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Superoxide Dismutase in Songbirds: Effects of Mercury, Exercise, and Sex on an Enzymatic Antioxidant

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APPROVAL PAGE

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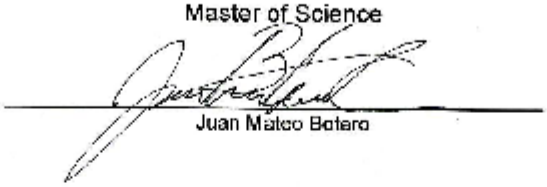

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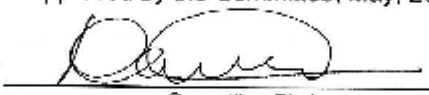

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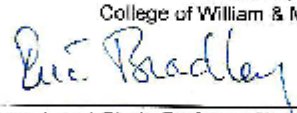

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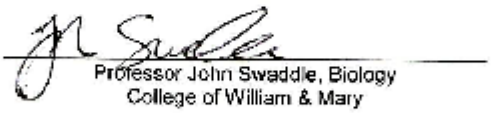

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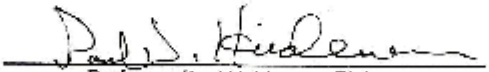

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COMPLIANCE PAGE

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ABSTRACT

Anthropogenic activities such as burning of fossil fuels, mining, and industrial processes continue to increase the levels of mercury circulating in the environment. Once methylated, mercury can be incorporated into animal tissues, causing it to biomagnify up the food web. Deleterious fitness impacts are commonly associated with increased mercury content in birds. The mechanistic pathway to those fitness effects needs further research in order to develop appropriate remediation efforts and predict future harm to bird populations. Oxidative stress is proposed as one of the primary ways though which mercury causes accumulation of cellular damage. Well-documented cases of mercury induced oxidative stress indicate an increased level of reactive oxygen species along with a depletion of protective antioxidant molecules. We assessed the relationship between dietary mercury exposure and activity levels of superoxide dismutase (SOD) isozymes to evaluate the ability of SOD to serve as a non-lethal biomarker of oxidative stress. We approached this goal in two ways. First, we monitored levels of extracellular SOD in the blood throughout the lifetime of zebra finches exposed to mercury only during development. We found no effect of developmental mercury exposure on SOD3 activity levels, but found significant effects of age and sex. Second, we examined the effect of lifetime mercury exposure and acute exercise on adult zebra finches. Again, mercury did not induce significant changes in SOD3 activity levels, however, sex did play a role in dictating SOD3 activity levels in the blood. We detected a small non-significant effect of mercury on liver, of similar effect size to that reported in Henry et al. (2014). This suggests that blood SOD3 activity levels may not be a viable option for quantifying mercury-induced oxidative stress; mammalian models suggest that this is because mercury interacts with each SOD isozyme differently. Mitochondrial SOD seems to be more susceptible to decreased activity levels from mercury than the extracellular SOD studied here. We found that sex is playing a crucial role in dictating SOD3 activity levels, likely due to estrogen's role as an antioxidant, as previously demonstrated in mammalian models. This is the first in-depth analysis of mercury, sex, exercise, and age activity levels as predictors of SOD3 in the plasma of songbirds. Because SOD3 activity sampled simultaneously in the liver and plasma of individuals was not correlated, SOD3 is not useful as a biomarker for oxidative stress in zebra finches.

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Chapter 1: Introduction to mercury-induced oxidative stress and superoxide dismutase

Mercury is a naturally cycling heavy metal found in liquid, vapor, and solid forms throughout the environment (Hooper et al., 2013). Anthropogenic activities such as fossil fuel burning, gold mining, and industrial amalgamation processes have dramatically increased mercury levels throughout the planet over the past 100 years (Boening, 2000). Once released, mercury becomes bioavailable through microbe-mediated reactions that methylate the toxin (Boening, 2000; Scheuhammer, 1987). Methylmercury has lipophilic characteristics, allowing it to easily move throughout the body and even cross the blood-brain barrier (Nuran Ercal, Hande Gurer-Orhan, & Nukhet Aykin-Burns, 2001). Ultimately, these processes lead to the bioaccumulation of mercury within individuals and its biomagnification towards the top of the food web (Boening, 2000; Cristol et al., 2008; Scheuhammer, 1987; Valko, Morris, & Cronin, 2005).

Researchers have been concerned with methylmercury's negative impact on avian fitness for over 50 years, but studies have disproportionally focused on piscivorous species (Scheuhammer et al., 2008). More recently, comparable mercury levels have been documented in terrestrial songbirds, originating in the invertebrates they eat (Cristol et al., 2008). Both captive and field studies have revealed that elevated tissue mercury levels correlate with a variety of reduced fitness parameters. Reduced reproductive success is one particularly well-studied result of mercury exposure. Clear connections have

been demonstrated between increased blood mercury content and reduced hatching/fledging success, egg size, urgency to breed, and other measures of reproductive output (Brasso & Cristol, 2008; Hallinger & Cristol, 2011; Varian-Ramos, Swaddle, & Cristol, 2014). Behavior is another parameter that can be significantly altered by mercury exposure, potentially decreasing fitness. Reduced spatial memory and altered singing behavior may result from mercury's impact on the nervous system. Significant damage to the nervous system is commonly documented in mercury-exposed birds through histological lesions, axon degeneration, and demyelination (Bennett, French, Rossmann, & Haebler, 2009; Heinz & Locke, 1976). Other impacts attributed to mercury include decreased immune function and endocrine disruption (Hallinger & Cristol, 2011; Henry, Cristol, Varian-Ramos, & Bradley, 2014; Moore, Cristol, Maddux, Varian-Ramos, & Bradley, 2014; Varian-Ramos et al., 2014; H. Wada, Cristol, Mcnabb, & Hopkins, 2009; Zhu, 2000)

To fully understand the scope of damage inflicted by mercury, we must better understand the mechanisms through which it causes this damage at the cellular level (Henry et al., 2014; Hoffman, Spalding, & Frederick, 2005; Zaman & Pardini, 1996). One proposed mechanism is oxidative stress (OXS) at the cellular level and the subsequent oxidative damage (OXD) that it causes. (Costantini, 2008; Hoffman, Eagles-Smith, Ackerman, Adelsbach, & Stebbins, 2011; Hoffman et al., 2005; Kenow et al., 2008; Zaman & Pardini, 1996). Reactive oxygen species (ROS; also referred to as free radicals and pro-oxidants) are common byproducts of a variety of biochemical reactions

that occur inside and outside of cells. All ROS share the characteristic of being extremely reactive, usually due to an unpaired electron (Sohal, Mockett, & Orr, 2002). This reactivity is nonspecific and can cause deleterious reactions with macromolecules, also known as OXD. It is crucial to clarify that ROS are commonly used in signaling pathways, both intra and extracellularly; ROS are abundant and sometimes intentionally produced, it is when they occur in excess that they can be harmful (Nuran Ercal et al., 2001).

Antioxidants are the body's natural defense against these reactive molecules and are produced internally as well as supplemented through the diet (Cohen, Klasing, & Ricklefs, 2007). An antioxidant is defined as any molecule that stabilizes a ROS without itself being permanently altered. They can be simple molecules like glutathione and vitamins, which are found in high concentrations throughout the body and act as generalists to stabilize a broad range of ROS (Costantini & Møller, 2008). Or, they can be large complex enzymes like superoxide dismutase (SOD) or catalase which can have their concentrations in various parts of the body modified through variable gene expression (Costantini, 2008; Hoogenboom, Metcalfe, Groothuis, de Vries, & Costantini, 2012; Metcalfe & Alonso-Álvarez, 2010b). At homeostasis, ROS and antioxidants maintain a balance in the bodies of all aerobic organisms. OXS is defined as a shift in that balance towards ROS (Costantini, 2008). When ROS concentrations surge, the likelihood that they react with important biomolecules like DNA, proteins and lipids, increases dramatically. OXD can manifest at the cellular level through mutations, loss of

protein-mediated pathways, and cell apoptosis (Costantini, 2008; Hoffman, Ohlendorf, Marn, & Pendleton, 1998). Accumulation of oxidative damage can alter the pathways, and ultimately, the function of whole systems (i.e. immune, nervous, etc.). The extensive system-wide impacts associated with mercury toxicity, may, at least in part, be explained by the cumulative damage caused by increases in OXS (Alonso-Álvarez, Bertrand, Faivre, & Sorci, 2007; Costantini, 2008, 2014; Kamiński, Kurhalyuk, & Szady-Grad, 2007). A more comprehensive understanding of mercury toxicity requires a better understanding of mercury's role in OXS and OXD (Nuran Ercal et al., 2001).

OXS and OXD research has been motivated by the possibility of better understanding longevity, and disease. In fact, the 'rate of living' hypothesis is based on the concept of OXS. The idea is that an organism with a higher metabolic rate lives a shorter life because high metabolic rates produce more ROS and accumulate eventually fatal OXD (Holloszy & Smith, 1986; Sohal et al., 2002). However there are two major exceptions to the empirical support for this hypothesis: birds and primates (Finkel & Holbrook, 2000; Sohal et al., 2002). When comparing mammals and birds of the same size, birds consistently have higher metabolic rates, higher glucose levels, and higher body temperatures. All of these characteristics typically correlate with higher ROS production and, in theory, should produce more OXS, OXD, and thus shorter lifespans (Ku, Brunk, & Sohal, 1993). However, the opposite is true, birds live longer than their mammalian counterparts. Birds have evolved to limit OXD accumulation, making them an interesting model species for OXS

research (Costantini, 2008). This paradoxical relationship also makes OXS-related comparisons between mammals and birds much more difficult.

Mercury induces OXS through two fronts: it depletes antioxidants and increases ROS production in the mitochondria (Nuran Ercal et al., 2001; Stohs & Bagchi, 1995; Zaman & Pardini, 1996). Most of mercury's damaging characteristics are attributed to its very high affinity for binding with sulfur (Hughes, 1957). Organic molecules that contain sulfhydryl groups (also known as thiols) commonly bind to free mercury and methylmercury within the body. The most common thiol in the body of most organisms is the antioxidant glutathione; a tripeptide composed of glycine, glutamate, and cysteine. The sulfhydryl group of the cysteine serves as the major electron donor to ROS, producing more stable molecules like water. However, when mercury is present, mercury's high affinity to the cysteine sulfhydryl group forms a highly stable mercury-cysteine complex (Ballatori & Clarkson, 1985; Dutczak & Ballatori, 1994). This complex is subsequently excreted through the kidneys and the liver (via biliary secretion) causing an overall decrease in total glutathione content of the body (Ballatori & Clarkson, 1985; Dutczak & Ballatori, 1994; Townsend, Rimmer, Driscoll, McFarland, & Iñigo-Elias, 2013). This reduction of antioxidants mainly occurs in the liver, but, since the liver is the source of most antioxidants in the body, it leaves the body more prone to OXS and OXD (Lawrence & Burk, 1976).

Mercury further exacerbates OXS by increasing ROS production (Lund, Miller, & Woodst, 1993; Stohs & Bagchi, 1995). In both its organic and

inorganic states, mercury disrupts the electron transport chain of the mitochondria. An in vivo study of rat kidneys demonstrated a two-fold increase in H_2O_2 production when mercury was introduced (Lund et al., 1993). In vitro studies suggest that this is due to a disruption of the ubiquinone-cytochrome b region of the electron transport chain (Stringari et al., 2010). Although ROS such as O_2^- and H_2O_2 are used and regularly produced during the respiration process, mercury causes a marked increase in their concentrations and therefore increases the likelihood that these ROS damage important macromolecules.

There are many ways to assay for OXS, however, there is no consensus on a single best biomarker (Costantini, 2008). Instead, it is recommended to assay more than one biomarker. Depending on the question of interest, researchers can pinpoint biomarkers indicative of antioxidant capacity, oxidative damage, or ROS production. Due to mercury's affinity to thiols and persistent ability to damage cysteine-containing proteins, we were interested in assessing mercury's effects on enzymatic antioxidant capacity, more specifically, the superoxide dismutase (SOD) group of enzymes. These three enzymes have a well-studied evolutionary history, variability in roles within the body, and integrative role in response to mercury toxicity, making them ideal for exploring the mechanisms underlying the effects of mercury (Ni et al., 2011; Zelko, Mariani, & Folz, 2002). OXS is a complex and ever changing state within and between individuals. Because the assay of SOD is focused on the activity of the enzyme only, and does not measure OXD or

ROS, we cannot be confident in making any broad conclusions about the oxidative state of the individuals under study. Instead, we focus on interpreting our data to better understand how SOD activity, and the many factors that go into its regulation (gene expression, transcription, etc.) correlate with sex, age, and mercury exposure

There are three isoforms of SOD currently identified in living organisms. Their evolutionary history has been traced back to the origins of photosynthesis by single-celled organisms, when oxygen concentrations began to increase dramatically (Zelko et al., 2002). In metazoa, the most common SOD found in the cytosol of cells is SOD1 (also referred to as Cu/Zn-SOD), which uses copper and zinc as cofactors. SOD2 is found in the mitochondrial matrix, where a majority of superoxide is produced. SOD2 is different from SOD1 and SOD3 in that it uses manganese as its cofactor. And finally, SOD3 is most commonly found in the extracellular matrix. It travels regularly through the blood plasma and the extracellular matrix of organs that experience high oxygen levels, such as the lungs and brain. The three forms of SOD vary widely in structure, although there is considerable conservation (40-60% in humans) in the structure and cofactors of SOD1 and SOD3 (Zelko et al., 2002). Genes for each of these SODs are found in different chromosomes of the human, mouse, rat, and zebra finch (*Taeniopygia guttata*). However, the expression of all three isozymes is mostly modulated by the Nuclear factor (erythroid-derived 2)-like 2 pathway (Nrf2) protein (Kumagai, Kanda, Shinkai, & Toyama, 2013; K. Wada et al., 2006).

The diversity in structure, localization, genetic promotors, transcriptional alterations, and cofactors of the three isozymes of SOD results in differing responses to toxins. In vitro studies using isolated enzymes indicate that mercury has a higher affinity for the exposed cysteine groups of SOD2 over of the more concealed cysteines of SOD1 (Kumagai, Shinyashiki, & Shimojo, 1997; Shinyashiki, Kumagai, Homma-takeda, Nagafune, & Shimojo, 1996). This affinity of mercury for SOD2 results in reduced SOD2 activity in the mitochondria of mercury-exposed cells, however, this does not correlate with decreased mRNA or total SOD2 protein content (Kumagai et al., 1997). The mercury-altered SOD2 enzymes can no longer perform superoxide dismutation, but they continue to be expressed. In the case of long-term mercury toxicity, they would continue to be modified and likely result in OXS.

There have been several conflicting reports on the response of SOD to mercury contamination. Increased expression of the SOD enzymes in response to mercury exposure has been connected to Nrf2 and its suppression mechanism, the Kelch like-ECH-associated protein 1 (Keap1) (Kumagai et al., 2013, 1997; Shinyashiki et al., 1996). At homeostasis, Keap1 binds to Nrf2 and suppresses its ability to localize in the nucleus and induce enzymatic antioxidant gene expression. Keap1 contains several cysteine residues that react with cellular stressors to release Nrf2 and allow for its nuclear localization. The fact that Keap1 uses a cysteine interaction to release Nrf2 has been suggested as an adaptive response to toxicity and

sudden spikes of OXS (Kumagai et al., 2013; Toyama et al., 2007). The activation of the Nrf2 transcription factor results in high levels of enzymatic antioxidant transcription including all three SOD proteins (Kumagai et al., 2013; Ma, 2013; Ni et al., 2011). This means that there are known mechanisms for both the decrease and increase in SOD activity in response to mercury exposure. The complexities of this mercury-enzyme interaction may result in varying responses between tissues and between individuals; more comprehensive studies are still needed. In vitro studies have also shown that cell type plays a major role on mercury-related SOD response (Ni et al., 2011). Although necessary, in vitro studies do not provide any insight into impacts on organismal fitness and survival. Furthermore, mammalian models are the focus of the in vitro work. Detailed comparisons are lacking between mammals and birds or between avian species, especially pertaining to chronic mercury exposure in contrast to acute toxicity.

Four major gaps in knowledge on the topic of SOD response to mercury-induced OXS are: (1) How do sex and age impact SOD expression and its response to mercury toxicity? (2) How do non-mammalian organisms vary in mercury-induced SOD response? For example, how does bird SOD activity respond to mercury contamination given that birds have higher metabolic rates, body temperatures, and blood glucose levels. (3) Is the response of the three SOD enzymes to mercury toxicity decoupled as suggested by in vitro studies? Will extracellular SOD3 levels correlate with intracellular SOD1 and SOD2? And finally, (4) what is the overall SOD

response of the whole organism to methylmercury exposure? Alternatively, are in vitro studies misleading because other cells in the body are compensating for SOD loss through increased SOD expression and transport? The goal of this study is to provide insight into the complexities of mercury-induced SOD response in vivo, using a model songbird species.

We focus on SOD3 with the goal of quantifying these complexities throughout development and into adulthood by using plasma in a longitudinal study as opposed to lethal tissue samples. This research does not provide a comprehensive answer to the questions above, instead, it sets a foundation for a more complete approach to research on avian SOD response to methylmercury exposure. We are interested in quantifying mercury's effects at levels that are non-lethal to our subjects. We also aim to provide insight into what wild songbirds are experiencing in response to ecologically relevant mercury levels. The insects that wild songbirds commonly prey upon have rarely been found to contain higher than 2ppm of mercury (Cristol et al., 2008; Henny, Hill, Hoffman, Spalding, & Grove, 2002; Scheuhammer et al., 2008), furthermore, these prey items are rarely the only source of food, individuals will feed on prey with varying mercury content. Therefore, we chose a high but ecologically relevant mercury content of 1.2ppm for all of our mercury exposure treatments.

Chapter 2: Tracking SOD3 throughout the lifetime of birds exposed to developmental mercury

Introduction

Most research on OXS in birds has used tissue from organs, collected after killing the subject. This method has the obvious shortcoming of making follow-up study of the same individual impossible. It has resulted in a very limited number of longitudinal studies quantifying OXS in an individual throughout its lifetime. This gap in knowledge makes it difficult to pinpoint periods of high OXD, or how other fitness-related parameters in adults may be correlated with that damage (Costantini, 2008). In addition, there are currently no studies on the effects of age and sex on plasma SOD3 levels in songbirds.

Studies using other OXS biomarkers may give us an idea of what to expect in terms of SOD3 response to age. During early development, nestlings experience high growth rates, which inherently means higher metabolism and levels of cellular respiration. Alonso-Alvarez et. al (2007) showed that higher growth rates in developing zebra finch nestlings negatively correlated with the blood's ability to withstand ROS attack, a proxy for antioxidant capacity. This suggests that younger birds should have lower levels of enzymatic antioxidants.

Mammalian case studies provide conflicting reports about the effects of age on SOD3. Activity levels of SOD3 in the lung of developing rabbits showed a continuous increase throughout development and stabilized into

adulthood (Nozik-Grayck, Dieterle, Piantadosi, Enghild, & Oury, 2000). Similarly, SOD3 mRNA levels in the lungs of mice increased immediately after birth and stabilized once sexual maturity was reached (Zelko et al., 2002). Furthermore, rat testicle SOD3 activity showed a spike just before sexual maturation, indicating SOD3 helps the gonads deal with the high oxidative cost of gamete production (Mruk et al., 1998). However, in humans, which have a longer development phase before sexual maturity, plasma SOD3 levels were higher in pre-pubescent children than adults (Adachi, Wang, & Wang, 2000).

Ours is the first study to examine SOD3 levels in a songbird throughout development and into adulthood. Studies on gender differences in SOD3 levels are lacking in songbirds, but mammalian models indicate that estrogen may play a pivotal role in antioxidant regulation. In fact, estrogen itself has antioxidant-like behavior, as demonstrated in neuroblastoma cells (Behl, Widmann, Trapp, & Holsboer, 1995). Furthermore, there is evidence of estrogen modulating gene expression of enzymatic antioxidants, including the SOD family of enzymes (mostly SOD2 in the mitochondria) (Olivieri et al., 2002; Vahter, Åkesson, Lidén, Ceccatelli, & Berglund, 2007). Although these are mammalian models, estrogen's role as an antioxidant modulator is well documented within the nervous system, possibly the site of mercury's most impactful damage (Nuran Ercal et al., 2001). The involvement of estrogen suggests that males and females may have different baseline levels of SOD,

however, support for this difference is lacking in birds and in SOD3, specifically.

Extensive research has been done to analyze the oxidative cost of reproduction. Captive studies on zebra finches have shown that females have a reduced capacity to withstand the attack of ROS when compared to males (Alonso-Álvarez et al., 2006). Researchers suggest that the cost of egg production, starvation during incubation, and higher workload during the nestling phase, are all reasons for females to incur higher OXS and OXD than males. In a brood size manipulation study, males and females with a larger brood size had 24% lower SOD activity (corrected for estimated energy expenditure) in the muscle than those with a small brood size. This study also indicated that females had higher SOD activity than males along with higher energy expenditure during the breeding effort. Whether the higher SOD activity is an adaptive response to the female-related costs of breeding is unclear, but sexual differences in SOD response have been documented in this species and require more investigation (Wiersma, Selman, Speakman, & Verhulst, 2004)..

Although we expect marked differences in SOD3 according to age and sex, little is known about how mercury might impact SOD3 activity or interact with age and sex effects. Our study is a part of a long-term analysis of the effects of mercury on bird fitness, including a test of the developmental stress hypothesis. The developmental stress hypothesis is based upon the assumption that adult fitness parameters are honest reflections of

developmental rearing conditions (Nowicki, Peters, & Podos, 1998). According to this hypothesis, early stressors, such as dietary restrictions or exposure to toxins, damage the development of fitness-related traits (Hoogenboom et al., 2012; Kennedy et al., 2005; Nowicki et al., 1998). Song learning is a thoroughly studied example of developmental stress. Buchanan and his team showed significant reductions in adult song motif duration and number of syllables per motif (two common ways to measure song complexity and attractiveness to mates) in zebra finches exposed to dietary restrictions and high corticosterone levels during development (Buchanan, Spencer, Goldsmith, & Catchpole, 2003). Studies have also applied this hypothesis to OXS and OXD during nestling development and shown that several OXS biomarkers work as predictors of fledging success, short-term survival, and even recruitment into the population as an adult (Losdat et al., 2013; Mangel & Munch, 2005; Noguera, Kim, & Velando, 2012). However, no such study exists with captive mercury-dosed populations. Therefore, finding correlations between mercury-induced OXS during different stages of development would have implications for the interpretation of early stress effects on adult parameters.

The purpose of this study was to measure the interactions of age, sex, and a developmental stressor (mercury) on SOD3 activity in the plasma of a model songbird, the zebra finch. A better understanding of SOD3 activity in the developing zebra finch may provide insight into how age and sex correlate

with OXS, and set the foundation for a future comprehensive assessment of OXS in a model songbird.

Methods

Subjects

We used an outbred colony of domesticated zebra finches maintained at The College of William and Mary. Birds were housed in cages (0.46 m width x 0.75 m length x 0.46 m height) where food, vitamin-enriched water, cuttlefish calcium supplement and grit were provided *ad libitum*. Rooms were maintained at an average temperature of 22°C and a 14:10 light:dark photoperiod. Only two dietary conditions were used in this study: control (0ppm control) or mercury (1.2ppm mercury). To prepare the mercury diet, commercially available finch food (ZuPreem®, Shawnee, KS) was mixed thoroughly in a rock tumbler with methylmercury (MeHg-cysteine) dissolved in water. Control food was mixed with cysteine and water at the same concentration as in the mercury diet. Individuals received the same diet throughout their entire lifetime and their parents had received the same diet; therefore, mercury exposure began *in ovo*.

To create a 2 x 2 cross-fostering study design, birds were paired and assigned to one of two categories: breeding pairs or foster pairs. When breeding pairs completed their clutch (stopped laying new eggs for several days), the eggs were moved to a foster pair of the same diet. The foster parents raised the offspring until 50 days post hatching (DPH). At 50 DPH,

the offspring were removed from the parental cage and placed in a single sex cage. At this point the offspring were either switched to the other dietary treatment, or maintained on the same dietary treatment for another 50 days, depending on treatment. At 100 DPH, all offspring were placed on control diets for the rest of their lives. This design created four kinds of developmental mercury-exposure treatments, denoted in the format "treatment at 0 to 50 DPH-treatment at 51 to 100 DPH": control-control, control-Hg, Hg-control, and Hg-Hg.

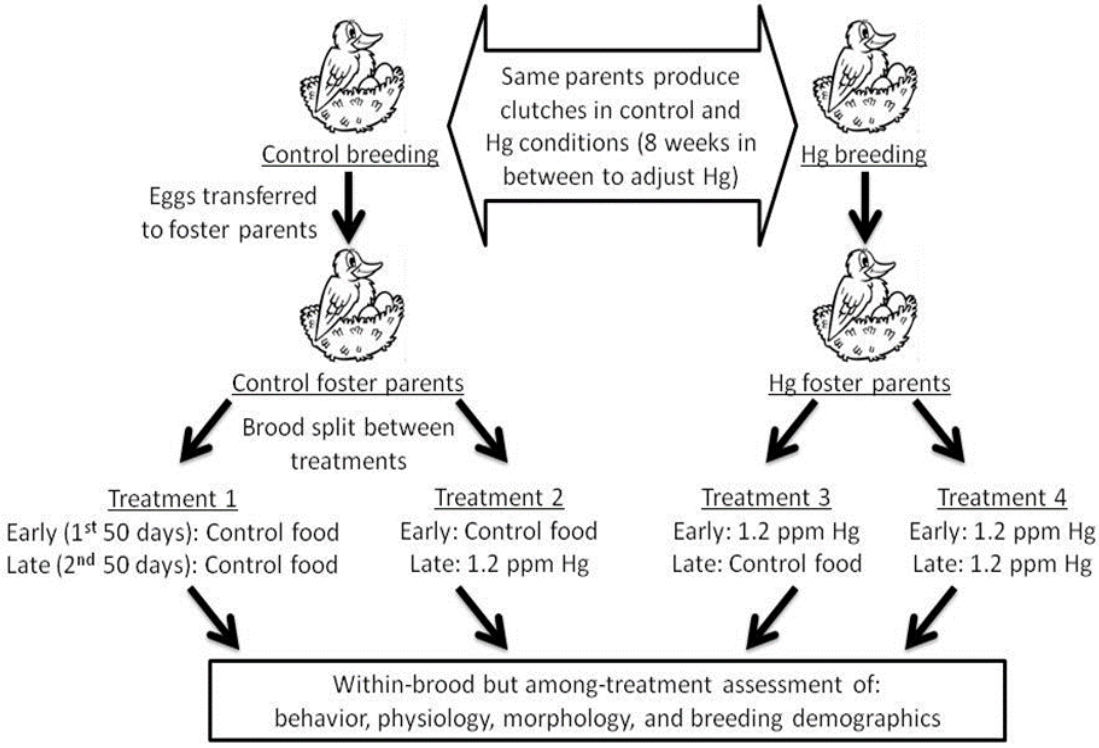


Figure 1. Schematic of developmental exposure design

Sample Collection

Husbandry and sample collection procedures were approved by The College of William and Mary's Institutional Animal Care and Use Committee

(2013-06-02-8721-dacris). Individuals were manually captured from their cages and a blood sample was collected by needle puncture of the brachial vein. Blood was drawn into two capillary tubes within 5 minutes of capture. One tube was used for mercury analysis and the other was centrifuged 13,000xg for 10 minutes to separate plasma and red blood cells. On average, 30µl of plasma were isolated per bird and used for SOD quantification. Blood samples were collected at 50 DPH, 100 DPH, and 350-400 DPH.

Mercury Analysis

Total mercury content in the blood was quantified using a Direct Mercury Analyzer 80 (Milestone, Monroe, CT). Since >90% of mercury in avian blood is MeHg (Wada et al., 2009), these total mercury values are representative of MeHg content. The mercury analyzer uses combustion-amalgamation cold vapor atomic absorption spectrophotometry per U.S. Environmental Protection Agency method 7473. A pair of duplicates was included every twenty samples along with blanks and two samples of standard reference materials (tuna and DORM-4, National Research Council of Canada, Ottawa, Ontario). Mercury levels in each batch of food were quantified using the same procedures, to assure no mercury contamination occurred in the control food and to maintain the 1.2ppm MeHg concentration within 10% of the nominal dose. Mean percent recoveries of THg for the tuna and DORM-4 were 103.09 ± 6.17% (n = 16) and 101.944 ± 1.57% (n = 16) respectively. All THg concentrations are reported on a wet weight basis ± SD.

Control birds had mean blood mercury concentration of 0.1031 ± 0.16 µg/g (range <0.01-0.72µg/g) and mercury-exposed birds averaged 19.25 ± 5.81 µg/g (Table 1; range 12.13192-38.49 µg/g).

SOD Assay

Enzymatic antioxidant capacity was calculated using the Superoxide Dismutase Assay Kit (Item No. 706002) purchased from Cayman Chemical Company (Ann Arbor, MI). This kit utilizes the catalytic reaction between the xanthine oxidase enzyme and a tetrazolium salt to produce large amounts of superoxide. Using bovine SOD, a standard curve allows us to quantify the amount of SOD units needed to exhibit 50% dismutation of superoxide per sample. Plasma samples are expressed as units/ml.

Plasma samples were thawed out individually at room temperature and diluted in a 1:5 ratio (or a dilution factor of 6) using sample buffer (706003). The diluted samples were placed on ice until the reaction was ready to begin. Standards were prepared using a provided bovine erythrocyte SOD (Cu/Zn) stock (706005) and diluted to cover a range of 0.005 to 0.05 U/ml. Standards were diluted in glass tubes using sample buffer, mixed thoroughly, and then added in duplicate to the corresponding wells. First, 10 µl of sample was added in triplicate according to a predetermined plate setup. Then, 200 µl of radical detector (706004) was diluted using assay buffer (706002) and was added to each well using a multipipettor. Finally, the reaction was started by adding 20 µl of diluted xanthine oxidase (706006) to each well except for the

third replicate of each sample, always columns 5, 8 and 11 of the plate. All of the xanthine oxidase was added within a minute and a timer was started upon first addition. Columns 5, 8 and 11 received 20 µl of sample buffer instead of xanthine oxidase; to calculate background absorbance from each sample. The plate was incubated on a plate shaker (Lab-line instruments inc.; Melrose Park, IL) and absorbance was read at 450nm (Multiskan FC, Fisher Scientific; Asheville, NC) exactly 30 minutes after addition of xanthine oxidase to column 1. A pool of plasma samples from 20 zebra finches was created to assess inter- and intra-assay repeatability.

To create a pool of birds that would approximate the mean of our eventual samples, 10 subjects were forced to fly back and forth for four hours and 10 were held in the same room without exercise. Plasma was pooled into one 15ml tube, thoroughly vortexed, quickly aliquoted into individual 600 µl tubes, and then snap frozen in liquid nitrogen. Six triplicates from this pool were assayed within each plasma SOD assay and calculations were performed as with any other sample.

SOD Calculations

Sample absorbance was calculated by subtracting sample background absorbance from the average absorbance of the two duplicates. The absorbance value of standard A, which contained no SOD, was then divided by the sample and standard absorbance to produce a linearized absorbance value for each bird sample and standard. This linearized standard value was

used to calculate a standard curve with known SOD U/ml on the x-axis and linearized rate on the y-axis. A linear regression was used to calculate a slope and intercept for each assay. Sample U/ml were calculated using the linearized rate for each sample, subtracting the calculated intercept, dividing by the calculated slope, multiplying by (0.23/0.01) and finally multiplying by the sample dilution factor. Sample dilution factor for all plasma samples was 6.

Statistical Analysis

We performed statistical analyses using both R and IBM SPSS Statistics v23. Three general linear models were developed for plasma SOD3 activity at each of the three ages sampled using type III sums of squares. The response variables were not normally distributed for any of the three ages when each was analyzed individually. We transformed these variables using the natural log in order to meet the assumption of normal distribution. All three models included sex and dietary treatment as fixed effects, and the interactive effects were included as well. The 50 DPH model only had two levels for diet, but the other two ages had four levels due to the design of the treatments. Average SOD activity values were calculated to perform comparisons within the factors of interest. 95% confidence intervals were reported and graphically depicted for all means.

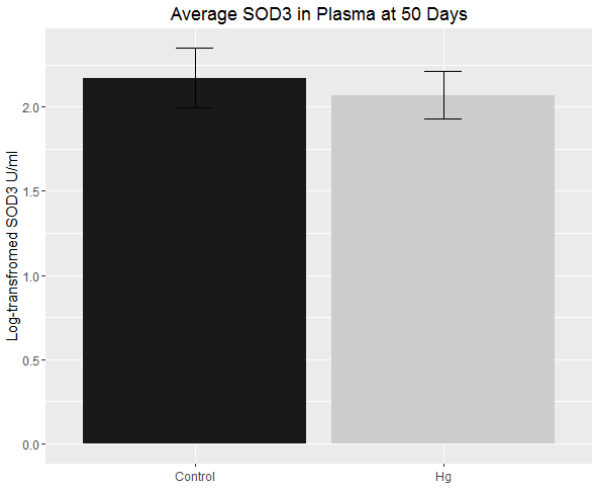
A repeated measures ANOVA was used to analyze the within-subjects effect of age on 17 individuals that were tracked throughout their lifetime.

Each individual was sampled for plasma SOD3 activity at three time points. The response variables met the assumption of sphericity, so no transformation was required for this analysis.

Results

50 DPH

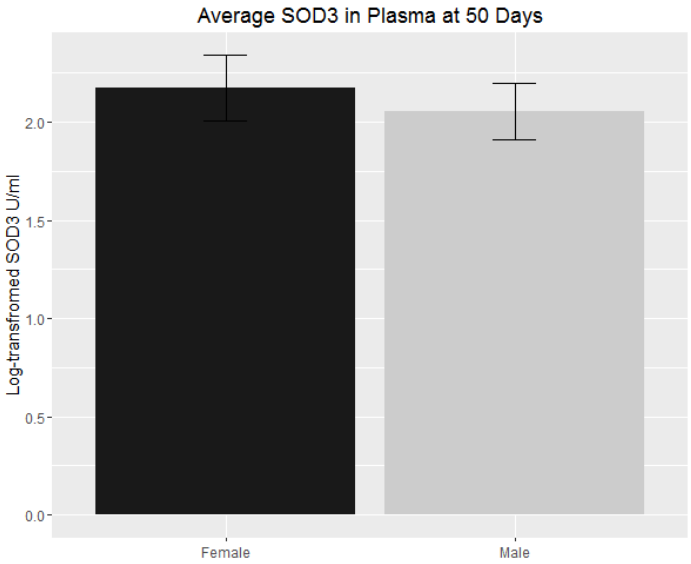
35 individuals were sampled for plasma SOD3 activity during the first 50 days of life. All birds were sampled during the 50 DPH treatment switch (the actual age at time of sampling ranged from 48 to 52 DPH). Of the 35 birds, 16 were on control diet and 19 on mercury diet. Control birds showed a slightly higher log-transformed SOD3 activity level (mean= 2.17 U/ml, SD= 0.355) in the plasma than mercury-exposed birds (mean= 2.07 U/ml, SD= 0.306), although the effect of diet was not significant ($F_{1,34} = 1.010$, $p = 0.323$).



Diet	Mean SOD3 ±CI (U/ml)	n
Control	2.17 ± 0.177	16
Hg (1.2ppm)	2.07 ±0.141	19

Figure 2: Average log-transformed SOD3 activity levels in the plasma of 50 DPH birds according to diet with 95% confidence intervals.

A similar, non-significant ($F_{1,34}$ = 1.358, p= 0.253) relationship was identified between the two sexes at 50 DPH. The 18 females had a slightly higher average (mean= 2.17 U/ml, SD= 0.353) than the 17 males (mean= 2.06 U/ml, SD= 0.299). There was no significant interaction between sex and dietary treatment ($F_{1,34}$= 0.060, p= 0.808).



Sex	Mean SOD3 ± 95%CI	n
Female	2.17 ± 0.166	18
Male	2.06 ± 0.145	17

Figure 3: Average log-transformed SOD3 activity levels in the plasma of 50 DPH birds according to sex with 95% confidence intervals.

100 DPH

The actual age at sampling for the 100 DPH group ranged from 98 DPH to 102 DPH. In total, 35 birds were sampled (4 in the control-control treatment, 8 in the control-Hg treatment, 12 in Hg-control, and 11 in Hg-Hg). The linear model for SOD3 activity at this age showed no significant effects of

sex ($F_{1,27}$= 0.246, p= 0.624), diet ($F_{1,27}$= 0.307, p= 0.307), or the interaction of the two ($F_{1,27}$= 0.453, p= 0.717). The control-control treatment had the highest SOD3 activity level (mean= 2.61 U/ml, SD= 0.332) with the Hg-Hg treatment having the lowest (mean= 2.15 U/ml, SD= 0.423).

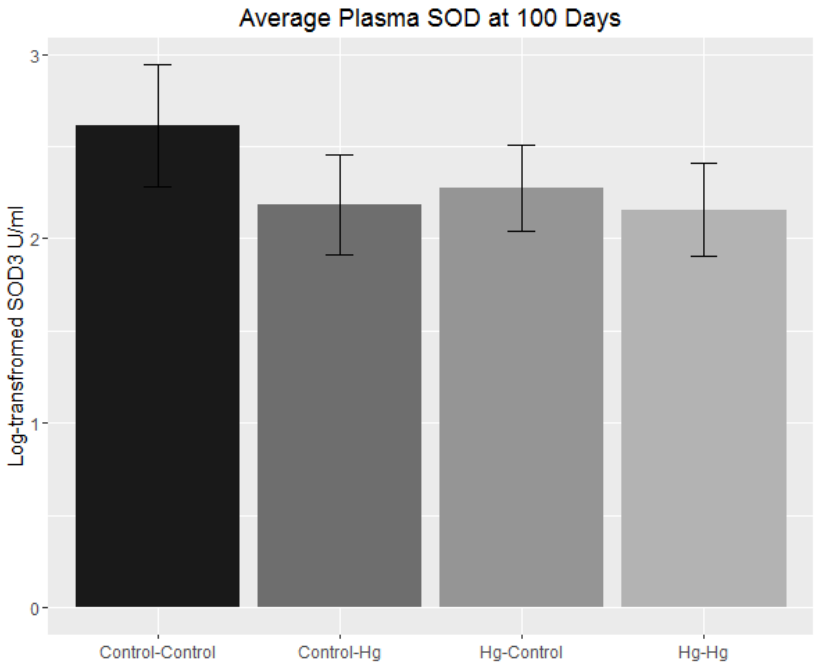


Figure 4: Average log-transformed SOD3 activity level in the plasma of birds sampled at 100 DPH according to diet treatment and 95% confidence intervals.

350-400 DPH

To get adult SOD3 activity levels in plasma, we sampled 34 individuals from 350-400 DPH. Of the birds sampled, 5 were in the control-control dietary treatment group, 8 in Control-Hg, 10 in Hg-control, and 11 in Hg-Hg. A

linear model analysis again showed no effect of sex ($F_{1,26}$= 0.942, P= 0.341), diet ($F_{1,26}$=0.889, P= 0.460), or their interaction ($F_{1,26}$= 0.906, P= 0.451).

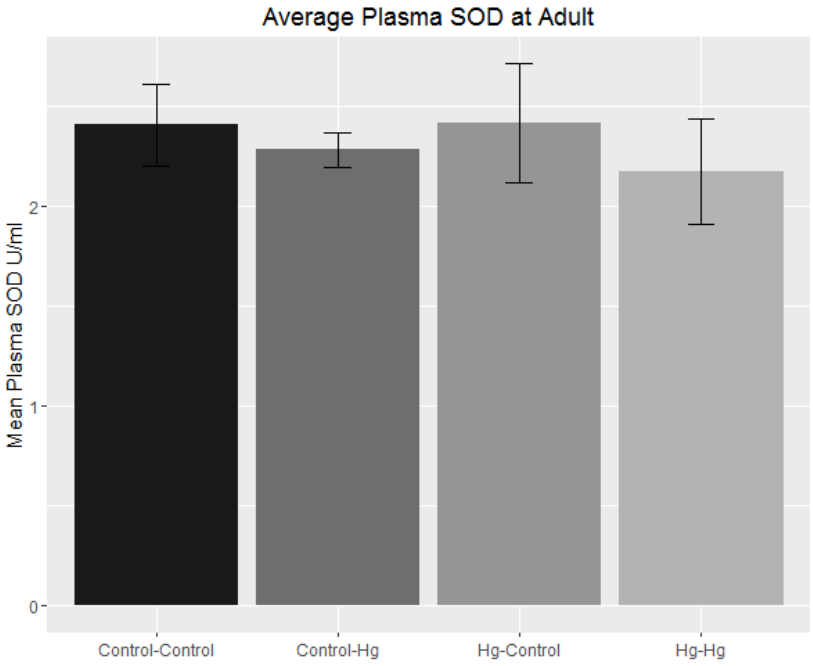


Figure 5: Average log-transformed SOD3 activity level in the plasma of birds sampled at 350-400 DPH according to diet treatment and 95% confidence intervals.

Repeated Measures Analysis

To analyze the effect of age on SOD3 activity, we tracked 17 individuals from birth to 400 DPH. Having sampled each individual three times, a repeated measures ANOVA was appropriate. The sphericity assumption was met, so there was no need to transform these data (Maulchy's test: W_2= 0.967, p= 0.876). Consistent with our analysis of the larger sample at each separate age, dietary treatment did not significantly

impact plasma SOD3 activity level ($F_{6,18}$= 4.709, p= 0.354) among individuals studied longitudinally. However, age was a significant predictor of SOD3 activity ($F_{2,18}$= 12.402, p< 0.001) in the blood with birds at 50 DPH exhibiting the lowest activity level (mean= 7.75 U/ml, SD= 2.699) when compared to the 100 DPH (mean= 10.34 U/ml, SD= 3.724) and 350-400 DPH (mean= 11.27 U/ml, SD=3.982) ages. Sex had a significant interaction with age ($F_{2,18}$= 3.847, p= 0.041). Females had a higher level of SOD3 activity than males at all three ages sampled with the largest difference found at 350-400 DPH when females had a mean value of 13.65 U/ml (SD=4.082) and males had 9.14 U/ml on average (SD= 2.524). A statistically significant three-way interaction was also detected between age, sex, and dietary treatment ($F_{2,18}$= 2.967, p= 0.0331).

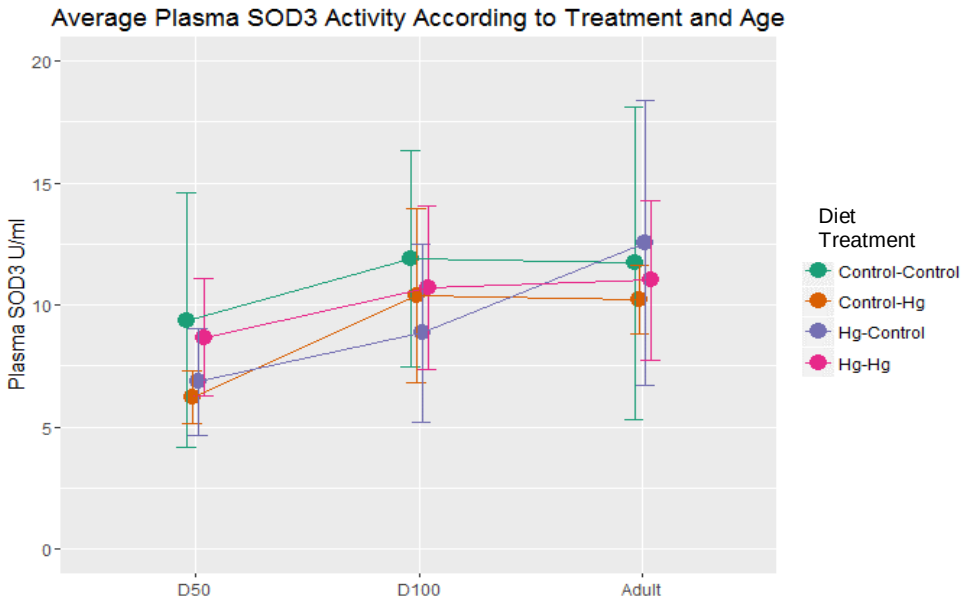


Figure 6: Average SOD3 activity, and corresponding 95% confidence intervals, in the plasma of 17 birds sampled at three different ages according to diet treatment.

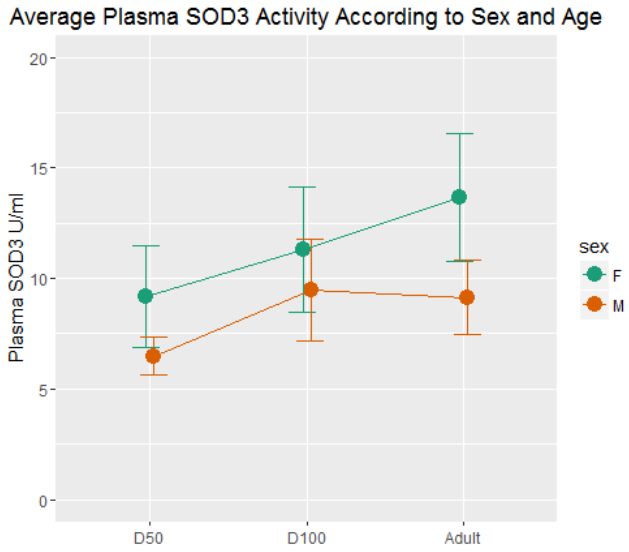


Figure 7: Average SOD3 activity, and corresponding 95% confidence intervals, in the plasma of 17 birds sampled at three different ages according to Sex.

Discussion

This study was designed to clarify the role of developmental mercury exposure, age, and sex on the activity levels of plasma SOD3. Interestingly, mercury-exposed birds did not show any differences from controls at 50 DPH or at 100 DPH. Developmental exposure to mercury for the first 50, second 50 or first 100 days also did not alter adult (350-400 DPH) SOD3 activity levels when all individuals had been off of mercury for many months. Finally, the repeated measures analysis of the same 17 birds through three ages (50 and 100 DPH and adult) showed no suggestion of an effect of mercury when compared to controls, although only 2 of the 17 birds were true controls that were never exposed to mercury. This admittedly low sample size of control

individuals likely decreased our power to detect differences between diets in the repeated measures analysis. However, sample sizes were much larger for the analyses done at each age, and no diet effects were ever detected there either. We conclude that developmental mercury is not altering plasma SOD3 activity in songbirds at this non-lethal and ecologically relevant level of exposure, a novel finding that could have implications for OXS and mercury toxicity research. Although our study was not designed to detect effects of higher mercury exposure, a higher dose might have much more drastic effects.

We predicted an overall depletion of plasma SOD3 activity in response to mercury exposure, based on previous findings that suggest mercury-cysteine interactions inhibit enzymatic activity (Henry et al., 2014; Nuran Ercal et al., 2001; Stohs & Bagchi, 1995). However, none of these previous studies have demonstrated the specific response of plasma SOD3 to mercury in songbirds. The most similar study was that of Henry et al (2014) using zebra finches on the same dose as the current study. But that study examined liver SOD, which is a combination of SOD1 and SOD2. Although we were surprised by the lack of relationship between mercury and SOD3, there are several mechanisms that could be responsible. In vitro studies have found extensive support for mercury's role in decoupling the electron transport chain and causing high ROS concentrations within the cell (Nuran Ercal et al., 2001). This includes an increase in superoxide radicals, which are the main substrate for SOD activity. A spike in the concentration of these molecules

could result in an overall depletion of antioxidants due to substrate overload and enzyme degradation. Alternatively, an adaptive response to toxic levels of ROS might induce enzymatic antioxidant gene expression and an increase in overall antioxidants including the SOD family (Stringari et al., 2010). These are two mechanisms known to occur in response to increased ROS production that would have opposite effects on SOD activity levels. Although our study was not designed to pinpoint which mechanism is driving a change, it is important to consider that a lack of effect of mercury on SOD activity may be explained by opposing response mechanisms.

The major enzymatic antioxidant transcription factor Nrf2, plays a protective role in response to mercury (Kumagai et al., 2013; Ni et al., 2011; Toyama et al., 2007). Nrf2 activity in the nucleus is modulated by its suppressor, Keap1, a protein that physically attaches to Nrf2 via cysteine-residues. Studies have shown that mercury interacts with these cysteines to separate the two proteins and induce antioxidant gene expression. We would therefore expect an increase in all antioxidants in response to dietary mercury exposure. In contrast, a majority of studies on mercury-induced OXS shows that mercury is more likely to deplete antioxidant content and activity (Custer et al., 1997; Henry et al., 2014; Hoffman et al., 2011; Nuran Ercal et al., 2001). It may be that the pathways between Nrf2 activation and the final modifications to SOD3 are responsible for the lack of response in SOD3 activity levels to mercury exposure.

The primary characteristic that differentiates SOD3 from other enzymatic antioxidants is its extracellular localization (the same characteristic that makes it a potential candidate for non-lethal OXS quantification through blood sampling). There is no evidence that the presence of mercury influences post-transcriptional modifications of SOD3 any differently than it would affect other enzymatic antioxidants, including SOD1 and SOD2. However, the cells that produce the highest amounts of SOD3 for the body are fewer than other enzymatic antioxidants. SOD3 plays a pivotal role as an extracellular antioxidant and can protect organs that experience high levels of oxygen transport like the lungs. Therefore, there is no need for all cells to express the SOD3 gene at the same rate as intracellular antioxidants (Zelko et al., 2002). Mammalian models indicate that the cells which produce the most SOD3 are alveolar cells, renal tubular cells, vascular smooth muscle cells, and lung macrophages (Folz et al., 1997; Loenders et al., 1998; Strålin, Karlsson, Johansson, & Marklund, 1995; Zelko et al., 2002). One study in particular ranked human organs according to SOD3 RNA levels and found that the heart had the highest level of expression followed by the placenta, pancreas, lung, kidney, skeletal muscle and finally the liver (Folz & Crapo, 1994). SOD1 and SOD2 have much more uniform expression in the tissues of mammals. In the liver in particular, SOD1 and SOD2 are continually produced due to the high level of metabolism requiring exceptional ROS protection (Zelko et al., 2002). Since the synthesis of SOD3 is highly localized to certain cell types in mammals, we might expect similar specificity in birds. Mercury

has also been shown to vary in concentration between cell types and organs (Whitney, 2014). It is possible that mercury is not found in high enough concentrations in SOD3-producing cells to impact its gene expression, but the mercury content of alveolar cells, lung macrophages, and renal tubular cells has never been reported, to our knowledge.

Post-transcriptional factors may also be to blame for the lack of relationship between mercury and plasma SOD3. Using mammalian models, Kumangai and his team (1997) isolated SOD enzymes according to their cofactor, producing both Mn-SOD (SOD2) and Cu/Zn-SOD (could include SOD3 but does not specify, likely isolated from SOD1). Each isozyme was then incubated with methylmercury and a stark difference appeared. After 24 hours, both Mn-SOD and Cu/Zn-SOD activity levels decreased significantly. However, Mn-SOD activity decreased by 60% while Cu/Zn-SOD only fell by 30%. These results suggest that mercury can have a direct impact on SOD activity and has a higher affinity for SOD2 than other SOD types.

The suggested mechanism for this differentiation in activity reduction involves the location of the cysteine groups of SOD2. Detailed analyses of the SOD2 structure show that the sulfhydryl groups of mammalian SOD2 cysteines are more exposed at the subunit surface than that of SOD1 and SOD3 (Kumagai et al., 1997). A more exposed cysteine group is more susceptible to mercury-caused denaturing, making SOD2 (Mn-SOD) more likely to be damaged by mercury exposure than the other SOD isozymes (Kumagai et al., 1997). This would explain why previous studies have

detected impacts of mercury on SOD activity in liver tissue, which includes SOD1 and SOD2 (Henry et al., 2014), while we did not detect the same effects in SOD3. Many more experiments are required to justify the suggested mechanisms for the discrepancy in the relationship between mercury and SOD isozymes; we suggest further analysis of gene expression in birds since we are forced to base our analysis on mammalian models. Also, exposure treatments with continuous increases in mercury levels may help to detect the point at which plasma SOD3 activity is significantly impacted. Our results suggest that at 1.2 ppm, zebra finches are able to compensate for the effects of mercury on plasma SOD3. How this trend continues at higher exposure treatments is unclear and requires further study.

Age was found to have a significant impact on activity levels of SOD3 in plasma as shown by a repeated measures model that included 17 individuals sampled at three ages. The youngest birds, 50 DPH, showed the lowest overall level of SOD3 activity, with a dramatic increase by 100 DPH and a further minimal increase by 350-400 DPH. This is the first study to show that activity levels of plasma SOD3 vary according to age in a songbird, although similar studies have shown this trend in mammals of similar size. Gradual increases in SOD3 during development tend to level out once sexual maturity is reached. These trends have been observed both in mice and rabbits (Nozik-Grayck et al., 2000; Zelko et al., 2002). Both of these studies pinpointed another important time point in SOD3 production, birth. A preemptive increase in SOD3 was observed in mammalian fetuses right

before birth to assist in dealing with the spike in ROS production that comes with the first breaths of oxygen outside of the womb. Aside from the obvious biochemical differences between oviparous and viviparous development, circulatory and respiratory systems also differ significantly and play a major role in SOD3 activity (Zelko et al., 2002). Comparative studies are imperative to understanding the role of OXS in dictating longevity and clarifying the reasons that songbirds are capable of living longer than their mammalian counterparts.

Antioxidant levels have commonly been shown to be reduced in younger songbirds compared to their adult counterparts (Alonso-Álvarez, Pérez-Rodríguez, García, Viñuela, & Mateo, 2010; Metcalfe & Alonso-Álvarez, 2010a; Noguera et al., 2012; Romero-Haro & Alonso-Álvarez, 2014). Although it is difficult to define the ultimate cause of this age-related difference, it is likely due to the increased metabolic cost that younger birds experience as they grow in size (Costantini, 2008). Our findings are consistent with that of most enzymatic antioxidant analyses done on birds, but opposite to what is found in humans, where SOD3 decreases as age increases (Adachi et al., 2000). This highlights the need for further research on the role of age and sex in dictating antioxidant activity levels.

Age was also shown to have a significant interaction with sex in our repeated measures analysis. Females consistently exhibited higher activity levels of SOD3 in the plasma at all three ages analyzed, with the largest difference being between the means of 350-400 DPH birds. When dealing

with sex effects, the most common explanation of differences in OXS biomarkers is breeding effort. It is crucial to highlight that our birds were always placed in single sex cages and were never allowed to breed. However, the two sexes were housed in the same room so there were visual and auditory interactions between the sexes. Furthermore, the housing conditions that we used, 14 hours of daylight and 10 hours of darkness, are commonly chosen to stimulate breeding efforts. Some females laid unfertilized eggs but these were never tracked per individual. The sex difference detected is therefore not attributable to breeding effort itself, but may indicate pre-emptive variation in preparation for a breeding effort.

Activity levels of SOD have been shown to differ between the sexes of rats. This correlation is mainly attributed to estrogen's antioxidant properties and role in gene expression (Behl et al., 1995; Olivieri et al., 2002). This is the first indication that plasma SOD3 activity levels differ in a similar way in songbirds. Studies repeatedly show that females incur a heavier workload, and thus more oxidative damage than males do during a breeding effort (Isaksson, Sheldon, & Uller, 2011; Linden & Moller, 1989). Therefore, it would be reasonable for females to have adapted defensive mechanisms to deal with these higher workloads. Interestingly, our other experiment showed the opposite trend with males having higher levels of SOD3 activity in the plasma than females. We discuss these discrepancies in more detail in chapter 3.

Ch3: Assessing the relationship between SOD3 in plasma and SOD1/SOD2 in the liver with birds experiencing exercise and lifetime mercury exposure

Introduction

Mercury increases ROS and decreases antioxidants in birds, as demonstrated by field and laboratory analyses (Henry et al., 2014; Hoffman et al., 2011, 2005; Hoffman & Heinz, 1998; Kenow et al., 2008). A common finding is the depletion of the non-enzymatic antioxidant glutathione (GSH). Levels of this important molecule have been shown to negatively correlate with blood mercury levels in ducks, terns and herons (Custer et al., 1997; Hoffman et al., 2011, 1998). Similarly, assays for higher levels of OXD such as TBARS and protein carbonyls positively correlate with increased mercury levels in gulls, owls and loons, among others (Espín, Martínez-López, León-Ortega, Martínez, & García-Fernández, 2014; Jenko, Karouna-Renier, & Hoffman, 2012; Kenow et al., 2008). However, all of these studies used liver samples to show changes in oxidative state of the organ. Similar studies on blood are much less frequent. Blood levels of the enzymatic antioxidant glutathione peroxidase decreased with increasing blood mercury in egrets fed an unnaturally high diet of 5ppm methylmercury (Hoffman et al., 2005). That study also found decreased albumin and total protein levels in the blood of these highly-dosed birds. Recently, Henry et al. (2014) found a relationship between mercury and OXS in a model songbird species, the zebra finch. By sampling liver tissue, they showed a decrease in GSH/GSSG ratio and

combined SOD1 and SOD2 activity with an increase in circulating blood mercury. These results showed that long-term mercury-exposed birds experience significant increases in OXS, however, because liver tissue was used, this study only indicates OXS levels at the time of the bird's death. There is little doubt that mercury induces OXS in birds, however, much more research is needed to understand which antioxidants are being affected, how OXS may be induced differently throughout the body, and how antioxidant levels in different organs correlate.

Another stressor that has been tied to inducing OXS is exercise. Exercise causes increased metabolism and thus an increase in ROS production. Healthy individuals have antioxidant levels that can cope with these increased ROS attacks, but when exercise is prolonged or another stressor is added, OXS is induced (Powers & Jackson, 2008). Jenni-Eiermann and colleagues (2014) found increased OXD and decreased enzymatic antioxidants in the blood of robins during migration. A comparison between short (20km) and long (60km) flights in homing pigeons, showed significantly higher OXS biomarkers in the blood of birds in the longer exercise treatment. Interestingly, older birds in that study were also more likely to show OXS-related biomarkers (Costantini, Dell'ariccia, & Lipp, 2008). In the lab, flight induction through an automated system was shown to increase oxidative damage and deplete thiol levels in the blood of zebra finches (Costantini, Mirzai, & Metcalfe, 2012). There seems to be enough evidence to suggest that extensive exercise induces OXS, However, the way

in which this OXS affects SOD3 in plasma has not been studied in birds. Further, the way in which exercise might interact with any effects of mercury to induce higher levels of OXS is unknown (Costantini et al., 2008, 2012; Jenni-Eiermann, Jenni, Smith, & Costantini, 2014). Mammalian models indicate that SOD3 gene expression as well as activity levels overall are upregulated in response to acute exercise (Hitomi et al., 2008).

Given the role of sex hormones (estrogen in particular) in modulating antioxidant behavior, it is crucial to consider that males and females may have different baseline levels of antioxidants. Support for sexual differences in baseline antioxidant levels are lacking in songbirds and in SOD3 in particular. Female rats showed higher levels of total SOD activity in macrophages than males of normal healthy individuals. When their ovaries were removed, the female SOD activity level dropped dramatically and resembled that of males. This effect was fully reversed by estrogen administration (Azevedo, Miyasaka, Chaves, & Curi, 2001). Given estrogen's role in antioxidant regulation and mammalian evidence for higher baseline antioxidant capacity, we suspect that sexual differences in SOD activity may arise in response to exercise. In chapter 2, we found that females consistently had increased levels of SOD. Here, we question how those levels may differ in response to exercise and mercury.

The concept of a dependable non-lethal biomarker to indicate mercury-induced OXS is something that researchers should be aiming for because it would provide the inherent benefit of keeping birds alive, allow for longitudinal

studies, and provide new ways to analyze the extent of mercury's impact on ecosystems. We know bird populations are declining across the world, and like the increases in mercury in the environment, the cause is mostly anthropogenic. As we move toward mitigating these effects, our goal should be to remain efficient in assessing mercury's impact and to minimize further damage during remediation efforts. Typically, when dealing with point-source mercury contamination, field researchers quantify mercury levels in nearby populations to identify which species are at risk from mercury exposure. Although information on mercury concentrations is useful, it does not provide any insight into how those mercury levels are actually impacting each species. A plasma biomarker for OXS may provide an opportunity to take that next step and identify which species are at greatest risk of fitness reductions. This would provide conservationists with more clarity on which species need relief and which ones may have adapted to deal with the high mercury levels. In order to do so, it is not ideal to euthanize wild birds in order to get an idea of their oxidative state. Instead, non-lethal assays should be further developed to work as dependable proxies for quantifying OXS in the whole individual.

The purpose of this study is to use exercise and mercury as known positive control inducers of OXS to assess the viability of SOD3 as a possible non-lethal biomarker of mercury-induced OXS. We aim to do this by using both liver and plasma samples to show their relationship within individuals. Furthermore, we hope to shed some light on the possible interaction between

mercury and exercise in inducing OXS in a model research species. Results will add to the basic understanding of OXS as it relates to these two specific stressors, and hopefully motivate the development of more non-lethal OXS biomarkers for applied research.

Methods

Subjects

Tissue samples were collected from an outbred colony of domesticated zebra finches maintained at The College of William and Mary. Birds were housed in single sex cages (0.46 m width x 0.75 m length x 0.46 m height) with no more than 4 individuals per cage. Food, vitamin-enriched water, cuttlefish calcium supplement and grit were provided *ad libitum* for each cage. Rooms were maintained at an average temperature of 22°C and a 14:10 light:dark photoperiod. Individuals were exposed to one of two diets: control (0ppm control) or mercury (1.2ppm mercury). To prepare the mercury diet, commercially available finch food (ZuPreem®, Shawnee, KS) was mixed thoroughly in a rock tumbler with methylmercury-cysteine dissolved in water. Control food was mixed with cysteine and water at the same concentration as in the mercury diet. Individuals received the same diet throughout their entire lifetime and their parents had received the same diet; therefore, mercury exposure began *in ovo*.

Exercise/No-Exercise regimen on adults

Adult birds were selected from the captive colony to create a factorial design experiment between the two factors of interest: diet and exercise. This design resulted in four final treatments: control+exercise, control+no-exercise, mercury+exercise, and mercury+no-exercise. When assigning individuals to each treatment group, we attempted to match for age, sex, and blood-Hg levels. Birds with a mercury diet had an average age of 489.2 DPH while the control-diet group had an average age of 471.8 DPH. If a bird could not complete the flight requirements it was removed from the study. A total of 64 individuals were selected to be a part of the study, however, only 56 completed the exercise/no-exercise regimen.

Diet	Sex	Blood Hg Level ± SE (mg/kg)	Exercise Treatment	n	Mean Age ± SE (DPH)
Mercury	Male	17.42307 ± 1.1298	Exercise	8	
			No-Exercise	6	
	Female	19.87691 ± 1.7878	Exercise	8	
			No-Exercise	7	
	Total	**18.6923 ± 1.0800**		**29**	**489.19 ± 22.81**
Control	Male	0.09747 ± 0.0500	Exercise	7	
			No-Exercise	7	
	Female	0.06144 ± 0.0105	Exercise	7	
			No-Exercise	6	
	Total	**0.08013 ± 0.0262**		**27**	**471.75 ± 25.69**

Table 1. Number of individuals that completed exercise regimen for each treatment group.

Two corridors (3.25m in length and 1.5m wide) were set up along opposite walls of an aviary room (4.5m X 3.25m). Three walls were part of the aviary room and plastic sheeting was used to create the fourth wall of each

corridor. The plastic wall (as depicted by the blue line in Figure 7) was 2m tall with a single door that was used as the only point of entry for the birds and technicians. The exercise corridor contained two perching locations on opposite sides. The only other opening in the plastic wall was a small circular hole at the center of the corridor for a DSLR camera (Canon; Long Island, New York) to film the birds as they flew past. On the opposite side of the camera we placed a 1m X 1m grid with 10cm X 10cm squares drawn on. This grid was used in the film analysis to confirm that all birds were flying relatively similar lengths during their exercise periods. The no-exercise corridor contained two cages so that birds were under similar conditions but did not fly back and forth to experience exercise. There was no food, water, grit or cuttlebone available in either of the corridors.

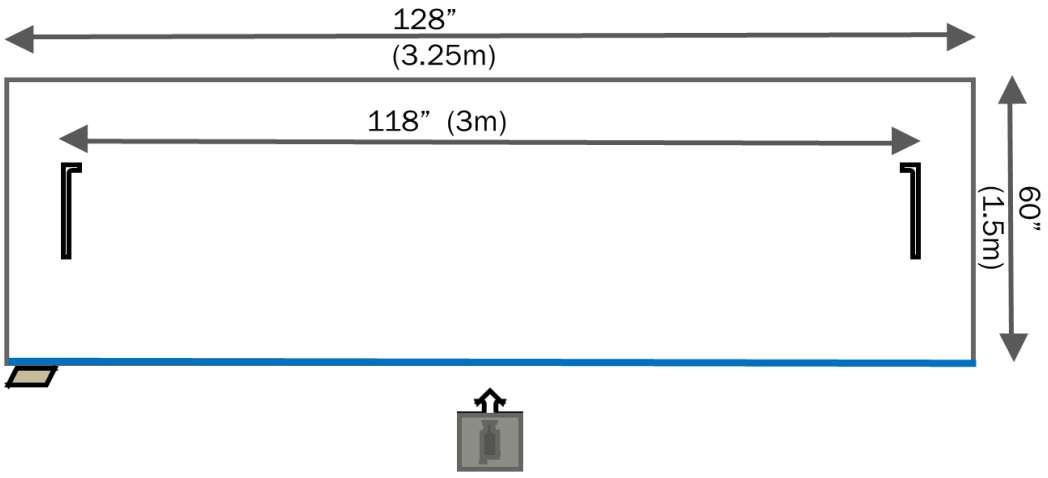


Figure 8. Aerial view of exercise corridors.

The exercise and no-exercise regimens consisted of 2 sessions within the corridors on consecutive days. On the first day, birds were weighed to get a baseline weight before they had a chance to feed after their overnight fast. After collecting mass, each individual was color marked in order to be identifiable in the videos. A thin layer of white paper correction fluid was painted on the bird's frontal auricular feathers below the eye and anterior to the ear opening. After it dried, a specific color combination was assigned to each individual and painted on top of the correction fluid using non-toxic markers as seen in Figure 3. The birds were then returned to their cage and allowed to feed. Minute-long observations were performed at each cage to assure that the marking technique did not alter behavior.

Figure 9 Photo of color marked bird.

After all birds were weighed and marked, individuals were placed into the exercise or no-exercise corridor. On the first day, all birds were placed into the corridors at the same time. Within the exercise corridor, flight was induced every 15 seconds by a technician via flushing from one perch location to the other. The exercise technician used a stopwatch to keep track of flushing interval, a clipboard to take note of any abnormal activity, and a

0.6m stick to nudge individuals that did not flush when the technician approached. The exercise technician was responsible for identifying any individual bird that was not capable of performing the exercise. A total of eight individuals had to be removed due to damaged feathers or overall lethargy and unwillingness to fly between perches every 15 seconds. No-exercise birds were placed in the no-exercise corridor at the same time but within single sex cages to limit movement. The no-exercise technician entered the room at the same time as the exercise technician but remained still, only taking notes of any abnormal activity. After 2 hours in the corridor all individuals were returned to their respective cages and remained undisturbed for the rest of the day.

On the second day of the two exercise regimens, individuals were again placed into their corresponding corridors, but this time in 10-minute intervals. A technician was already inside the corridor and each regimen began with the addition of the first bird. After 4 hours in the corridor, one individual was removed at a time by a third technician. The 10-minute interval provided a gap of time for technicians to collect all samples while assuring that all birds experienced the same length of time within the corridors.

Sample Collection

Husbandry and sample collection procedures were approved by the College of William and Mary Institutional Animal Care and Use Committee (2013-06-02-8721-dacris). Individuals were manually captured from their

respective exercise regimen and a blood sample was collected by needle puncture of the brachial vein. Blood was drawn into two capillary tubes within 3 minutes of exercise regimen completion. One tube was used for mercury analysis and the other was centrifuged at 13,000xg for 10 minutes to separate plasma and red blood cells. On average, 30µl of plasma was isolated per bird and used for SOD quantification. Birds were euthanized by rapid decapitation within 5 minutes of exercise treatment, and immediately dissected using alcohol-cleaned tools. Liver samples were removed first, placed in 1.5ml cryogenic vials and dropped into liquid nitrogen in order to halt all enzymatic activity. All sample collection was performed within 10 minutes of euthanasia. Samples were snap frozen and later transferred to -80°C freezer.

Mercury and SOD Quantification

Mercury content and plasma SOD levels in the blood were quantified using the methods detailed in chapter 2. Liver samples were removed from the -80°C freezer one at a time and a piece was dissected on ice and weighed with the goal of reaching 50 mg. The actual weight was recorded to be used for dilution factor calculations. The tissue was placed in 250 µl of HEPES buffer prepared using 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose and homogenized for one minute by hand with a plastic pestle. The samples were then centrifuged at 3,000 x g for 5 minutes at 4°C. A serial dilution was performed to optimize absorbance readings within sample wells to fall within the standard curve. 2 µl of the supernatant was further diluted in

1990 µl of HEPES (996 dilution factor) buffer and placed on ice until assayed. A pool was created by combining 25 µl of supernatant from two liver samples, vortexing thoroughly and then diluting in 2ml tubes that could be taken out individually during each assay. Six pool samples from this same dilution were included in each of the two assays run.

SOD Calculations

Using the same procedures as detailed in chapter 2, we calculated blank-subtracted linearized rates for each sample. However, dilution factors for the liver samples had to be adjusted according to the amount of liver mass homogenized per sample. Working under the assumption that 1 g of liver mass is approximately equal to 1 ml of water, we calculated the dilution factor of the homogenate by adding the volume of the liver to the 250 µl of HEPES buffer in which it was homogenized. That sum was then divided by the initial volume of the liver to produce a homogenate dilution factor. To calculate the final dilution factor, we then multiplied the homogenate dilution factor by 996. All liver SOD samples are expressed as U/g.

Statistical Analysis

We performed statistical analyses using both R and IBM SPSS Statistics v23. General linear models were developed for plasma SOD3 and liver combined SOD1/SOD2 activity using type III sums of squares. In both cases the first model fit included weight and age as covariates, which were

subsequently removed because they did not have significant effects and were not of interest under the original design of this experiment. Both models included sex, diet, and exercise treatment as fixed effects, and the three interactive effects were included as well. The three-way interaction was not included because there is no current research that suggests its importance to our questions.

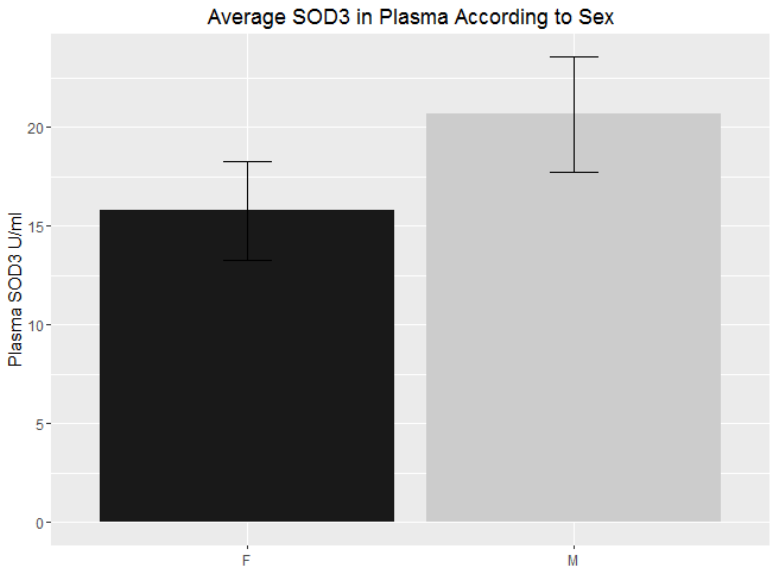
Average SOD activity values were calculated to perform comparisons within the factors of interest, ignoring the other factors. We used these raw mean values instead of estimated marginal means from the models because there was a lack of significant fit from both models and we were interested in comparing effect sizes which are calculated from the original mean values and standard deviation. Cohen's D was used to estimate effect size and 95% confidence intervals were reported and graphically depicted for all means. A correlation analysis was used to compare the relationship between SOD3 in the plasma and combined SOD1/SOD2 activity in the liver of 30 individuals for whom we collected both tissues.

Results

Plasma SOD3 Activity

Preliminary analyses showed no covariate effects of weight or age on plasma SOD3 activity, so those components were removed from the model. Instead, the final model included diet, exercise treatment, and sex as the factors of interest, as well as their two-way interactions. Only sex had a

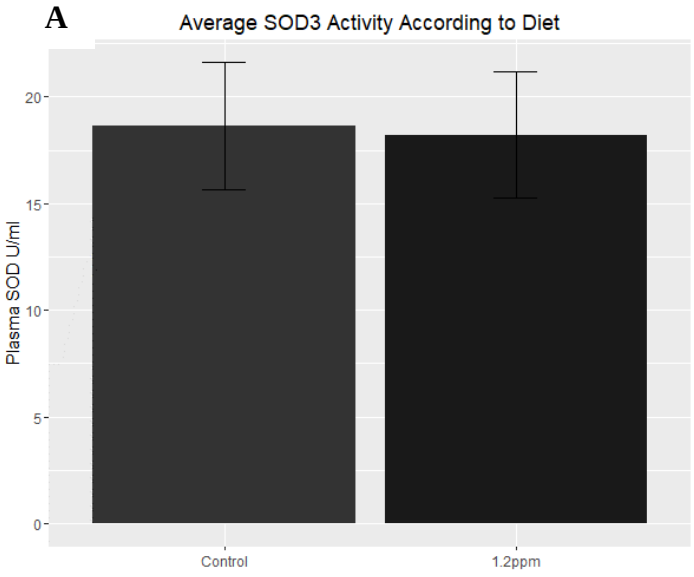
significant effect on SOD3 activity in the plasma ($F_{1,38}$= 6.870, p= 0.013), with the 18 males (mean= 20.65 U/ml, SD= 6.667) having a higher average than the 21 females (mean= 15.78 U/ml, SD= 5.341).



Sex	Mean SOD3 U/ml ± 95%CI	N
Female	15.78 ± 2.518	18
Male	20.65 ± 2.910	17

Figure 10: Average plasma SOD3 activity and 95% confidence intervals according to sex, independent of diet and exercise treatment.

Diet ($F_{1,38}$= 0.040, p= 0.842), exercise treatment ($F_{1,38}$= 1.469, p= 0.235), and their interaction ($F_{1,38}$= 1.019, p= 0.321) did not have a significant effect on plasma SOD3 activity. Figure 10 shows further independent analyses of each factor of interest. These visualizations support the finding that diet and exercise did not impact SOD3 in the way that we predicted. The three-way interaction between sex, diet, and exercise treatment was also not significant ($F_{1,38}$ = 2.050, p = 0.162) and is visualized in Figure 12. Although statistically significant differences were not detected, it is important to highlight that the birds in the exercise regimen (mean= 19.35, SD= 6.031) had a higher level of SOD3 activity than the no-exercise regimen (mean= 17.40, SD= 6.976), with a moderate effect size (Cohen's D = 0.301). Most surprising was the lack of diet effect. The treatments showed a near-zero effect size of 0.064 (Cohen's D). A slightly lower level of SOD3 activity was seen in the mercury treated birds (mean= 18.21, SD = 6.783) compared to controls (mean= 18.63, SD= 6.337), but this is almost certainly neither biologically or statistically significant.



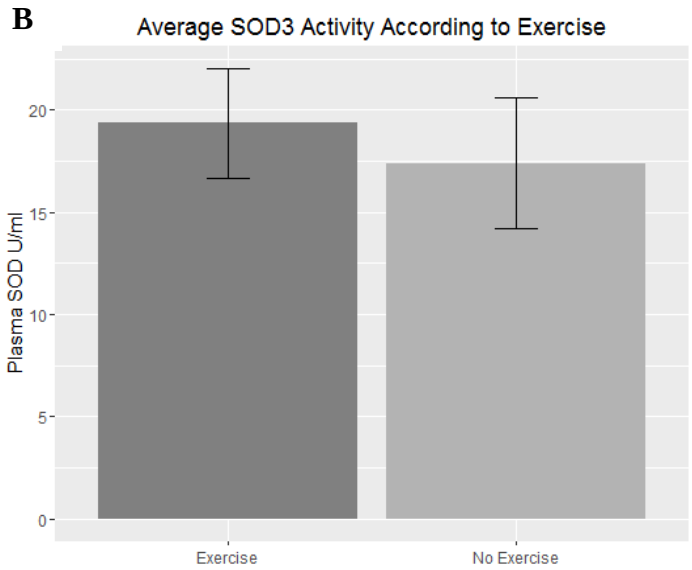


Figure 11: (A) The mean plasma SOD3 activity and 95% confidence intervals for each dietary treatment independent of exercise treatment. (B) The mean plasma SOD3 activity and 95% confidence intervals for each exercise treatment independent of diet.

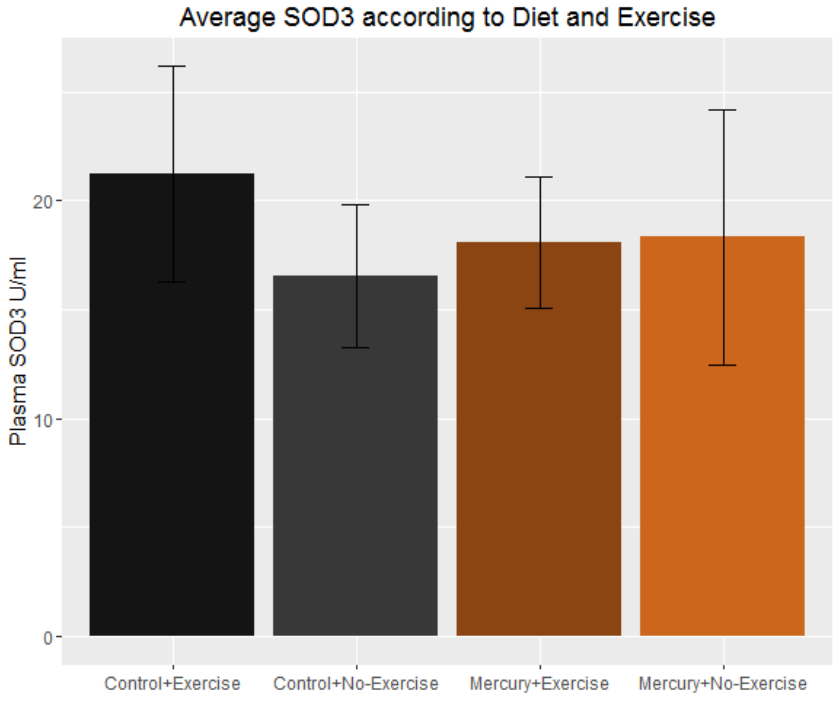


Figure 12: Comparison of average plasma SOD3 activity level and 95% confidence intervals of the four treatments that resulted from the factorial design.

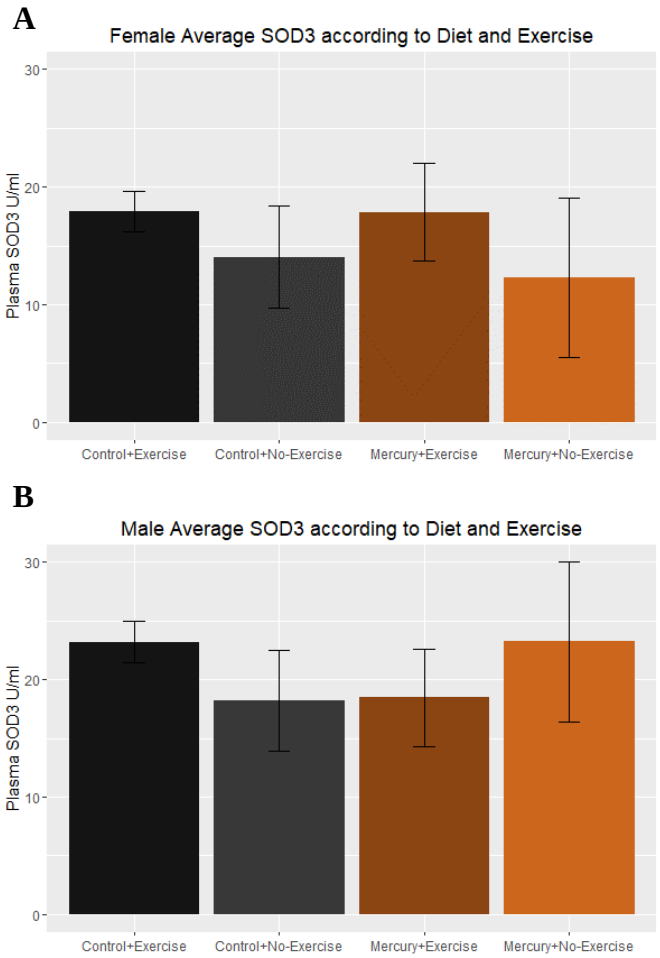


Figure 13: Graphical representation of how diet and exercise treatment affected the (A) females differently than (B) males. Average plasma SOD3 activity level and 95% confidence intervals shown.

Liver SOD1/SOD2 Activity

No covariate effects of weight or age on SOD1/SOD2 in the liver (hereafter “liver SOD”) were detected, therefore, those components were removed from the final model. The final model included diet, exercise treatment, and sex as the factors of interest, the two-way interactions of these three factors were also included. This model showed no effect of exercise treatment ($F_{1,33}$= 1.243, p = 0.275), sex ($F_{1,33}$= 0.034, p= 0.855), or diet ($F_{1,33}$= 0.349, p= 0.560) on liver SOD. There were also no significant interactions detected. We performed analyses within each factor of interest independent of the other factors, similar to plasma analysis. The mercury-exposed birds exhibited a non-significant lower average (mean= 4565.85 U/g, SD= 1080.018) liver SOD activity than control individuals (mean= 4816.70 U/g, SD= 1299.229) and an effect size of 0.212 (Cohen’s D). Exercised birds (mean= 4872.48 U/g, SD= 1066.202) had a higher liver SOD activity level than no-exercise birds (mean= 4461.74 U/g, SD= 1318.263) when dietary treatment was ignored. The difference in these two means is slightly larger than the difference between the two dietary treatments as expressed by the effect size (Cohen’s D= 0.347). Finally, there was no significant effect of sex on liver SOD activity. Males had a slightly higher level (mean= 4751.82 U/g, SD= 1225.85) of SOD activity than the females (mean= 4630.72 U/g, SD= 1173.608), with a very small effect size (Cohen’s D= 0.102).

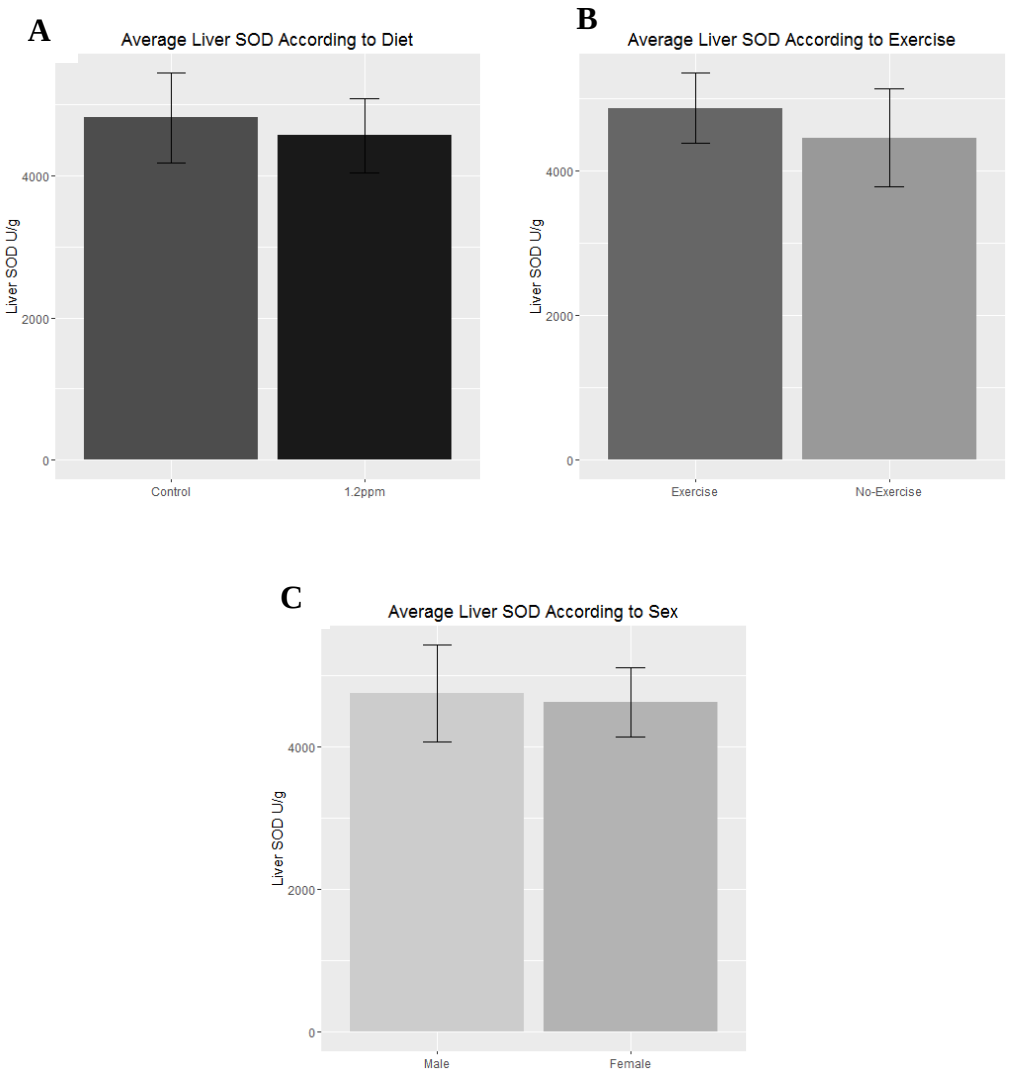


Figure 14: (A) Average liver SOD and 95% confidence intervals according to diet independent of sex and exercise treatment. (B) Average liver SOD and 95% confidence intervals according to exercise treatment independent of sex and diet. (C) Average liver SOD and 95% confidence intervals according to sex independent of diet and exercise treatment.

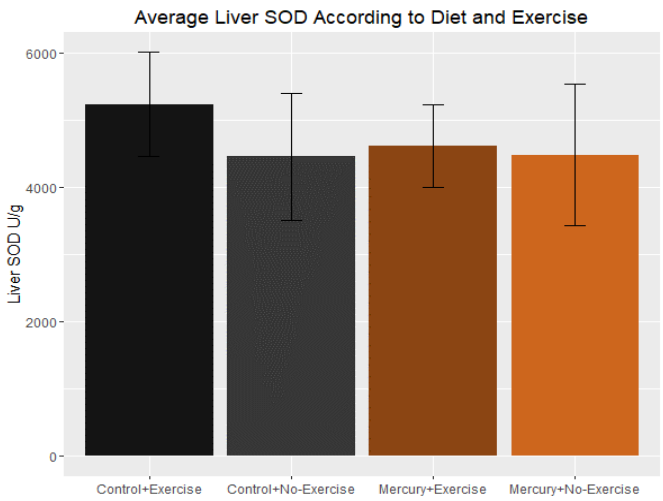


Figure 15: Average liver SOD and 95% confidence intervals per treatment combination of diet and exercise.

Relationship Between Plasma and Liver SOD Activity

With the goal of assessing plasma SOD3 activity as a non-lethal biomarker of bird antioxidant content, we performed an analysis comparing plasma to liver SOD activity levels. Table 2 shows the mean values for each factor considered to have a possible effect on SOD activity. As previously mentioned, sex was the only factor that had a significant effect on plasma SOD3. This effect was only seen in the plasma of the birds sampled and a small effect size (Cohen's D= 0.102) was seen in the liver, with the males showing higher SOD activity than the females. Plasma SOD3 activity was virtually equal (Cohen's D= 0.064) between the dietary treatments, while Liver SOD showed an increased level of activity in control birds (Cohen's D= 0.212). A similar effect size was reported by Henry et al. (2014) in younger

zebra finch treated with the same dose (Cohen's D= 0.250). Exercise seemed to impact SOD levels in the plasma and liver similarly, with exercised birds showing a higher average level of activity than non-exercised birds.

Factor	Level	Mean Plasma SOD3± 95%CI (U/ml)	n	Mean Liver SOD± 95%CI (U/g)	n	Cohen's Effect Size	
						Plasma	*Liver*
Sex	*Female*	15.78± 2.518	18	4630.72± 569.283	17	**0.750**	0.102
	Male	20.65± 2.910	21	4751.82± 594.626	17		
Diet	*Control*	18.63± 2.987	18	4816.70± 596.127	17	0.064	0.212
	Mercury	18.21± 2.960	21	4565.85± 557.718	17		
Exercise Treatment	*Exercise*	19.35± 2.697	20	4872.48± 489.207	19	0.301	0.347
	No-Exercise	17.40± 3.201	19	4461.73± 680.747	15		

Table 2: Comparison of mean values for plasma SOD3 and liver SOD activity for each factor of interest. Cohen's D values are used to represent effect size, values in bold represent significant effect of the corresponding factor on the corresponding tissue sampled.

We sampled SOD activity in both plasma and liver from 30 birds in this experiment. These birds were used to analyze the relationship between SOD3 in the plasma and liver SOD. There was no linear relationship between the SOD content of these two tissues (Fig. 15). Correlation analyses on all 30 birds ($F_{1,28}$=0.578, p=0.4536), as well as each diet individually (Mercury: $F_{1,12}$= 0.001, p= 0.979; Control: $F_{1,14}$= 0.553, p= 0.2665), showed non-significant relationships.

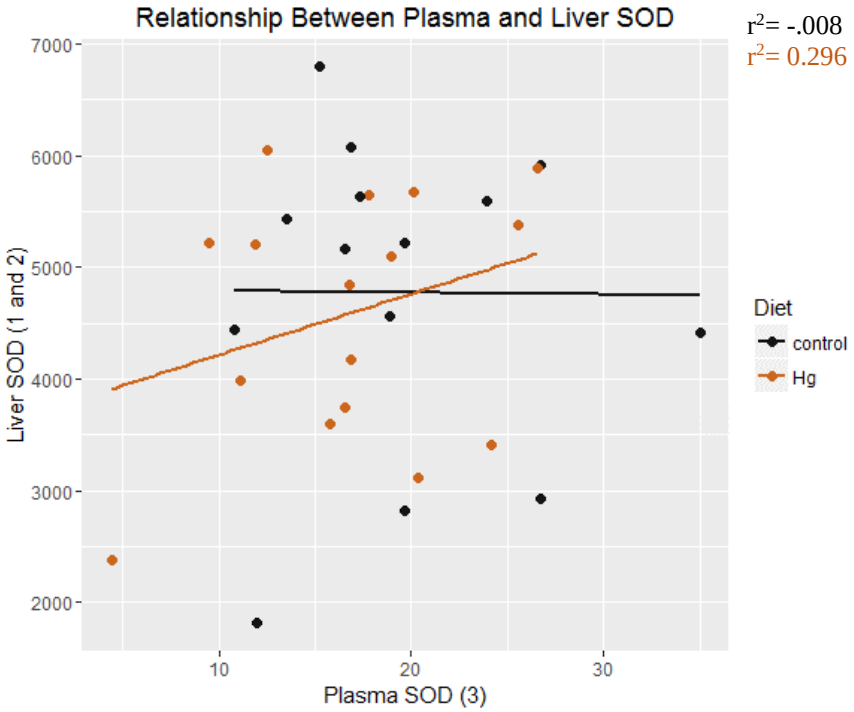


Figure 16: Plasma SOD3 activity and liver SOD for 30 individuals; colors indicate dietary treatment; lines indicate linear regression for each diet although neither was found to fit the points significantly.

Discussion

The primary goal of our study was to clarify the relationship between mercury and plasma SOD3 activity. We detected no effect of mercury on plasma SOD3 activity throughout development (chapter 2), in adulthood, nor in combination with another stressor, exercise. Pre- and post-transcriptional mechanisms that may be driving this unexpected finding were detailed in chapter 2. Briefly, we suspect that mercury is affecting the organs and cell types that specialize in SOD3 production differently than it is affecting the source of other enzymes, including SOD1 and SOD2. Also, mercury's post-

transcriptional damage may be decreasing SOD2 activity more than SOD1 and SOD3 activity due to structural differences between the isozymes (Kumagai et al., 1997). Although we did not detect significant effects of mercury or other factors such as age and sex, we hope to provide analysis and interpretation on our results that may serve as the foundation for future studies. We cautiously use effect size (Cohen's D) throughout this analysis with the clear understanding that it is not indicating significant effects on our response variables. The effect size comparisons may provide us with insight for future projects that could be designed with the power to show significant effects.

Henry et al. (2014) provided evidence for a negative correlation between circulating blood mercury and SOD activity in the liver ($t_{73.0}$ =-2.214, P = 0.030). Mercury circulating in plasma had a tight positive relationship with liver mercury content in the that study. One difference between that study and the present study is that Henry's zebra finches were exposed to one of five different mercury doses (0, 0.3, 0.6, 1.2, 2.4 ppm), as opposed to two levels (0 and 1.2ppm). A regression analysis was possible in the Henry et al. (2014) study because the mercury content of the birds spanned 0 to 30 ppm. In contrast, our control birds had a blood mercury content tightly distributed around 0 ppm and our mercury-exposed were tightly distributed around 20 ppm. Using only those two doses from the Henry et al. (2014) study gives a similar negative result as the present study (t-test: t_{38}= 0.814, p= 0.421). We also found a similar effect size of mercury on liver SOD activity (Henry et al:

0.25, n= 40; present study: 0.21, n= 39). This comparison between studies suggests that our design did not have the power to detect the effects of mercury on liver SOD. A more continuous distribution of mercury exposure, along with the inclusion of an unrealistic high mercury dose (2.4ppm), revealed a significant effect of mercury on liver SOD, but examining just a portion of that relationship does not. The similar effect size on liver SOD between the two studies suggests that we may be at least picking up similar overall effects; reduced liver SOD activity in mercury (1.2ppm) exposed birds. When we make a similar comparison to the effect size calculated for plasma SOD3 activity (Cohen's D=0.064), it is clear that this effect is not occurring in the plasma. Given what we know about the tissue-specific production of SOD3, especially that the liver is not one of the primary producers of it, it is reasonable to expect this dissimilarity between SOD isozymes in response to mercury.

Linear models on liver SOD and plasma SOD3 detected no significant effects of exercise. We predicted an overall increase in plasma SOD3 in response to exercise, given mammalian model accounts for an exercise-induced response in SOD3 specifically (Fukai et al., 2000; Hitomi et al., 2008; Rush, Turk, & Laughlin, 2003). It is important to note that several studies on birds have demonstrated increased OXD along with decreased antioxidant capacity, including the enzymatic antioxidant GPx (Costantini et al., 2008, 2012; Jenni-Eiermann et al., 2014). These studies provide conflicting reports

of enzymatic antioxidant response to exercise and highlight the need for more in-depth analysis of our results.

Independent of dietary treatment and sex, there was a non-significant trend for exercised birds to have higher SOD activity than controls, in the plasma (Cohen's D=0.301) and in the liver (Cohen's D=0.347). We imposed exercise on zebra finches for a total of six hours over two days. A similar experiment by Costantini et al. (2011) exercised zebra finches for eight hours over three days and quantified several OXS biomarkers. OXD was increased significantly in exercised birds, while total plasma thiols, a non-enzymatic measure of antioxidants, were significantly depleted. This is an interesting difference in antioxidant response to similar levels of exercise within the same species; we show an increasing trend in SOD (liver and plasma SOD3) activity, while Costantini et al. (2014) found a decreasing trend in non-enzymatic antioxidants. If non-enzymatic antioxidants are being depleted but enzymatic antioxidants are not, then gene expression may be playing a key role in response to exercise. The more extensively studied mammalian models indicate that this may indeed be the case.

There are several compelling studies on mammals that shed light on the mechanisms for an effect of exercise on SOD. Fukai et al. (2000) suggests that exercise can increase circulating levels of nitric oxide, which is known to induce gene expression of enzymatic antioxidants, including the SOD isozymes. Mice exercised on a treadmill for 150 minutes showed increased levels of SOD3 mRNA in the skeletal and aortic muscle (Hitomi et

al., 2008). Meanwhile, other enzymatic antioxidants (SOD1, SOD2, and GPx) did not change in relation to exercise. Similar studies are lacking in songbirds and although we detected only a non-significant trend in exercise induced upregulation in plasma SOD3, this might be indicative of a protective role for this enzyme during acute exercise stress. Future studies should implement mRNA analyses in order to confirm that the increased activity that we detected is indeed due to gene expression. Important differences between mammalian and avian respiratory, circulatory, and muscular systems, as well as new evidence that suggests that certain bird species do not need to upregulate exercise in preparation for migration, highlights the pivotal role that bird research may play in better understanding OXS (Pennisi, 2017).

Another interesting trend is apparent when we analyze the effects of exercise on SOD separately between control birds and mercury-exposed birds. As depicted in Figure 11, control+exercise birds exhibited the highest level of plasma SOD3 activity of any group in our factorial design. The lowest overall average level of plasma SOD3 activity was found in the control+no-exercise group. These trends were also found in the liver SOD, as seen in Figure 14. In response to exercise, control birds exhibited an effect size of 0.741 (Cohen's D, n=17), while mercury-exposed birds differed from non-exercised birds by the much smaller effect size of 0.037 (Cohen's D, n=21). A similar pattern occurred when comparing SOD in the liver, with the control birds exhibiting an effect size of 0.600 (Cohen's D, n=17) in response to exercise, while mercury-exposed birds differed from non-exercised birds by

an effect size of 0.125 (Cohen's D, n=17). Although we did not find a significant effect of the interaction between diet and exercise treatment in our model, analysis of the effect sizes suggests that mercury exposed birds may have been unable to upregulate SOD levels in response to exercise.

Mercury causes a similar effect on the vital stress hormone, corticosterone. Zebra finches exposed to dietary mercury had lower levels of stress-induced corticosterone than control birds (Moore et al., 2014). Although corticosterone and SOD are very different molecules, it is interesting that mercury can act as an inhibitor to a stress response. The implication that mercury may be affecting exercise-induced SOD response is not statistically supported by our study, but we believe that these trends set up preliminary data for more research on this topic. Using non-lethal sampling of SOD3 in the plasma, a longitudinal study is possible to get baseline values and stress induced values for the same individuals.

Sex was the only factor that had a significant impact on predicting plasma SOD3 activity according to the linear model. However, this effect was not significant in the liver and its effect size was small (Cohen's D=0.103), although in the same direction. Males had a higher average SOD activity level than females. These results are in contrast to our results from chapter 2, where females were found to have a significantly higher activity level of SOD3 in adults. Housing conditions were identical for both studies; individuals were in single-sex cages but in the same room as birds of the opposite sex. Only one male (HG4620; SOD3 = 18.914 U/ml), was a part of a breeding effort

before being chosen for this experiment and the SOD values that he showed were not outliers. Other housing conditions were set to mirror wild bird breeding conditions (ad libitum resources, 14:10 light:dark photoperiod), and both sexes commonly exhibit breeding behavior (e.g. male song, female egg laying) even though they are in separate cages. Since we did not measure any breeding behavior in either of these experiments, we cannot interpret any changes between the sexes to be related to breeding effort, as many OXS studies do. However, comparisons to studies analyzing oxidative strategies between the sexes may shed some light on the mechanisms at work behind these results.

There are some crucial differences between our two experiments (chapter 2 and 3) that must be taken into consideration to understand the contradictory effects of sex on plasma SOD3 activity. The mean age of adult birds sampled in the study described in chapter 2 was 378.1 DPH (SD=28.2) while the mean age of birds sampled in the study described in chapter 3 was 480.5 DPH (SD=134.5). Although there is a large difference between average age, the large variation in age of birds in chapter 3 was necessary for us to test for a covariate effect of age. Age did not affect SOD3 content and was subsequently removed from the model.

To analyze an interactive role of mercury with sex we must again clarify that mercury exposure in the present study (chapter 3) occurred *in ovo* and continued throughout the lifetime of the bird. The study described in chapter 2 involved only developmental mercury exposure; adults were no

longer on mercury when sampled for the 350-400 DPH age. Although we never detected a significant effect of dietary treatment on SOD3, there are a number of deleterious fitness effects caused by sub-lethal mercury exposure (Whitney, 2014). Impacts on endocrine, immunological, and neurological function could have indirect influence on SOD3 expression and activity (Zelko et al., 2002). Furthermore, we know that these impacts are dependent on dose and time/length of exposure, so we can predict that lifetime exposure (chapter 2) and developmental exposure (chapter 3) will affect individuals differently. We did not design the studies with the intention of identifying those indirect effects, nor how they may vary between the two exposure types. There are too many confounding variables to be sure that the difference in timing of mercury exposure between the two studies is the driving force behind the contrasting response of SOD3 between the sexes. However, given the empirical evidence for differences in susceptibility to environmental stressors between males and females, it is worth considering (reviewed in Jones, Nakagawa, & Sheldon, 2009).

Further graphical representations (Fig. 12) show that exercise impacted females in the control diet in the same way that it impacted those in the mercury diet; exercised individuals expressed higher levels of SOD3 activity. Males on the control diet showed the same trend, however, males on the mercury diet showed the opposite trend with birds in the no-exercise group having the highest average level of SOD3 activity. We approach this post hoc analysis with caution, the experimental design reduces our sample

size significantly when we analyze treatment interactions by sex (n: female= 18, male= 21, four levels within each one). Our model did not find a significant three-way interaction between diet, sex, and exercise treatment. Nevertheless, the abnormal trend that mercury-exposed males exhibited in response to exercise may be playing a role in masking the expected result of increased SOD3 activity in response to exercise. Further support for this unique role of sex in dictating response to stressors comes from the repeated measures analysis in chapter 2. This model, did find a significant three-way interaction between dietary treatment, sex, and age, suggesting that the effects of diet on plasma SOD3 activity may be dependent on sex in differing ways as birds age.

The possibility of a three-way interaction between mercury exposure, sexual differences, and exercise is a complex and difficult concept to elucidate. We believe that there are three empirically supported mechanisms that, when analyzed together, provide support for such an interaction. (1) Circulating mercury levels in females fluctuate more throughout their lifetimes than male mercury levels, due to the mercury depuration that occurs when an egg is laid, although this trend varies by species (Robinson, Lajeunesse, & Forbes, 2012). Although the exact type of effect that these fluctuations have is unclear, we must consider that this variation is pivotal to any impact mercury may have. (2) There is also evidence, from mammalian models, that suggest females have an antioxidant advantage due to estrogen's antioxidant properties, along with elevated mitochondrial levels of enzymatic antioxidants

(Behl et al., 1995; Olivieri et al., 2002; Vahter et al., 2007). This basal antioxidant advantage may indicate that response to an acute stressor, such as exercise, would be different between the sexes, because the starting points are different. (3) Finally, Jones et al. (2009) suggests that inherent differences between the sexes such as size, development of the endocrine system, and metabolic requirements, can lead to varied responses to chronic environmental stressors, including mercury. When synthesized, these three mechanisms show that circulating levels of mercury vary between the sexes (1), chronic toxin exposure itself can elicit different responses between the sexes (2), and the differences in basal oxidative state results in different responses to acute stressors such as exercise (3). These mechanisms could combine for a three-way statistical interaction in SOD3 activity response.

Another important factor to consider is the physical state of the birds prior to of sample collection. Plasma collection in this study (chapter 3) occurred after 4 hours in the exercise or the no-exercise corridors. Regardless of exercise treatment, these birds had no access to water or food for the four hours prior to sampling. In the study detailed in chapter 2, sampling occurred within 5 minutes of capture at random times throughout the day, so the birds had *ad libitum* access to water and food prior to sampling. Therefore, we must realize that both the exercise and no-exercise treatments received additional stress from food and water deprivation, placement in a novel surrounding (corridor), and proximity to humans. When we compare the activity levels of SOD3 in the plasma of the adult females

from experiments of chapter 2 to that of females described in chapter 3 we found two very similar means (chapter 2: 13.654 U/ml ± 2.887, chapter 3: 15.775 U/ml ± 2.518). However, when we compare the mean SOD3 values for the males between the two experiments, we found a notable difference (chapter 2: 9.144 U/ml ± 2.881, chapter 3: 20.651 U/ml ± 2.910). The males are solely responsible for the contrasting results of the effect of sex on plasma SOD3 values since the females show almost identical means between the two experiments. As detailed in Appendix B, the assay kits used for chapter 2 and chapter 3 did contain different batches of enzyme as provided by the company and may be accounting for some of the differences. However, the change in male SOD3 content exceeds the 17% inter-assay variation that we experienced using these kits, suggesting that the male difference is more than just error. All of the birds in the chapter 3 study were subject to significant prolonged stress, while the birds in chapter 2 were not. However, it is only the males that differed dramatically between the studies. Perhaps the prolonged stress of being in the exercise or no-exercise corridor elicited a varied response in SOD3 activity levels between males and females. Given that females have higher baseline levels of antioxidant capacity due to estrogen's role in modulating antioxidants, variability in antioxidant response to stress between the sexes is a reasonable expectation. Unfortunately, without baseline levels of the same individuals that went through the chapter 2 study, we cannot be certain that this is the case.

The overarching goal that spanned our two studies was to assess the viability of using plasma SOD3 activity to detect OXS without euthanasia. The chronic stressor, mercury, did not have any detectable impact on the activity levels of SOD3 in plasma. Only a three-way interaction with sex and age was detected, but to have confidence in that interaction would require a much more mechanistic approach. A non-significant trend in upregulation of SOD3 activity was detected in response to the acute stressor, exercise. Finally, a clear difference, although not in a consistent direction, between sexes was detected in plasma SOD3 activity levels. These results do not support the viability of SOD3 in plasma as a good non-lethal biomarker of OXS. The main takeaway is that SOD3 was only significantly affected by an innate trait, sex. The application of a non-lethal OXS biomarker heavily depends on its ability to detect environmental stress on the oxidative state of the individual and our experiments on SOD3 did not meet that requirement (Costantini, 2008). Given that we measured activity levels in the plasma, we only saw a snapshot of the complex process that is responding to an environmental stressor. Finally, our analysis of correlation between liver SOD and plasma SOD3 activity showed that within an individual, these enzymes are not linearly related. These findings further support that the long evolutionary history of the SOD family of enzymes has resulted in a significant divergence in their structure, role, and regulation within the body. Each isozyme should always be considered individually, and quantification of each enzyme should be done in a very specific manner.

Appendix A: Other assays attempted

The original approach to this study was intended to produce a comprehensive assessment of oxidative status within each individual. In order to do this, we wanted to quantify several biomarkers of OXS for comparison as is suggested by most OXS research (Costantini, 2008). We experienced significant issues with the accuracy and precision of these assays and decided to focus only on SOD. For future reference, we will detail which assays were attempted and the issues experienced with each in this appendix. We attempted all assays on whole blood or plasma samples with the goal of doing non-lethal OXS quantification.

Glutathione Ratio Assay (GSH/GSSG)

Glutathione is the major non-enzymatic antioxidant in the body of most organisms. When reduced glutathione (GSH) reacts with a ROS it becomes oxidized glutathione (GSSG). The reduction back to functional GSH is guided by an enzyme named glutathione reductase (GRx) (Kenow et al., 2008; Patrick, 2002). The ratio of GSH to GSSG is a commonly used biomarker for oxidative stress, since a downward shift in the ratio would indicate a higher concentration of oxidized glutathione. Furthermore, mercury is known to create a non-reversible bond with GSH that ultimately results in its depletion in the body, also leading to a decreased ratio (Ballatori & Clarkson, 1985). A negative relationship between liver glutathione ratio and blood mercury content was clear in zebra finches fed several diets of mercury concentrations

(Henry et al., 2014). We are unaware of any attempt at identifying this same trend in the blood of songbirds. We used the microplate assay for GSH/GSSG kits (GT40) from Oxford Biomedical Research (Rochester Hills, MI).

Results

Samples from a pool of blood thoroughly mixed were expected to show similar calculated values of glutathione ratio. In fact, we saw a very large amount of variation between these pooled samples. The lowest glutathione ratio quantified from these pool samples was 17.57 while the largest value from the same pool was 341.41 (Table 3). A coefficient of variation (CV) of 57.13% was calculated and indicated the lack of repeatability between assays. Our results indicate that this assay does not reliably show oxidative status in the blood of zebra finches.

Assay Run	Pool Ratio
1	241.84
1	17.57
1	57.42
2	44.39
2	160.91
2	230.25
3	133.92
3	341.41
3	235.85
4	241.47
4	234.78
4	144.39
5	19.16
5	215.02
5	134.62
avg	163.53
sd	93.43
CV	57.13

Table 3: Calculated ratio values for all pools included in 5 assays of glutathione ratio. Average, standard deviation, and coefficient of variation reported.

Protein Carbonyls

A commonly used biomarker of OXD is the protein carbonyl content of protein. Attack by ROS can result in the carbonylation of several amino acids (i.e. lysine, arginine, histidine) (Veskoukis, Nikolaidis, Kyparos, & Kouretas, 2009). These carbonyls can be quantified spectrophotometrically by the production of hydrazones when plasma samples are incubated with 2,4-dinitrophenylhydrazine (DNPH). We purchased Protein Carbonyl Colorimetric Assay Kits (10005020) from Cayman Chemical Company (Ann Arbor, MI). This kit uses the difference in absorbance between a DNPH-incubated sample and an HCl-incubated sample to calculate carbonyl content.

We followed the procedures as detailed on the kit booklet for plasma samples but failed to produce expected results. After DNPH incubation, this assay requires protein purification and wash. We identified this step as the issue; protein levels of our samples were dramatically depleted after the three washes were executed. Therefore, the difference in absorbance between DNPH-incubated samples and HCl-incubated samples were negligible and inconsistent. Several modifications were attempted but we were never able to retain high enough protein levels throughout the assay. Since enough protein was not present, we were unable to measure carbonyl content reliably.

Total Antioxidant Capacity

The final assay that we attempted is a kit produced by Cayman Chemical Company (Ann Arbor, MI) that attempts to quantify the total

antioxidant capacity (AOX) of a plasma sample. This kit quantifies the ability of a sample's antioxidants in a plasma sample to prevent the oxidation of 2,2'-Azino-di-[3-ethylbenzthiazoline suphonate] by metmyoglobin. Trolox is used to create a standard curve so resulting AOX is quantified as millimolar Trolox equivalents. The kit instructions were followed carefully for plasma samples and the standard curves consistently showed similar spectrophotometric levels as examples given by Cayman. Given our previous troubles with repeatability and the low volume of plasma needed for this assay (2ul) we decided to assay 21 samples in two consecutive runs. We then quantified the Trolox equivalent units for each and gave them a rank order from lowest to highest. If the assay was dependable then we would expect each sample to show a similar rank value in each run. Instead, we see rank values that seem randomly assigned to each sample (Table 4). This indicates a lack of repeatability between assays and was the main reason for not trying more runs on this kit.

Band #	Rank Assay 1	Rank Assay 2
4208	7	17
4302	20	6
4355	2	1
4385	8	11
4620	17	18
4831	16	10
4875	5	8
4875	15	2
4968	11	9
4972	3	3
4977	9	19

5113	18	15
5116	1	16
5120	19	20
5126	12	14
5134	6	7
5147	21	21
5230	4	13
5250	13	4
5252	10	12
5276	14	5

Table 4: Rank order for plasma samples run in two spate assays quantifying AOX.

Appendix B: Repeatability Measures for Cayman SOD Assay Kit

Plasma

Eight assays were performed on plasma SOD samples, two for chapter 2 and six for chapter 3. Overall, Cayman Chemical Company (hereafter “Cayman” Ann Arbor, MI) provided us with two batches of enzymes as a part of these kits. All the chapter 2 assays used batch 1 of bovine erythrocyte SOD (Cu/Zn) stock (706005) and xanthine oxidase (706004), while the chapter 3 assays used batch 2 of these same enzymes. All enzymes were shipped from Ann Arbor, MI to Williamsburg, VA overnight in dry ice containers, contrary to Cayman’s preference of shipping in wet ice. Cayman does not provide repeatability measures of the same sample between assay runs. They did however provide a standard curve for each of the two batches, batch 1 resulted in a slope of 55.15 and batch 2 in a slope of 49.13. These differences in slope provide an informal indication of variability between enzyme batches.

Furthermore, correspondence with Cayman's quality control department indicates that intra-batch variation of this slope is about 15% on average. How this variation affects our calculated levels of SOD is not clear, we therefore created our own measure of repeatability by implementing a pooled sample analysis.

We included 5 or 6 pool samples in each colorimetric microplate run. We collected plasma from twenty birds, centrifuged each sample, and combined all their plasma into a single tube. One pool sample is a 6ul aliquot of this plasma mixture after thorough homogenization. In theory, each of the samples will result in the same calculated value of SOD activity. The coefficient of variation provides a comparable value of variability for the pool samples within an assay (intra-assay variation) as well as between assays (inter-assay variation).

Table 5 includes the calculated CV for each assay run performed and included in both chapter 2 and 3. We experienced significant variability in this intra assay variation measure ranging from 0.48% to 12.15%. The average overall intra assay variation was 7.64%. When we combine all of our pool samples to calculate the inter-assay variation we see a higher variability between the assays. Final inter-assay variation was 18.54%.

Assay Run Name	Mean SOD U/ml	Standard Deviation	n	CV%
Chapter2-1	15.76	1.86	5	11.84
Chapter2-2	17.12	0.63	6	3.69
Chapter3-1	12.03	1.46	6	10.19
Chapter3-2	13.64	0.67	6	6.94
Chapter3-3	15.24	1.55	6	12.15

Chapter3-4	14.97	1.03	5	4.92
Chapter3-5	11.22	0.54	5	0.48
Chapter3-6	10.20	1.15	6	11.36

Table 5: Intra-assay variation for each assay run in both chapters

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