per year (Nriagu & Becker 2003). Non-eruptive volcanoes may still contribute significantly to global atmospheric mercury

concentrations via degassing, with estimates of 37.6 tons per year (Nriagu & Becker 2003). Volcanic mercury emissions can vary significantly, for example a single volcano was responsible for approximately 94 tons of mercury emissions over a 20-year period (Nriagu & Becker 2003). However, due to the complex chemical behavior of mercury once released into the atmosphere, volcanic emissions remain understudied (Bagnato et al. 2009). Current estimates indicate that volcanic activity is responsible for 20-40% of natural emissions, although uncertainties regarding transport mechanisms, complex reaction pathways, and variable residence times in volcanic plumes preclude a more accurate approximation of volcanic mercury emissions (Pyle & Mather 2003).

Anthropogenic Sources

In the past two centuries, human activity has resulted in a two- to three-fold increase in global mercury deposition into air, water, and soil (Driscoll et al. 2007). Primary anthropogenic sources of mercury include mine tailings, industrial effluent, waste incineration and disposal, and atmospheric deposition from electric power generation (Driscoll et al. 2007; Pacyna et al. 2006; Wolfe et al. 1998). Additional sources include biofuel combustion, cement production, mining, wastewater treatment plants and improper disposal of consumer products such as batteries, fluorescent light bulbs, and mercury switches (Driscoll et al. 2007; Lohman et al. 2008). Annual global mercury emission into the atmosphere is 6600 metric tons, with an estimated 33 to 36% a direct result of anthropogenic emissions (Driscoll et al. 2007). However, mercury deposition is not geographically uniform; some United States regions have been identified as mercury 'hotspots'. Many 'hotspots' resulting from atmospheric deposition

can be found in northeastern North America, where deposition can be six times greater than levels documented in 1990 (Evers et al. 2007). These hotspots may be of small extent adjacent to an emissions source or of large extent associated with methylation, such as the presence of aquatic systems (Evers et al. 2007).

Fossil fuel combustion is the largest source of global mercury emissions, of which coal-fired power production is the largest contributor (Pacyna & Pacyna 2002). Worldwide, coal combustion is responsible for an estimated 50% of anthropogenic mercury emissions (Hylander 2001). Coal-fired power plants are especially prevalent in China (Tian et al. 2010), a country with a history of severe mercury contamination. It is estimated that mercury emissions from coal combustion in China from 1978 to 1995 totaled 2493.8 tonnes (Wang et al. 2000). In a study of mercury contamination in China's Xiamen western sea area, researchers quantified mercury levels in seawater adjacent to a coal-fired power plant and found total mercury concentrations greater than 70 ng/L in the area near the outfalls of the power plant (Liang et al. 2010). Despite widespread environmental contamination, documented health effects, growing concern regarding prenatal mercury exposure (Zhang & Wong 2007), China has significantly increased construction of coal-fired power plants in recent years, with few restrictions placed on emissions (Shindell & Faluvegi 2010). Use of coal in China has increased dramatically from 1995 to 2006, with estimates that China is now responsible for 10% of mercury emissions worldwide (Shindell & Faluvegi 2010).

Anthropogenic Use: Mining

Mining has contributed significantly to the contamination of ecosystems worldwide. Mining activity in some areas dates back to ancient times, with extensive mining operations present in Almadén, Spain dating back to the Roman Era (Martinez-Cortizas et al. 1999). Cinnibar (HgS), the primary ore of mercury, was mined and used as a red pigment long before mining of elemental mercury became prevalent (Wiener et al. 2003). Mercury mining has created thousands of polluted sites around the world (Spiegel & Veiga 2010), the ecological impacts of which include diversion of rivers, water siltation, deforestation, degradation of terrestrial and aquatic habitat, as well as severe mercury contamination (Mol & Ouboter 2004). Mercury has long been used as an amalgamator in the extraction of gold and silver, largely because it is both a fast and inexpensive means of extracting desired metal particles (Spiegel & Veiga 2010). However, poor management practices and a lack of regulation of artisanal and small-scale gold mining result in the discharge of approximately 1000 tonnes of mercury each year (Spiegel & Veiga 2010). Mercury use in artisanal and small-scale gold mining is estimated to account for one-third of anthropogenic emissions (Swain et al. 2007), approximately 3,000 tonnes of which are volatized directly to the atmosphere, while 700 tonnes are discharged into soil, rivers, and lakes with associated mine tailings (Spiegel & Veiga 2010).

Wastes from mining operations release mercury for tens, even hundreds, of years after activity has ceased (Johnson et al. 2009). For example, researchers investigated the biogeochemical cycle of mercury in California's San Carlos Creek, a wetland habitat that

receives runoff from surface mine waste from the New Indria mercury mine (Ganguli et al. 2000). Water 1.2 km downstream from the New Indria mine, which has remained closed for over 30 years, was found to have methylmercury concentrations ranging from 1.1-1.7 ng/L (Ganguli et al. 2000). In a similar study, researchers quantified mercury levels in California's Walker Creek intertidal delta, an important winter habitat for migratory waterfowl that has been contaminated by runoff from the Gambonini mercury mine (Johnson et al. 2009). The Walker Creek delta is thought to receive mercury from over-bank and floodplain deposits, salt marshes, and intertidal mudflats that continue to store mercury despite the closing of the Gambonini mine in 1972 (Johnson et al. 2009; Whyte & Kirchner 2000). Using sediment cores, researchers estimated over 40 kg/year of mercury continued to flow into the delta at the time of sampling in 2003 (Johnson et al. 2009). Continued mercury contamination for decades after mining operations have ceased attests not only to the extent of the contribution of mining to the global mercury crisis, but the dire need to regulate current operations.

Mercury use often presents extremely hazardous conditions for humans. Serious long-term health effects have been documented for both miners and individuals inhabiting mining regions (Spiegel & Veiga 2010). Unfortunately, artisanal and small-scale mining have been difficult to regulate, as policymakers are unsure how to properly address associated hazards and subsequently, which practices to limit (Spiegel & Veiga 2010). Such extraction activities are increasing, as mining provides a readily accessible source of income for communities and populations plagued by poverty (Spiegel & Veiga 2010). Despite risks of artisanal and small-scale mining, it seems unlikely that operations

will become less prevalent until economic development policies are successfully implemented.

Speciation and Deposition

Mercury can be a local, regional, or global pollutant depending upon the chemical state after release into the atmosphere (Dastoor & Larocque 2004; Driscoll et al. 2007). Mercury is released from point sources in one of three forms; elemental mercury (Hg^0), ionic mercury (Hg II), and particulate mercury (PHg) (Driscoll et al. 2007). Atmospheric mercury is transported variable distances, with potential to travel tens of thousands of kilometers before deposition on the earth's surface (Driscoll et al. 2007). Understanding physical forms and chemical speciation is crucial in assessing environmental impacts and determining fate of mercury once emitted from sources (Bagnato et al. 2009). Reactions of various mercury states determine the solubility, mobility, and toxicity of mercury in aquatic ecosystems (Ullrich et al. 2001), topics that warrant further investigation before mercury speciation, transport, and fate are adequately understood.

Elemental mercury is the most prevalent form of mercury in the atmosphere, where it exists in a gaseous state (Dastoor & Larocque 2004; Schroeder et al. 1998). The global mean concentration of gaseous elemental mercury is estimated to be 1.6 ng m^{-3} in surface air (Selin 2009). Elemental mercury is in its least reactive state, where it has low solubility in water and is slowly oxidized, all of which contribute to its persistence in the atmosphere and ability to be transported on both local and global scales (Dastoor & Larocque 2004; Driscoll et al. 2007). Elemental mercury can persist in the atmosphere for up to two years (Driscoll et al. 2007; Fitzgerald et al. 1986; Schroeder et al. 1998).

However, studies of local deposition in the Arctic and Antarctic have revealed the potential for rapid oxidation and deposition of atmospheric elemental mercury under specific conditions (Lindberg et al. 2002). For example, mercury can persist in the environment for very short durations, such as 1-2 days in the springtime Arctic environment (Dastoor & Larocque 2004; Schroeder et al. 1998).

Gaseous ionic mercury, largely composed of gaseous chloride and oxide forms, is highly soluble in water and is often deposited near its source (Driscoll et al. 2007). The exact form of ionic mercury is unknown, yet thought to result primarily in mercury chloride (Selin 2009). With a residence time of approximately 0.5 to 2 days, considerable deposition of gaseous ionic mercury can occur near emission sources (Driscoll et al. 2007). Similarly, particulate mercury resides in the atmosphere for relatively short periods of time, from an estimated 0.5 to 3 days (Driscoll et al. 2007). The distance traveled before deposition is dependent upon the diameter/mass of the particulate matter released (Schroeder & Munthe 1998). Particulate mercury exhibits seasonal variation, with higher atmospheric concentrations found in the winter months (Keeler et al. 1995).

Atmospheric deposition is primarily responsible for the input of mercury into watersheds and terrestrial environments (Driscoll et al. 2007; Selin 2009). Rain and snow events are associated with wet deposition, while dry deposition occurs when particulate and gaseous ionic mercury are deposited as a result of fog or cloud formation (Driscoll et al. 2007). Rates of wet deposition can vary significantly due to seasonal and geographical shifts in relative concentrations of elemental and reactive gaseous mercury (Gratz et al. 2009). In one of the longest running wet deposition measurement efforts to date, researchers quantified mercury content of rainfall in Underhill, Vermont, from 1995 to

2006 (Gratz et al. 2009). The range in sample concentration was 0.9 to 90.5 ng L^{-1}, which was dependent not only on amount of precipitation, but also influenced by temperature as well as precipitation type (Gratz et al. 2009).

Dry deposition may also occur via gas exchange by plants, which are known to be sinks for atmospheric mercury (Stamenkovic & Gustin 2009). It is believed that stomatal uptake of atmospheric gases results in the sorption of atmospheric elemental mercury at the surface of the leaf (Stamenkovic & Gustin 2009). However, the air-surface exchange of elemental mercury and subsequent dry deposition is difficult to quantify, as meteorological, biological and soil conditions introduce significant variation into measurement efforts (Zhang et al. 2009). Ambient concentrations of mercury and atmospheric chemistry also influence dry deposition rates (Zhang et al. 2009), with greatest deposition in forested ecosystems versus areas with less foliage (Driscoll et al. 2007).

In addition to identification of mercury species, of particular interest is the deposition surface type (i.e. soil or vegetation), as well as the length of time since deposition. Of the mercury deposited onto the earth's surface via wet and/or dry deposition, newly deposited mercury is more susceptible to volatilization and methylation than previously stored mercury (Hintelmann et al. 2002). Such behavior of newly deposited mercury was determined by the Mercury Experiment to Assess Atmospheric Loading In Canada and the US (METAALICUS), in which researchers introduced a stable isotope of mercury (^{202}Hg) into a boreal forest subcatchment to monitor the dynamics of the newly deposited pollutant (Hintelmann et al. 2002). After 3 months, approximately 66% of ^{202}Hg remained present in vegetation, as compared to <5% for

native mercury (Hintelmann et al. 2002). Data suggest that mercury enters soils from surrounding vegetation only once decomposition of plant matter occurs (Hintelmann et al. 2002). While it is still unclear if soils behave as sources or sinks for atmospheric mercury, re-emission from soil may occur slowly and over long periods of time (Gustin et al. 2008), thus soil has the potential to be a significant source of the heavy metal.

Globally, foliar uptake is an important sink for atmospheric mercury (Gustin et al. 2008). Uptake is influenced by plant species and age, as well as air mercury concentrations (Gustin et al. 2008). However, vegetation is also associated with the re-emission of atmospherically deposited mercury (Gustin et al. 2008). Using a gas exchange chamber, researchers measured multiple processes at foliar surfaces, including mercury emission, deposition, and re-emission, thus indicating that exchange at foliar surfaces is more dynamic than previously believed (Millhollen et al. 2006). Additionally, deciduous species may facilitate mercury cycling to a greater extent than evergreen species (Millhollen et al. 2006), likely contributing to the variation in extent of mercury contamination within and among ecosystems. Although our current understanding of such factors remains far from complete, our knowledge of mercury emissions sources, pathways, and pools in the environment has greatly increased in recent years (Wiener et al. 2003).

Methylation and Transfer to Terrestrial Systems

Methylation, which refers to the conversion of inorganic mercury to methylmercury by a methyl group donor (Weiner et al. 2003), is thought to be driven primarily by sulfate-reducing bacteria in the water column (Jeremiason et al. 2006).

Although the majority of mercury found in both terrestrial and aquatic environments is present in inorganic forms, methylation is most closely associated with aquatic ecosystems (Wiener et al. 2003). Methylation of inorganic mercury occurs inside the sulfate-reducing bacteria via an enzyme-mediated transfer of a methyl group to mercuric ions (Choi & Bartha 1993). Aside from concentration of inorganic mercury in the aquatic environment, the presence of mercury-complexing ions also influences the extent of methylation in the water column (Benoit et al. 1999). Both chloride and sulfide ions bind to ionic mercury (Hg (II)), creating neutral species more capable of crossing the cellular membranes of methylating bacteria (Benoit et al. 1999). Additionally, chemical methylation of inorganic mercury is also possible in the presence of methyl donors (Ullrich et al. 2001). For example, the photochemical methylation of mercury may occur in the presence of sewage effluent or industrial wastewater, as both have been identified as methyl sources (Ullrich et al. 2001). While it is believed that methylation is primarily a microbially mediated reaction, both biotic and abiotic methylation warrant further research, as the relative importance of each process in aquatic systems remains elusive (Ullrich et al. 2001).

Ecosystems with conditions conducive to methylation are associated with greatest risk for predatory species, as it is predominantly methylmercury that is accumulated in the tissues of organisms (Hammerschmidt et al. 1999; Scheuhammer et al. 2007). Generally, systems with greatest rates of methylation include low-pH lakes, surface waters with large upstream or adjoining wetlands, water bodies with upstream or adjoining terrestrial areas with high frequencies of flooding, and dark-water lakes and streams (Scheuhammer et al. 2007). However, recent evidence indicates that methylation

also occurs in terrestrial systems (Driscoll et al. 2007; Evers et al. 2005; Rimmer et al. 2005); insectivorous montane passerines have a high proportion of methylmercury in their blood (90-100%). Although total blood mercury levels were relatively low for the terrestrial species studied, Bicknell's thrush (*Catharus bicknelli*), yellow-rumped warbler (*Dendroica coronata*), blackpoll warbler (*Dendroica striata*), and white-throated sparrow (*Zonotrichia albicollis*), passerines not closely associated with aquatic systems may be at risk for methylmercury exposure (Rimmer et al. 2005). Unfortunately, many of the chemical and biological processes that influence mercury methylation and bioaccumulation are not yet understood (Ullrich et al. 2001).

Because of its ability to enter cells, methylmercury is of great concern due to its high bioavailablity, known neurotoxicity, and propensity for rapid bioaccumulation (Driscoll et al. 2007; Scheuhammer et al. 2007; Wolfe et al. 1998). Beginning in 1991, an experimental reservoir was established in northwestern Ontario's Experimental Lakes Area (Gerrard & St. Louis 2001). Reservoir creation, which increased decomposition of organic matter and significantly increased methylation rates, facilitated the transfer of methylmercury to the terrestrial ecosystem (Gerrard & St. Louis 2001). In Virginia's contaminated South River, researchers found that spiders were largely responsible for the transfer of mercury from aquatic to terrestrial systems (Cristol et al. 2008). Spiders consume aquatic invertebrates, thus were found to have the highest concentrations of mercury, the majority of which was present as bioavailable methylmercury (Cristol et al. 2008). Of the prey items delivered to three species of passerine nestlings, spiders comprised ~20-30% of prey biomass (Cristol et al. 2008), thus substantial concentrations of methylmercury were being consumed by nestlings. As methylmercury is most

prevalent in aquatic ecosystems, the aforementioned studies reveal the significance of food web interactions in the introduction of methylmercury to terrestrial habitats and the subsequent risks faced by insectivorous avifauna.

Summary

Mercury emissions occur as a result of multiple natural processes, including volcanic activity, wildfires, and geothermal activity. Thus, human and/or wildlife exposure to the heavy metal occurred long before the advent of industrial activity. Anthropogenic mercury emissions, however, have markedly changed deposition patterns and have contributed significantly to global mercury pollution. Currently, direct anthropogenic emissions account for an estimated 36-66% of mercury released into the environment annually (Driscoll et al. 2007). Despite widespread concern about the effects of environmental mercury, global emissions are expected to worsen, largely due to increases in coal combustion (Shindell & Faluvegi 2010). Coal-fired power production is the largest contributor of anthropogenic mercury emissions (Pacyna & Pacyna 2002) and is associated with the release of elemental mercury into the atmosphere (Dastoor & Larocque 2004; Schroeder et al. 1998). Elemental mercury, the least reactive state of the heavy metal (Dastoor & Larocque 2004; Driscoll et al. 2007), is deposited by precipitation into aquatic environments. Here, the conversion of elemental mercury into toxic methylmercury occurs via sulfate-reducing bacteria in the water column (Jeremiason et al. 2006). Although the majority of mercury found in both terrestrial and aquatic environments is present in inorganic forms, methylation is most closely associated with aquatic ecosystems (Wiener et al. 2003). As methylmercury is highly

bioavialable, it is of great concern due to its propensity for rapid bioaccumulation (Driscoll et al. 2007; Scheuhammer et al. 2007; Wolfe et al. 1998). Although methylmercury is most prevalent in aquatic systems, recent research has revealed that surrounding terrestrial systems are also at risk for contamination (Cristol et al. 2008). Therefore, terrestrial passerines once thought to be invulnerable to methylmercury exposure are now known to be at risk for bioaccumulation (Gerrard & St. Louis 2001). Insectivorous passerines are among the least studied taxa; little is known about the risks associated with mercury exposure and potential population level effects (Rimmer et al. 2005).

Chapter II: Effects of Mercury on Wildlife

Section I: Mercury in Living Organisms

Bioaccumulation and Biomagnification

Methylmercury readily accumulates in the tissues of living organisms, thus rapidly biomagnifies up the food chain. Biomagnification, the concentration of contaminants with increasing trophic level, is well documented for mercury (Bowles et al. 2001; Eagles-Smith et al. 2009; Kim & Burggraaf 1999). Most of the metal found in sediment and surface water is inorganic mercury, while the majority found in the tissues of living organisms that occupy higher trophic levels is methylmercury (Scheuhammer et al. 1998). For example, the proportion of total mercury present as methylmercury increased from <1% in plants to 94% in piscivorous fish in New Guinea (Bowles et al. 2001). The total concentration of methylmercury also increases from water to lower trophic levels, with highest concentrations found in fish and piscivores (Wiener &

Suchanek 2008). For example, concentrations of methylmercury in trout inhabiting New Zealand lakes were 3 to 8 times greater than that of their prey (Kim & Burggraaf 1999). Additionally, the greatest bioaccumulation occurred in a low trophic level of the trout food web, with a bioaccumulation factor of $10^{4.72}$ between zooplankton and water (Kim & Burggraaf 1999). However, bioaccumulation factors were much lower between forage fish and zooplankton, with a bioaccumulation factor between $10^{0.73}$ and $10^{1.06}$ (Kim & Burggraaf 1999). Trophic position is believed to be the most important factor in assessing the risk of methylmercury bioaccumulation to a given organism (Bowles et al. 2001).

In determining susceptibility to bioaccumulation, the length of the food web must also be considered. Individuals that occupy top-level trophic positions of long food webs usually have higher levels of methylmercury than those occupying the same position of shorter food webs (Wiener et al. 2003). For example, Cristol et al. 2008 documented blood mercury levels of terrestrial passerines which surpassed that of piscivores from the same location in Virginia's contaminated South River ecosystem. Of the prey items consumed by terrestrial passerines, predatory spiders had the highest levels of methylmercury (Cristol et al. 2008). Thus, by consuming a diet rich in spider biomass, terrestrial songbirds in the contaminated area increased the length of their food webs, subsequently increasing susceptibility to biomagnification (Cristol et al. 2008).

Section II: Effects of Mercury on Avifauna

Routes of Elimination

Once ingested, mercury circulates throughout the bloodstream before deposition in tissues or excretion from the body. However, birds can eliminate mercury from their

bodies through several mechanisms; for example, by deposition in growing feathers (Condon & Cristol 2009; Spalding et al. 2000a). Estimates of excretion into growing feathers have been measured at 70-93% of total body burden for some species (Burger 1993). As mercury will bind nearly irreversibly to sulfhydryl groups (Crespo-Lopez et al. 2009), the presence of disulfide-rich keratin proteins render growing feathers a prime target for mercury accumulation (Eisler 2006). The amount of mercury accumulated in feather keratin is proportional to the concentration ingested by the individual at the time of feather growth (Braune & Gaskin 1987). For example, black-headed gull (*Larus ridibundus*) chicks were dosed with methylmercury and elimination in multiple tissues was measured (Lewis & Furness 1991). Higher mercury concentrations were found in all feather types as compared to internal tissues. Of the mean 71% of administered mercury during the fledgling period, 49% was deposited in plumage (Lewis & Furness 1991). Additionally, amount of mercury ingested did not affect the proportion excreted in either feces or feathers (Lewis & Furness 1991). In another captive study, 5 week-old great egret (*Ardea alba*) chicks were dosed with 0.5 or 5 mg methylmercury chloride/kg in fish (Spalding et al. 2000a). Mercury concentrations in growing feathers were found to most closely reflect cumulative mercury consumed per weight, with feathers showing the highest concentrations of mercury as compared to internal organs (Spalding et al. 2000a). Blood mercury concentrations increased after feather growth ceased in week 9, associated with a significant decline in appetite and weight index in both dosed groups (Spalding et al. 2000a). Results indicate an increased risk of mercury toxicity when egret feather growth is complete, a period which overlaps with additional challenges of leaving the natal colony, foraging independently, facing more predators, and exposure to disease

(Spalding et al. 2000a). Excretion via feather growth has also been shown to be an important protective mechanism against mercury toxicity in free-living eastern bluebirds (*Sialia sialis*) (Condon & Cristol 2009). Nestling bluebirds had significantly lower blood mercury levels than adults, however, shortly after fledging feather growth ceased and blood mercury levels of young approached that of adult birds (Condon & Cristol 2009). Later, blood mercury levels of young birds decreased upon the onset of additional molt, confirming the importance of feather growth as an excretory mechanism for mercury-exposed songbirds (Condon & Cristol 2009). However, the period of molt is transient, thus mercury elimination from the body is terminated along with feather growth, subsequently causing mercury levels to increase (Braune & Gaskin 1987).

Egg laying is an additional important mechanism by which females may reduce total body burden of mercury (Eisler 2006; Evers et al. 2003). Mercury excreted into eggs by breeding females is most closely correlated with blood mercury levels, which reflect dietary mercury levels during the time of egg development (Evers et al 2003). In a study in which hens (*Gallus domesticus*) were orally administered 500 or 100 μg per Kg body weight methylmercury chloride, mercury residues were detected in eggs 2 days after exposure, with a mean of 50% and 46% of administered methylmercury deposited in eggs in the high and low dose group, respectively (Kambamanoli-Dimou et al. 1991). Lewis et al. 1993 estimated that female herring gulls may excrete over 20% more mercury than males as a direct result of egg laying. For free-living seabirds and shorebirds, mercury excreted into the first-laid egg is greatest, with a subsequent decrease across the clutch (Becker 1992; Lewis et al. 1993). Comparison of mercury residues in first and last laid eggs revealed concentrations 39% greater in first laid eggs of herring gulls, 37% greater

in common terns, and 10% greater in American oystercatchers (Becker 1992). However, no decline in egg mercury concentrations was detected across clutches of an insectivorous songbird, the great tit (*Parus major*) (Dauwe et al. 2005). Another passerine, the tree swallow, also produced clutches with no significant effect of laying order on egg mercury levels (Brasso et al. 2010). Researchers suggest that lack of intra-clutch variation may be attributed to a balance between elimination of mercury into eggs and intake via dietary exposure (Brasso et al. 2010). The mechanism by which passerines excrete mercury into eggs may differ from that of other species, yet factors influencing mercury deposition in eggs across avian taxa have yet to be investigated (Brasso et al. 2010)

Neurotoxicity

Methylmercury is a potent neurotoxin capable of crossing both the placental and the blood-brain barriers (Schroeder & Munthe 1998; Wolfe et al. 1998). Mercury-associated damage to neuronal axons has been observed in a number of avian species (Fimreite & Karstad 1971; Spalding et al. 2000b). Red-tailed hawks (*Buteo jamaicensis*) fed a diet of mercury-contaminated chicks exhibited obvious neurological symptoms including weakness in extremities, inability to coordinate muscle movements, uncontrolled attempts to fly or walk, and difficulty eating (Fimreite & Karstad 1971). Upon pathological examination, Fimreite & Karstad (1971) noted swelling of axons of myelinated nerves in the spinal cord, with dilation of myelin sheaths and loss of myelin. The axons of the brain stem, midbrain, cerebellum, and cerebrum of some birds exhibited similar lesions. The most adversely affected axons were swollen, fragmented, at times

contracted or curled, and undergoing lysis in digestion chambers (Fimreite & Karstad 1971). Two of three hawks fed a diet of 7.2 mg/kg mercury for 4 weeks showed lesions of axons and myelin sheaths, with all hawks fed a greater amount of mercury affected, but no low-dose or control birds exhibiting any neurological abnormalities (Fimreite & Karstad 1971). Similarly, Spalding et al. (2000b) fed captive juvenile great egret chicks a diet of methylmercury chloride at low and high doses, 0.5 and 5 mg/kg wet weight respectively. During week 10, high-dose birds had tremors while perching, a delayed righting time, exaggerated wing response to twisting the body, as well as an exaggerated response to touching of eyelids (Spalding et al. 2000b). In weeks 10-12 birds exhibited head tremors, drooped wing posture, uneven gait, compromised ability to fly and land on a perch, exaggerated wing response to falling, and some were unable to stand (Spalding et al. 2000b). The most severe lesions in high-dosed birds with a mean brain mercury concentration of 35 mg/kg were seen in peripheral nerves of the spinal column, with lesser lesions in the midbrain, followed by the cerebellum and cerebrum (Spalding et al. 2000b). Interestingly, mercury appeared to have differential effects on the brachial and sciatic peripheral nerves, where sciatic nerves had little inflammation and lesions consisted of neuronal loss, atrophy, and demyelination (Spalding et al. 2000b). Brachial nerves, however, showed significant Wallerian degeneration, indicative of a more acute damage (Spalding et al. 2000b).

Immune Function

Scientists have long suspected that mercury interferes with avian immune function (Scheuhammer et al. 2007). Many researchers have noted a correlation between

severe disease and/or mortality and high concentrations of mercury in tissues. Between the years 1987 and 1991, Sundlof et al. (1994) collected 144 species of Ciconiiform found dead in the Florida Everglades. Of birds potentially capable of breeding, 30-80% were found to have mercury concentrations high enough to impair reproduction (Sundlof et al. 1994). Additionally, 4 great blue herons (*Ardea herodias*) had liver mercury concentrations ≥ 30 mu g/g, levels associated with overt neurological symptoms (Sundlof et al. 1994). In southern Florida, Spalding et al. (1994) followed 3 adult and 19 juvenile radio-tagged great white herons (*Ardea herodias occidentalis*) until their death. They found a correlation between a liver mercury concentration > 6ppm and death from chronic disease. Generally, birds with less liver mercury died from acute causes, whereas birds with high liver mercury died as a result of chronic, frequently multiple, diseases (Spalding et al. 1994). Although evidence is correlative, associations between mercury exposure and disease have encouraged additional studies regarding the effects of mercury on avian immune function.

Immune effects of mercury exposure have been documented in multiple avian species, both in captive and free-living birds. Captive great egret chicks fed a high-dose diet of 5 mg/kg methylmercury chloride (wet weight in fish) became ataxic and had hematologic, neurologic, and histologic changes, the most severe of which were in neurological and immune tissues (Spalding et al. 2000b). Low-dosed egrets fed 0.5 mg/kg had lower packed cell volumes, increased lymphocytic cuffing in a skin test, increased bone marrow cellularity, decreased bursal cell wall thickness, decreased thymic lobule size, fewer lymphoid aggregates in the lung, increased perivascular edema in lung, and decreased lung phagocytosis of carbon as compared to control birds (Spalding et al.

2000b). Additionally, researchers noted that sublethal hematologic, neurologic, and immunologic effects occurred at lower concentrations in captive than wild birds, a pattern attributed to reduced variation in the small population of control birds (Spalding et al. 2000b). However, severe effects such as death and disease occurred at lower levels in wild than captive birds, likely due to additional sources of stress for wild birds (Spalding et al. 2000b). Although free-living double-crested cormorant (*Phalacrocorax auritus*) chicks along Nevada's Lower Carson River had lower organ concentrations of mercury than adults, Henny et al. (2002) documented toxicity to immune (spleen, thymus, bursa), detoxifying (liver, kidneys), and nervous systems. Fledglings also exhibited signs of oxidative stress, which were most pronounced in those with highest mercury concentrations. Additionally, juvenile snowy egrets (*Egretta thula*) had increased activity of protective hepatic enzymes, presumably a compensatory effect of mercury-induced oxidative stress (Henny et al. 2002). In one of the first attempts to study the effects of mercury on the songbird immune response, researchers used two standardized immune assays, a mitogen-induced swelling response to phytohaemagglutinin (PHA) and the antibody response to sheep red blood cells (SRBCs), to investigate the immune response of female tree swallows inhabiting a mercury contaminated riparian habitat in western Virginia (Hawley et al. 2009). In both years of the study, females breeding in contaminated areas mounted a significantly weaker response to PHA tests as compared to those inhabiting reference sites. However, blood mercury concentration alone did not account for the degree of mitogen-induced swelling. No differences were observed in the antibody response to SRBCs between birds in contaminated versus reference cites, although researchers notepattern of response was similar to that for PHA (Hawley et al.

2009). Contrary to results obtained by Hawley et al. (2009), Kenow et al. (2007) dosed captive common loon (*Gavia immer*) chicks with methymercury chloride and found a significant relationship between blood mercury level and antibody response to SRBCs. Kenow et al. (2007) noted evidence for mercury-associated lymphoid depletion in the bursa, which may partially explain the reduction in antibody response to the SRBC antigen. However, nonlinear responses obscured results, as antibody responses to mercury exposure were observed only in low dose birds. Additionally, no relationship was observed between blood mercury and swelling response to PHA (Kenow et al. 2007). Although mercury is a known immunosuppressor (Hawley et al. 2009), results from previous studies reveal the need to further investigate both lethal and sublethal immune effects of mercury across avian taxa. While evidence is correlative, associations between mercury exposure and disease have stimulated additional studies regarding the effects of mercury on avian immune function.

Behavior

Effects of mercury exposure may be manifested as acute symptoms of toxicity or via physiological alterations that can modify behavior (Heath and Frederick 2005). Birds exposed to mercury may exhibit a variety of abnormal behaviors. Mallards (*Anas platyrhynchos*) fed a diet containing 0.5ppm methylmercury for three generations produced fewer eggs and ducklings than controls (Heinz 1979). Dosed females also laid a greater proportion of eggs outside of nest boxes, while young produced by treated pairs exhibited reduced responsiveness to maternal calls as well as a hypersensitivity to a threatening stimulus (Heinz 1979; Nocera & Taylor 1998). In one of the most heavily

contaminated common loon populations in North America, a correlation was found between chick blood mercury level and a decrease in back-riding behavior. Additionally, exposed young spent a greater amount of time preening, although birds did not compensate for increased energy expenditures with increases in begging or feeding (Nocera and Taylor 1998). In populations of breeding common loons in Maine and New Hampshire, Evers et al. (2006) noted striking differences in the nest attendance behavior of more and less exposed common loon adults. As blood mercury levels increased from ≤ 1.0 ug/g to ≥ 3.0 ug/g, average time spent brooding nests declined from 99% to 85%. Loons with higher levels of mercury exposure showed increased lethargy and spent less time foraging for themselves and/or for their young (Evers et al. 2006). Although manifested as a variety of symptoms that vary across taxa, substantial evidence indicates that mercury exposure can significantly alter behavior.

Summary

The effects of mercury exposure vary in severity across species, age classes, and studies. The effects of physiological processes including molt and egg laying are known to reduce blood mercury levels; however, long-term effects of seasonal mercury buildup and excretion remain unknown. Similarly, breeding seabirds and shorebirds have shown decreasing egg mercury levels across clutches (Becker 1992; Lewis et al. 1993), while no declines in egg mercury concentrations were detected across clutches of insectivorous songbirds (Dauwe et al. 2005). Brasso et al. (2010) suggested that lack of intra-clutch variation may be attributed to a balance between elimination into eggs and intake via

dietary exposure. However, additional research is needed to elucidate physiological mechanisms behind observed egg mercury deposition patterns across taxa.

Conflicting results have also been found with regard to the effects of exposure on neurologic and immune function. Severe effects associated with mercury exposure, including death and disease, occurred at lower levels in wild than captive birds, while sublethal hematologic, neurologic, and immunologic effects occurred at lower concentrations in captive than wild birds (Spalding et al. 2000b). Additionally, researchers noted exposed tree swallows mounted a significantly weaker response to a PHA test and a similar, but non-significant pattern of response in antibody response to SRBCs (Hawley et al. 2009), whereas mercury exposed loons showed no relation between blood mercury levels and extent of swelling response to PHA yet did show a reduced antibody response to SRBCs (Kenow et al. 2007). However, in addition to small sample sizes, tree swallows were sampled in the field whereas loons were sampled as part of a captive dosing study. As such, birds in the wild may have been exposed to other pathogens or may have exhibited reduced immune function as a result of breeding activity. The innumerable variables associated with field studies, physiological differences in taxa evaluated, and breeding status of individuals sampled, hinder the ability to draw meaningful conclusions about the effects of mercury on immune function. Existing literature is indicative of the lack of consensus on mercury's effects on avian physiology and the need for additional captive dosing research.

Section III: Reproductive Effects

Piscivores

The effects of methylmercury on avian reproduction remain poorly understood, yet reproduction is believed to be among the most sensitive endpoints of mercury toxicity (Brasso & Cristol 2008; Heinz & Hoffman 1998; Wolfe et al. 1998). The common loon is a relatively well-studied bird with regard to mercury contamination and reproductive effects of mercury exposure. Researchers have noted a correlation between mercury contamination and decreased reproductive success for breeding loons in Wisconsin (Burgess & Meyer 2008; Meyer et al. 1998), Canada (Burgess & Meyer 2008), Maine (Evers et al. 2006), and New Hampshire (Evers et al. 2006). In Wisconsin, increased blood mercury levels were associated with decreased survival in chicks (Meyer et al. 1998b), while exposure was also associated with decreased reproductive success for breeding pairs both in Wisconsin and Canada (Burgess & Meyer 2008). Data indicate a 50% reduction in maximum productivity when female blood levels were 4.3 μg/g (Burgess & Meyer 2008). Additionally, in an extensive study of loon populations of Maine and New Hampshire, Evers (2006) found that territorial breeding pairs at high-risk for mercury contamination produced 41% fewer fledglings than the reference group. Reproductive effects occur at significantly lower dietary levels than those known to result in obvious symptoms of toxicity (Scheuhammer 1988), thus reproductive output is a useful indicator of the sublethal effects of mercury exposure.

Evers et al. (2003) have established four risk categories based on loon egg mercury concentration. The first (low risk) category includes egg mercury ranging from 0-0.60 μg/g, levels not associated with reproductive impairment (Evers et al. 2003). Such

egg concentrations are associated with background levels from environments that do not receive large inputs of mercury or have increased natural or anthropogenic mercury but lack the factors necessary for high rates of methylation (Evers et al. 2003). The second category (moderate risk) is associated with egg mercury concentrations of 0.60-1.30 μg/g, which reflect increased environmental contamination that is associated with significant reproductive impairment in some individuals (Evers et al. 2003). The high-risk category is associated with egg concentrations of >1.30 μg/g, which indicates a heavily contaminated environment in which loons are at risk for significant declines in reproductive success (Evers et al. 2003). Accordingly, females with blood mercury concentrations of 3.0 μg/g laid eggs containing >1.30 μg/g and subsequently experienced marked reproductive impairment (Evers et al. 2003).

A captive dosing study in which juvenile white ibises (*Eudocimus albus*) were chronically exposed to low doses of methylmerucry revealed marked reproductive impairment (Jayasena 2010). Birds were fed relatively low, environmentally relevant doses of methylmercury from 2005 to 2008, with the highest dose being 0.3 ppm ww (Jayasena 2010). In the final year of the study, 35% of high-dose females failed to produce any nestlings and of those producing nestlings, 35% had fewer fledglings (Jayasena 2010). Additionally, mercury exposure was associated with increased incidence of homosexual pairing, which by the final year of the study, had increased to an average of 18-26% greater for dosed birds (Jayasena 2010). Authors conclude that in a worst-case scenario, a 13% reduction in successful breeding attempts as a result of male-male pairing combined with a 35% decrease in fledgling production could lead to a 48% decrease in reproductive output (Jayasena 2010). Such reproductive impairment would

have significant effects at the population level, a result which attests to the potential effects of even low, environmentally relevant methylmercury exposure on reproductive output (Jayasena 2010).

Passerines

Recent research has revealed susceptibility of terrestrial passerines to mercury biomagnification, and thus, a risk for reproductive impairment. An investigation of mercury levels in terrestrial songbirds inhabiting a contaminated site in western Virginia revealed levels similar to that of fish-eating species (Brasso & Cristol 2008). Brasso et al. (2008) present the highest mercury concentrations recorded for tree swallows; levels equal to that of the piscivorous belted kingfisher (*Ceryle alcyon)* from the same location. Decreased reproductive success for second-year tree swallows breeding in the contaminated area was also reported (Brasso & Cristol 2008). Custer et al. (2007) sampled populations of tree swallows and house wrens (*Troglodytes aedon)* breeding on a river above and below a historical mining site in Nevada, where mercury concentrations were 15-40 times higher in the eggs of birds breeding below the contaminated site. Results indicated a reduction in hatching success in both species, where mercury levels in tree swallow clutches that showed reduced hatching were twice that of clutches in which all eggs hatched (Custer et al. 2007). In New Jersey, mercury levels in the eggs of breeding marsh wrens (*Cistothorus palustris)* were higher than eggs of some fish-eating birds (Tsipoura et al. 2008). Increased mercury levels were found in unhatched eggs when compared with randomly selected eggs from the site (Tsipoura et al. 2008). Although much research has yet to be completed, there is a clear link between passerine

mercury exposure and decreased reproductive success. Although the risk of mercury-associated reproductive impairment in passerines is a relatively recent finding, substantial evidence suggests that many wild species could experience declines in productivity associated with mercury exposure (Brasso & Cristol 2008; Custer et al. 2007; Longcore et al. 2007).

Embryotoxicity

Egg injection studies have allowed researchers to study the effects of controlled methylmercury concentrations on developing embryos. A recent injection study helped to elucidate the relationship between egg methylmercury level and embryotoxicity for many wild bird species for which no such data existed (Heinz et al. 2009). When mallard ducks were fed diets containing 5, 10, or 20 μg/g ww, ducklings hatched from eggs containing an estimated 2.3 μg/g mercury exhibited neurological signs of methylmercury toxicity, while mortality occurred in eggs with levels as low as 0.74 μg/g (Heinz & Hoffman 2003). However, in this same study, some embryos survived in eggs containing more than 30 μg/g, indicating a wide range of sensitivity in mallard embryos exposed to methylmercury (Heinz & Hoffman 2003). Using values from the LC_{50} and survival through 90% of incubation, embryos were characterized as having a low, medium, or high sensitivity to injected methylmercury (Heinz et al. 2009). Embryos of two passerine species, the common grackle *(Quiscalus quiscula)* and tree swallow, were reported to have medium sensitivity, while mallards were among the species found to have the lowest sensitivity to methylmercury (Heinz et al. 2009).

Despite the relative tolerance of mallards to in-ovo methylmercury exposure, investigators use egg methylmercury levels derived from mallard breeding studies to predict adverse effects in wild birds (Heinz et al. 2009). This concentration of egg mercury, 0.8-1.0 μg/g ww (Heinz 1979) is used as a default value by investigators but is unlikely to provide sufficient protection for passerines (Heinz et al. 2009). On Nevada's Carson River, tree swallow clutches that experienced reduced hatching success were found to have mean egg mercury levels of 7.88 μg/g dw (approximately 0.788-0.985 μg/g ww), whereas clutches in which all eggs hatched had a mean of 3.86 μg/g dw (approximately 0.386-0.483 μg/g ww) (Custer et al. 2007). Researchers monitored breeding tree swallows in Maine and Massachusetts and found mean egg mercury levels ranging from a mean of 0.316-1.280 μg/g per egg per clutch (Longcore et al. 2007). Investigators collected 11 clutches that contained 0.885-1.313 μg/g mercury in the 3rd egg, four of which had reduced hatching success when mercury levels in the third egg were ≥ 0.916 μg/g (Longcore et al. 2007). In summary, substantial evidence exists that wild passerines experience reduced reproductive output with elevated egg mercury levels. Although the threshold concentration of egg mercury associated with embryotoxicity may be above the level of exposure of many wild bird embryos, it is likely that sensitive species are being impacted (Heinz et al. 2009). Furthermore, discrepancies in the lethality of injected vs. maternally deposited mercury preclude reliable interpretation and synthesis of results regarding embryotoxicity.

Summary

Thorough study of the effects of mercury exposure on reproduction is imperative in the effort to provide protection to wild birds, as reproduction is believed to be among the most sensitive endpoints of mercury toxicity (Brasso & Cristol 2008; Heinz & Hoffman 1998; Wolfe et al. 1998). Additionally, reproductive effects occur with less exposure than those known to result in obvious symptoms of toxicity (Scheuhammer 1988), thus impaired reproduction may be used as a hallmark of mercury exposure. Despite the potential usefulness of reproductive physiology, behavior, and output in determining acceptable environmental levels of mercury, significant gaps exist in the study of mercury-associated reproductive impairment. For example, it is unclear if reproductive impairment occurs as a result of embryotoxicity, endocrine disruption, behavioral abnormalities, or a synergy of the adverse physiological effects of mercury. Establishment of a sensitive threshold of environmental mercury exposure for passerines should be made a priority, as researchers have documented mercury levels in insectivorous songbirds greater than those of fish-eating species (Brasso et al. 2008) and subsequent reproductive impairment (Brasso et al. 2008; Tsipoura et al. 2008).

Evers et al. (2003) have established four risk categories of egg mercury concentrations, however levels have been determined using loon eggs and it is unlikely that these levels may accurately be extrapolated to eggs of wild passerines. Additionally, current egg mercury levels used to predict adverse effects in wild birds have been determined using eggs of mallard ducks, a species known to have one of the lowest sensitivities to egg mercury (Heinz et al. 2009). Passerine eggs were reported to have a medium sensitivity to mercury, yet the concentration of egg mercury, 0.8-1.0 μg/g ww

(Heinz 1979) used as a default value by investigators is based on studies from mallard eggs. This threshold, determined largely from egg mercury injection studies, is unlikely to provide sufficient protection for passerines and sensitive species (Heinz et al. 2009). Significant variability in the tolerance of different taxa to egg mercury, discrepancies in the lethality of injected vs. maternally deposited mercury, and the range of tolerances observed in mallard studies indicate a clear need for additional research. Studies of both wild and captive passerines are imperative, as this taxon remains significantly understudied (Seewagen 2010). It is clear that current thresholds of egg mercury do not provide sufficient protection for passerines (Heinz et al. 2009); however, embryotoxic thresholds of environmental mercury will be elucidated only with large-scale, multi-generational, and carefully controlled studies on songbird species.

Chapter III: Endocrine Effects of Mercury

Section I: Interference of Hormonal Activity

General

Abnormal steroidogenic activity has been associated with mercury exposure in both mammals (Burton & Meikle 1980; Friedman et al. 1998) and fish (Hontela et al. 1992; Leblond & Hontela 1999). However, few studies have investigated effects in birds (Heath & Frederick 2005). Inappropriate steroidogenic activity has the potential to alter the HPA (Hypothalamic-Pituitary-Adrenal), HPG (Hypothalamic-Pituitary-Gonad), or HPT (Hypothalamic-Pituitary-Thyroid) axes (Tan et al. 2009) and subsequent hormonal processes responsible for normal stress regulation, growth, metabolism, thermoregulation, and reproductive function. As mercury has been shown to

preferentially accumulate in the pituitary gland in both humans and primates (Cornett et al. 1998; Vahter et al. 1995), it is reasonable to suspect that mercury also accumulates in the avian pituitary, resulting in compromised function and abnormal synthesis and/or release of both endocrine and neuroendocrine secretions. Recent data indicate an affinity of mercury for the endocrine system, although the mechanism by which mercury preferentially accumulates in such glands remains unknown (Tan et al. 2009). Mercury, an "endocrine active" contaminant (Tan et al. 2009), has the potential to act via many mechanisms, including action as a hormone agonist or antagonist, alteration of hormone production at the endocrine source, interference with the release of stimulatory or inhibitory hormones from the pituitary or hypothalamus, alteration of biosynthetic pathways via disturbance of enzyme activity, and/or the disruption of serum-binding proteins (Guillette & Gunderson 2001).

Mechanism

Growing evidence indicates that mercury is an endocrine disrupting pollutant; however, potential mechanisms behind observed endocrine effects remain elusive. Mercury is a cation, thus has the potential to react with most intracellular ligands, including thiol, phosphate, amino, and carboxyl groups (Clarkson 1987). Mercury also interferes with enzyme activity, disrupts cell membranes, damages structural proteins, and affects genetic integrity through nucleic acid interactions (Clarkson 1987). Genotoxicity, which is manifested primarily as teratogenesis and carcinogenesis (Crespo-Lopez et al. 2009), is associated with compromised integrity of a cell's DNA. As steroid hormones exert their effects through the regulation of transcription and translation, such

effects on DNA have the potential to affect the function of all steroid hormones.

Oxidative stress is associated with DNA damage caused by free radicals, an unstable and highly reactive chemical form (Crespo-Lopez et al. 2009). Free radicals most closely associated with genotoxic events are reactive oxygen forms (ROS) (Crespo-Lopez et al. 2009). Mercury compounds have been shown to increase ROS levels (Ercal et al. 2001), an effect which has been implicated as a mechanism behind mercury-induced genotoxicity (Di Pietro et al. 2008; Lee et al. 1997; Rao et al. 2001; Schurz et al. 2000). Possible effects of free radicals associated with DNA damage include direct action on amino acids to cause genetic mutations (Schurz et al. 2000) as well as conformational changes in protein structure (Crespo-Lopez et al. 2009). Such conformational changes may compromise proteins responsible for the maintenance of DNA, including repair enzymes, DNA-polymerases, and the tubulin and kinesin motor proteins (Cebulska-Wasiewska et al. 2005; Crespo-Lopez et al. 2009; Halliwell 2007; Stohs & Bagchi 1995; Stoiber et al. 2004).

As early as the 1970s, it was believed that mercury preferentially binds to tubulin and kinesin, thus the influence of mercury on mictotubules has been a topic of great interest (Crespo-Lopez et al. 2009). Researchers evaluated the effect of inorganic mercury salts on tubulin assembly and kinesin-driven motility in V79 Chinese hamster fibroblasts (Thier et al. 2003). Thier et al. (2003) found a dose-dependent inhibition of in vitro tubulin assembly as well as a decrease in kinesin-driven motility at concentrations as low as 0.1 μM. Results provide support for the hypothesis that mercury genotoxicity may result from interference with cytoskeletal proteins (Thier et al. 2003). As cytoskeletal proteins are involved in cell movement, mitotic spindle formation,

chromosomal segregation, and nuclear division, mercury's effects on mictrotubules may promote inappropriate chromosome separation (Crespo-Lopez et al. 2009), resulting in a host of possible cellular abnormalities.

Mercury may compromise genetic integrity via interference with DNA repair mechanisms (Crespo-Lopez et al. 2009). For example, mercury may inhibit the action of DNA polymerase via free radical production (Crespo-Lopez et al. 2009; Stohs & Bagchi 1995) and may also bind to the "zinc fingers" core of DNA repair enzymes (Crespo-Lopez et al. 2009). Mercury has a high affinity for sulfhydryl groups, which are present on cysteines (Crespo-Lopez et al. 2009). Thus, as "zinc fingers" contain one atom of zinc in addition to four cysteines and/or histidines, these specific sequences of protein chains are likely susceptible to mercury interference (Crespo-Lopez et al. 2009). Mercury's affinity for the sulfur-hydrogen bond characteristic of the sulfhydryl functional group may interfere with the integrity and subsequent action of DNA repair enzymes, contributing to potential genotoxic effects (Cebulska-Wasiewska et al. 2005; Crespo-Lopez et al. 2009; Gomez et al. 2007).

Additionally, mercury may exert direct effects on DNA molecules. Li et al. (2006) investigated the effects of inorganic mercury (HgII), methylmercury (MeHg(I)), ethylmerury (EtHg(I)), and phenylmercury (PHg(I)) on DNA using capillary electrophoresis with on-line electrothermal atomic absorption spectrometric detection (CE-ETAAS). The CE-ETAAS assay indicates the level of DNA damage associated with mercury species, provides kinetic and thermodynamic information regarding the interactions of mercury species and DNA, and provides evidence of mercury-DNA covalent bonding (Li et al. 2006). Methylmercury bound to DNA with the greatest

affinity and speed, indicating the potential for rapid formation of MeHg-DNA complexes (Li et al. 2006). Researchers also note an affinity of mercury for both endocyclic and exocyclic nitrogen sites of DNA bases, yet such binding did not result in conformational change, as effects were observed only in secondary structure (Li et al. 2006). Data were obtained using an in vitro study and indicate the ability of mercury species to cross the nuclear membrane and thus exert direct effects on DNA (Crespo-Lopez et al. 2009).

Finally, mercury may interfere with genetic integrity through inappropriate methylation. Pilsner et al. (2009) used the LUminometric Methylation Assay (LUMA) for a genetic methylation study in mercury-exposed polar bears. DNA methylation in the lower brain stem region was characterized for 47 polar bears hunted in East Greenland from 1999-2001 (Pilsner et al. 2009). Although not statistically significant ($P = 0.17$), a positive relationship was seen between brain mercury concentration and genomic DNA methylation in male bears (Pilsner et al. 2009). Although further investigation of the relationship between mercury exposure and DNA hypermethylation is needed, epigenetics is an important consideration in the study of environmental factors and disease (Skinner et al. 2010). Environmental factors, such as endocrine disrupting contaminants, may cause a cascade of molecular and physiological processes, beginning with epigenetic effects that lead to genomic alterations, inappropriate gene expression, and eventually a disease state (Skinner et al. 2010).

Summary

Recent research indicates an affinity of mercury for the endocrine system, although the mechanism by which mercury preferentially accumulates in these glands

remains unknown (Tan et al. 2009). Mercury-associated interference with steroidogenic activity has been documented in mammals (Burton & Meikle 1980; Friedman et al. 1998) and fish (Hontela et al. 1992; Leblond & Hontela 1999), yet little is known about compromised endocrine function in birds (Heath & Frederick 2005a). While research has yet to determine if mercury is a true Endocrine Disrupting Contaminant (EDC), mercury has the potential to disrupt cellular processes via many mechanisms. Mercury may interfere with enzyme activity, disrupt cell membranes, damage structural proteins, and effect genetic integrity through nucleic acid interactions (Clarkson 1987), specifically interference with proteins containing sulfhydryl groups, which are present on cysteines (Crespo-Lopez et al. 2009). With regard to effects on endocrine cells, mercury may act as a hormone agonist or antagonist, alter hormone production at the endocrine source, interfere with the release of stimulatory or inhibitory hormones from the pituitary or hypothalamus, alter biosynthetic pathways via disturbance of enzyme activity and/or disrupt serum-binding proteins (Guillette & Gunderson 2001).

Research regarding the effects of mercury on avian endocrine function represents a significant gap in our knowledge of the physiological effects of mercury exposure. There are multiple pathways by which mercury may interfere with endocrine processes, some of which may be synergistic and therefore, difficult to determine without extensive future study. It is likely that mercury affects avian reproductive physiology (Heath and Frederick 2005), however existing research indicates that endocrine function may be compromised at exposure levels lower than levels currently known to cause adverse effects (Scheuhammer 1987). Future research that involves careful monitoring of hormone levels in mercury-dosed individuals, the creation of toxicological models

applicable to specific taxa, and the establishment of a threshold level of environmental mercury exposure to protect wild passerines should be made a priority.

Section II: The Effects of Mercury on Sex Hormones

Lutenizing Hormone

Lutenizing hormone is a pituitary hormone associated with estrogen production in females and Leydig cell production of testosterone in males. In an extensive study of over 6,000 women, multiple biomarkers were used to investigate the effects of chronic mercury exposure on a subset of the US population (2009). One such biomarker for the pituitary was the level of LH, which was found to vary inversely with blood mercury level in women aged 35-49 (Laks 2009). Mercury is known to bind almost irreversibly to free sulfur bonds, characteristic of cysteines (Crespo-Lopez et al. 2009; Laks 2010). The enzyme thioredoxin, which contains a specific sequence of cysteine resides, has been shown to have a high affinity for inorganic mercury (Carvalho et al. 2008; Laks 2010). In 1990, researchers revealed a homologous tetrapeptide (Cys-Gly-Pro-Cys) between the active site of thioredoxin and the catalytic β subunit of LH (Boniface & Reichert 1990; Laks 2010). Thus, LH would be expected to preferentially bind to inorganic mercury and hence be a prime target for bioaccumulation (Laks 2010). LH is the only hormone with a rare high affinity binding site for mercury, thus levels of this hormone may be an early and especially sensitive indicator of exposure (Laks 2010).

Testosterone

Methylmercury may suppress the sex hormones responsible for secondary sexual characteristics and the stimulation of gonadal development and gametogenesis (Klaper et al. 2006). Accordingly, markedly reduced serum testosterone levels have been associated with methylmercury exposure, most notably in mammals. Male Sprague-Dawley rats treated chronically with injections of 0.26 mg methylmercury chloride every 48 hours for 6 weeks or acutely with a single injection of 7.0 mg methylmercury chloride, showed markedly lower testosterone levels than control animals (Burton & Meikle 1980). Hypertrophy of interstitial Leydig cells was seen in experimental animals, along with elevated levels of pituitary LH, presumably secreted to raise testosterone levels (Burton & Meikle 1980). Testosterone synthesis was profoundly impaired in acutely treated rats, as administration of HCG resulted in a mean serum testosterone level of 23.3 μg/dl in control animals and a mean of 4.0 μg/dl in treated rats, indicating a significant impairment of testosterone synthesis (Burton & Meikle 1980). Similarly, Brown Norway rats (*Rattus norvegicus*) administered 80 μg/kg methylmercury twice weekly had 44% reduced intratesticular testosterone levels compared to controls (Friedman et al. 1998). A 17% reduction in number of sperm in the cauda epididymides was seen, as well as a negative correlation between fertility and mercury concentration in the testes (Friedman et al. 1998). Although the mechanism behind mercury's effect on male reproductive function remains unknown, mercury may alter androgen metabolism or directly compromise Leydig cell function (Friedmen et al. 1998). Mercury may also impair hypothalamic or pituitary control of steroidogenesis (Friedman et al. 1998), which may help to explain altered steroid hormone levels in mercury-exposed wildlife, as endocrine

processes are highly conserved across vertebrate taxa.

Estrogens and Progesterone

As compared to testosterone, sex hormones estrogen and progesterone have received little attention with regard to the potential endocrine disrupting effects of mercury. Progesterone binding capacity in the presence of methylmercury has been shown to be significantly reduced in cytoplasmic receptors obtained from the eggshell gland mucosa of domestic fowl (Lundholm 1991). In comparing the effects on progesterone binding capacity of inorganic mercury and methylmercury, the inorganic mercury yielded a standard dose-response curve, while methylmercury dramatically altered the shape of the curve to a nearly flat line (Lundholm 1991). In humans, inorganic mercury activated estrogen receptors through the formation of a complex in the binding domain of receptors in MCF-7 breast cancer cells (Martin et al. 2003). Likewise, methylmercury was found to dose-dependently modulate 17β-estradiol-dependent growth in MCF-7 breast cancer cells within a narrow concentration range of 0.5-1 μM (Sukocheva et al. 2005). Within this range, methylmercury significantly stimulated growth of MCF-7 cells, induced Ca2+ mobilization, and activated extracellular signal-regulated kinase ½ (Erk ½), thus indicating a strong potential for interference with multiple growth-related signaling pathways (Sukocheva et al. 2005). Methylmercury may be a potentially estrogen-disrupting contaminant with the capacity to alter intracellular signaling pathways in MCF-7 cells, resulting in abnormal estrogen-receptor activity.

Multiple Hormone Studies

Studies in which researchers investigate the effects of mercury on multiple endocrine endpoints are few, as most have focused on a single hormone. However, as biosynthetic pathways often render the presence of one hormone dependent on another, such multiple hormone studies may provide key insight into the mechanism behind mercury-associated endocrine disruption. In one such study, researchers compared body-feather mercury concentrations of free-living white ibises with multiple hormone concentrations (Heath & Frederick 2005). Estradiol levels in pre-breeding females were negatively correlated with mercury exposure, while no relationship was reported between mercury and female testosterone, progesterone, and corticosterone concentrations (Heath and Frederick 2005). Incubating males were shown to have a positive relationship between mercury concentration and testosterone. These researchers suggest mercury exposure may cause fewer birds to attempt reproduction and/or an increase in abandonment rates as a result of impaired endocrine function (Heath & Frederick 2005).

To further investigate correlative evidence between mercury exposure and endocrine dysfunction in the aforementioned field study, Jayasena (2010) fed juvenile white ibises 0.00 (control) 0.05 (low), 0.1 (medium), or 0.3 (high) ppm methylmercury in a controlled setting. Fecal concentrations of estradiol, testosterone, and corticosterone were measured in both males and females. Mercury exposure was associated with abnormal concentrations of reproductive hormones estradiol and testosterone in both breeding males and females (Jayasena 2010). With regard to estradiol concentrations in dosed females, correlations were always negative, although effects varied non-linearly with dose regime (Jayasena 2010). Negative, nonlinear correlations between female

estradiol and mercury exposure were also seen in the comparable field study (Heath & Frederick 2005a). Dosed male ibises showed an increase in estradiol during display, the greatest of which occurred in the high-dose group (Jayasena 2010). Similar to results seen in estradiol concentrations, testosterone in female ibises generally decreased in a non-linear manner (Jayasena 2010). However, in the 2008 breeding season, high dose females showed increased testosterone during laying, incubation, and chick-rearing, which was also associated with decreased reproductive success in that year (Jayasena 2010). Male birds also showed decreased testosterone in all breeding stages in 2007 as well as in pre-laying stages of 2008 (Jayasena 2010). In 2008, males showed increased testosterone during laying, a result complementary to that of the field study by Heath and Frederick (2005). Similar to females, high-dose males also showed increased testosterone during incubation and chick-rearing in 2008, which was associated with reduced reproductive success (Jayasena 2010). With regard to basal corticosterone concentrations during breeding, both male and female ibises showed an overall decrease in the stress hormone as compared to controls (Jayasena 2010). During an experimental reduction of food availability, Jayasena (2010) reduced *ad libitum* food by 20% for seven days and re-measured corticosterone levels. Incubating males in both the medium and high-dose group had higher corticosterone levels as compared with control males; however, such increases were not associated with nest abandonment (Jayasena 2010). Researchers also note the magnitude of food stress imposed was likely not indicative of the severity of the reduction in food availability that wild birds may encounter. Finally, results from the field and captive dosing study should not be compared directly, as researchers measured hormone concentrations in the former using plasma levels (Heath and Frederick 2005),

whereas fecal metabolites were used in the later study (Jayasena 2010).

Summary

Endocrine processes are highly conserved across vertebrate taxa, therefore indications that mercury may impair hypothalamic or pituitary control of steroidogenesis in mammals (Friedman et al. 1998) should also be considered in the study of mercury's effects on avian endocrinology. Studies of other taxa are helpful in predicting effects of mercury exposure on avian communities, however discrepancies in results preclude reliable extrapolation. For example, marked reductions in serum testosterone levels have been associated with methylmercury exposure in rats (Burton & Meikle 1980). In a two-year captive study, exposed male white ibises also showed decreased testosterone in all breeding stages (Jayasena 2010). In the subsequent year, however, dosed males had decreased testosterone levels during the pre-laying phase, followed by increased testosterone concentrations during the laying phase (Jayasena 2010). In the same year, high dose females also showed increased testosterone during laying, incubation, and chick-rearing (Jayasena 2010). Non-linear and variable results with regard to sex and stage of reproduction in white ibises prevent researchers from establishing reliable dose-response relationships between mercury exposure and hormone concentrations.

Additionally, the comparison and synthesis of results from existing studies is precluded by marked differences in experimental design, dosing regimen, and method of sampling. Current understanding of the effects of mercury exposure on the white ibis, one of the most extensively studied species with regard to effects of mercury on reproductive physiology, is limited due to inconsistencies in experimental setup as well as sampling

design. In the study by Heath and Frederick (2005), exposed wild birds were monitored throughout the breeding phase, however researchers were unable to determine with certainty the length of mercury exposure, accumulation in tissues, age of individuals sampled, or exposure to additional environmental contaminants. Adverse weather events also obscured their ability to correlate mercury exposure and possible endocrine disruption with reproductive effort. In a subsequent study, Jayasena (2010) monitored the effects of mercury on multiple hormones and breeding success in captive white ibises. While some trends in hormone levels were consistent with the field study, many hormones increased or decreased in directions opposite of predictions from results of the wild ibis study. Additionally, results should not be directly compared due to striking differences in sampling methodology. Heath and Frederick (2005) measured hormones in plasma samples, whereas Jayasena (2010) used concentrations found in feces, which are influenced by differences in gut passage rate, integration of gonadal or adrenal synthesis, variable binding to plasma proteins, and hepatic biotransformation and clearance (Guillette & Gunderson 2001).

Despite marked differences in the aforementioned research, multiple-hormone studies provide critical insight into mercury-associated endocrine effects. Studies in which researchers investigate the effects of mercury on multiple endocrine endpoints are few; most have focused on a single hormone. However, as biosynthetic pathways often render the presence of one hormone dependent on another, such multiple hormone studies may provide key insight into the mechanism behind mercury-related endocrine abnormalities.

Additionally, the effects of mercury exposure on hormone levels often show non-

linear dose responses, indicating the need for improved toxicological and biochemical understanding through further study. Correlations between mercury exposure and hormone levels from field studies should be used to guide controlled captive dosing studies, where researchers may simultaneously monitor levels of multiple hormones while controlling for life-history stages and environmental variables.

Section III: Stress in Avifauna

Significance of Corticosteriod Activity

Corticosterone, the primary avian glucocorticoid, is necessary for optimal fitness, successful reproduction, and survival (Schoech et al. 2009; Wingfield & Kitaysky 2002; Wingfield et al. 2008). Stress, which can be physical, behavioral, or social, is a threat to an animal's internal physiological balance known as homeostasis (Wingfield et al. 1997). Because the vertebrate stress response both constitutively and facultatively redirects energy away from stressors (Wingfield et al. 1997), there is an important adaptive value of glucocorticoid secretion (Remage-Healey & Romero 2001; Wingfield & Kitaysky 2002; Wingfield et al. 1998). Corticosterone is secreted from the zona fasiculata and reticularis cells of the adrenal cortex upon binding of the precursor hormone ACTH, which is secreted from the pituitary gland in response to CRH release from the hypothalamus. If the stressor is transitory, there will be a temporary spike in corticosterone. However a prolonged stimulus would elevate plasma corticosterone accordingly, until adrenal exhaustion, negative feedback, or termination of the stressor causes corticosterone to return to pre-stress levels (Schoech et al. 2008). While chronically elevated levels of glucocorticoids are known to have many adverse effects

including reduced cognitive ability (Hodgson et al. 2007; Kitaysky et al. 2003), compromised immune function (Buchanan 2000; McEwen et al. 1997; Sapolsky et al. 2000), and reproductive impairment (Schoech et al. 2008; Wingfield et al. 1998), the primary function of glucocorticoid secretion is to facilitate glucose release under challenging situations (Schoech et al. 2008). A transient elevation in corticosteriod level will redirect energy to allow the avoidance of challenging situations such as adverse weather, social instability, or the presence of a predator (Astheimer et al. 1992; Breuner et al. 1998). If avoidance is not possible, glucocorticoid secretion will assist with glucose regulation under stressful situations (Astheimer et al. 1992; Buttemer et al. 1991; Schoech et al. 2008). Although local populations may experience occasional high mortality, the adaptive nature of the stress response will confer greater individual fitness in the long term (Wingfield et al. 1997).

In understanding the physiological states associated with varying levels of glucocortiods, it is imperative to distinguish between the source of stress, severity of the stressor, and the degree to which the stressor is predictable. Wingfield et al. (1997) identifies three hypothetical levels of corticosterone secretion in birds, level A, B, and C, all of which correspond to varying degrees of unpredictable environmental events. Level A represents baseline corticosterone secretion, which exhibits daily and seasonal variation as stimulated by periodic secretion of ACTH (Wingfield et al. 1997). Level B is associated with corticosterone secretion dictated by predictable environmental change, such as the shift from night to day or winter to spring (Wingfield et al. 1997). Finally, level C is associated with the 'transitory emergency state', whereby deactivation of the current life history stage frequently occurs (Wingfield et al. 1997). Surges in

corticosterone into level C are temporary but will remain elevated above level B until social instability is resolved, weather becomes more favorable, etc.; however, if corticosterone levels remain elevated for extended periods of time, adverse effects characteristic of chronic stress may result (Wingfield et al. 1997).

In attaining a complete understanding of the physiological, behavioral, and reproductive effects of corticosterone, an important question centers around the differential effects of corticosterone at physiological states A, B, and C. It is hypothesized that glucocorticoids produce concentration-dependent effects as a result of differences in types and sensitivities of binding receptors (Landys et al. 2006). Evidence for three glucocorticoid receptor types exists, two of which are classic genomic receptors which include the low-affinity glucocorticoid receptor (GR) and the high-affinity mineralcorticoid receptor (MR) (Landys et al. 2006). Multiple studies have shown full occupation of MR receptors at low plasma glucocorticoid concentrations, while significantly higher concentrations yield binding activity of GR receptors (Breuner & Orchinik 2001; Kalman & Spencer 2002; Landys et al. 2006; Reul & Dekloet 1985). Breuner and Orchinik (2001) measured both neuronal cystolic and membrane receptor levels in wild male and female house sparrows (*Passer domesticus*) and report values for the dissociation constant K_d, or the concentration of hormone required to bind half the receptors. The K_d for the binding of corticosteroids to the GR and MR receptors was 11.5nM and 0.4nM, respectively (Breuner & Orchinik 2001). Breuner & Orchinik (2001) also measured baseline and stress-induced corticosterone during three different life history stages- nesting, molting, and winter. Researchers found seasonal differences in both baseline and stress-induced plasma corticosterone secretion and seasonal variability

in the number of high and low affinity corticosteroid receptors in the brain. Combined with reported differences in dissociation constants for GR and MR receptors, results provide substantial evidence, not only for seasonal behavioral and physiological variability in corticosteroid response (Breuner & Orchinik 2001; Breuner & Wingfield 2000), but also for the differential effects of corticosterone at hypothetical physiological states A, B, and C (Landys et al. 2006).

Stress & Reproduction

The onset of a stressor inhibits reproductive activity, both physiologically and behaviorally (Sapolsky et al. 2000). Prolonged elevations in plasma corticosterone may trigger changes that interrupt or arrest reproduction (Schoech et al. 2008), while broad anti-reproductive effects can occur very shortly after a stressor is encountered (Sapolsky et al. 2000). Many studies have shown strong correlations between increased glucocortiocid activity and reduced levels of reproductive hormones and breeding activity (Love et al. 2004; Schoech et al. 2008; Silverin 1998). Male pied flycatchers (*Ficedula hypoleuca*) were easily stressed during early stages of the breeding cycle, as both experimental birds with corticosterone implants and control birds that had been handled abandoned territories during the period of territorial establishment (Silverin 1998). Free-living European starlings that abandoned nests had significantly higher free baseline corticosterone levels than non-abandoning birds in each stage of reproduction measured: laying, incubation, and chick-rearing (Love et al. 2004). In an experimental study, silastic implants were used to raise plasma corticosterone of female zebra finches (*Taeniopygia guttata*) to that of a stress-induced state (Salvante & Williams 2003). Implanted females

exhibited decreased vitellogenin production, a 44% reduction in pairs initiating breeding as compared to control pairs, and an 8-day delay in clutch initiation in pairs that did attempt reproduction (Salvante & Williams 2003). Due to the high anabolic cost of reproduction, notably for females, breeding is expected to halt with onset of a stressor (Sapolsky et al. 2000). Thus, long-term elevations in corticosterone are associated with the delay or complete arrest of reproductive physiological and behavioral activity (Schoech et al. 2008).

Mercury and Stress Regulation

Environmental contaminants may affect endocrine function by increasing baseline stress hormone levels (Wingfield 2008). Endocrine-disrupting chemicals are likely to pose added stress that may interfere with life history stages including migration, reproduction, and molt (Wingfield 2008), thus potentially compromising the stability of certain avian populations. Evidence suggests that mercury is associated with increases in stress hormone levels, while some animals show a mercury-associated decrease in glucocorticoid concentrations. Wada et al. (2009) documented reduced responsiveness of the HPA axis in 13-17 day-old tree swallow nestlings in mercury-contaminated sites in western Virginia (Wada et al. 2009). Late stage nestlings in the contaminated area had baseline corticosterone levels 103% greater and stress-induced levels 27% lower than nestlings of the same age from reference sites (Wada et al. 2009). Results reveal an approximate 14-fold increase in stress-induced plasma corticosterone in reference late stage nestlings, whereas those in contaminated sites show an approximate 8 fold difference between baseline and stress-induced corticosterone, an indication of potential

endocrine disruption (Wada et al. 2009). Contrary to results obtained from nestlings in Virginia, both adult and nestling tree swallows in mercury- contaminated sites in Massachusetts showed a significant negative relationship between blood mercury level and baseline corticosterone (Franceschini et al. 2009). However, no relationship was seen between blood mercury concentration and stress-induced plasma corticosterone in either adult or nestling birds (Franceschini et al. 2009).

Compromised adrenal function in mercury-treated rats has also been documented. While cortisol is the main stress hormone in most mammals, the primary glucocorticoid in rats is corticosterone. Researchers treated male Sprague-Dawley rats with injections of 0.26 mg methylmercury chloride every 48 hours for 6 weeks (Burton & Meikle 1980). No effect on baseline cortiosterone was seen in mercury-treated rats, however exposure to both ether stress and ACTH stimulation caused corticosterone concentrations to more than double in control animals, whereas serum levels of experimental rats were not significantly elevated (Burton & Meikle 1980). The adrenal glands of the experimental group weighed more than twice that of controls, with marked hyperplasia of the zona fasiculata (Burton & Meikle 1980), suggesting that the integrity of one of two primary corticosterone-releasing cell types of the adrenal gland was significantly compromised. However, administration of cortisone acetate to suppress ACTH secretion caused adrenal atrophy, strongly suggesting that adrenal enlargement was caused by ACTH stimulation (Burton & Meikle 1980). Additionally, researchers followed the conversion of *in vitro* radiolabeled cholesterol, the starting molecule for steroid hormone synthesis, and found significant impairment in the conversion of cholesterol to pregenelone (Burton & Meikle 1980). The exact mechanism behind reduction in levels of the corticosterone precursor

pregnenelone is unknown, however chronic methylmercury treatment may impact the formation of cyclic AMP stimulated by ACTH and/or interfere with activity of the enzyme responsible for such conversion (Burton & Meikle 1980). It should be noted that unlike previously discussed captive studies in which birds were fed dietary mercury, rats were directly injected with methylmercury chloride. To date, it is unknown if injected versus dietary doses of mercury have differential effects in severity, affected organs, or efficiency of excretion.

Summary

Corticosterone, the primary avian glucocorticoid, is secreted in response to stress, or a threat to homeostasis (Wingfield et al. 1997). As the vertebrate stress response both constitutively and facultatively redirects energy away from stressors (Wingfield et al. 1997), glucocorticoid secretion is highly adaptive (Remage-Healey & Romero 2001; Wingfield & Kitaysky 2002; Wingfield et al. 1998). However, chronic elevations in basal levels of corticosterone are associated with reproductive impairment (Schoech et al. 2008; Wingfield et al. 1998). Elevations are associated with the delay or complete arrest of reproductive physiological and behavioral activity (Schoech et al. 2008). The onset of stressors that precipitate a "stress-induced" response are also associated with reproductive impairment, as this 'transitory emergency state' is associated with the deactivation of the current life history stage, including complete cessation of reproductive activity (Wingfield et al. 1997). However, the transitory emergency state is temporary and results in predictable negative effects on reproductive success, whereas much less is known

about the correlation between chronically increased baseline corticosterone and subsequent reproductive impairment.

Mercury may act as a physiological stressor (Adams et al. 2009), thus exposure may stimulate increases in basal corticosterone concentrations. However, conflicting results have emerged from field-based studies of passerine birds. Researchers documented reduced responsiveness of the HPA axis in tree swallow nestlings in mercury-contaminated sites in western Virginia and found that exposed late stage nestlings had baseline corticosterone levels 103% greater than nestlings in reference sites (Wada et al. 2009). However, both adult and nestling tree swallows in mercury-contaminated sites in Massachusetts showed a significant negative relationship between blood mercury level and baseline corticosterone (Franceschini et al. 2009). Despite the fact that researchers measured corticosterone concentrations in the same species, opposite patterns in the correlations between mercury exposure and basal corticosterone concentrations were found. While significant discrepancies between the two studies are the intensity of mercury exposure and age of individuals sampled (i.e., duration of exposure), results indicate a clear need for additional research on the relationship between mercury exposure and basal corticosterone for both adult and nestling passerines.

Section IV: Thyroid Function

General

The function of the avian HPT (hypothalamic-pituitary-thyroid) axis appears to be analogous to that of other vertebrate taxa (McNabb 2007). The HPT axis is imperative in

regulation of metabolism (Danforth & Burger 1984), development, and thermoregulation (McNabb & Olson 1996). Thyroid hormone activity is necessary for appropriate central nervous system (CNS) development and affects gene expression in nearly every vertebrate tissue (Soldin et al. 2008). Two forms of thyroid hormone are stored and released from the thyroid follicle, T4 (thyroxine) and T3 (triiodothyronine). T3 is converted to T4 by peripheral tissues via deiodinase enzymes. T3 is believed to be the metabolically active hormone responsible for most thyroid hormone activity in mammals (McNabb 2007), as the mammalian thyroid hormone receptor (TR) shows a higher affinity for T3 than T4 (Zoeller et al. 2007). While T4 is generally thought of as a prohormone, T4 can also bind to TRs and thus repress or enhance transcription. Regulation of thyroid hormone activity is achieved through a negative feedback of T3 and T4 on the hypothalamus and anterior pituitary gland, which causes a decrease in both the release of TRH from the hypothalamus and TSH from the pituitary gland. Conversely, when T3 and T4 levels drop, a release of TRH from the hypothalamus will result in pituitary release of TSH, which will act on the follicle cells of the thyroid gland to produce and secrete T4 and T3.

Generally, adult birds have plasma or serum T4 concentrations of 5-15 ng/mL and T3 concentrations of 0.5-4 ng/mL (McNabb 2007). Once secreted from the thyroid follicle, the predominance of T4 and T3 is significantly influenced by deiodinase enzyme activity. As seen in the mammalian thyroid gland, the high concentration of T4 relative to T3 and the increased ratio of T3 and T4 in the circulation indicate that the majority of avian T3 is produced in extrathyroidal tissues (McNabb 2007). Both type I and II deiodinases (5'D I and 5'D II, respectively) convert T4 to bioactive T3, while type III

deiodinase (5D III) inactivates T3 via inner ring deiodination to yield T2 (McNabb 2007). Type I deiodinase may also convert T4 to reverse T3 (rT3), which will subsequently prevent T3 formation (McNabb 2007). In addition to the moderation of thyroid hormone levels via deiodinase activity, physiological and environmental conditions also have significant effects. Food availability and temperature appear to have the most significant influence on thyroid hormone levels, although T4 and T3 levels are also affected by iodine availability, the quality of food resources obtained, as well as season and reproductive condition (McNabb 2007).

Mercury and Thyroid Hormone

The HPT axis is susceptible to significant methylmercury accumulation, with concentrations approaching that of the liver and kidneys (Tan et al. 2009). As mercury has been shown to preferentially accumulate in the pituitary gland of humans and other primates (Cornett et al. 1998; Vahter et al. 1995), it is reasonable to suspect that mercury also accumulates in the avian pituitary, resulting in compromised function, abnormal synthesis, and/or release of both endocrine and neuroendocrine secretions.

Through selective effects on thyroid homeostasis, mercury may perturb neurodevelopmental processes (Soldin et al. 2008). Selenocysteine is a vital component of all three iodothyronine deiodinase active sites and is thus necessary for the activation and deactivation of thyroid hormones (Soldin et al. 2008). As methylmercury has a high affinity for the sulfhydryl groups present in cysteines (Crespo-Lopez et al. 2009), such deiodinating enzymes may be targets for the formation of a methylmercuric-cysteinyl complex (Soldin et al. 2008). Generally, the effects of methylmercury on both T4 and T3

levels have been inconsistent, likely due to variation in species, age of experimental animals, and length of exposure (Tan et al. 2009). At present, only one study exists in which researchers examined the effects of methylmercury exposure on TH levels in an avian species. Wada et al. (2009) measured T3 and T4 levels in tree swallow nestlings at different developmental stages. Differences in plasma T4 levels between birds inhabiting reference and contaminated sites were most pronounced in late stage nestlings, where exposed nestlings exhibited depressed T4 levels (Wada et al. 2009). Similarly, plasma T3 levels in exposed nestlings were consistently lower than that of reference birds, with the most significant difference evident in late stage nestlings (Wada et al. 2009). Authors propose that suppression of the HPT axis is an effect of mercury exposure and suggest that mercury may interfere with deiodinase activity (Wada et al. 2009). Specifically, mercury may suppress 5'-DI and hinder the conversion of T4 to T3, or may enhance 5-DI, thus increasing the conversion of bioactive T3 into inactive D2 (Wada et al. 2009). Although the mechanisms by which mercury may interfere with HPT axis function are yet to be understood (Tan et al. 2009), developmental exposure to mercury may permanently compromise HPT axis function, thus reducing future fitness and survival (Wada et al. 2009).

Summary

Thyroid hormones are imperative in regulation of metabolism (Danforth & Burger 1984), development, and thermoregulation (McNabb & Olson 1996), thus have significant effects on fitness. The function of the avian HPT (hypothalamic-pituitary-thyroid) axis appears to be analogous to that of other vertebrate taxa (McNabb 2007), yet

remains understudied, notably for passerine birds. The HPT axis is susceptible to significant methylmercury accumulation, with concentrations approaching that of the liver and kidneys (Tan et al. 2009). As mercury has been shown to preferentially accumulate in the pituitary gland of primates (Cornett et al. 1998; Vahter et al. 1995), it is reasonable to suspect that mercury also accumulates in the avian pituitary.

However, understanding of the effects of mercury exposure on passerine T3 and T4 concentrations represents a significant research gap, as only one study exists. Researchers measured T3 and T4 levels in tree swallow nestlings in reference and mercury contaminated sites and found marked reductions in concentrations of both hormones in mercury-exposed nestlings (Wada et al. 2009). Additionally, effects were most prominent in late stage nestlings, suggestive of differential developmental effects by age (Wada et al. 2009). Authors proposed that suppression of the HPT axis is an effect of mercury exposure and suggest that mercury may interfere with deiodinase activity (Wada et al. 2009). Although the mechanisms by which mercury may interfere with HPT axis function are yet to be understood (Tan et al. 2009), developmental exposure to mercury may permanently compromise HPT axis function (Wada et al. 2009). As developmentally appropriate thyroid hormone levels are imperative in early ontogeny, growth and thermoregulation (McNabb 2007), altered levels may reduce fitness and survival (Wada et al. 2009) of offspring. Additionally, as thyroid status is an integral element of successful adult reproduction (McNabb 2007), compromised thyroid function may contribute to reproductive impairment in mercury-exposed adults. As thyroid hormone levels are influenced by season, reproductive condition, and food availability (McNabb 2007), the attempt to determine the effects of mercury exposure on T3 and T4 levels in

adult and nestling birds must be done in a captive setting.

Chapter IV: Research Question and Captive Dosing Study

Section I: Experimental Design

Corticosterone

Prior to the completion of the present research, the existing literature was void of research regarding the effects of environmentally relevant mercury exposure on basal corticosterone concentrations in captive songbirds. Additionally, no attempt has been made to document the effects of duration of mercury exposure on basal corticosterone levels. Finally, insufficient information existed concerning the relationship between baseline stress hormone levels and reproductive output. Field-based studies of mercury-exposed passerine birds have shown both positive and negative relationships between exposure and baseline corticosterone levels, indicating the need for additional research in a captive setting.

Previous research has shown strong correlations between increased basal corticosterone levels and reproductive impairment in songbirds (Love et al. 2005; Saino et al. 2005; Schoech et al. 2008). Additionally, it has been suggested that mercury may act as a physiological stressor (Adams et al. 2009), which would be expected to increase basal corticosterone levels. I hypothesized that mercury exposure poses a physiological strain on individuals, thus increasing basal corticosterone levels, and that reduced reproductive output would be correlated with increased basal corticosterone levels in pre-breeding adults. With regard to the effect of duration of exposure, I hypothesized that decreases in circulating corticosterone would be observed as a result of adrenal

exhaustion and/or negative feedback of corticosterone on the pituitary and/or hypothalamus. To control for stage of reproduction, each pair was sampled during the late fledgling period of the second clutch produced. In addition to investigating the effects of treatment group and duration of exposure on basal corticosterone levels, I also asked whether differential effects of mercury exposure would be seen on male and female zebra finches.

Thyroid Hormones

Prior to the completion of the present research, there had been only one attempt to investigate the effects of mercury exposure with T3 and T4 levels in a passerine. Thyroid hormones play vital roles in both adult and developing birds (McNabb 2007) and the HPT axis is known to accumulate concentrations of mercury approaching that of the liver and kidneys (Tan et al. 2009). Studies of T3 and T4 concentrations in poultry have shown correlations between reproductive impairment and hyper- and hypothyroidism (McNabb et al. 2007), indicating a clear role for normal thyroid function in optimal reproduction. Additionally, the significant effects of thyroid hormones on metabolism (Danforth & Burger 1984) suggest that any mercury-related interference may compromise the ability of breeding females to provide the energetic investment necessary to produce eggs, incubate, and provide adequate parental care. With regard to developing young, appropriate thyroid hormones are imperative in early ontogeny, growth and thermoregulation (McNabb 2007). As such, altered levels may reduce fitness and survival (Wada et al. 2009) of offspring.

As field-based correlations have shown a negative relationship between mercury

exposure and thyroid hormones (Wada et al. 2009), I hypothesize that both adults and developing young will show reduced concentrations of circulating T3 and T4 levels. Additionally, I hypothesize that reproductive impairment will be correlated with reduced circulating concentrations of T3 and T4 in breeding pairs.

Captive Dosing Study

As field studies are subject to uncontrollable variables such as exact age of individuals sampled, length and severity of exposure to mercury, and exposure to additional environmental pollutants, it is imperative to isolate the effects of mercury exposure using a captive dosing experiment. An experimental study allows for the control of length and duration of mercury exposure, age, genetic similarity of breeding pairs, and enables researchers to control all environmental variables of the housing facility. Additionally, researchers are able to regularly monitor levels of blood mercury and carefully measure behavioral, physiological, and reproductive endpoints that cannot be readily studied in the field. Most importantly, researchers assign subjects to treatments, thereby eliminating the need to rely on correlative data.

We used a model organism songbird, the Australian zebra finch, for a captive dosing study established at the indoor aviary at the College of William and Mary in Williamsburg, VA. Australian zebra finches are a suitable study organism, as they breed readily in captivity and are well-studied in terms of their neurobiology, genetics and behavior (Zahn 1996). Our captive dosing study represents one of the first controlled experiments that aims to determine the effects of mercury exposure on multiple reproductive, physiological, and behavioral endpoints in a songbird.

MATERIALS AND METHODS

Section 1: Experimental Design

Captive Dosing Study

Australian zebra finches were maintained at the indoor aviary at the College of William and Mary in Williamsburg, VA. Finches were housed in one of three rooms according to mercury treatment (control, methylmercury chloride, or methylmercury cysteine). Pairs were randomly assigned to one of four dosing groups; 0.5 ppm methylmercury chloride, 1 ppm methylmercury chloride, 0.5 ppm methylmercury cysteine, or 1 ppm methylmercury cysteine. Each treatment group had 7 pairs and 10 pairs were assigned as controls. Finches were given mercury-dosed food (*ad libitum*), which was prepared by dissolving aqueous methylmercury in water to obtain the appropriate concentration. The MeHgCl or MeHgCys solution was thoroughly mixed into finch feed (ZuPreem Avian Maintenance) using a large tumbler. To ensure the appropriate concentration of mercury, food was used only when the average of 10 samples was within 20% of the expected mercury concentration.

For the first 10 weeks of dosing birds were housed in single sex cages. Each wire cage contained multiple wooden perches, food, water, and grit for proper digestion. On a daily basis, birds received food and fresh water with approximately 200 μL soluble vitamins per 300 mL water. Grit was replaced as needed. All personnel wore a mask, latex gloves, and disposable polypropylene shoe covers, which were disposed of upon exiting each treatment room. Each room was maintained at a temperature of approximately 21°C and a constant photoperiod of 14 hours light: 10 hours dark. All birds were sampled biweekly to monitor increases in blood mercury concentration. Blood

samples were collected from the cutaneous ulnar vein, which was pricked using a 30 gauge needle (Becton, Dickinson and Co. [BD], Franklin Lakes, New Jersey). Approximately 20-50 μL of blood was collected from each bird in a 70 μL heparinized capillary tube. Crito-caps ® were placed at the end of each tube, which was stored in a pre-labeled 10 cc BD ® Vacutainer and frozen until analysis. Once blood mercury level had reached a plateau at week 10, birds were sampled for blood mercury concentration once every 3 weeks. Breeding pairs were allowed to produce three clutches and raise offspring from the final clutch until the late fledging stage. Dosing continued for 22-35 weeks, a duration which was determined by the breeding activity of individual pairs. Following independence of fledglings, adults were humanely sacrificed and all organs harvested for mercury analysis. Research has been approved by the College of William and Mary's IACUC (protocol # 05-03-6516-dacris).

Mercury Analysis

All blood samples were analyzed for mercury using a Milestone Direct Mercury Analyzer-80 (DMA; Microwave Laboratory Systems). The DMA-80 uses cold vapor atomic absorption spectroscopy to measure total mercury concentration. Blood samples are placed in nickel boats, 40 of which fit into a carousel in the DMA-80. Samples are then dried by a stream of oxygen while passing through a heated coil. Samples are then combusted at 750 °C, which releases elemental mercury (Hg^0). Mercury collects on a gold trap and subsequently enters an atomic absorption cell, where light from a mercury vapor lamp is absorbed by mercury ions. Using absorbance readings from known samples used for calibration, the absorption of light by mercury ions is converted into a mass of

mercury in the sample, which is then converted into a concentration based on weight of sample. The sensitivity of the DMA-80 was .0055 ppm.

Breeding Pair Establishment

Birds were removed from single sex cages and housed with a pre-assigned mate once mercury levels reached a plateau around 10 weeks after commencement of dosing. Mates were randomly selected within dosing groups, with no known siblings paired. Pairs were placed in identical wire cages (18 x 18 x 30 in), which contained food, water, perches, a cuttlebone for bill sharpening, grit for digestion, nest box, a small bundle of hay for nest-building, and cotton for nest lining. Hay was replaced as needed. For three consecutive days, 10 breeding pairs were established beginning at 08:00 and ending at approximately 11:00. On the fourth day, 8 pairs were established, for a total of 38 breeding pairs.

Section 2: Corticosterone Sampling

Baseline Corticosterone Sample Collection in Pre-breeding Adults

After a 3-day mate acclimation period, birds were sampled for baseline corticosterone. A 3-day interval was chosen based on results of a previous study which indicated that after 48 hours, corticosterone returned to basal levels in a male-female zebra finch pair that had been separated and reunited (Remage-Healey et al. 2003). As dosed finches had not been in the presence of a mate before pairing, an additional 24 hours was allowed for acclimation before basal corticosterone sampling. The 3-day interval before sampling was intended to avoid effects of changes in HPA axis activity

associated with pair bonding.

Prior to sampling, the left and right sides of each treatment room were visually separated. A series of opaque black curtains were hung from the ceiling, providing a visual barrier between the right and left sides of each treatment room. Additional curtains were hung in a "V" in front of the point of entry in each room, allowing for entry to either side of the treatment room without visual cues of activity for birds on the opposite side. Curtains were hung approximately 5 inches from the ceiling and extended to the floor, thus finches were unable to detect any movement by researchers on the opposite side of the treatment room. Prior to sampling, researchers observed finch behavior on one side of treatment rooms while excessive movement was made on the opposite side and confirmed that curtains provided a highly effective visual barrier. However, as curtains did not provide an acoustic barrier, researchers proceeded with sampling as silently as possible. Two pairs from both sides of each treatment room were sampled for 3 consecutive days, with the final 2 pairs sampled on day 4. As low-dose birds were housed on one side of the room and high-dose birds on the other, the side of treatment room sampled first was randomly chosen immediately prior to sample collection.

Each bird was sampled within 3 minutes of the onset of disturbance (entrance to housing room) to minimize any disturbance-related changes in circulating corticosterone (e.g. (Romero & Reed 2005; Romero & Romero 2002). Time upon sample completion was used as a covariate in statistical analyses. Sampling began at least 3 hours after "dawn", as basal corticosterone in a passerine bird has been shown to peak immediately prior to start of the light phase, fall to low levels with the onset of activity, and stabilize for several hours (Breuner et al. 1999). All samples were completed between 0800 and

0830.

Blood samples were collected as previously described for measurement of blood mercury concentration. Each sample was kept on ice until blood was transferred into a 0.5 μL Eppendorf tube and centrifuged for 5 minutes. Serum was removed using a 20 μL pipette and transferred into a new, pre-labeled 0.5 μL Eppendorf tube. All samples were frozen at -4 °C until analysis.

Baseline Corticosterone Sample Collection in Breeding Adults

After the collection of pre-breeding basal corticosterone, pairs were allowed to breed for 22-35 weeks. While offspring from the third clutch were in the late fledgling stage, an additional baseline corticosterone sample was taken. To control for the effect of breeding stage on glucocorticoid activity, each pair was sampled during the late fledgling stage. As such, pairs were sampled over a range of exposure durations due to variability in breeding activity. Sampling was completed following the same methodology as for pre-breeding corticosterone, with the exception that 1-3 pairs were sampled per day over the course of many weeks as they reached the appropriate reproductive stage.

Section 3: Thyroid Hormone Sampling

T3 and T4 Sample Collection: Adults and Juveniles

Blood samples from adult pairs and juvenile males were obtained immediately prior to sacrifice. Each of 38 the adult pairs from the corticosterone study was sacrificed. One 55-day-old juvenile male from the second clutch produced by each breeding pair was also sacrificed. (Juveniles were hatched from eggs laid by dosed females and had

been dosed until sacrifice). A 26 G needle was used to collect approximately 400 µl of blood from the jugular vein from each individual. Blood was centrifuged directly after collection and serum was frozen at -4 °C until analysis.

Section 4: Hormone Analysis

Corticosterone Radioimmunoassay

Corticosterone ^{125}I-double antibody radioimmunoassay (RIA) kits (MP Biomedicals, Salon, OH, USA) were used to measure plasma corticosterone. Total (free and bound) corticosterone was measured, providing a measurement indicative of total adrenal synthesis. A limited amount of corticosterone (antibody) is reacted with the corresponding radioisotope-labeled hormone. As additional corticosterone antigen is added from unknown samples, the added corticosterone competes with the radiolabeled antigen for binding sites on the antibody. With increasing amount of the corticosterone, a correspondingly decreasing fraction of radiolabeled hormone is bound to the antibody. Bound radiolabeled antigen was read using a Scintillation counter (Beckman Coulter Inc., Fullerton California, USA) and the unknown corticosterone concentrations of the samples were calculated from a standard curve. Standard curves were created using six corticosterone standards (25 to 1000 ng/mL) included in the RIA kits. Final counts from known standards were used to plot a standard curve (relationship between binding affinity and total corticosterone concentration), from which unknowns were determined. Validation of the assay included the serial dilution of zebra finch serum in steroid diluent to determine the optimal concentration of serum to diluent, 10 ul of serum to 220 ul diluent. An optimal ratio of serum to diluent was indicated by a final hormone

concentration range that fell within the most linear region of the standard curve. Serum used in assay validation was obtained from a random group of zebra finches not used in the dosing experiment. Final finch corticosterone concentrations were compared to those presented in the literature for basal corticosterone concentrations in passerine birds. A dilution of the lowest standard, 25 ng/mL, was serially diluted to allow determination of the lowest detectable corticosterone concentration, 12.5 ng/mL. All samples were run in duplicate. Intra-assay variation was calculated as the average coefficient of variation of sample duplicates within each assay. Inter-assay variation was calculated as the average coefficient of variation of Control I and II (provided by the manufacturer, Control I= 63-93 ng/mL, Control II= 440-668 ng/mL) across all assays. Intra- and inter-assay coefficients of variation were 3.02% and 21.35%, respectively.

T4 Radioimmunoassay

Total T4 radioimmunoassay (RIA) kits (MP Biomedicals, Salon, OH, USA) were used to determine the concentration of total T4 in plasma samples obtained from adult and juvenile finches. Total concentrations of T4 were measured, as circulating levels are indicative of whole body exposure and thus provide the best measure of organismal thyroid status (McNabb 2007). Sample unknowns and a fixed amount of radiolabeled antigen were added to tubes coated with monoclonal T4 antibody. Unlabeled hormone present in the sample unknowns competes with the radiolabeled antigen for a limited number of antibody binding sites. Thus, the level of radioactivity bound is inversely related to the concentration of T4 in the sample. Radioactivity bound to tubes is counted and a standard curve is used to determine hormone concentrations in unknown samples.

Standard curves were created using five T4 standards included in RIA kits. Standards, which ranged from 2 to 20 ug/dL, were included in the assay along with unknown samples. Final counts from known standards were used to plot a standard curve (relationship between binding affinity and total T4 concentration), from which unknowns were determined. All adult samples were run in duplicate. Intra-assay variation was calculated as the average coefficient of variation of sample duplicates within each assay. In the few cases where the value of one duplicate gave biologically unrealistic concentrations for total T4, these inconsistent values were eliminated and not included in variability calculations. Inter-assay variation was calculated as the average coefficient of variation of standards (0 ng/mL, 2 ng/mL, 4 ng/mL, 8 ng/mL, 12 ng/mL, and 20 ng/mL, provided in RIA kit) across all assays. Intra- and inter-assay coefficients of variation were 11.73% and 28.04%, respectively.

T3 Enzyme Immunoassay

Total T3 enzyme-linked immunosorbent assay (ELISA) kits (MP Biomedicals, Salon, OH, USA) were used to determine the concentration of total T3 in plasma samples obtained from adult finches. As with T4, total concentrations of T3 were measured, as circulating levels are indicative of whole body exposure and are thus provide the best measure of organismal thyroid status (McNabb 2007). ELISA, also known as enzyme immunoassay (EIA) is a biochemical technique whereby a known amount of antigen, T3, is pre-bound to each of the wells on the plate. Samples with unknown concentrations of T3 antigen are added and immobilized via adsorption to the side of the well. The antibody, which is linked to an enzyme, is added and forms a complex with the antigen.

An enzymatic substrate is added, producing a visible signal indicative of the concentration of antigen in each well. Antigen concentration is calculated from the absorbance measured using a Multiskan plate reader (Fisher Scientific). Standards provided in ELISA kit included 0 ng/mL, 0.75 ng/mL, 1.5 ng/mL, 3 ng/mL, 6 ng/mL, and 10 ng/mL. Due to low circulating concentrations of T3 in the study population, kit standards were modified to include standards of 0.0937 ng/mL, 0.1875 ng/mL, and 0.375 ng/mL. All adult samples were run in duplicate. Intra-assay variation was calculated as the average coefficient of variation of sample duplicates within each assay. Inter-assay variation was calculated as the average coefficient of variation of standards (0 ng/mL, 0.09 ng/mL, 0.18 ng/mL, 0.37 ng/mL, 1.5 ng/mL, and 3.0 ng/mL) provided in ELISA kit) across all assays. Intra- and inter-assay coefficients of variation were 10.55% and 15.21%, respectively.

Section 5: Data Analysis

Statistical Analyses

I used regression to determine the relationships between time since initial disturbance and corticosterone, as well as blood mercury and corticosterone or thyroid hormones. Reduced Major Axis regressions were used to determine the relationships between time since disturbance and corticosterone, and blood mercury level and corticosterone. Mean Square Model regressions were used for thyroid hormone data. Regressions were performed using PAST (RMA regressions) and SPSS (MSM regressions) and graphs were created using Microsoft Excel. I used a one-way ANOVA to determine the effect of mercury treatment on corticosterone, T4, and T3 concentrations

when males and females were combined. I used a two-way ANOVA to determine the effect of mercury treatment and sex on corticosterone, T4, and T3 concentrations. All ANOVA statistical analyses were performed using SPSSx Statistical Software. For each analysis, an alpha level of α=0.05 was used.

RESULTS

Section 1: Corticosterone

Pre-breeding Baseline Corticosterone

Circulating baseline corticosterone concentration was not related to the time elapsed between entering the bird's room and completing the sampling of its blood, when those samples were taken within 3 minutes ($F_{1,68}$=1.694, P=0.191, y=0.404x -25.24, R^2= 0.0246) (Fig. 1). Therefore, all samples collected within 3 minutes of room entry were included in analyses. In all statistical analyses, methylmercury cysteine and methylmercury chloride treatments were combined by dose level based on lack of evidence that form of mercury influenced tissue accumulation (pers. comm. C Ramos).

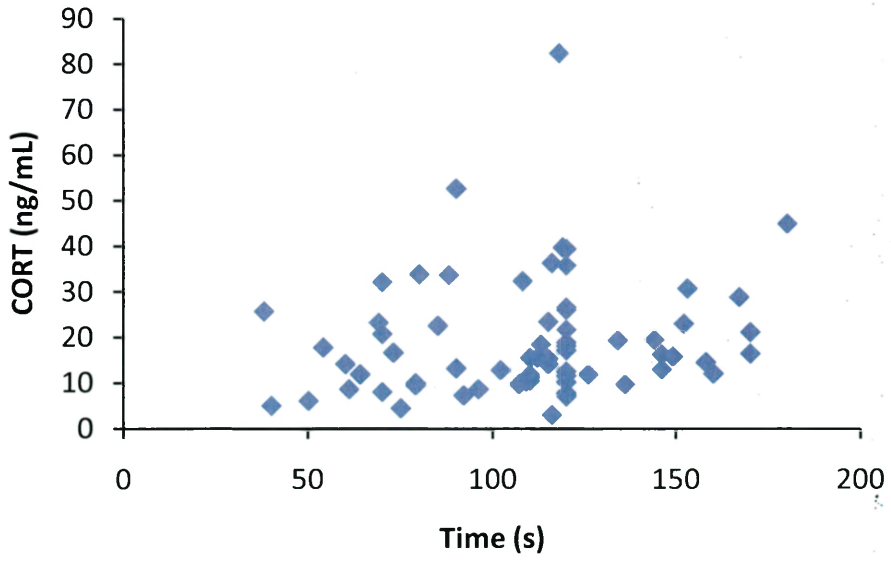

Figure 1. Time elapsed between room entry and completion of sample versus corticosterone in pre-breeding birds. R^2= 0.0246, Corticosterone =0.404 * Time -25.24.

There was no reliable relationship between blood mercury and circulating baseline corticosterone levels ($F_{1,67}$=0.001, P=0.942, y= 3.133x+ 2.179, R^2=0.000092) (Fig. 2). When control birds were removed, results did not change qualitatively.

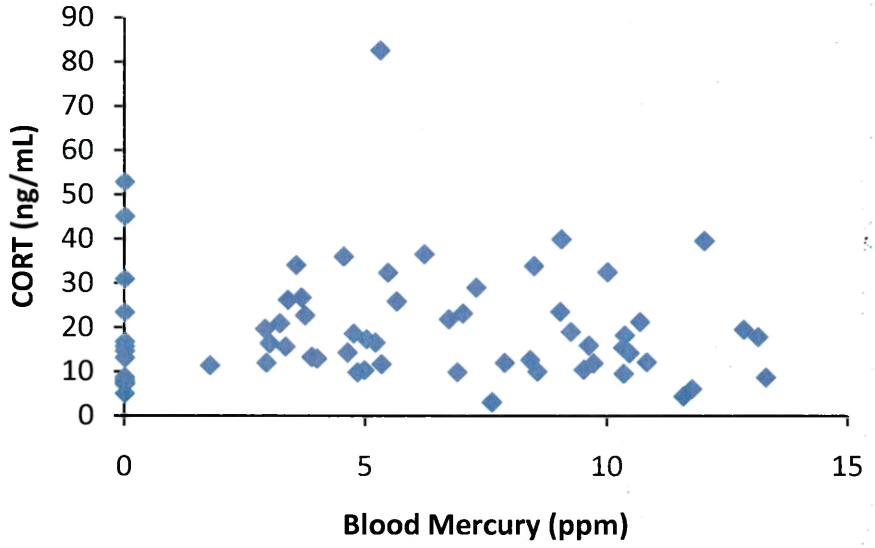

Figure 2. Blood mercury level versus corticosterone in pre-breeding adults. R^2= 0.000092, Corticosterone = 3.1326 * Blood Mercury + 2.1791.

There was little consistent effect of mercury treatment group ($F_{2,63}$=3.064, p=0.216), sex ($F_{1,63}$=0.078, p=0.780), or the interaction of sex-by-treatment ($F_{1,63}$=0.078, p=3.660, p=0.160) on corticosterone (Fig. 3).

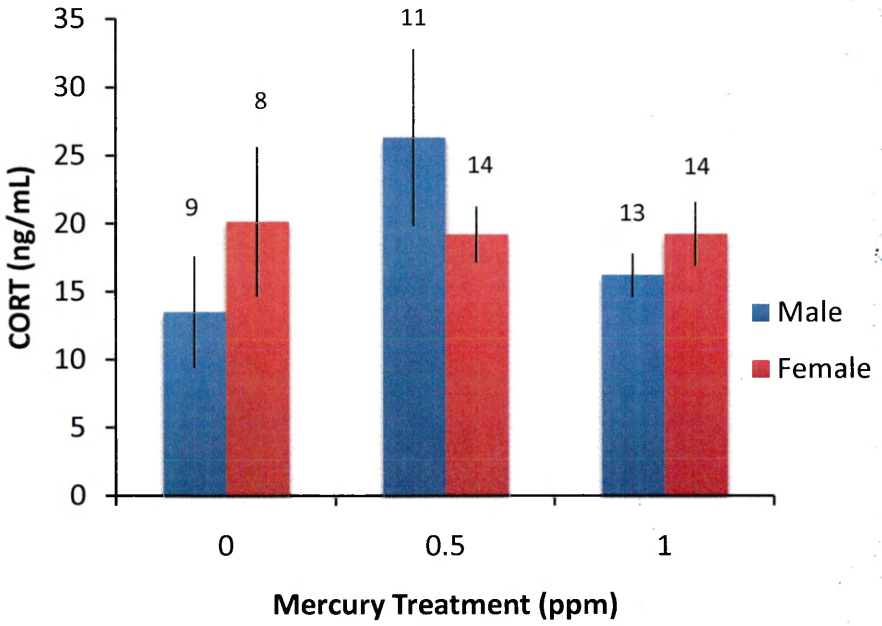

Figure 3. Corticosterone versus mercury treatment and sex in pre-breeding adults. Graph shows mean +/- standard error. Sample sizes are indicated above the bars.

Breeding Baseline Corticosterone

As with pre-breeding samples, circulating baseline corticosterone concentration was not related to the time it took to sample blood, including entering the housing room, when those sample were taken within 3 minutes ($F_{1,75}$=2.704, P=0.106, y=0.0512x - 33.65, R^2= 0.0352) (Fig. 4). All individuals were sampled, however, some pairs failed to reproduce successfully. To control for the effect of reproductive stage on basal corticosterone, infertile pairs (n=6) were eliminated from analyses.

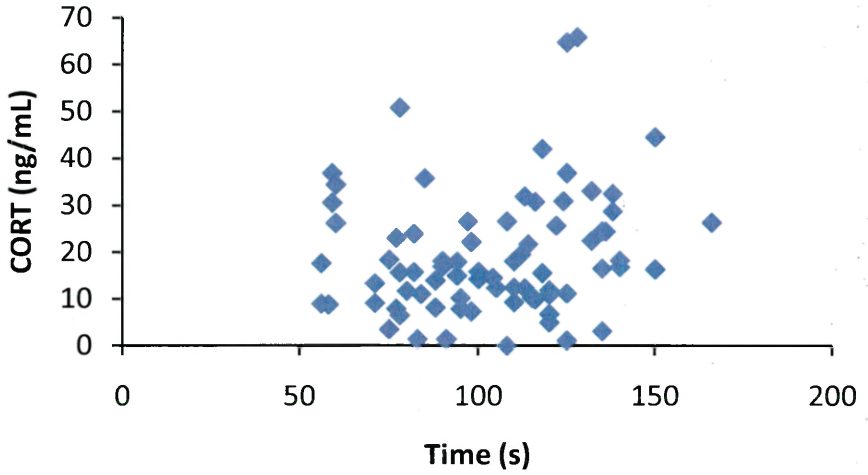

Figure 4. Time sample completed versus total corticosterone in breeding adults. R^2= 0.0352, Corticosterone = 0.512 * Time + -33.652.

There was no reliable relationship between blood mercury and circulating corticosterone ($F_{1,74}$=0.002, P=0.961, y=2.388x + 2.574, R^2= 0.00003) (Fig. 5). When control birds were removed, results did not change qualitatively.

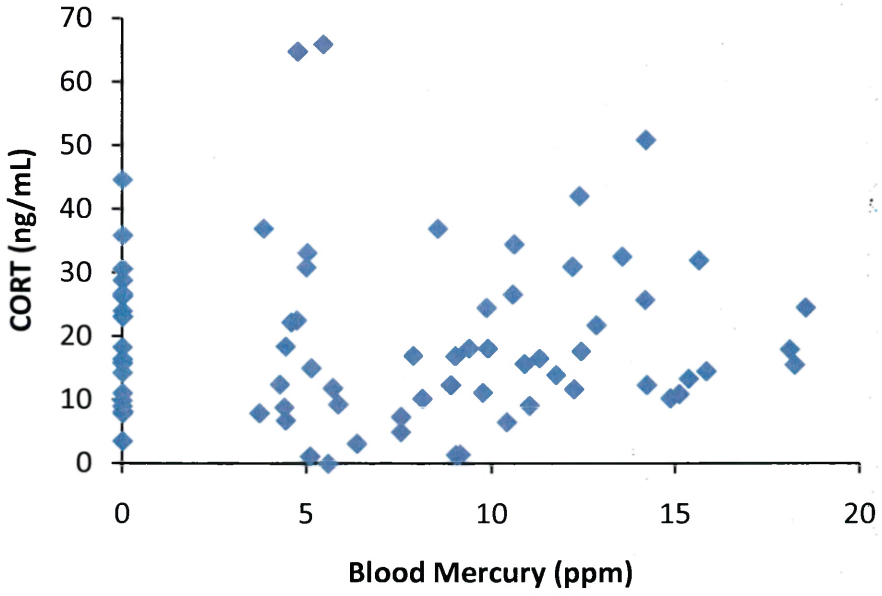

Figure 5. Blood mercury level versus corticosterone in breeding adults. R^2= 0.00003, Corticosterone = 2.388 * Blood Mercury + 2.574.

There was little effect of mercury treatment group ($F_{2, 62}$= 0.208, p= 0.902) on basal corticosterone in breeding adults. A statistically significant effect of sex ($F_{1, 62}$= 4.608, p= 0.032) was detected. A trend was found for the interaction of sex-by-treatment ($F_{2, 62}$= 5.128, p= 0.077) on circulating corticosterone (Fig. 6). Males and females showed opposing trends in corticosterone responses, where males decreased in circulating corticosterone while females increased.

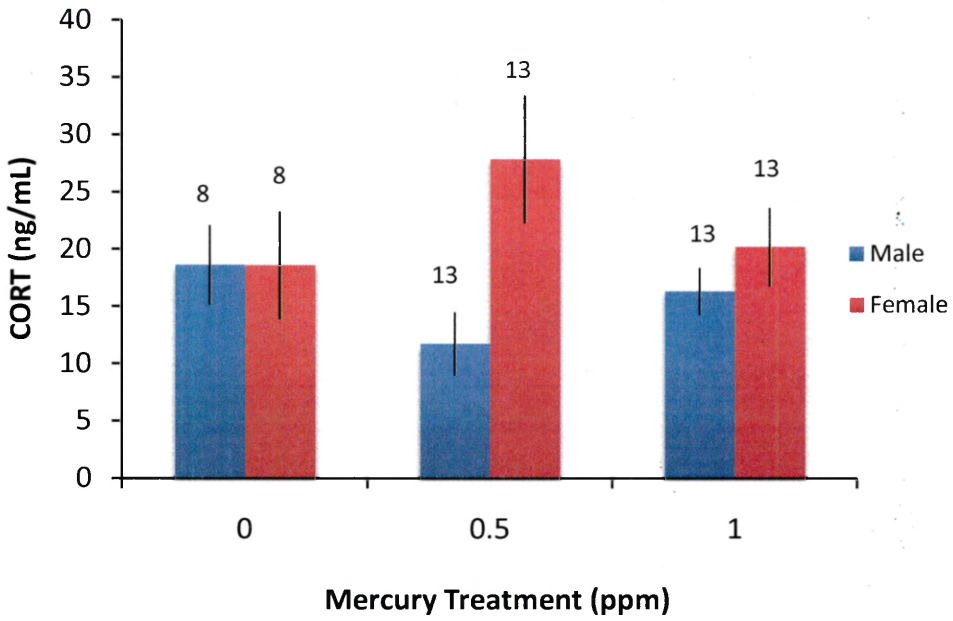

Figure 6. Corticosterone versus mercury treatment and sex in breeding adults. Graph shows mean +/- standard error. Sample sizes are indicated above the bars.

Change in Baseline Corticosterone Between Pre-breeding and Breeding Period

The change in basal corticosterone between the first and second sampling was calculated by subtracting the pre-breeding level from that measured during the relevant breeding period. It is important to note that while stage of reproduction was controlled for, the variability in reproductive timing did not allow for the control of duration of

mercury exposure. As birds were dosed throughout the experiment, pairs that reproduced more slowly were consequently exposed to dietary mercury for a greater length of time.

There was little effect of mercury treatment group ($F_{2, 63}$= 1.179, p= 0.314), sex ($F_{1, 63}$= 1.369, p= 0.246), or the interaction of sex-by-treatment ($F_{2, 63}$= 0.143, p= 0.867) on the change in basal corticosterone with increased duration of mercury exposure (Fig. 7). The interaction of sex-by-treatment over time ($F_{2, 63}$= 4.635, p= 0.013) revealed a statistically significant effect on changes in basal corticosterone. Post- hoc Bonferroni comparisons reveal a significant difference in the corticosterone response of low dose males and females. Males showed a mean decrease and females a mean increase in basal corticosterone over the exposure period.

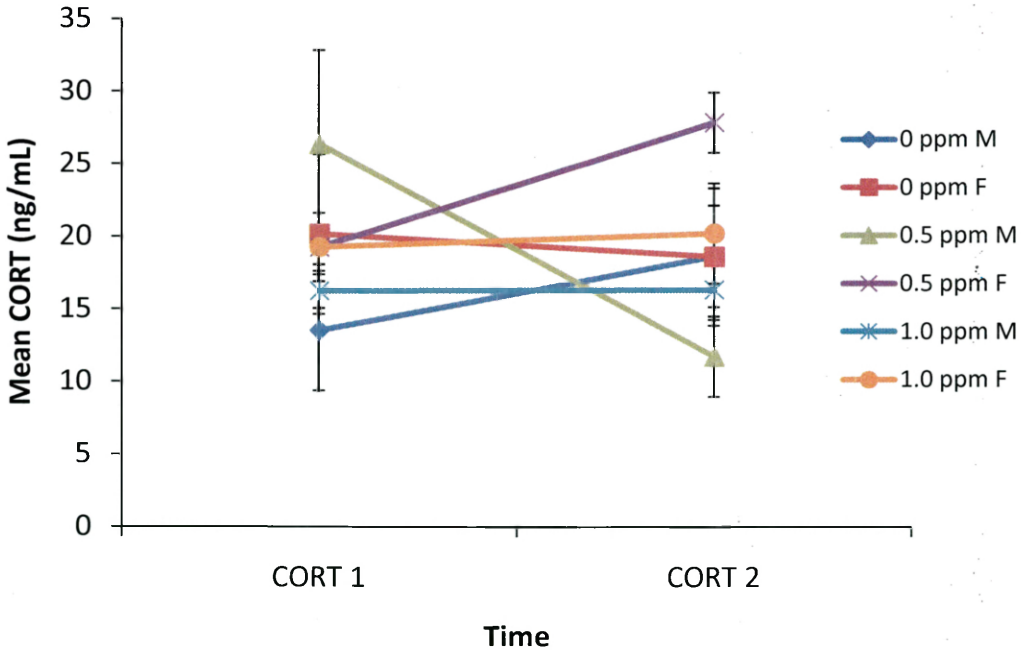

Figure 7. Mercury treatment versus change in corticosterone over dosing period by sex. Graph shows mean +/- standard error.

Section 2: Thyroid Hormones

Adult T3 & T4

There was no apparent effect of blood mercury on circulating T3 ($F_{1,73}$=0.152, P=0.698, y=0.0047x + 1.0587, R^2= 0.002) or T4 ($F_{1,68}$=0.297, P=0.588, y=0.0291x + 2.0051, R^2= 0.0041) concentrations (Fig. 8, 9).

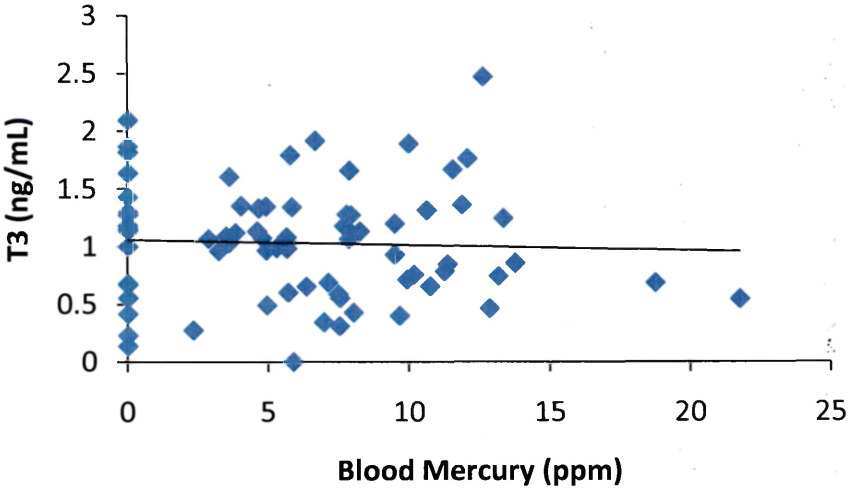

Figure 8. T3 versus blood mercury level. R^2= 0.002, T3 = 0.0047 * Blood Mercury + 1.0587.

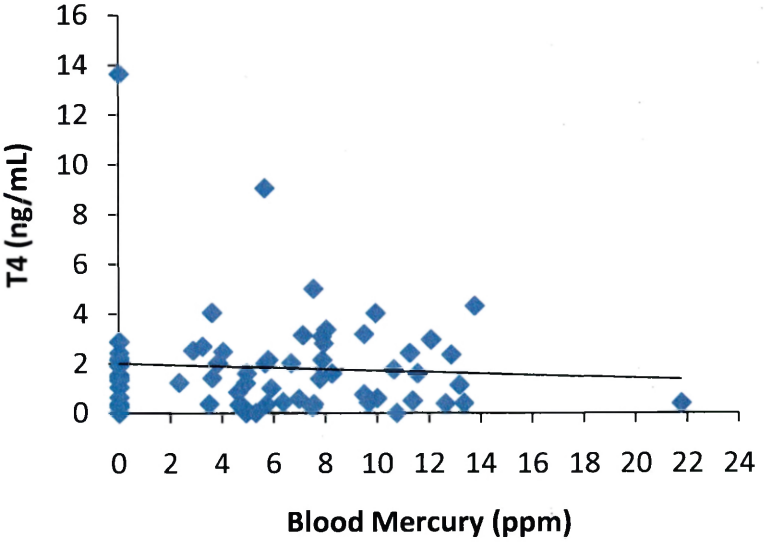

Figure 9. T4 versus blood mercury level. R^2= 0.0041, T4=0.0291 * blood mercury + 2.0051.

There was no statistically significant effect of mercury treatment group ($F_{2, 59}$= 2.423, p=0.298) or the interaction of sex-by-treatment ($F_{2, 59}$= 0.345, p= 0.842) on circulating T3 (Fig. 10). However, a highly statistically significant effect of sex ($F_{1, 59}$= p=0.004) on total T3 was detected (Fig. 10). In each treatment, males had higher total T3 concentrations than females.

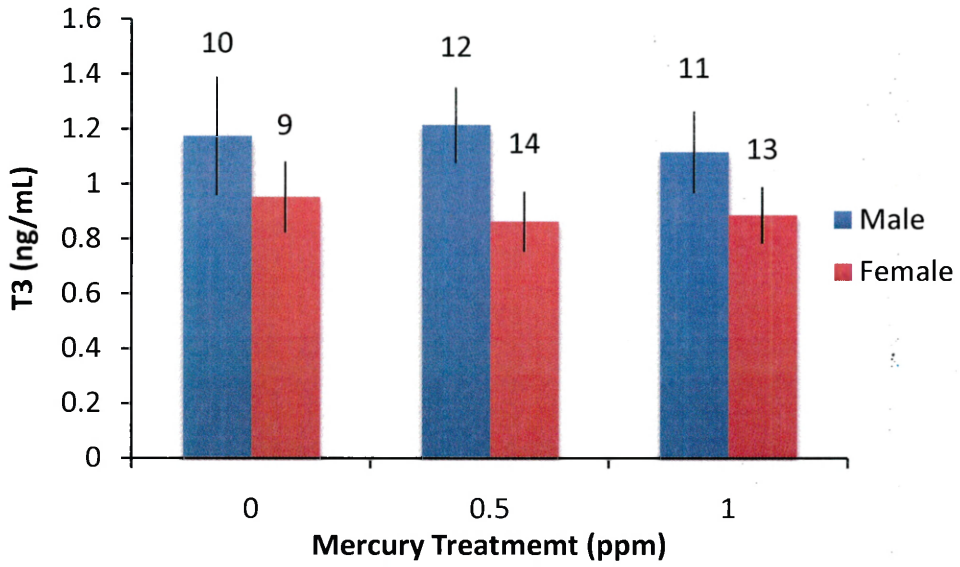

Figure 10. T3 versus mercury treatment and sex. Graph shows mean +/- standard error. Sample sizes are indicated above the bars.

There was no significant effect of mercury treatment group ($F_{2, 63}$= 0.104, p=0.949), sex ($F_{1, 63}$= 0.674, p=0.412), or the interaction of sex-by-treatment ($F_{2, 63}$= 2.207, p= 0.332) on circulating T4 (Fig. 11). In the control and low dose groups, females had lower total T4 concentrations than males, a pattern that was reversed in high dose birds.

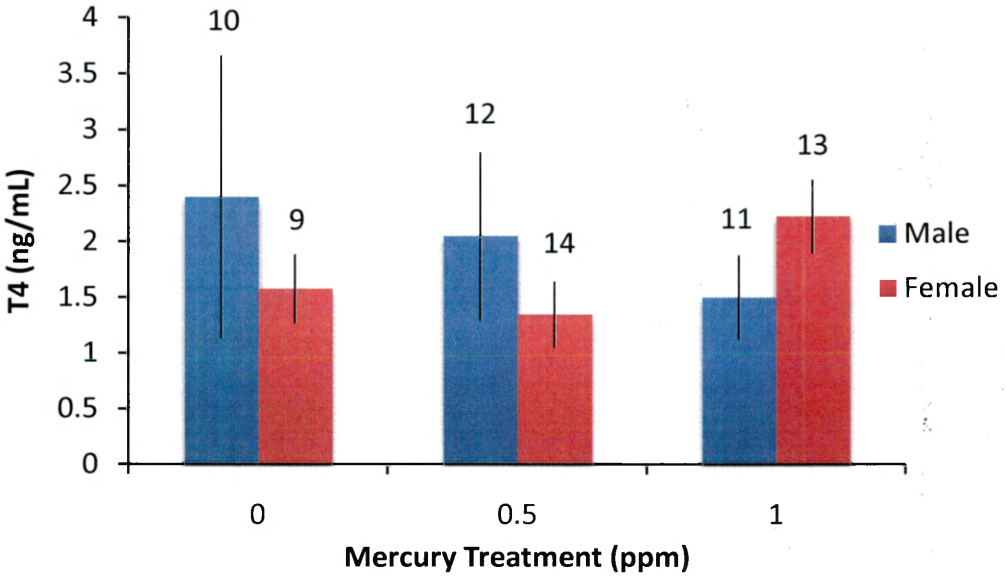

Figure 11. T4 versus mercury treatment and sex. Graph shows mean +/- standard error. Sample sizes are indicated above the bars.

Juvenile T3 & T4

Regressions of blood total T3 and T4 concentrations as a function of blood mercury in juvenile males revealed a trend toward increasing total T3 with increasing blood mercury level ($F_{1,25}$=6.658, P=0.16, y=0.1009x + 0.8626, R^2= 0.217) (Fig. 12) but no significant relationship between blood mercury level and T4 ($F_{1,28}$=2.098, P=0.159, y=0.0301x + 0.8801, R^2= 0.072) (Fig. 13).

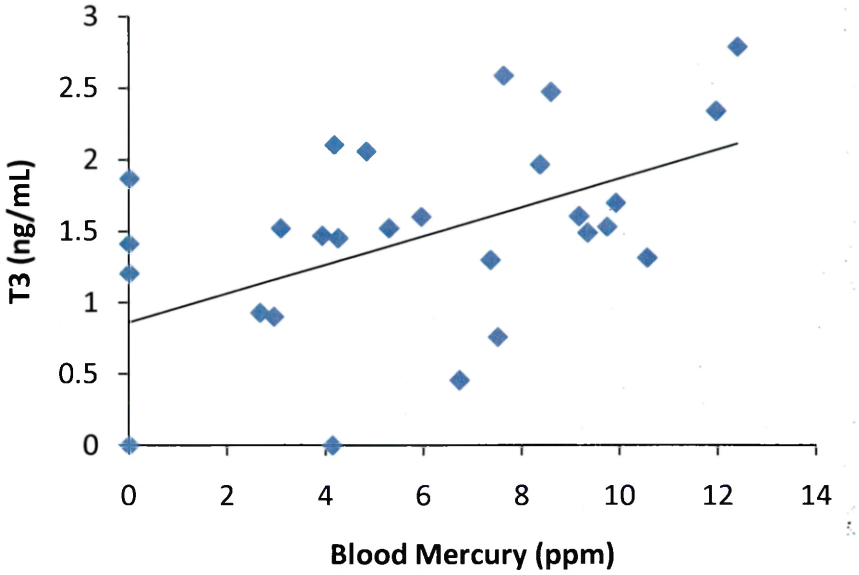

Figure 12. T3 versus blood mercury level in juvenile males. R^2= 0.217, T3=0.1009x * Blood Mercury + 0.8626.

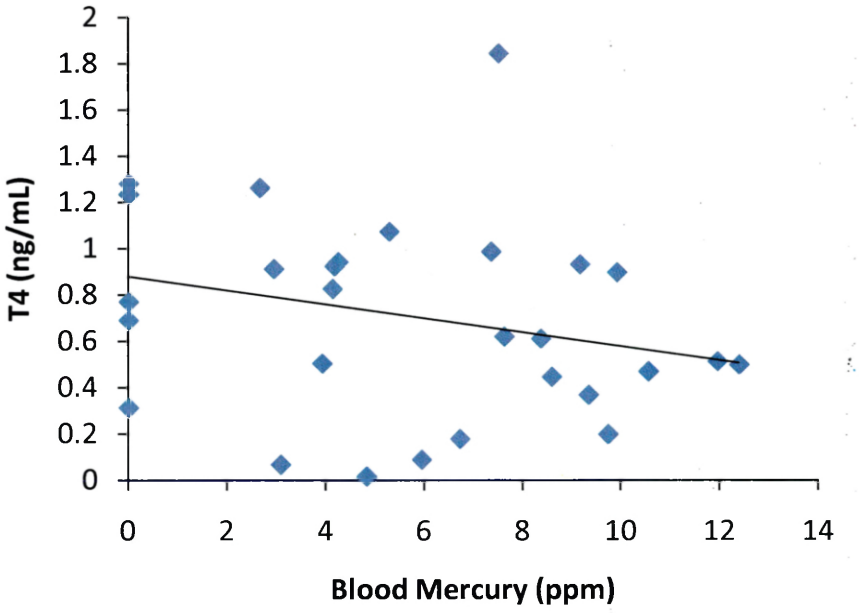

Figure 13. T4 versus blood mercury level in juvenile males. R^2= 0.072, T4=-0.0301 * Blood Mercury + 0.8801.

There was a trend ($F_{2,24}$=2.656, P=0.091) toward increasing total T3 concentration with increasing mercury exposure in juvenile males (Fig. 14). Conversely, total T4

concentrations appeared to decrease with increasing mercury exposure ($F_{2,29}$=0.987, P=0.386) (Fig. 15).

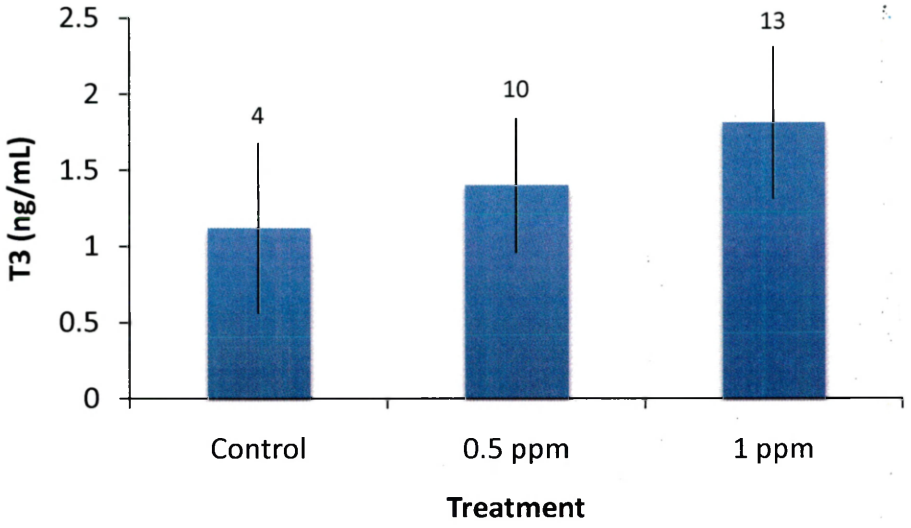

Figure 14. T3 versus mercury treatment in juvenile males. Graph shows mean +/- standard error. Sample sizes are indicated above the bars.

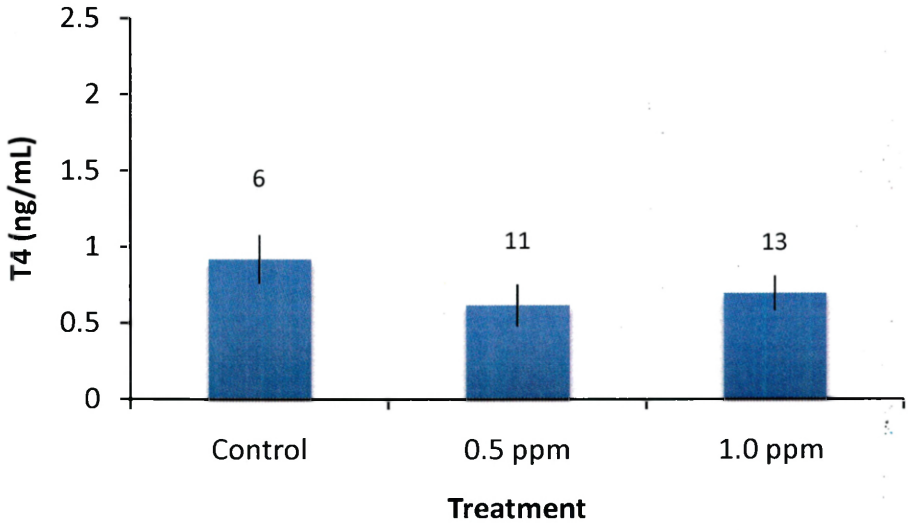

Figure 15. T4 versus mercury treatment in juvenile males. Graph shows mean +/- standard error. Sample sizes are indicated above the bars.

DISCUSSION

Section I: Adults

Thyroid Hormones

No significant differences were found in T3 or T4 concentrations among treatments, however a highly statistically significant difference was found between male and female circulating triiodothyronine concentrations (P=0.004). Few studies have reported circulating T3 and T4 levels in non-mammalian vertebrates, notably in passerine birds. To our knowledge, the present study is the first in which significant differences in total T3 concentrations between reproductively active males and females have been reported. Little information exists on thyroid function in passerine birds, which precludes thorough comparison of present data to related studies. Reproduction is especially metabolically costly for females (Trivers 1972) and thyroid hormones play significant roles in the regulation of metabolism (Danforth & Burger 1948), thus increased physiological demand may render increases in circulating T3 adaptive for breeding females. A current lack of data on thyroid hormone concentrations in passerines hinders my ability to make robust biological conclusions, except to recommend that sex-based differences in thyroid function should be incorporated into future studies.

No effect of mercury treatment was detected on circulating T3 or T4 levels, therefore results were inconsistent with initial hypotheses. In previous experiments, methylmercury has been reported to inhibit the production of Thyroid Stimulating Hormone (TSH), subsequently lowering the production of thyroid hormones T3 and T4 (Nishida et al. 1989). However, results of the same study indicate that inorganic mercury inhibits the production of the enzyme responsible for the addition of iodine onto

thyroglobulin, thyroid peroxidase (Nishida et al. 1989). The interference of thyroid peroxidase decreases thyroidal synthesis, leading to an initial net increase in peripheral conversion of T4 to T3 to maintain appropriate levels (Nishida et al. 1989). In a similar study, circulating concentrations of both T4 and T3 in mercuric chloride-fed mice were significantly reduced. Researchers suggest mercury binds to liver glutathione (GSH) and likely to the enzymes involved in the biosynthesis of T3 (Sin et al. 1990). As such, it is possible that liver mercury would bind to and inhibit 5' thyroxine deiodinase type II, reducing conversion of T4 to T3 (Sin et al. 1990). However, it should be noted that these effects of mercury exposure on thyroid hormone concentrations were reported in rodents. Striking differences exist between metabolic demands and life history events in mammals and birds, thus mercury exposure may not affect the two taxa in a similar manner. It is possible that the passerine thyroid is more resilient to mercury exposure and/or has a higher threshold for accumulation before thyroid pathways are affected. Comparison of the present study with related research in passerines will be an important element in the assessment of ecological implications; however, there are no additional known reports of the measurement of plasma T3 and T4 concentrations in relation to mercury exposure in adult birds.

While no effect of mercury exposure on circulating T4 concentrations was detected, increased concentrations of T4 in high-dose females should be investigated further. Mounting evidence indicates that mechanisms behind and effects of plasma T3 and T4 may not be as highly conserved across vertebrate taxa as once believed; unique mechanisms may have evolved in passerine birds (Pant & Chandolasaklani 1995). Because T4, a compound containing four iodine molecules, is converted to T3 by

deiodinase enzymes present in target tissues, mammalian endocrinologists contend that the effects of T4 are mediated by its conversion to T3 (Pant & Chandolasaklani 1995). However, T4, which is associated with the onset of molt, migration, and reproduction in songbirds, may have an independent hormonal role (Pant & Chandolasaklani 1995). Songbirds are generally iodine deficient, which may be an important adaptation that precludes high concentrations of circulating T4. More iodine would facilitate higher levels of circulating T4, which may stimulate the overlap of physiologically demanding seasonal events (Pant & Chandolasaklani 1995). Accordingly, suppression of T4 via increased peripheral conversion to T3 in the summer months is associated with gonadal development and fat deposition in preparation for breeding (Pant & Chandolasaklani 1995). Females in the high-dose group exhibited a mean increase in circulating concentrations of T4 as compared to control and low-dose females. As suppression of T4 is believed to be an important endocrine adaptation used to facilitate reproduction, increased levels of T4 in high-dose females may be maladaptive. Reproduction is metabolically costly, therefore female finches would likely benefit from the suppression of a hormone which is known to stimulate the onset of additional energetically costly life history events. In the current study, high-dose pairs did not experience reproductive impairment (unpublished data), yet effects of increased T4 may be significant for reproductively active females living in the wild.

As avian endocrinologists have yet to determine differential roles of T3 and T4 and their biological and physiological effects in passerines, there is a significant research gap yet to be filled. In addition to the need for research on passerine thyroid function, future research regarding the potential endocrine-disrupting effects of mercury is

imperative. Most data regarding circulating thyroid hormone concentrations in birds exposed to potential thyroid-disrupting chemicals is highly variable and difficult to interpret in the context of thyroid disruption (McNabb 2007). As thyroid hormones play instrumental roles in life history processes and almost every tissue in vertebrates is thyroid hormone sensitive (Hulbert 2000), effects of mercury that result in small changes in circulating thyroid hormone levels have the potential for significant physiological effects. Adult birds have plasma or serum T4 concentrations of approximately 5-15 ng/mL and T3 concentrations of approximately 0.5-4 ng/mL (McNabb 2007). As vertebrates have evolved mechanisms to maintain set balances in total T3 and T4 concentrations, small biochemical changes are likely to have major physiological effects. In addition to measuring thyroid status, future research should also incorporate measurement of metabolic activity, such as stored liver glycogen and/or basal metabolic rate in mercury-exposed passerines. Thyroid hormone levels should also be measured during major life history stages, including molt, migration, and reproduction, both with and without exposure to mercury. Only with a greater understanding of thyroid status in passerine birds will researchers have the resources necessary to biologically interpret variations in T3 and T4 concentrations that may result from mercury exposure.

Corticosterone

Pre-breeding serum corticosterone concentrations did not vary significantly between treatment groups when sampled a few days after pairing. Mean baseline corticosterone showed little variation between treatment groups among females, whereas males treated with 0.5 ppm mercury showed elevated levels of the hormone compared to

both control and 1.0 ppm mercury treatment groups. In post-breeding birds, the mean corticosterone concentration was nearly identical for both males and females in the control group. Likewise, both sexes in the 1.0 treatment group showed similar mean baseline corticosterone, with females having a marginally higher concentration than males. Both sexes in the 0.5 treatment group exhibited the greatest deviation in mean corticosterone as compared to control birds, with males showing a marked decrease and females showing a marked increase.

Changes in mean baseline corticosterone over the breeding period reveal a non-linear dose-response to mercury treatment. In each treatment, a biphasic response was seen, whereby males and females responded differently to exposure. Control females showed a slight decrease of 7.73% in mean serum corticosterone, whereas males showed an average increase of approximately 0.38%. Birds in both the 0.5 and 1.0 ppm treatment groups showed the same pattern with regard to sex, with males showing a decrease and females an increase in baseline serum corticosterone concentration. However, the magnitude of increase/decrease was greater in the 0.5 ppm treatment group than the 1.0 group. Females fed 0.5 ppm dietary mercury showed an average increase in serum corticosterone 2.5 times greater than that of females in the 1.0 ppm group. Males fed 0.5 ppm dietary methylmercury showed an average decrease in serum corticosterone 33.7 times greater than males in the 1.0 ppm treatment group.

Several biological mechanisms should be considered in the interpretation of the non-linear, sex-specific patterns of variation in baseline serum corticosterone concentration. Non-linear dose-responses to contaminants have been observed in previous studies in which birds were exposed to mercury (Adams et al. 2009; Welshons

et al. 2003), thus were not unanticipated. Multiple biological processes involved in glucocorticoid regulation may be targets of alteration by mercury with potentially variable thresholds (Adams et al. 2009), thus complex and non-linear responses are expected. Glucocorticoid concentrations represent a dynamic interaction between adrenal synthesis, binding to plasma proteins, and hepatic biotransformation and clearance (Adams et al. 2009; Guillette & Gunderson 2001). As such, it is possible that mercury differentially affects each of the aforementioned processes. Variability in effects may result from sex-based physiological differences, which may be heightened by the disparity in reproductive investment by males and females. A range of mechanisms by which mercury may alter stress hormone regulation is possible and it is important to consider all biological explanations for observed effects.

The role of plasma binding globulins is an important consideration in the interpretation of steroid hormone concentrations. Finch corticosterone levels were measured as total concentration, thus include both free and protein-bound hormone. Total corticosterone concentrations are indicative of the rate of adrenal synthesis, whereas the concentration of free hormone determines the physiological concentration, or the amount of hormone available to target tissues (Welshons et al. 2003). The primary function of basal corticosterone is to facilitate glucose release under stressful situations (Schoech et al. 2008), therefore the free concentration of corticosterone will determine the extent to which such physiological effects are exerted. Corticosteroid Binding Globulin (CBG) binds to corticosterone with high affinity, rendering the steroid biologically inactive. CBG also influences metabolic clearance rates, delivery of corticosteroids to specific tissues, and binding of the CBG-corticosteroid complex to binding globulin receptors

(Rosner 1990). The onset of a stressor may decrease CBG levels (Lynn et al. 2010), increasing the proportion of steroid available to bind to receptors. Thus, if mercury reduces CBG production, the biologically active concentration of corticosterone could be elevated. For example, although pre-breeding concentrations are similar for females across treatments, it is possible that the total concentration of free hormone is elevated in exposed birds. Likewise, although average male total corticosterone is highest in birds dosed with 0.5 ppm mercury, it is unknown if the amount of CBG increased, decreased, or remained stable in low dose males as compared to control and high dose birds. Changes in corticosterone over the breeding period would have greater implications for the fitness of exposed birds if CBG levels were found to increase for groups in which mean corticosterone decreased markedly. CBG was not measured in the present study, but concentrations of both free and bound corticosterone and corresponding CBG levels should be incorporated into future research.

Some reports suggest that environmental contaminants may compromise endocrine function by increasing baseline glucocorticoid concentrations (Wingfield et al. 2008). If mercury acts as a physiological stressor, exposure could lead to an up- or down-regulation of corticosterone (Adams and Frederick 2009). Duration of exposure is a pertinent variable in the assessment of mercury as a physiological stressor. If exposure is of shorter duration, an organism would likely respond by increasing glucocorticoid synthesis to regain homeostasis. However, if exposure is chronic, an initial increase in stress hormone levels might be followed by an eventual downregulation of glucocorticoid concentration as a result of pituitary/hypothalamic feedback, interference with hormone production, and/or endocrine mediated pathways acting to reduce glucocorticoid levels,

thereby preventing adverse effects on immune function, growth, and/or reproduction. As such, variable responses to mercury would be expected depending on the severity and duration of exposure. The age, developmental stage, physiological condition, and the setting under which the stress response to mercury exposure is measured (e.g. wild or captive) would also be expected to introduce variability in basal corticosterone concentrations. In western Virginia, tree swallow nestlings sampled at 13-17 days of age showed a significant increase in baseline corticosterone concentrations with increasing blood mercury levels (Wada et al. 2008), whereas an adult population sampled in Massachusetts had a significant negative relationship between blood mercury and corticosterone (Franceschini et al. 2009). Although results of the aforementioned studies indicate highly variable effects of mercury on baseline corticosterone levels, hormone concentrations may be directly related to mercury level and duration of exposure to. For the present study, it is possible that pre-breeding male finches in the 0.5 treatment group responded by increasing corticosterone synthesis in response to exposure to a physiological stressor. Males in the high dose group may display comparatively lower basal corticosterone concentrations as a result of the activation of pathways responsible for the maintenance of homeostasis. Such pathways may be activated when a certain mercury burden threshold is reached, which occurred only in high-dose birds. Although no mechanisms were suggested, the activation of similar pathways may explain the negative relationship between blood mercury level and corticosterone in adult tree swallows.

The pattern seen in pre-breeding male finches is suggestive of a "hormetic" response. Hormesis is a biological phenomenon characterized by low-dose stimulation

and high-dose inhibition in response to a stressor (Calabrese 2010; Calabrese & Baldwin 2002). It is proposed that the mechanism behind hormesis is an initial toxicity and disruption in homeostasis followed by a compensatory response (Stebbing 1998). As it is not possible for a single proximate mechanism to be responsible for all hormetic responses (Calabrese 2010), mercury may stimulate low-dose corticosterone concentrations via an unknown mechanism. Stimulation in the low dose males may also result from the alteration of endocrine pathways disrupted by mercury, such as binding globulin production, steroid synthesis, metabolism, and/or feedback pathways. Little is known about how mercury affects the physiology of stress, therefore it is not yet possible to identify the mechanisms by which mercury exposure produces nonlinear and hormetic responses.

To our knowledge, the present study is the first in which researchers have measured basal corticosterone in mercury-exposed birds at two distinct treatment intervals. Sampling birds at both pre-and post-breeding stages allowed for potential change in corticosterone with increasing duration of mercury exposure to be quantified. The dose-time response is also an imperative element in the consideration of hormesis. Only studies that include multiple doses and a repeated measures component are suited to quantify the effect of time on response (Calabrese 2010). It is possible that the increase in basal corticosterone in low-dose pre-breeding males represents the overcompensatory response, indicating that the initial toxicity phase occurred within the first 10 weeks of exposure, prior to sampling. Conversely, it is also possible that the initial increase in basal corticosterone in low dose pre-breeding males represents the initial toxicity. As such, the subsequent overcompensatory response would be the marked decrease in mean

basal corticosterone seen in post-breeding birds. Low-dose males exhibited a mean decrease in baseline corticosterone of 55.5% over the course of the designated breeding period. Although it is not possible to confirm that such a decrease is related to the hormetic dose-response, basal corticosterone patterns shown by low dose males support hormesis as a possible mechanism.

Patterns in mean female corticosterone do not reveal a mechanism behind the observed individual responses. Pre-breeding females showed similar mean basal corticosterone across mercury doses; differences were not significant. Patterns in post-breeding levels were opposite that of males, as low-dose females displayed a marked increase in mean corticosterone and high dose females showed a slight increase. While the disparity in the direction of corticosterone change between males and females suggests that hormesis may not be the mechanism behind observed trends, males and females may respond differently to both mercury exposure as well as reproductive activity. A significant effect of sex in the response of low dose birds was seen, thus variability in the male versus female response may be due to differential effects of reproduction on stress physiology. As reproduction is an energetically demanding stage, results may be partially attributed to physiological changes induced by breeding. Reproduction is generally more metabolically costly for females (Trivers 1972), thus exposure to a stressor may cause females to respond by increasing basal corticosterone. In some birds e.g. American kestrels (*Falco sparverius*), females exhibit a higher susceptibility to stress than males (Bortolotti et al. 2002). Presently, no conclusions may be drawn regarding potential mechanisms behind observed sex-based and non-linear patterns in mercury-treated finches. Non-linear responses to potential Endocrine

Disrupting Contaminants (EDCs) highlight the complexity of physiological responses (Ottinger et al. 2008), illustrating the need for future research into mechanisms driving observed patterns. Although relationships between EDCs and long-term effects on avian populations can be challenging to prove (Ottinger et al. 2008), elucidation of non-linear results should be made a priority in the effort to establish a sensitive endpoint indicative of mercury exposure.

As many wild passerines contend with challenges such as migration, reproduction, molt, adverse weather conditions and social instability, extrapolating stress physiology of captive birds to their wild relatives must be done with caution. With such dramatic differences in living conditions, the direct comparison of absolute levels of basal corticosterone between captive and wild birds may not be appropriate. The production of glucocorticoids mediates physiological and behavioral responses to unpredictable events, increasing chances of survival (Wingfield & Kitaysky 2002). However, chronically elevated corticosterone reduces immune function (McEwen et al. 1997; Sapolsky et al. 2000), interferes with reproductive activity (Schoech et al. 2008; Wingfield et al. 1998), and compromises cognitive ability (Hodgson et al. 2007; Kitaysky et al. 2003). Thus, as chronically elevated corticosterone has adverse effects on fitness, domesticated zebra finches may have evolved alterations in stress physiology in response to the nurturing conditions in which they are housed. Despite any adaptations by captive finches that may alter total concentrations of corticosterone, net changes in stress hormone levels in contaminant-exposed captive birds are still relevant in predicting effects on wild birds, as the same biochemical pathways would likely be affected. While small net changes in total hormone concentration did not affect reproductive parameters

in captive finches, relatively minor alterations in hormone concentrations may have significant ecological effects. For example, basal stress levels of wild song sparrows breeding in areas with artificially increased food availability and reduced predator numbers were compared to those of individuals breeding under unmanipulated conditions (Clinchy et al. 2004). Birds at the unfed sites with higher predator abundance had significantly higher levels of basal corticosterone, which was correlated with significant reductions in brood size (Clinchy et al. 2004). Despite significant effects on brood size, the total difference in basal corticosterone between birds in food supplemented sites with reduced predator density and birds with no additional food and natural densities of predators was approximately 7 ng/mL (Clinchy et al. 2004). In the present study, control female finches showed a decrease in basal corticosterone of 1.56 ng/mL (7.73%) over the mercury exposure period, whereas low and high dose females showed increases of 6.46 ng/mL (44.83%) and 2.50 ng/mL (44.83% and 4.89%) respectively. While mercury did not have significant effects on either baseline corticosterone or reproductive output in our study, it is possible that observed changes in circulating stress hormone levels would yield important physiological changes in a wild bird. Future research should incorporate systematic measurements of circulating hormone levels in both mercury-exposed captive and wild passerines, along with measurements of multiple physiological, behavioral, and reproductive endpoints.

Section 2: Juvenile Males

Thyroid Hormones

No significant effect of mercury on plasma T3 or T4 was found in juvenile males. A trend was found for increasing total T3 with increased mercury exposure ($p=0.091$), whereas total T4 concentrations showed the opposite pattern. However, the tendency for control birds to have higher concentrations of circulating T4 was not statistically significant ($p=0.347$). Few studies exist in which researchers have measured thyroid hormone levels in relation to mercury exposure in young birds. Nestling white storks (*Ciconia ciconia*) exposed to heavy metals did not show any effect of contamination on plasma T3 and T4 levels (Baos et al. 2006). However, late-stage nestling tree swallows in mercury-contaminated sites in Virginia showed reduced concentrations of both plasma T3 and T4 (Wada et al. 2009). Mean plasma T3 levels in all three age classes sampled showed a 15-40% reduction in T3 as compared to nestlings of the same age in reference sites (Wada et al. 2009). No differences in plasma T4 levels in 3-12 day-old nestlings were found between contaminated and reference sites (Wada et al. 2009). However, late stage nestlings exposed to mercury showed reduced concentrations of circulating T4 as compared to that of nestlings of the same age in reference sites (Wada et al. 2009).

Results of the current study suggest similar effects of mercury exposure on T4 in wild tree swallow nestlings and captive zebra finch juveniles. As vertebrates show a surge of T4 during development (Hulbert 2000), the observed mercury-associated reductions in circulating thyroxine are believed to be maladaptive. It is not known if the increase in total T3 with mercury exposure in juvenile finches may be a compensatory effect resulting from decreased total T4. As T4 is converted to T3 via deiodinase enzyme

activity, it seems unlikely that increased T3 concentrations would occur as a compensatory mechanism. Additionally, as T3 and T4 have been demonstrated to have independent hormonal roles in songbirds (Pant & Chandolasaklani 1995), replacement of one hormone with the other would not be sufficient to restore normal growth, development, and/or metabolic activity. Inappropriate stimulation of deiodinase enzyme activity may be the mechanism behind increased T3 and reduced circulating T4 levels in exposed birds. While reduced circulating levels of T4 in exposed birds is parsimonious with the existing literature, increased levels of total T3 in exposed birds was unexpected. Inappropriate stimulation of deiodinase enzyme activity provides a reasonable explanation, however, evidence suggests that mercury would act to inhibit, not stimulate, such enzyme activity. Both type I and II iodothyronine deiodinase enzymes have a selenocysteine residue at the active site (Hulbert 2000). Mercury is known to inhibit the function of many enzymes that contain sulfhydryl residues of cysteine (Naganuma et al. 2000), therefore inhibition of type I and II deiodinses would be expected. Type II deiodinase was inhibited by methylmercury in mouse NB4A3 neuroblastoma cells, an effect that was not mitigated by removal of methylmercury from the cell culture (Mori et al. 2006). If the catalytic subunit of type I and II deiodinase enzymes is involved in mercury-associated inhibition, this effect would be expected in type III deiodinase activity as well. Type III deiodinase also contains a selenocysteine residue at the active site (Hulbert 2000), rendering this enzyme susceptible to inhibition by mercury. Unlike type I and II deiodinases that convert T4 to T3, type III deiodinase converts T3 to the inactive reverse T3 via inner ring deiodination (Hulbert 2000). As such, elevated T3 levels may result if type III deiodinase enzyme activity is inhibited.

Mechanisms driving observed differences in the thyroid hormone responses of adults and juvenile males are unknown. Differences may be a direct result of the age, sex, and/or breeding stage of the individuals sampled. In our study, juvenile finches were hatched from eggs laid by dosed females and fed a mercury diet throughout development, whereas adults were raised to maturity before mercury exposure was begun. While the effects of mercury exposure on T3 and T4 levels in juvenile males were not statistically significant, effects are important to consider from both a physiological and ecological perspective. In an attempt to determine the effects of mercury exposure on wild passerine fitness, discussion of results should not be dictated by statistical significance. Rather, researchers should consider the potential effects of small, statistically nonsignificant changes in hormone concentrations and determine if such changes are enough to compromise normal physiological processes.

Appropriate concentrations of thyroid hormones are imperative for the normal differentiation and maturation of many major systems, including the skeletal system, heart, and body musculature (McNabb 2007). Additionally, thyroid hormones are necessary for the formation of synaptic interconnections between brain neurons and the architecture of all brain regions (McNabb 2007). Therefore, altered thyroid hormone states during development would be expected to have lasting negative effects on many systems, notably central nervous system function (McNabb 2007). Studies of inappropriate thyroid hormone levels in both male and female developing chicks have also shown adverse effects on reproductive potential. In female chicks, hypothyroidism during embryonic development was associated with a decrease in oocyte volume, nuclear size, and mitchondria (Roda-Moreno et al. 2000). In post-hatch male chicks treated with

thyroid hormone for several weeks, hyperthyroidism resulted in precocious puberty, larger testis size, and abnormal spermatogenesis (Kirby et al. 1996; Knowlton et al. 1999). As abnormal thyroid hormone levels during development may not compromise growth or survival, it is possible for reproductive physiology or other developmental endpoints to be negatively affected. The small changes in total T3 and T4 concentrations in juveniles sampled for this study may not be sufficient to result in significant interference with major developmental processes; however, even minor changes in circulating levels may be physiologically significant.

CONCLUSION

Results of the current research do not provide evidence that mercury is an endocrine disrupting contaminant. This is the first study, to my knowledge, that has attempted to measure the effect of duration of mercury exposure on corticosterone in an avian species. While no effect of mercury treatment on basal corticosterone was found, differences in sex-based responses of low-dose birds should be investigated further. It has been suggested that non-linear dose-responses are characteristic of endocrine disruption, however, additional research regarding the effects of mercury on the passerine adrenocortical response are needed before conclusions may be drawn. No effect of mercury exposure was found on T4 or T3, however the significant difference between the sexes in circulating T3 concentrations in breeding adults has not been reported in the literature. Further investigation of the effects of environmentally relevant mercury exposure on passerine hormonal activity is imperative in establishing a sensitive endpoint of toxicity. Multifarious physiological responses indicate the complexity of hormonal

responses to mercury exposure and illustrate the need to measure multiple hormones over an extended period of time and over a range of dosing regimens. Further research should incorporate measurement of plasma binding globulins, biosynthetic pathways, and the metabolism and clearance of hormones. Results of the current study will assist in the establishment of a threshold level of mercury exposure for songbirds and will be widely applicable as global mercury pollution worsens and passerine populations continue to experience precipitous declines.

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