The effect of sub-lethal methylmercury exposure on corticosterone hormone and the glucocorticoid receptor in the Australian zebra finch (Taeniopygia guttata)

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The effect of sub-lethal methylmercury exposure on corticosterone hormone and the glucocorticoid receptor in the Australian zebra finch (*Taeniopygia guttata*)

A thesis submitted in partial fulfillment of the requirement for the degree of Bachelor of Science in Biology from the College of William and Mary

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Abstract

Mercury can disrupt the endocrine systems of mammals and fish, but little is known about its effects on the avian stress response. An experimental manipulation was used to show that methylmercury suppresses the stress-induced corticosterone response in birds, an effect previously unreported in the literature. Corticosterone regulates many normal metabolic processes, such as the maintenance of proper blood glucose levels during stressful daily fasting; an inability to increase corticosterone levels in response to stressors renders a bird less able to face a wide array of environmental challenges.

Reproductively mature zebra finches that had been exposed to 0.0, 0.3, 0.6, 1.2, or 2.4 μg/g Hg (wet weight, ww) dietary methylmercury throughout their life (i.e. from the egg onwards) were the subjects of this study. In contrast to some field studies, no significant change in baseline plasma corticosterone concentrations was attributable to chronic methylmercury exposure. However, a comparison between the baseline corticosterone levels and levels after 30-minutes of handling stress revealed that the ability of birds to mount a stress response was reduced with increasing blood total mercury concentration. These results are consistent with adrenal corticoid disruption due to chronic mercury exposure, and mirror a similar study on free-living nestling songbirds exposed to environmental mercury. In addition, the glucocorticoid receptor in 50-day old juvenile zebra finches was studied to determine if this facet of the stress response pathway was also disrupted. No change was detected by quantitative PCR analysis in the expression of the glucocorticoid receptor in the brains of juvenile zebra finches. This result is consistent with the conclusion that mercury exposure does in fact have a significant effect on the
stress response pathway, as the system is not compensating by altering receptor expression in response to abnormal hormone concentrations. More research will need to be done to determine whether the system is truly affected by mercury exposure, and whether or not it is compensating in some way for the disturbance of hormone concentrations. This project also studied the expression of the glucocorticoid receptor in zebra finch embryos of various stages, both through qPCR analysis and in situ hybridization. The purpose of studying embryos was not to compare expression among mercury treatment groups at this time, but rather to begin to characterize expression of the glucocorticoid receptor during the developmental stages of the Australian zebra finch, as there is currently no literature on this subject. The glucocorticoid receptor appears to be fairly ubiquitously expressed in zebra finch embryos from stage 17-30: more work will need to be done to continue the characterization of the expression of this receptor during the embryonic development of this species.
I. Introduction

1.1 Overview of the project

Mercury is a widely recognized environmental toxin that affects various systems within a number of different organisms. Mercury is released into the environment naturally at relatively low levels, but additional industrial facilities may cause environmental mercury levels to rise to the point where damage is done to exposed organisms. Once deposited, mercury has the potential to harm ecosystems at multiple levels. Mercury has been shown, through a number of research studies discussed below, to affect one facet of many ecosystems, bird populations, in a number of ways. Research on mercury’s effects on birds is important for a number of reasons. The most obvious of these reasons is that, depending on the level of exposure, mercury intake may be potentially lethal and may represent a threat to whole populations of birds within a particular area. Birds are present at a number of trophic levels within many ecosystems, and disrupting whole populations of birds may jeopardize the balance of these systems due to the specific interactions between trophic levels. Birds help to introduce or increase diversity within and among ecosystems. Certain species of birds transport fish eggs between bodies of water, introducing fish species to areas where they were not previously present or increasing the size of populations in an area where these populations were not particularly large. Other species of birds help distribute the seeds of various plant species, increasing plant diversity in the regions they inhabit. In addition to benefiting the numerous species listed above, maintaining a diversity of bird species is especially
important for human populations, as a diversity of bird species decreases the risk of human infection with such zoonotic diseases as West Nile virus (Swaddle and Calos 2008).

One way that birds cope with daily stress and survive threats in the wild is through the hormonal stress response mediated by the endocrine system. Corticosterone, the major stress hormone in birds, is released from the adrenal glands in response to stress and allows the bird to funnel energy into survival-related processes. The regulation of this response is key to the animal’s health and survival: misregulation of the pathway can decrease the animal’s fitness. Currently, there is no clear consensus on mercury’s effects on this hormonal stress response pathway in birds, as literature on the subject reports various conflicting results. This variability in results may be a product of the fact that most of the previous studies of mercury’s effects on corticosterone have been field studies, the results of which may have been affected by any number of confounding variables. In an attempt to elucidate mercury’s effects on this vital response pathway, this project studied different aspects of the stress response in a controlled laboratory setting. Australian zebra finches (Taeniopygia guttata) were divided into five, sub-lethal dosage treatment groups and both corticosterone and its receptor were studied across the assigned treatment groups using various molecular techniques. The Australian zebra finch is a widely used songbird model, and thus the results of this study not only have implications for wild populations of songbirds exposed to excess mercury, but also will contribute to a growing body of knowledge on the basic science of this important research species.
1.2 Review of the literature

1.2.1 The endocrine system and corticosterone

The endocrine system controls a variety of hormones that, in turn, control numerous processes within the body. Processes under endocrine control range from reproduction to thirst and hunger. One key function of the endocrine system is to mediate an organism’s response to stressors. A stressor can be any physical, behavioral, or social factor that disrupts an organism’s internal balance or homeostasis (Sapolsky et al. 2000). Homeostasis is restored through activation of a stress response pathway that proceeds through the Hypothalamic-Adrenal-Pituitary (HPA) axis. This stress response pathway originates in the hypothalamus and anterior pituitary of the brain, involves a cascade of signaling and effector molecules such as adrenocorticotropic hormone (ACTH), and ends, in birds, with the release of the glucocorticoid hormone corticosterone from adrenal cortical cells in the adrenal glands (Wingfield et al. 2008). Corticosterone, a hydrophobic steroid hormone, is able to pass freely through cellular membranes and exert its effects on cells expressing the proper intracellular receptors for it. The release of glucocorticoids activates an emergency life history stage that promotes coping and survival in the presence of a stressor (Romero and Romero 2002, Wingfield et al. 1997, Wingfield et al. 1998, Wingfield et al. 2008).

Emergency life history stages activated by corticosterone are characterized by suppression of non-vital processes such as reproduction, as well as increased activity and glucose catabolism (Remage-Healey and Romero 2001, Wingfield et al. 1998, Wingfield
et al. 2008). Levels of corticosterone will increase in the bloodstream as long as an organism continues to receive stimuli from the perceived threat. Levels will remain elevated as the organism attempts to cope with a stressor, but will fall back to baseline levels once the stressor has been removed, overwhelming negative feedback is received, or the organism has reached the point of “adrenal exhaustion” (Schoech et al. 2009). Corticosterone feeds back negatively on the hypothalamic region of the brain, so once the stressor no longer stimulates the stress response pathway, elevated corticosterone levels end the production of additional corticosterone hormone. It is important that corticosterone concentrations return to baseline levels as soon as the organism no longer perceives a stressor because continuously high circulating levels of corticosterone can have deleterious effects on the organism (Dickens et al. 2009). Such reported effects of chronic stress include inhibition of the immune and reproductive systems, neuronal damage, and, in extreme cases, death (Sapolsky et al. 2000, Wingfield et al. 1998). Even during times when an unusual stressor is not present (i.e., when corticosterone is at a baseline level in the bloodstream), corticosterone is a key regulator of such everyday biological processes as glucose metabolism and tissue repair (Wingfield et al. 1998, Wingfield et al. 2008).

1.2.2 The glucocorticoid receptor

There are two receptors commonly associated with corticosteroid reception by the cell, the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR), which are closely related to other hormone receptors such as the estrogen and androgen receptors
These two receptors are both intracellular, and bind corticosterone with different affinities (Schmidt et al. 2010). The mineralocorticoid receptor regulates basal fluctuations in corticosterone and the sensitivity of feedback to the hypothalamus, while the glucocorticoid receptor regulates the changes that occur within an organism in response to stress, as well as the return to homeostasis that follows (de Kloet 1991). Corticosterone, because it is a lipid-soluble steroid hormone, easily crosses the cellular membrane and binds to either of these cellular receptors. Ligand-binding events change the conformation of the intracellular corticosteroid receptors, allowing them to dissociate from chaperone proteins and be transported to the nucleus by exposure of a nuclear localization signal (Caamano et al. 1998). Within the nucleus, glucocorticoid or mineralocorticoid receptors can act as transcription factors that affect gene expression (Schmidt et al. 2010). There has been little research on the downstream targets of ligand-activated corticosterone receptors in birds, but studies in mammals have revealed neural and immune genetic targets of activated corticosteroid receptors. These genes that are regulated indirectly by corticosterone levels are associated with the hippocampal neuronal and anti-inflammatory response to stress (Chatterjee and Sikdar 2014, Kassel et al. 2001). Regulation of the entire pathway is key, as alterations to any part can have a number of effects on the organism’s fitness.

Chronic stress has been shown to alter levels of corticosterone, as well as the feedback pathway in which it participates in birds (Rich and Romero 2005). This alteration of corticosterone in response to chronic stress, in turn, has been shown to alter receptor expression in birds (Dickens et al. 2009). Dickens et al. showed that chronically stressed European starlings (Sturnus vulgaris) showed lower mRNA expression of both
the glucocorticoid and mineralocorticoid receptors, suggesting feedback on the stress response pathway causing down-regulation of receptors in response to chronically elevated levels of corticosterone (Dickens et al. 2009). Hodgson et al. found this same decrease in mineralocorticoid receptor mRNA expression in response to abnormally high levels of corticosterone in Australian zebra finches (*Taeniopygia guttata*), but no decrease in glucocorticoid receptor expression (Hodgson et al. 2007). The birds used in the research by Hodgson et al., which were bred to respond to acute stressors with abnormally high plasma levels of corticosterone, also were impaired in their performance on spatial tasks in comparison to birds that responded with normal elevation of corticosterone levels in response to an acute stressor (Hodgson et al. 2007). This supports the notion that corticosterone and the stress response pathway play a role in neural development, specifically in spatial learning.

1.2.3 Sources of mercury in the food chain

Mercury is initially released into the atmosphere as either a byproduct of natural processes such as volcanic activity and wildfires, or through anthropogenic activities such as mining and coal combustion (Pacyna and Pacyna 2006, Shroeder and Munthe 1998, Spiegel and Veiga 2010). Small-scale gold mining alone can release up to 1000 tons of excess mercury into the environment either through atmospheric routes or discharge into water systems, if the proper precautions are not taken (Spiegel and Veiga 2010). In general, industrial sources release three to four times the amount of mercury into the environment as natural (preindustrial) sources (Jeremiason et al. 2006).
Mercury, as a metal, is unusual in that it exists readily in ambient air in the vapor phase, whereas most other metals exist as solids (Shroeder and Munthe 1998). This raises concern as mercury in the air is much more mobile, with the elemental form able to travel distances on the order of tens of kilometers or more in the air, and can persist in the atmosphere for a year or more once released (Shroeder and Munthe 1998). Once elemental mercury (oxidation state 0) has been deposited into an aquatic system, either by wet deposition or dry deposition of particulate mercury, it can be methylated by sulfate-reducing bacteria resulting in the more biologically toxic form, methylmercury (Jeremiason et al. 2006, Shroeder and Munthe 1998). Methylation of mercury by sulfate-reducing bacteria during anaerobic respiration generally occurs under anoxic conditions in sediments and wetlands (Gilmour et al. 1992, Jeremiason et al. 2006). Once in the environment in this more biologically active form, methylmercury can bioaccumulate and biomagnify up to one million times original concentrations in organisms throughout the food chain (Shroeder and Munthe 1998). Methylmercury causes particular concern for exposed organisms because it has the ability to affect a wide range of systems: mercury readily crosses the blood-brain barrier and there exerts its effects as a neurotoxin (Gilmour et al. 1992, Shroeder and Munthe 1998).

1.2.4 Mercury’s widespread effects on animals other than birds

Methylmercury harms organisms throughout an ecosystem in a variety of ways. Specifically, methylmercury disrupts cell function in a few key ways: it interferes with calcium homeostasis, induces oxidative stress, and interacts with sulfhydryl groups to
form thiol-containing compounds, disrupting or preventing the activity of key molecules such as glutathione (Atchison and Hare 1994, Ceccatelli et al. 2010, Clarkson 1972, Sarafian and Verity 1991). On a larger scale, this cellular disruption due to methylmercury exposure has widespread effects ranging from neurotoxicity to changes in reproductive success (Ceccatelli et al. 2010, Hopkins et al. 2013). Specifically, a number of studies have shown that methylmercury acts as a neurotoxin by increasing the rate of caspase-dependent apoptosis in neural cells (Castoldi et al. 2000, Charleston 1996, Nagashima 1996). This neurotoxic activity, among other things, makes methylmercury exposure of particular concern for developing fetuses, as mercury can easily cross the placental barrier from mother to fetus (Lauwerys et al. 1978).

Mercury also disrupts the endocrine system in a variety of species. As mentioned above, mercury affects reproduction, which is controlled largely by hormones regulated by the endocrine system. In a study by Hopkins et al. methylmercury exposure decreased the success of hatching in snapping turtles (Chelydra serpentine) by both increasing egg infertility and embryonic mortality (Hopkins et al. 2013). Methylmercury was also shown to affect reproductive function by decreasing adrenal, testicular, and epididymal function in rats (Burton and Meikle 1980, Friedman et al. 1998).

Besides controlling reproduction, the endocrine system also controls an organism’s stress response. Methylmercury exposure has been shown in a number of studies to affect cortisol concentrations in a variety of fish species. Hontela et al. found that yellow perch (Perca flavescens) and northern pike (Esox lucius) at mercury-contaminated sites were unable to mount a cortisol stress response in response to acute stress (i.e. increase their plasma levels of cortisol) compared to fish at reference sites.
These fish were also found to have atrophied corticotropes, or cortisol-producing cells (Hontela et al. 1992). Another study in rainbow trout found that fish exposed to mercury had trouble with steroidogenesis, or the synthesis of the cortisol hormone itself (Leblond and Hontela 1999). Mercury exposure also affects the other side of the stress response pathway by changing the binding affinity of the stress hormone (cortisol/corticosterone) receptor, the glucocorticoid receptor. A study carried out in rats showed that mercury exposure decreased glucocorticoid receptor binding abilities due to the formation of complexes of mercury with thiol groups present on the receptor (Brkljacic et al. 2004). The formation of these complexes changes the receptor’s structure and lowers its affinity for its substrate, cortisol. Another rat study showed that mercury exposure increased the association of the glucocorticoid receptor with certain heat shock proteins (Brkljacic et al. 2007). Heat shock proteins act as chaperones allowing proteins to maintain their normal functions in suboptimal cellular conditions. The association of the glucocorticoid receptor with heat shock proteins in response to mercury suggests that the protein’s function is put at risk by the exposure (Brkljacic et al. 2007). The effects of mercury exposure on the glucocorticoid receptor has been studied in mammals, but few, if any, studies have investigated the effect of mercury exposure on the glucocorticoid receptor in birds.

1.2.5 Mercury’s effect on corticosterone in birds

A limited number of studies have addressed the potential effect of mercury exposure on the avian stress response. The outcomes of these studies provide inconsistent
results and highlight the need for additional research. One study of environmentally exposed Forster’s tern (\textit{Sterna forsteri}) chicks found a negative correlation between baseline plasma corticosterone concentrations and blood mercury concentrations, possibly due to the down regulation of the HPA axis in response to a chronic mercury stressor (Herring et al. 2012, Rich and Romero 2005). A similar negative correlation between baseline corticosterone concentration and blood mercury concentration was found in both adult and nestling tree swallows (\textit{Tachycineta bicolor}) exposed to environmental mercury (Franceschini et al. 2009). There was, however, no relationship between the induced stress response and blood mercury levels in these tree swallows. In contrast, a study of nestling tree swallows (13-17 days old) at a more highly contaminated site found that circulating baseline corticosterone concentrations were elevated in mercury-exposed birds compared to unexposed reference birds, and that the induced stress response was depressed (Wada et al. 2009). All of these studies were field studies and thus were only correlational, as exposure was dependent upon environmental factors. These studies were also subject to a number of confounding variables, as field studies do not allow for the control of outside variables. There has been one previous attempt to experimentally manipulate mercury exposure to determine the effects on fecal corticosterone (which may be analogous to baseline plasma concentration) (Adams et al. 2009). In that study, captive white ibises were fed 0, 0.05, 0.1 or 0.3 µg/g Hg (wet weight) beginning at 90 days of age. Approximately two months later, only the highest-dosed birds had elevated baseline corticosterone, whereas after another five months all three doses tended to have elevated corticosterone relative to controls, possibly due to the chronic stress of longer term exposure to mercury. Though the results of this study
provide a basis for comparison with baseline levels, they tell us little about the effects of exposure to methylmercury on the avian stress response.
II. Materials and Methods

2.1 Sample collection

2.1.1 Animal housing and care

Captive-bred zebra finches were housed indoors under long days to stimulate breeding (14h light: 10h dark) on the campus of the College of William and Mary in Williamsburg, Virginia, USA. Unrelated finches that had been exposed to mercury through natural maternal deposition in the egg and throughout their lives were randomly paired within their life-long treatment group (same as parental treatment group), 0.0, 0.3, 0.6, 1.2 or 2.4 µg/g (wet weight) dietary methylmercury, and allowed to breed. Breeding stages were recorded daily and nestlings resulting from these pairs were banded at 10 days old. Each treatment group contained 18 original pairs (for a total of 90 pairs) and identical numbers of birds of multiple treatments were randomly assigned to each of the four housing rooms. Each zebra finch pair was housed in a wire cage (45 height x 45 width x 75 length cm) containing two wooden perches, a nest box with hay for nest building, *ad libitum* nutritionally complete food (ZuPreem, Shawnee, Kansas, USA), water, digestive grit, and a cuttlebone for calcium. Finches were given fresh food daily containing the designated concentration of dietary methylmercury dissolved in water and mixed with the absorbent food pellets. Each batch of finch food was tested to ensure that it was within 10% of the nominal dosage (wet weight). Because the dosed food contained 13.9% moisture when tested and consumed, the equivalent dry weight dosages of
methylmercury were 0.00, 0.35, 0.70, 1.39 and 2.78 µg/g Hg. Water was changed daily and contained 200 µL soluble vitamins (Vita-Sol UltraVite, 8 in 1) per 300 mL.

2.1.2 Blood collection for analysis of mercury concentration

For the corticosterone study, the blood of all adult birds was collected monthly to assess blood mercury concentration. Approximately 20-40 µL of blood was collected from each bird in a 70 µL heparinized capillary tube after pricking the brachial vein with a 30-gauge needle (Becton-Dickenson, Franklin Lakes, NJ). For the glucocorticoid receptor study, blood samples were taken as described above from the offspring of the original 90 pairs at 10 days when they were banded, and at approximately 50 days when they were sacrificed. All blood samples were frozen at -20 °C until analysis on a wet weight basis by atomic absorption spectroscopy using a Direct Mercury Analyzer-80 (DMA, Milestone, Shelton CT). The DMA-80 was calibrated before the experiment and re-calibrated as necessary to keep standard reference materials within 7.5% of the manufacturer-provided values. A blank, an empty sample container, a duplicate, and two aliquots of each standard reference material (DORM-3 and DOLT-4) were run with every 20 samples. Recovery of total mercury was 96.05% ± 0.65% for DORM-3 (n=21) and 98.48% ± 0.23% for DOLT-4 (n=21) for the birds whose blood mercury values were compared to measured corticosterone concentrations. All other quality control measures were also within acceptable limits.

2.1.3 Blood collection for analysis of corticosterone concentration
Adult zebra finches were sampled for corticosterone beginning at 10:00 AM on each day of collection to control for the natural fluctuation of corticosterone levels throughout the day. Collection of samples from all adult birds (from the original 90 pairs) occurred over approximately one month (11 June-20 July, 2012). Because a researcher entering a room could cause a spike of corticosterone within a few minutes, each room was entered only once per day for the purpose of collecting samples. All daily animal care was performed after corticosterone sampling was completed. Both members of each pair were sampled at the same time, and pairs were randomly chosen from each of the four housing rooms each day until all pairs had been sampled. Baseline samples were taken within three minutes of the first researcher entering a given room. Approximately 35 µL of blood was collected as described above for mercury analysis, except that the blood was immediately stored on ice for later centrifugation. After the baseline sample was taken, each bird was placed into an opaque cloth bag to produce standardized capture stress. Exactly 30 minutes after first entering the room, the brachial vein on the opposite wing was punctured to obtain another 35 µL sample in the same manner as the first. Immediately after completing collection each day blood samples were spun on a microcentrifuge at 10,000 rpm for three minutes to separate the plasma. The plasma supernatant was then drawn off with a pipette, placed into a pre-labeled 1.5 mL eppendorf tube, and stored at -80 °C until assayed for corticosterone.

2.1.4 Brain dissection and storage
Brains were collected from approximately 50-day old fledglings from all treatment groups to be analyzed by quantitative polymerase chain reaction (qPCR). The birds were sacrificed by rapid decapitation with large, sharp surgical scissors. Immediately after removal of the head the brain was dissected so as to preserve the integrity of the RNA. The skin covering the head was peeled back to reveal the skull. The neck muscles at the base of the skull were severed with fine dissecting scissors and the skull was cut up the midline with scissors pointed upwards so as not to damage the brain underneath. The skull was cut up the midline from the base to the very top of the skull right above the eye line. A cut was then made sideways from the base of the skull to the ear cavity on both sides of the skull. The halves of the skull could then be peeled away from the brain underneath with forceps. The dura, or membrane covering the brain, generally peeled away from the brain with the skull, but in cases where it did not, fine forceps were used to gently pull the dura off the brain tissue without damaging the latter. The bottom sections of the skull below the sideways cuts towards the ear cavities were also pulled down to expose the cerebellum. The brain was gently pushed away from the skull cavity with forceps, and fine scissors were used to sever the optic nerve and all other smaller connective nerves. The brain was then free to be pushed gently out of the skull with forceps and into a pre-labeled 15 mL Falcon tube (BD Falcon). The Falcon tube was immediately placed in liquid nitrogen and all tubes were kept in boxes at -80 °C until RNA could be extracted for qPCR.

2.1.5 Embryo dissection and storage
Previously dissected embryos were used for whole mount in situ hybridization analysis of glucocorticoid receptor expression. Eggs at the appropriate stage were cut longitudinally and the contents were allowed to flow out onto a clean glass dish. Under a dissecting microscope (Olympus SZ61), the embryo was separated from all embryonic membranes and either put into a glass vial of 4% paraformaldehyde in PBS buffer at 4 °C, or put into an empty, pre-labeled 1.5 mL eppendorf tube and then flash frozen in liquid nitrogen. Embryos to be analyzed by quantitative PCR were flash frozen and then kept at -80 °C until analysis. Embryos to be analyzed by in situ hybridization, after fixing for 24 hours in 4% PFA at 4 °C, were dehydrated using a methanol series (100% 1xPBS, 75/25 1xPBS/MeOH, 50/50 1xPBS/MeOH, 25/75 1xPBS/MeOH, 100% MeOH). These embryos were then stored at -20 °C in methanol until processing.

2.2 Radioimmunoassay for plasma corticosterone concentrations

2.2.1 Validation of assay for use in zebra finch

The Double Antibody Corticosterone 125-I RIA Kit (MP Biomedicals, LLC) was validated for use in zebra finches. A standard curve was created using the logit transformation of the B/Bo for the standards versus the log concentration of the standards (range: 25 ng/ml to1000 ng/ml). The assay kit was validated for use with both zebra finch and starling (another species of songbird, Sturnus vulgaris) plasma by demonstrating that the slope of the logit/log transformed serial dilution of authentic corticosterone (Sigma-Aldrich; chromatographed and recrystallized), brought up in a pool of plasma from the
appropriate undisturbed bird species, was not significantly different from the standard curve. Appropriate dilutions of baseline and stressed zebra finch samples were determined to be 10 µL sample: 220 µL steroid diluent and 10 µL sample: 500 µL steroid diluent.

2.2.2 Corticosterone radioimmunoassay

Serum corticosterone was measured in 10 µL heparinized plasma diluted to the concentrations determined above with the Double Antibody Corticosterone 125-I RIA Kit (MP Biomedicals, LLC). The kit contained enough reagents for 100 tubes and 48 or 52 tubes were run in each assay (baseline and stressed samples from seven or eight birds, respectively, run in duplicate). Standards, controls, and samples were set up according to the manufacturer’s protocol. 200 µL I$^{125}$ radioactively labeled corticosterone reagent was added to each tube followed by 200 µL anti-corticosterone to all tubes except the non-specific binding tubes. Tubes were incubated for two hours and then precipitation solution was added to each tube to precipitate out corticosterone bound to the antibody. The tubes were centrifuged at 2500 rpm for 15 minutes and the supernatant was disposed of. The pellet was re-suspended in scintillation fluid and the samples were analyzed for radioactive decay using a scintillation counter (Beckman Coulter, Fullerton California). Amount of radioactive decay provided a direct measure of the amount of bound synthetic corticosterone, and an indirect measure of actual corticosterone bound from the original plasma sample. A total of 14 assays were performed. The kit high and low controls (78±15ng and 554±114ng), were all from the same lot (RCBC 1101L), and were run in
every assay to provide a measurement of between-assay variability. Intra-assay variation was calculated as the average coefficient of variation between duplicates with readable values across all assays and was 14.8%. Inter-assay variation was calculated as the coefficient of variation for the high control (provided by the kit manufacturer) for each assay and was 12.2%.

2.2.3 Minimum detection limit assay

The minimum detection limit for this assay in zebra finches was determined using a corticosterone RIA wherein a charcoal-stripped zebra finch plasma pool was spiked with a serial dilution (25.0 ng/ml to 2.5 ng/ml) of authentic corticosterone. The lowest spiked RIA sample that was demonstrated as different from the zero standard provided by the kit manufacturers was the 5.0 ng/ml duplicate sample. A more conservative estimate of the limit of detection, which was the value obtained by subtracting two times the standard deviation of the zero standard duplicates from the mean value of the duplicates (limit of detection=5.7 ng/ml), was used in analyzing corticosterone data. All corticosterone concentration values obtained from all assays that fell below this minimum limit of detection were assigned the 5.7 ng/ml value.

2.3 Quantitative polymerase chain reaction (qPCR)

2.3.1 Primer design
Primers for qRT-PCR were designed specifically by Life Technologies to match the sequence of either the glucocorticoid receptor or control gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), mRNA. Primers were designed to contain approximately 50% GC content, have a theoretical annealing temperature around 60 °C, and be approximately 20 base pairs long. Target gene size was selected to range from 100-200 bp. Primer sets were ordered as part of the Taqman Gene Expression Assay Kit and were stored at – 20 °C until use.

2.3.2 RNA extraction and purification from flash-frozen juvenile brains

Total RNA from zebra finch brains was extracted using MagMax-96 for Microarrays as outlined in manufacturer’s protocol (Ambion). Brains were removed from -80 °C and placed in liquid nitrogen. Large glass homogenizers were filled with 5 mL Tri-Reagent (Sigma-Aldrich), and each brain was quickly transferred to a glass homogenizer and homogenized fully to allow Tri-Reagent to fully inactivate RNAses before moving to the next sample. After a brain was fully homogenized, the 5 mL homogenate was transferred to a 5 mL eppendorf tube, and then 1200 µL of the mixture was transferred to each of four Lysis Matrix H tubes (MP Biomedicals). These samples were homogenized with an Omni Bead Ruptor Homogenizer (OMNI International) on a 20 second cycle. The homogenate from the Lysis Matrix H tubes was then transferred to fresh 1.5 mL eppendorf tubes pre-filled with 240 µL 1-bromo-3-chloropropane (BCP) phase separating reagent (Molecular Research Center, Inc.). Samples were thoroughly vortexed for 30 seconds and then placed on ice for five minutes. The samples were
inverted periodically throughout the incubation to maintain homogeneity of the mixture. The samples were then centrifuged at 10, 500 rpm at 4 °C for 10 minutes to separate the organic and aqueous phases.

The MagMax plate was prepared by adding 100 µL of the aqueous phase from each sample into row A. To each sample in row A, 50 µL isopropanol was added, and then 10 µL RNA Binding Beads were added to the surface of the mixture. Rows B and C of the plate were filled with 150 µL of Wash Solution #2, and row D was filled with 30 µL Elution Buffer (both provided with the MagMax kit). The AM1839 Spin Program was selected on the MagMax system (Applied Biosystems) to elute purified RNA. To further purify the samples, the eluted RNA in row D was transferred to fresh, pre-labeled eppendorf tubes and precipitated by the addition of 1/10 volume sodium acetate (NaOAc) and three volumes of cold ethanol. Samples were stored overnight at -80 °C. The following day, RNA was centrifuged at 4 °C at 14,000 rpm for 30 minutes to pellet precipitated RNA. The pellet was washed once with 1 mL 70% ethanol and centrifuged again for 5 minutes at room temperature. Samples were dried using a speedvac at low heat for 1-2 minutes until no residual ethanol remained. Samples were re-suspended in 20 µL of nuclease-free water. Yield and purity were assessed using a Nanodrop ND 1000 spectrophotometer according to the manufacturer’s protocol (Nanodrop). This RNA was then further purified using the Turbo DNA-free DNase system. RNA was combined with buffer, RNAsin, and DNase and allowed to incubate at 37 °C for 20 minutes on a heating block. After 20 minutes 1/10 of the total volume of inactivation reagent was added and the mixture was incubated at room temperature for five minutes with periodic flicking of the tube to keep the mixture homogenous. The samples were then spun on a
microcentrifuge at room temperature at 13,200 rpm for 1.5 minutes. The clear supernatant was drawn off and purity of the RNA was assessed again using the Nanodrop system. 1 µL of purified RNA was also run on a 0.75% agarose gel (50 mL Tris-Acetate EDTA (TAE): 0.35 g agarose: 2.5 µL ethidium bromide) to additionally assess purity. After heating the gel mixture to create a homogenous solution, the gel was allowed to solidify. After solidification the gel was covered in 1x TAE solution, and run for 20-40 minutes at 170 V with a maximum current of 500 milliamps. The first lane of the gel was loaded with 10 µL of 1 kb+ ladder (Invitrogen), which was used to create a length standard, and 2 µL 6X DNA dye. The remaining lanes were loaded with 1 µl of the purified RNA, 9 µL of nuclease-free water, and 2 µL of 6X DNA dye for each sample. Gels were imaged using ultraviolet transillumination in a FluorChem light box (Alpha Innotech) and photographed and annotated using FluorChem HD2 software. Purified RNA was stored at -80 °C for cDNA synthesis by RT-PCR.

2.3.3 RNA extraction from flash-frozen embryos

Zebra finch embryos flash frozen and stored at –80 °C were removed from storage and placed in liquid nitrogen. Whole embryos were then quickly transferred from the tube in liquid nitrogen to a Lysis Matrix H tube on ice filled with 1200 µL of Tri-Reagent. The Lysis Matrix H tubes were loaded into an Omni Bead Ruptor Homogenizer (OMNI International) on a 20 second cycle to homogenize the whole embryo and extract RNA. All homogenate was transferred to fresh, pre-labeled 1.5 mL eppendorf tubes containing 240 µL BCP as described above for zebra finch brain RNA extraction. The
embryo RNA extraction and purification processes from this point were identical to the brain RNA extraction and purification processes described in the previous section.

2.3.4 cDNA synthesis

cDNA was synthesized using the iScript cDNA synthesis kit (BioRad). Each reaction consisted of 5 µL 5X iScript RT Supermix (reverse transcriptase included), 1 µg extracted RNA (volume depending on concentration of purified RNA from Nanodrop), and nuclease-free water up to 20 µL. Reactions were run with the following cycle on a GeneAmp PCR System 9700 thermocycler (Applied Biosystems): 5 minutes at 25 °C, 60 minutes at 42 °C, 5 minutes at 85 °C, and a final hold at 4 °C. Once the cycle had been completed, aliquots containing 4 µL of cDNA were made and stored at -80 °C for use in qPCR.

2.3.5 qPCR

Quantitative PCR was performed using StepOne v2.3 machine and software. A quantitation-comparative experiment was selected in the software setup, with standard analysis speed and the Taqman reagents used. For each experiment glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was the endogenous control and was run in parallel with every sample being analyzed for glucocorticoid receptor expression. cDNA from a control dose bird was run in every assay and served as a reference sample. All samples were run in triplicate. Each well contained 1 µL 20x Taqman Gene Expression Assay
(primer set for either glucocorticoid receptor or GAPDH), 10 µL Taqman Gene Expression Master Mix, 8 µL nuclease-free water, and 1 µL of either cDNA or nuclease-free water for negative controls. Total well volume was 20 µL. 4 µL cDNA aliquots were diluted before 1 µL cDNA was added to the qPCR plate wells. Aliquots were diluted by addition of 4 µL nuclease-free water for a concentration ½ of the original. Diluted cDNA aliquots were used because initial results from calibrating qPCR runs showed that ½ diluted cDNA (final concentration of 100 ng) allowed for amplification of the target genes during the appropriate cycle range. cDNA concentration was adjusted to allow amplification around the 15th-20th amplification cycle, as the StepOne software removes any earlier amplification as background in the analysis of gene expression. Analysis of gene expression was performed using DataAssist software from Life Technologies.

2.4 Whole-mount in situ hybridization

2.4.1 PCR of embryo cDNA

Glucocorticoid receptor cDNA was amplified using primers designed in Primer3Plus as described above for qPCR, except that target gene length was set to approximately 1000 base pairs. The reaction mix was composed of 4 µL dNTP mix, 5 µL 10X buffer, 2.5 µL each of forward and reverse primers, 1 µL cDNA, 0.3 µL SuperTaq polymerase, and 34.7 µL nuclease free water. The reaction was run with the following cycle on a GeneAmp PCR System 9700 thermocycler (Applied Biosystems): 2 minutes at 94 °C; 35 cycles of 30 seconds at 94 °C, 30 seconds at 60 °C and 3 minutes at 68 °C; 7
minutes at 68 °C; and a final cool down to 4 °C. RT-PCR products were run on a 1% agarose gel (50 mL TAE: 0.5 agarose: 2.5 µL ethidium bromide). The gel was visualized as described above for RNA extraction and purification.

2.4.2 Gel extraction of glucocorticoid receptor cDNA

PCR product of the appropriate length was run and then extracted from a 0.8% agarose gel to avoid contamination with unwanted sequences possibly amplified by the primer set during PCR. The 0.8% agarose gel was prepared as described above but with 0.4 g agarose to 50 mL TAE. 25 µL of the RT-PCR product was run with 3 µL 6X DNA dye against the 1 kb+ ladder as described above. A transilluminator was used to visualize the bands after the gel had run for approximately 30 minutes. The band at the appropriate length for the glucocorticoid receptor (approximately 1.2 kb) was cut out of the gel using a scalpel and weighed in a pre-weighed 1.5 mL eppendorf tube. cDNA was extracted from the gel using a Qiaquick Gel Extraction Kit (Qiagen) following the manufacturer’s instructions with the provided reagents. Yield and purity of the purified cDNA were assessed using the Nanodrop ND 1000 spectrophometer according to the manufacturer’s protocol (Nanodrop).

2.4.3 Cloning of glucocorticoid receptor cDNA

Ligation reaction containing 1 µL Strataclone vector mix, 2 µL PCR product (purified from the gel as described above), and 3 µL Strataclone cloning buffer was
allowed to incubate at room temperature for five minutes before transformation. During the incubation one tube of Strataclone SoloPack competent cells were thawed. 1 µL of ligation reaction mixture was added to the tube of thawed competent cells and incubated on ice for 20 minutes. After 20 minutes the reaction mixture was heat shocked for 45 seconds at 42 °C and then incubated on ice again for two minutes. After the incubation period, 250 µL of Luria broth medium was added to the transformation reaction and the cells were allowed to recover for at least two hours at 37 °C with agitation. After two hours the reaction mixture was plated with a glass spreader on two Luria broth (LB)-ampicillin plates spread with 40 µL of X-gal. The reaction mixture was plated in the amounts of 100 µL pure reaction mixture and 5 µL pure reaction mixture in 45 µL Luria broth medium, respectively. Plates were incubated at 37 °C for 16-18 hours to allow colony growth. Once colonies had grown, four colonies showing insertion of the target gene (white colonies) were chosen for analysis using the CloneChecker System (Life Technologies). The PCR reaction mixture was prepared following the manufacturer’s instruction using the reagents provided. The reaction mixture was run with the following cycle on a GeneAmp PCR System 9700 thermocycler (Applied Biosystems): 30 seconds at 100 °C, 6 minutes at 25 °C, 10 minutes at 37 °C. During the 6 minutes at 25 °C the machine was stopped and 1 µL of buffer H and 1 µL of EcoR1 were added to the reaction mixture. The machine was then allowed to continue running. Once the machine had finished running the products were analyzed for purity using gel electrophoresis as described above for RT-PCR. Pure samples that had been appropriately transformed gave bands at the expected length for the insert (1.2 kb for the glucocorticoid receptor).
After verification of the insertion of the gene of interest, host cultures containing plasmids with the inserted gene (white colonies on initial culture plate) were grown for 12-20 hours in a shaking incubator at 37 °C and 250 rpm. These cultures contained Luria broth medium (150 mL) inoculated with 150 µL of 50 µg/mL ampicillin and 3 µL of a solution containing re-suspended, transformed colonies in Luria broth medium. Plasmids were isolated from the host using the PureYield Plasmid Midiprep System (Promega) according to the manufacturer’s directions for a bacterial culture volume of 101-250 mL. Cultures were pelleted by centrifugation for 10 minutes at 4 °C at 5500 rpm using a Fiberlite F14 rotor. The supernatant was removed and cells were re-suspended by pipetting up and down with a sterile transfer pipette in 6 mL cell re-suspension solution. 6 mL cell lysis solution was then added and samples were inverted to mix and incubated at room temperature for three minutes. 10 mL neutralization solution was added and the mixture was inverted 5-10 times. Solution was centrifuged at 4 °C at 7700 rpm for 15 minutes using the HB6 rotor.

A column stack was created with a blue PureYield Clearing column on top of a PureYield Binding column and the two connected columns were placed on a vacuum manifold. Supernatant from the centrifuged solution was then passed through both of the columns. The clearing column was removed and to the binding column on the vacuum, 5 mL of Endotoxin Removal Wash and then 20 mL of Column Wash Solution were added. The column was dried for 30-60 seconds by allowing the vacuum to pull all moisture through the column membrane. The eluator vacuum elution device was assembled on the vacuum manifold according to the manufacturer’s protocol and samples were eluted in 600 uL of nuclease-free water. The yield of plasmid DNA was analyzed using a
Nanodrop ND 1000 spectrophotometer (Nanodrop) as described in the above procedures. Purified DNA samples were submitted for analysis of insert orientation by sequencing. The mixture submitted for sequencing contained 0.75 µL plasmid DNA, 6.93 µL nuclease-free water, and 0.32 µL forward or reverse M13 primers (correspond to promoter sites surrounding the insert site on the Strataclone vector plasmid).

2.4.4 Linearization

Plasmids were linearized for in situ hybridization RNA probe synthesis. Restriction digests were set up containing 20 µg DNA, 2 µL of the appropriate restriction enzyme (20 U/µl, Promega; HindIII for sense probe, BamHI for antisense probe), 10 µL 1x restriction buffer (Promega), and nuclease-free water up to 100 µL. Digests were incubated for 2-3 hours at 37 °C with an additional 1 µL of enzyme added after 60 minutes of incubation. DNA was extracted with an equal volume of phenol/chloroform: isoamyl alcohol (25:24:1) and chloroform: isoamyl alcohol (24:1). To precipitate linearized DNA, 1/10 volume 3M sodium acetate (NaOAc) and two volumes of cold 100% ethanol were added before freezing at -80 °C for at least 30 minutes (the linearization solutions were often left overnight to aide in time management). Precipitated DNA was collected by centrifugation at 4 °C for 20 minutes at 14,000 rpm. Pellets were washed in 70% ethanol and resuspended in 20 µL 1x TE. Linearized plasmids were analyzed on 1% agarose gel by combining 1µl of linearized plasmid, 2 µl 6X DNA dye, and 9 µl sdd H₂O as described above for analysis of purity and yield by gel electrophoresis.
2.4.5 RNA probe synthesis for *in situ* hybridization

Both antisense and sense probes were synthesized for *in situ* hybridization analysis of glucocorticoid receptor expression. A 51.5 µL transcription reaction containing 10 µL 5x transcription buffer (Promega), 5 µL 0.1 M dithiothreitol (DTT), 3.75 µL of each of 10 mM rCTP, rATP, and rGTP, 2.45 µL 10 mM rUTP (Promega), 1.3 µL 10 mM labeled rUTP (dig-11 UTP, Roche), 4 µg linearized DNA (dependent upon Nanodrop concentrations), 0.5 µL RNAsin (20 U/µl, Promega), 3 µL of the appropriate RNA polymerase (20 U/µl, Promega; T7 for sense probe, T3 for antisense probe), and nuclease-free water up to the final volume was combined in 1.5 mL eppendorf tube. Reactions were incubated at 37 °C for 2 hours, with an additional 1 µL of enzyme added after the first hour. To digest the DNA template, 1 µL RQ1 DNase (1 U/µl, Promega) was added at the end of the second hour and the reaction was incubated at 37 °C for an additional 10 minutes. The integrity and yield of the transcription reaction was assessed by combining 2.5 µL transcription reaction, 7.5 µL nuclease-free water, and 2 µL 6X DNA dye and running the sample on a 1% agarose DNA gel as described previously.

After beginning the gel as described above, RNA probes were purified using lithium chloride (LiCl). 30 µL 7.5 M LiCl precipitation solution was added and the solution was placed at -20 °C for one hour. The probe was centrifuged at 4 °C for 25 minutes at 14,000 rpm and then washed with 70% ethanol. After the ethanol wash, samples were centrifuged for an additional five minutes at 4 °C at 14,000 rpm and the ethanol removed. Samples were then dried in a speed vacuum for 1-2 minutes or until all
residual ethanol had evaporated. The RNA probe pellet was re-suspended in 20 µL nuclease-free water. The integrity and yield of the transcription reaction was assessed by combining 1 µL transcription reaction, 9 µL nuclease-free water, and 2 µL 6X DNA dye and running the sample on a 1% agarose DNA gel as described above. The yield and purity were assessed using a Nanodrop ND 1000 spectrophotometer (Nanodrop). A 10x stock of RNA probe was made by adding an appropriate volume of ISH buffer to the concentrated probe solution. RNA probes were stored at -20 °C.

2.4.6 Whole mount *in situ* hybridization with zebra finch embryos

Whole mount *in situ* hybridization using the glucocorticoid receptor probe was performed on zebra finch embryos of various stages according the Cepko/Tabin core lab protocol for RNA whole mount *in situ* hybridization, with the following modifications. On day two of the modified procedure, probe was removed from the embryo vials and conserved for later use (up to four uses for one probe stock; storage at -20 °C). The embryos were rinsed with pre-heated ISH buffer four times for five minutes each at 60-65 °C in a hot water bath with gentle shaking. The ISH buffer was discarded in the appropriate hazardous liquid trash and wash solution 1 (20 mL formamide, 2 mL 20x SSC, 40 µL Tween-20, 17.96 mL RNA-free water) was added to the vials. Embryos were washed with this solution twice for thirty minutes each at 60-65 °C in a hot water bath with gentle shaking. The wash solution was discarded and replaced with 1:1 wash solution 1: MABT (maleic acid buffer with Tween-20 added). The embryos were then washed for 10 minutes at 60-65 °C in a hot water bath with gentle shaking. This solution
was removed and the embryos were washed in MABT for at room temperature for one hour with vertical nutation. After the MABT wash the embryos were washed with a series of the following blocking solutions: MABT + 2% BMB (Boehringer Mannheim blocking reagent), MABT + 2% BMB + 20% heat-inactivated goat serum, MABT + 2% BMB + 20% heat-inactivated goat serum + 1/2000 anti-dig-AP antibody. All washes were performed with vertical nutation and the wash times were one hour, two hours and overnight for the three washes, respectively. The first two washes were performed at room temperature, while the third, overnight incubation was performed at 4 °C. The antibody solution was prepared using zebra finch chick powder as an additional blocking agent. Days three and four of the Cepko/Tabin protocol were combined in the modified protocol used by omitting the overnight incubation at the end of day three and going straight to day four washes. Throughout the procedure MABT was used in place of TBST (Tris-buffered saline solution with Tween-20).

Once the in situ hybridization was complete, embryos were imaged whole using a dissecting scope (Olympus SZX7). All images were taken using Olympus imaging software.

2.5 Statistical Analysis

2.5.1 Analysis of hormone data

Linear regression was used to examine the relationship between time and baseline corticosterone to ensure that time between disturbance (entering room) and completion of
blood collection was not a confounding factor. For data analysis, the monthly mercury blood sample that was closest in date (typically within one week) to that of the collection of the corticosterone blood sample from the same bird was used. In all cases blood mercury samples and corticosterone blood samples were collected less than three weeks apart. Baseline corticosterone and stress response (change in concentration from baseline to stressed levels) were analyzed two ways, using either treatment group or individual blood mercury levels. Analysis by treatment group is important because the experimental manipulation relied on randomly assigning subjects to one of five treatments. However, analysis by individual blood mercury proved to be informative as well because there was a large amount of variation in blood mercury levels within treatment groups due to individual differences among the finches, which was not necessarily reflected by the assigned treatment group. For both types of analyses a linear mixed model with a scaled identity covariance matrix was used to examine the effect of methylmercury on corticosterone concentration. Date of collection was classified as a random effect and sex, breeding stage (nesting or not nesting), and either dose or blood mercury level as fixed effects. These analyses were performed using SPSS Statistical Software (Version 11.0, Chicago, SPSS Inc.).

2.5.2 Analysis of receptor data

Analysis of qPCR data was completed using DataAssist (Life Technologies). The program was used to generate box plots of expression data across the five treatment groups for brain cDNA, and for the cDNA from whole embryos of various stages and
treatment groups. Expression of genes was measured using $C_T$ (cycle threshold) values, or
the cycle of amplification at which the target or control gene began to amplify (i.e. a
certain level of fluorescence was detected) above a threshold level. The $C_T$ value is
inversely proportional to the abundance of the target or control gene: a gene with a lower
$C_T$ value is relatively more abundant than a gene with a higher $C_T$ value. Relative
quantitation (RQ) values for each of the methylmercury treatment groups were calculated
based on the average expression of the glucocorticoid receptor in the control juvenile
birds for brain cDNA and based on the average expression in the youngest stage (stage
17) for whole embryo cDNA. P-values for relative quantitation numbers were obtained
by the DataAssist software using a standard t-test that compared differences in expression
between each of the four mercury treatment groups (0.3, 0.6, 1.2 and 2.4 µg/g Hg) and
the control group for juvenile brain cDNA, and differences in expression between the
youngest stage embryos and two older stages of embryos for whole embryo cDNA.
III. Results

3.1 Corticosterone results

The length of time between initial disturbance and sample collection did not significantly affect baseline corticosterone concentrations within the range of three minutes (Figure 1; $r^2 = 0.02$, $F_{1,95} = 1.53$, $p = 0.21$), therefore time between disturbance and collection was not included in further analyses.

Stress response (difference between baseline and stressed corticosterone concentration measured in the same bird 30 minutes later) decreased significantly with increased individual blood mercury levels (Figure 2a; $F_{1,86.06} = 3.88$, $p = 0.05$). Here, breeding stage was a significant factor with nesting birds having a greater increase in corticosterone after being captured ($F_{2,84.08} = 3.41$, $p = 0.038$), while sex was not ($F_{1,82.80} = 0.01$, $p = 0.93$). The average stress response observed in birds fed the three highest doses of dietary methylmercury appeared to be decreased compared to the control and lowest mercury dose (0.3 µg/g, ww) groups, but this difference was not statistically significant overall (Figure 2b; $F_{4,79.52} = 2.29$, $p = 0.07$). Breeding stage was again a significant factor, with actively nesting birds having a bigger increase in corticosterone after handling stress ($F_{2,82.95} = 7.58$, $p = 0.001$), but sex was not a significant factor ($F_{1,78.19} = 0.41$, $p = 0.52$).

Baseline corticosterone was not significantly related to individual mercury level across the full range of blood mercury concentrations (Figure 3a; $F_{1,83.22} = 1.77$, $p =
Breeding stage was a significant factor with nesting birds having a lower baseline level of corticosterone, \((F_{2,83.95} = 7.94, p = 0.001)\) but sex was not a significant factor \((F_{1,81.88} = 0.51, p = 0.48)\). Comparing treatment groups rather than individual blood levels, there was a suggestion that methylmercury exposure may have increased baseline corticosterone, as the average corticosterone levels of the birds in the mercury treatments tended to be higher than control birds, but this relationship was not statistically significant (Figure 3b; \(F_{4,83.54} = 1.76, p = 0.15\)). As with the individual blood concentration analysis, baseline corticosterone did vary significantly with breeding stage \((F_{2,82.98} = 3.96, p = 0.02)\), with nesting birds having lower corticosterone levels, whereas sex was not a significant factor \((F_{1,80.06} = 0.01, p = 0.93)\).
Figure 1. Lack of significant relationship between time of collection (sec) and baseline corticosterone concentrations. A strong response would indicate that 180 sec from initial disturbance was too long a time period for collecting baseline samples, as “baseline” samples taken would start to actually represent stressed levels of corticosterone.
Figure 2a. Stress response, measured as change in corticosterone concentration from baseline after 30 minutes of standardized stress, across individual blood mercury levels (µg/g Hg wet weight blood). The stress response is significantly decreased with increasing blood mercury concentration.
Figure 2b. Mean stress response, measured as change in corticosterone concentration from baseline after 30 minutes of standardized stress, across dietary methylmercury treatment groups (µg/g Hg wet weight food) with standard error bars. Methylmercury treatment group does not significantly affect the average stress response.
**Figure 3a.** Baseline corticosterone concentrations across individual blood mercury levels (µg/g Hg wet weight blood). Baseline corticosterone is not affected by blood mercury concentration.
Figure 3b. Mean baseline corticosterone concentration across dietary methylmercury treatment groups (µg/g Hg wet weight food) with standard error bars. Methylmercury treatment group does not significantly affect average baseline corticosterone concentration.
3.2 Glucocorticoid receptor results

3.2.1 Whole mount in situ hybridization of zebra finch embryos

Whole mount in situ hybridization was performed on whole, approximately stage 31 (all embryos from actual range of stages 30-32) to characterize the areas of expression of the glucocorticoid receptor during mid-development. The range of stages (~30-32) represents stages encountered on the 7th day of development: the zebra finch total developmental period from the egg being laid to hatching is 14 days. Figure 4 shows results from incubation of whole, control (0.0 µg/g Hg) embryos with the sense probe. The sense probe represents the complementary RNA sequence to the antisense probe, and has the same sequence as the mRNA for the glucocorticoid receptor. Embryos were incubated with sense probe to control for non-specific binding. All sense embryos show relatively little non-specific binding, though some can be seen in the heads of the embryos. This may either be non-specific binding or the failure to completely wash away all of the reagents necessary for color production from this area of dense tissue. Embryos from both the 0.0 and 2.4 µg/g Hg treatment groups (Figures 5 and 6) were incubated with antisense probe, which binds to the glucocorticoid receptor mRNA if present (i.e. if the receptor is being expressed). Expression of the glucocorticoid receptor can be seen throughout the head and torso regions of the embryos of both treatment groups, beyond the background, non-specific binding seen in the sense embryos. There may be slightly less expression in the 2.4 µg/g Hg embryos, but a quantitative analysis of expression
would need to be performed to confirm or deny this as whole mount *in situ* hybridization
can only provide qualitative data on the expression of a gene.
**Figure 4.** Whole mount *in situ* hybridization for the glucocorticoid receptor of stage 31 zebra finch embryos: sense embryos (0.0 µg/g Hg). Embryos are approximately 1 cm from head to tail.
Figure 5. Whole mount \textit{in situ} hybridization for the glucocorticoid receptor of stage 31 zebra finch embryos: antisense embryos (0.0 µg/g Hg). Embryos are approximately 1 cm from head to tail.
Figure 6. Whole mount *in situ* hybridization for the glucocorticoid receptor of stage 31 zebra finch embryos: antisense embryos (2.4 µg/g Hg). Embryos are approximately 1 cm from head to tail
3.2.2 qPCR of 50-day-old juvenile brains

The expression of the glucocorticoid receptor in juvenile (50 day) zebra finch brains was determined using the comparative delta-delta CT method that compares the relative cycle of amplification of a target gene (above a threshold) to a reference gene and reference sample. GAPDH was selected as the endogenous control and a male control bird, HG 3820, was selected as the reference sample and cDNA from this reference bird was run in all assays. Relative quantitation values were adjusted for the expression of the housekeeping gene and calculated based on the average expression of all birds within the control group. Each mercury treatment group (the average expression of all birds within the group) was compared to the control group’s average expression using a simple t-test. Expression of the glucocorticoid receptor was not significantly decreased or increased in any of the mercury treatment groups compared to the control group (see Table 1 for RQ and p-values).
<table>
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*Table 1.* Relative quantitation and p-values from average expression of the glucocorticoid receptor in the control and each of the four mercury treatment groups.
Figure 7. Boxplot of glucocorticoid receptor expression in individual 50-day-old juvenile zebra finch brains across all mercury treatment groups (µg/g Hg wet weight food) (n=4 for each). Circles represent the mean while black lines represent the median amplification cycle (C_T) above the threshold. Red=0.0 µg/g Hg, blue=0.3 µg/g Hg, green=0.6 µg/g Hg, yellow=1.2 µg/g Hg, purple=2.4 µg/g Hg.
3.2.3 qPCR of various stage whole zebra finch embryos

The expression of the glucocorticoid receptor in whole zebra finch embryos was determined using the comparative delta-delta $C_T$ method as described above for the analysis of zebra finch brain cDNA. One of the youngest stage embryos, a stage 17 embryo, was selected as the reference sample. Relative quantitation values were adjusted for the expression of the housekeeping gene and calculated based on the average expression of all stage 17 embryos. The average expression of each of the two older stages (stages 25 and 30) was compared to the average expression of the youngest, stage 17 embryos to produce relative quantitation (RQ) values by a simple t-test. Expression of the glucocorticoid receptor was not significantly decreased or increased in older embryos compared to younger embryos, though there appears to be a possible decreasing trend with increased embryonic stage (see Table 2 for RQ and p-values).
<table>
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</table>

**Table 2.** Relative quantitation and p-values from average expression of the glucocorticoid receptor in the three embryonic stages selected for analysis.
**Figure 8.** Boxplot of glucocorticoid receptor expression in individual stage 17, 25, and 30 zebra finch embryos of various treatment groups (n=2 for each). Circles represent the mean while black lines represent the median amplification cycle ($C_T$) above the threshold. Red=stage 17, blue=stage 25, green=stage 30.
4.1 Corticosterone hormone

In this experimental test of the relationship between methylmercury exposure and avian corticosterone, lifelong dietary dosing decreased the corticosterone stress response in adult, captive-bred zebra finches. This may have been due in part to chronically elevated baseline corticosterone levels in exposed birds, although baseline levels were not statistically different between treatments at the time we measured them. The weak response in corticosterone after 30 minutes of capture stress in birds exposed to higher doses of methylmercury may have been the result of adrenal corticoid disruption due to the chronic stress of methylmercury exposure. Life-long exposure to mercury may have induced chronically elevated circulating levels of corticosterone that fed back to reduce the number of receptors so as to maintain homeostasis. A change in the activity of glucocorticoid receptors may be the mechanism behind the observed decrease in the stress response in mercury-dosed birds. Additionally, altered hepatic metabolism with a concomitant change in plasma binding proteins would alter the concentration of free to bound plasma corticosterone, thus acting to reduce circulating stress-induced corticosterone. Clearly, more research is needed here to determine how mercury may be negatively impacting the stress response. Our finding of a decreased stress response is similar to that observed in free-living nestling tree swallows at a heavily mercury-contaminated site (Wada et al. 2009). Both studies found a significant negative relationship between exposure to mercury and the magnitude of the stress response.
Methylmercury exposure through dietary dosing may have increased circulating baseline concentrations of corticosterone, but the effect was statistically non-significant. Baseline hormone level was also not significantly related to individual blood mercury concentration. This lack of a negative relationship between baseline corticosterone and mercury contrasts with two previous studies that reported decreased baseline corticosterone concentrations with increased blood mercury levels in free-living birds exposed to moderate levels of non-point-source environmental mercury (Franceschini et al. 2009, Herring et al. 2012). There were, however, a number of differences between the present study and those finding decreased baseline corticosterone. Herring et al. points to the possible down-regulation of the HPA axis as an explanation for the negative correlation between baseline corticosterone and blood mercury (Herring et al. 2012). While this could be the mechanism in wild populations, the zebra finches used in the present experiment were not under food shortage nor were they at any risk for predation and thus the stressors they experienced were different. In the only other dosing study on captive birds, juvenile white ibises showed an increase in baseline corticosterone similar to the non-significant trend reported here (Adams et al. 2009). The zebra finches used in this experiment, though developmentally exposed to methylmercury, might not have been experiencing a level of stress that was high enough to cause down regulation of the HPA axis, as wild birds incur many more environmental stressors than those in a captive environment. Another difference between the present study and those showing reduction in baseline corticosterone is the degree of mercury exposure. All but our lowest treatment dose were higher than the likely exposure levels at the sites in the above-mentioned field studies or the captive dosing study.
In all analyses, corticosterone concentrations (both baseline and stress response) varied significantly with breeding stage. Nesting birds had significantly lower baseline levels of corticosterone and a significantly higher stress response than non-nesting birds. All birds were paired shortly before the onset of sampling (6 June, 2012), so the birds that were farthest along in breeding at the time of sampling were those that had begun breeding most rapidly after being paired. Birds that were less stressed by the experiment or the captive environment may have bred more quickly, which could explain why nesting birds had significantly lower baseline corticosterone levels (indicative of a less-stressed organism) than non-nesting birds. Birds that could not cope as well with ambient stress likely nested later, leading to the finding of higher baseline corticosterone and a weaker stress response in non-nesting birds. The relationship between mercury and corticosterone found, however, was not likely an effect of mercury-induced changes in nesting activity, as the proportion of nesting birds in each treatment group at the time of sampling appeared to be unrelated to dose, with the proportion nesting as follows: 0.0>2.4>0.6>1.2>0.3 µg/g Hg (ww). Further research will be necessary to explain the relationship between nesting activity and corticosterone.

The results suggest that methylmercury has the potential to disrupt one facet of the endocrine system, the regulation of corticosterone, and thus acts as an endocrine-disrupting contaminant, i.e., a chemical agent that in some way affects the release and regulation of one or more parts of the endocrine system (Diamanti-Kandarakis et al. 2009). High baseline levels of corticosterone in exposed birds can negatively affect the body in a number of ways, from reduced reproduction to inability to repair vital tissues. High circulating levels of corticosterone also appear to affect the ability of a bird to
respond to stressors. If a bird is unable to mount an appropriate stress response then it will be less able to deal with a stressor, such as a predator or storm, and will thus have a lowered chance of survival in the wild. Chronic stress by developmental and lifelong exposure to methylmercury may cause a disrupted adrenal corticoid response, a state that affects not only the release and regulation of corticosterone, but other key hormones as well.

This is the second study to demonstrate that mercury disrupts corticosterone regulation in birds, and the first to show an experimental effect on the stress response (Adams et al. 2009). The dietary mercury exposures used in the present study ranged from realistic levels for an insectivore at a typical contaminated site (0.3-0.6 µg/g Hg, ww) to a top-level predator at a highly contaminated site (1.2 µg/g, ww) and beyond the environmentally relevant range (2.4 µg/g Hg, ww), although blood mercury levels were considerably higher than found in the field by the end of the study, likely due to duration and constancy of exposure (Cristol et al. 2008). More research is needed to determine the threshold for endocrine disruption, the mechanism of action, whether this is an activational or organizational effect, and whether other currently unidentified hormonal pathways are disrupted in mercury-exposed birds. In addition to corticosterone, low doses of methylmercury have already been linked to disruption of reproductive hormones in birds (Jayasena et al. 2011). The effect of mercury on corticosterone is enough to cause additional concern for the health of wild populations of songbirds living near contaminated industrial sites. On the other hand, the apparent lack of effect on birds exposed to the lowest level of mercury is good news for most populations of birds exposed to only atmospheric mercury deposition at low concentrations.
4.2 Glucocorticoid Receptor

4.2.1 Results of in situ hybridization

A successful probe for the glucocorticoid receptor was synthesized and an in situ hybridization on whole zebra finch embryos was carried out. Expression of the glucocorticoid receptor was seen throughout the stage 30-32 zebra finch embryos, both in the 0.0 and 2.4 µg/g Hg treatment groups. This result is not surprising, as corticosterone affects systems throughout the body and thus receptors must be present within most cells. This result also agrees with the above qPCR data, as the receptor was shown by qPCR analysis to be expressed at this stage in zebra finch embryos. No quantitative conclusions can be drawn from the qualitative in situ hybridization data, but there appears to be decreased expression of the receptor in the 2.4 µg/g Hg embryos compared to the control embryos of the same stage. Quantitative qPCR data would need to be obtained to determine whether or not this observation is real. In addition, viewing the signal from the glucocorticoid receptor in cryosectioned embryos on slides would allow more exact determination of the location of expression of the receptor within the zebra finch embryos, and may allow better qualitative comparison of mercury treatment groups. Some embryos were sectioned using a cryosectioning machine following earlier in situ hybridizations with this probe, but the signal was not strong enough to be observed within individual sections on slides. Allowing the color reaction portion of the in situ hybridization protocol to proceed further (producing a darker color) may help with this
problem, as the signal might darken enough to be seen. However, allowing the color reaction to proceed further would also produce more background, non-specific binding of the probe.

4.2.2 qPCR analysis

The qPCR analysis of the effects of lifelong exposure to methylmercury on the expression of the glucocorticoid receptor in the brains of juvenile (50 days old) zebra finches showed that methylmercury dosage does not appear to affect expression of the receptor in the brain. qPCR analysis of zebra finch brain total RNA (made into cDNA) revealed that across methylmercury treatment groups there was no significant change in the average cycle of amplification, which is indicative of expression of the gene, of the glucocorticoid receptor. While, at times, negative results can be disappointing, in this case the result of no change across treatment groups could have real implications for these birds in light of the effects of mercury exposure on the corticosterone hormone. Compensation by the HPA (hypothalamic-adrenal-pituitary) axis has been suggested to explain decreases in corticosterone concentrations in response to chronic stress by exposure to methylmercury (Herring et al. 2012). Certainly, this may be the explanation for the results described above showing that mercury exposure decreases the stress response: these zebra finches may be downregulating corticosterone production in response to chronic stress, so that they can continue to carry out everyday processes. From the glucocorticoid data obtained, though, it does not appear that any compensation is occurring on the receptor side of the pathway. Though glucocorticoid receptors are
present in numerous places throughout the body, one of the most important regions containing these receptors is the brain, where feedback from the system is received and adjustments are made. Despite the potentially elevated levels of corticosterone hormone due to chronic mercury exposure and the inability of mercury-exposed birds to mount a stress response as strong as unexposed birds, there are no changes seen in receptor expression in the brains of dosed zebra finches.

Though this lack of a difference between exposed and unexposed birds may very well be real, it is important to note that corticosterone hormone data was obtained from adult developmentally exposed birds (>50 days old) while glucocorticoid receptor data was obtained from juvenile developmentally exposed birds (~50 days old). It is possible that the system does in fact compensate, but that this compensation does not occur as early as 50 days and thus the differences in glucocorticoid receptor expression was not seen in the qPCR analysis performed because of the age of the birds. A study of adult (>50 days old) zebra finch brains would shed light on whether or not compensation is occurring, just not at the juvenile stage. It is also a possibility that compensation for increased levels of corticosterone due to the stress of mercury exposure may be occurring in the expression of the receptor elsewhere in the body, not in the brain. Analysis of tissue from different vital organs by qPCR would shed light on whether or not compensation is occurring elsewhere in the zebra finch body. Finally, the stress response pathway is much more complicated than just the receptor and hormone. Corticosterone binding globulins regulate the bioavailability and metabolic clearance of corticosterone downstream from its production (Breuner and Orchinik 2001). A number of variables, such as seasonality and body condition, have been noted to cause fluctuations in both the
binding affinity and concentration of corticosterone binding globulins, which then, in turn, affects the amount of corticosterone available to the organism (Breuner and Orchinik 2001, Fassler et al. 1986). It is possible that our zebra finches that are experiencing higher levels of baseline corticosterone, and that are unable to mount as large a stress response as birds unexposed to mercury, are compensating for this, not by adjusting receptor expression, but by adjusting the binding affinity and/or concentration of corticosterone binding globulins. More research will need to be done in all of the above-mentioned areas to fully determine the effects of lifetime methylmercury exposure on the stress response pathway.

An analysis by qPCR was also performed on whole zebra finch embryos of various stages. This analysis showed that there is no significant difference in the expression of the receptor among the three stages used (17, 25, and 30), though there appears to be a non-significant decrease in expression with increasing embryonic stage. The sample size for each stage is low, though, so an increased data set may bolster or diminish this apparent trend. The data from this analysis, though, does provide a starting point for analysis of the expression of this receptor in this species, *Taeniopygia guttata*. To this date I have no knowledge of any other study on expression of the glucocorticoid receptor in zebra finch embryos, and thus this study most likely provides the first data on the timing and relative level of expression of the glucocorticoid receptor in this species. The receptor is expressed at all three embryonic stages studied, so further research could be performed with even younger stages to determine when expression of the glucocorticoid receptor is first “turned on” in zebra finch embryos. This embryonic qPCR
data provides an intriguing first look into the expression of the glucocorticoid receptor during development in a common laboratory model system, the Australian zebra finch.

4.3 Conclusions and future directions

The ability to respond to stressors with a surge of corticosterone is vital to the maintenance of health and survival in birds. Chronic exposure to mercury at environmentally relevant dietary concentrations reduced this vital stress response in captive Australian zebra finches. These finches are less able to mount an appropriate stress response with higher blood mercury concentrations, and thus would be more susceptible to threats in the wild. More highly exposed finches also appeared to have (non-significantly) increased levels of baseline corticosterone, indicative of chronic stress. Higher circulating levels of corticosterone could also affect multiple other bodily systems (other than the endocrine) that are sensitive to circulating levels of this hormone, such as the reproductive and immune systems. In addition, it does not appear that there was any compensation for altered levels of corticosterone hormone within the stress response pathway of exposed zebra finches. Expression of the glucocorticoid receptor, which is subject to feedback from the rest of the stress response pathway, was not significantly different across methylmercury treatment groups in juvenile (50-day old) zebra finches.

While the corticosterone hormone study was fairly extensive, there are still conflicting results reported across the field and thus more repetitions of studies such as the one carried out at William and Mary, as well as additional field studies of
contaminated sites, will need to be performed to further elucidate mercury’s effects on corticosterone. The receptor, on the other hand, is not well studied in avian species especially in regards to the effects of mercury exposure on its function and expression. There is a need for much more research in this area, though the results provided in this study offer an interesting first look. A number of variations on the glucocorticoid study reported here, including using larger samples sizes, comparing sexes, and using and comparing finches of different ages, may help to determine whether or not the lack of effect of mercury exposure on expression of this receptor is real. In addition, there is another important aspect to this system, corticosterone-binding globulins, which have been reported to fluctuate under certain conditions and which are important in determining actual active levels of plasma corticosterone. These binding proteins were not studied in this project. However, compensation for altered corticosterone concentrations may be occurring within this facet of the system in mercury exposed zebra finches.

In addition to investigating the effects of mercury exposure on aspects of the stress response pathway, expression of the glucocorticoid receptor was also studied in zebra finch embryos. Again, there is a general lack of literature on the developmental expression of the glucocorticoid receptor in avian species, and thus the in situ hybridizations and quantitative PCR analyses performed here provide an interesting first look. The receptor appears to be expressed throughout the body of stage 30 embryos, and is expressed in whole embryos as early as stage 17. No earlier stages were analyzed, so a good next step would be qPCR analysis of various younger stages to determine when
expression begins, and if the pattern of expression changes with embryonic
developmental stage.

Mercury causes concern for the conservation and welfare of many species, and
birds are not unique in this regard. Low dose mercury exposure disrupted the stress
response pathway in Australian zebra finches, a model songbird, by decreasing the ability
of the exposed birds to respond to stress. Compensation for altered hormone levels did
not occur by altered expression of the receptor. It is possible that compensation is
occurring elsewhere, but more research is needed on this system to determine that. In
general, precautions should be taken with songbird populations exposed to mercury, as
they may be less fit due to a decreased ability to deal with stress.
V. References


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