Dietary mercury and stress responses: how lifetime exposure to mercury alters stress responses and their relationship to spatial cognition

by

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Abstract

Methylmercury is a widespread environmental contaminant that negatively impacts both humans and wildlife at sublethal levels. While environmental sources of methylmercury exist, the primary sources are anthropogenic, and most animals are exposed to methylmercury via diet. In my thesis, I examine how lifetime methylmercury exposure affects stress and cognition using the zebra finch model. My first goal is to examine the relationship between methylmercury and translocation stress. A captive colony of zebra finches from the College of William and Mary were transported to Auburn University. Two weeks before departure, birds were bled for baseline and stress-induced CORT. One day after arrival, birds were bled again in the same manner. Methylmercury exposed-birds experienced a significant increase in baseline CORT; however, methylmercury did not appear to impact stress-induced CORT. All birds experienced significantly lower stress-induced CORT as a result of translocation. My next goal is to examine how the interaction between methylmercury and CORT impacts spatial cognition. Zebra finches were put through a battery of spatial memory tests using baited wooden blocks with covers the birds were trained to remove. In general, the more stressed the bird, the longer it took to learn the procedure and the location of the food reward.

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List of Abbreviations

ACTH	adrenocorticotropic hormone
CORT	corticosterone
CRF	corticotropin releasing factor
HPA	hypothalamic-pituitary-adrenal
MeHg	methylmercury
PVN	paraventricular nucleus
ZEFI	zebra finch

Chapter 1: An introduction into the toxicology and significance of methylmercury

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Keywords: zebra finch, methylmercury, stress, cognition

Abstract

Methylmercury is a widespread environmental contaminant that is well-documented to induce negative impacts on physiology and behavior in living organisms. Methylmercury exposure primarily occurs via diet in terrestrial organisms. It is a historically significant environmental contaminant, with infamous exposure incidences occurring in Japan and Iraq. For my thesis, I chose the zebra finch model in part because birds are the only species other than humans that go through the process of learning vocalizations, suggesting that similar brain areas in both birds and humans have converged functionally over their evolution. Additionally, zebra finches are reasonably easy to care for, easily accessible, and have been used in methylmercury studies prior to my own.

Introduction

Chemical structure of mercury (organic vs. inorganic)

Methylmercury ($[CH_3Hg]+$) is an organometallic cation that bioaccumulates in the environment. It is comprised of a methyl group bonded to a mercury ion. It has a particularly high affinity for sulfur-containing anions, especially thiol in cysteine, and readily combines with chloride, hydroxide, and nitrate anions (Boening 2000). The fact that methylmercury binds readily with sulfur can be helpful in estimating risk in certain bodies of water, such as saltmarshes (Mitchell and Gilmour 2008). It is because of its high affinity for the thiol groups in cysteine, suggesting that this is the way methylmercury would occur in tissue of prey items, that the birds used in my experiments were fed pellets containing methylmercury cysteine.

In contrast, inorganic mercury (Hg^0), popularly known as quicksilver, colloidal mercury, and liquid silver, is the most volatile form of mercury. It poses less of a risk to humans because it cannot readily cross cell membranes or the blood-brain barrier. In developed countries, most of

its presence in everyday products, such as in dental amalgams and thermometers, has been eliminated, but it is still widely used in developing economies, particularly in the small-scale mining of gold. While inorganic mercury can be avoided, methylmercury from fossil fuel combustion that has circulated in the atmosphere and deposited in the ocean is found in most species of fish, including widely consumed species such as tuna (World Health Organization 2014).

Methylmercury

Methylmercury is a widespread environmental contaminant that bioaccumulates, causing detrimental effects to both humans and wildlife. Methylmercury, or organic mercury, is formed via the actions of microbes in aquatic ecosystems, such as lakes, wetlands, and rivers (Ulrich et al. 2010). While natural mercury emission occurs through volcanoes and forest fires, the source contributing to the most lethal levels are anthropogenic (Tewalt et al. 2004). In fact, the amount of methylmercury present in the atmosphere has quadrupled since the age of industrialization (Boening 2000). Even the level of detriment caused to organisms at sublethal levels is problematic. While inorganic mercury can produce harmful effects at levels as low as 5 ug/l, methylmercury can exert the same effects at levels 10 times lower (Boening 2000).

Humans and wildlife are also exposed to methylmercury via diet. Most commonly, people are exposed when consuming predatory species of fish. Because methylmercury biomagnifies, the amount found in predatory fish exceeds the amount produced by the microbes in aquatic environments. There are two well-known historical examples of methylmercury poisoning via diet, Minamata, Japan and Iraq, that provide evidence for the neurological and physiological effects of methylmercury on humans.

Historical Significance

Methylmercury poisoning was first characterized on a wide scale when what is now known as Minamata Disease was discovered in Minamata city in Kumamoto prefecture in Japan in 1956. From 1932 to 1968, Chisso Corporation released methylmercury via industrial wastewater, which bioaccumulated in the fish in the bay (Harada 1995). Those fish were then consumed by the populace, ending in the poisoning of the city on a large scale. Symptoms included ataxia, vision loss, and skin numbness. It was described as "dancing cat fever" because cats in Minamata that consumed the poisoned fish were debilitated by ataxia and, unable to control their movements, would appear to be dancing and would frequently fall into the water and drown. Minamata also demonstrated the vulnerability of the fetus to environmental contaminants, as pregnant women who had consumed the poisoned fish were minimally harmed compared to the severe birth defects their children were born with from exposure in utero (Harada 1995). To this day, patients experiencing chronic methylmercury poisoning decades earlier still complain of paresthesia in the libs and distal areas of extremities (Ekino et al. 2007).

A similar mass poisoning incident occurred in 1971 in Iraq when grain treated with a methylmercury fungicide and not intended for human consumption was imported into Iraq and consumed by its citizens and residents during a famine. As the labeling on the bags of poisoned grain were in Spanish, the consumer had no way of knowing how dangerous it was. Patients experienced similar symptoms to those in Minamata, including damage to hearing and speech, ataxia, and numbness. Hundreds of people were killed, and even more were grievously debilitated (Greenwood 1985).

The zebra finch model

Methylmercury research has traditionally focused on fish-eating vertebrates, but it is now known that environmental methylmercury can bioaccumulate in arthropods which are eaten by insectivorous birds, thereby negatively impacting species that are not directly part of aquatic food webs (Cristol et al. 2008). Songbirds have been suggested to be a valuable sentinel species for mercury (Jackson et al. 2015). Yet, physiological consequences of dietary mercury are still understudied. In my thesis, I use zebra finches, a model organism for songbirds, to examine the impacts of methylmercury on response to translocation stress and spatial cognition. Zebra finches are an appealing model organism for neurobehavioral research because song birds share an ability with humans that only few organisms do: the ability to learn vocal patterns through imitation of a tutor, indicating that similar brain areas in humans and song birds have converged functionally throughout our evolution (Mello 2014).

It is well-documented that lifelong methylmercury exposure impairs spatial cognition in zebra finches, but the exact mechanism is unknown (Swaddle et al. 2017). It is also known that lifetime exposure to methylmercury affects levels of corticosterone (CORT), the primary stress hormone in birds; specifically, birds exposed to methylmercury are less able to mount a stress response (Moore et al. 2014). My thesis project tests the hypothesis that dietary methylmercury negatively impacts spatial cognition via altering CORT secretion. Specifically, the overarching goals of my thesis are to examine how methylmercury exposure through diet impacts an organism's stress and spatial cognition and how these may be affecting each other. Chapter 2 describes how lifetime methylmercury exposure impacts translocation stress in zebra finches. Chapter 3 describes how lifetime methylmercury exposure combined with a stressful event (translocation) impacts spatial cognition in a battery of spatial memory tests.

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Chapter 2: Effects of dietary mercury and translocation on adrenocortical responses in zebra finches

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Keywords: zebra finch, corticosterone, methylmercury, translocation, stress

Abstract

Translocation stress includes of physical and psychological stressors that culminate to negatively impact an organism. It is known that methylmercury exposure can also modulate circulating corticosterone (CORT) levels. Here, I examine the effects of methylmercury on the magnitude of adrenocortical response due to translocation. A captive colony of zebra finches exposed to dietary methylmercury for their lifetimes was used. Zebra finches were transported via van from Williamsburg, Virginia to Auburn, Alabama for 10 hours overnight. Birds were bled 2 weeks before translocation and 1 day after arrival. Overall, translocation stress had a strong effect on plasma corticosterone levels. Irrespective of treatment, birds had higher baseline CORT and lower stress-induced levels of corticosterone after translocation. When each treatment was examined separately, only birds on dietary mercury had higher baseline while only control birds had a lower stress response (factor increase in CORT) after translocation. This points towards a new hypothesis that methylmercury disrupts the negative feedback loop of the hypothalamic-pituitary-adrenal axis. Further studies are needed to explore this possibility.

Introduction

Stress is defined as an actual disruption of homeostasis or a perception of a threat to an organism's well-being (Ulrich Lai and Herman 2009). Appropriate response and adaption to stress is pivotal in the survival of an organism. It is well documented that stress negatively impacts an organism, and in general, the mechanism by which stress works physiologically is well-understood (Tsigos et al. 2009).

Glucocorticoids, the stress hormone, are released by the adrenal cortex as a part of the hypothalamic-pituitary-adrenal (HPA) axis (Stephens and Wand 2012). Within the paraventricular nucleus (PVN) of the hypothalamus, neurons release two neurohormones: corticotropin releasing factor (CRF) and arginine vasopressin. These neurohormones are released

into the blood vessels that connect the hypothalamus and the pituitary gland and subsequently stimulate the anterior pituitary to produce adrenocorticotropic hormone (ACTH) into the bloodstream. ACTH then causes the synthesis and release of glucocorticoids from the adrenal glands on the kidneys (Stephens and Wand 2012). The primary glucocorticoid in birds is corticosterone (CORT) (Noguera et al. 2018).

Many environmental contaminants are well-understood to alter hormone levels in organisms exposed to them (Hothckiss et al. 2008). Specifically, methylmercury impairs endocrine functionality by inhibiting hormone-receptor binding or hindering biosynthesis of enzymes needed to produce hormones (Rice et al. 2014). Further, methylmercury exposure is correlated with lower levels of plasma CORT levels, which can trigger a series of compensatory responses that can cause lasting damage (Iavicoli et al. 2009).

It has been shown that corticosterone inhibits protein synthesis and degrades proteins in birds, and birds with increased corticosterone experience slower feather growth and an abnormal period of poor flight as a result (Romero et al. 2005). In hatchlings, increased corticosterone levels are correlated with increased food aggressiveness and begging behavior, and while this may immediately increase a hatchling's ability to obtain food, it ultimately compromises its cognitive functioning including the association of food with aggressive behavior (Kitaysky et al. 2003).

Examining the effects of transportation stress are important regarding the wellbeing of animals. Routine handling and transportation can result in chronic stress in an animal. Psychological stress, including novelty, transportation, and handling can negatively impact an animal. Additionally, other stressors can compound on transportation stress, such as hunger,

thirst, temperature variation, high volume sounds, and fatigue. My goal in this chapter of my thesis is to examine the effects of methylmercury exposure on transportation stress.

It has been well-documented that methylmercury exposure is associated with a change in blood CORT levels (Evers et al. 2003, Whitney and Cristol 2017); however, the relationship between methylmercury exposure and transportation stress has not been examined. Multiple stressors can have additive, synergistic, or antagonistic effects on physiology. As animals in the wild rarely encounter only one isolated stressor, understanding the relationship between multiple stressors is important, yet understudied. To understand how a chronic dietary mercury exposure alters stress response to translocation, we exposed captive zebra finches to dietary mercury or control diet throughout their lives. We then subjected the birds to translocation stress to characterize an interplay between the two stressors. The goal of this experiment is to determine if lifetime methylmercury exposure interacts with and exaggerates the adrenocortical response. We hypothesized that birds exposed to methylmercury will experience an inability to mount a stress response (lower stress-induced CORT) compared to their control counterparts.

Methods

Adult male zebra finches used in this study were obtained from a captive colony at the College of William and Mary. Parents of the experimental birds were fed either 0 or 1.2 ppm wet weight methylmercury cysteine that had been added to Zupreem food pellets and provided ad libitum. Birds within each treatment group were paired and allowed to mate in home cages (0.46 m width x 0.75 m length x 0.46 m height) with access to nesting material and nest box, water, and grit as described in Paris et al (2018). Upon fledging and being assigned to new cages with 3-5 other same-sex individuals, experimental birds were maintained on the same diet (mercury or

control pellets) until the end of the study. Thus, all birds were exposed to either mercury or control diet throughout their lives, beginning in ovo.

A total of 32 males were used in this study (post hatch days 580.2 ± 67.5). Two weeks before transportation, birds were bled via puncturing brachial vein to quantify baseline and stress-induced CORT levels. Initial blood samples were collected within 3 minutes of entering the animal room before circulating CORT starts to raise (Romero and Reed 2005). This sample represents baseline levels of CORT. After the first collection, birds were placed singly in an opaque cloth paper bag for 30 minutes, then bled again to quantify an increase in CORT levels in response to capture, handling, and restraint. Upon completion of blood sampling, birds were returned to their home cages.

Two weeks after the initial blood sampling, zebra finches were transported by van from College of William and Mary in Virginia to the Auburn University aviary in Alabama. To prevent additional nutritional stress from food deprivation, birds were transported at night, between 10pm EST to 7am CST, when zebra finches do not feed. Birds were transported approximately 8 per cage for the duration of the trip, about 10 hours. Birds were given access to regular Zupreem during transportation. Additionally, the cages were covered with cloth to prevent street lights from waking and disturbing the birds. The birds were taken from the College of William and Mary at 10pm EST. Birds arrived at the Auburn University aviary in the morning at 7am CST and were sorted by sex and treatment and put into home cages with no more than 3 birds per cage. Birds were given ad libitum access to their normal mercury or control Zupreem as well as water and grit. The following morning at 8:00am, a day after the arrival, males were bled in the same manner as described above to quantify changes in plasma CORT in response to translocation.

Blood CORT levels were analyzed using commercial EIA CORT assay, following instructions in the manufacturer's manual (Enzo Life Sciences, cat no. ADI-901-097). This assay has been validated for zebra finch plasma previously (Wada et al. 2009). We ran an additional optimization assay and determined that plasma dilution of 1:20 with 2.5% steroid displacement buffer is an alternative to 1:40 dilution with 1.5% steroid displacement buffer for zebra finch plasma. This allowed us to use a lower plasma dilution compared to the previous assay condition to detect low levels of corticosterone while maintaining low interference of the plasma.

Statistical analysis

All statistical analyses were performed using SPSS 21. Baseline and stress-induced levels of corticosterone, and factor increase between baseline and stress-induced levels were analyzed using a linear mixed model. Factor increase in corticosterone levels was calculated by dividing stress-induced levels of corticosterone by baseline corticosterone levels. Values outside of 4 standard deviations away from the mean were considered an outlier, thus were removed from the statistical analysis. This excluded values from one control male whose baseline and stress-induced levels were 5 to 8 times higher than the average values of respective corticosterone levels. Final sample size for control and mercury groups were 14 and 17 before translocation and 13 and 17 after translocation (one control bird died shortly after translocation). Treatment and location were used as fixed factors. In all models, subject ID and assay plate number were included as random effects. Body mass measured immediately after translocation was used as a covariate in initial models but was later removed because it did not significantly contribute to the model ($p = 0.15$). Interaction terms with covariates were removed when $p > 0.15$ in a stepwise fashion, starting from the most complex 3-way interactions. Means ± standard errors are presented in the text and the figures.

Results

Effects of dietary mercury and translocation on baseline corticosterone levels

When the effect of translocation was examined within each treatment group, only the mercury treated birds had a significant increase in baseline levels due to translocation stress ($p = 0.022$) while control birds did not ($p = 0.529$). On average, mercury treated birds increased their baseline corticosterone levels from 3.34 ± 0.63 ng/mL to 6.75 ± 2.14 ng/mL while control birds increased from 3.58 ± 0.70 ng/mL to 4.42 ± 0.82 ng/mL. When birds from both treatment groups were examined together, birds had a higher baseline corticosterone levels after translocation ($F = 4.122$, $p = 0.047$; Fig. 2.1) with no significant effect of dietary mercury treatment ($F = 0.426$, $p = 0.517$). In addition, we detected no significant interaction between treatment and location ($F = 1.158$, $p = 0.287$).

Effects of dietary mercury and translocation on stress-induced corticosterone levels

Dietary mercury treatment did not have a significant effect on stress-induced levels of corticosterone ($F = 0.761$, $p = 0.387$). However, birds had significantly lower stress-induced levels of corticosterone after translocation compared to before ($F = 4.344$, $p = 0.042$; Fig. 2.2). The effect of translocation did not differ between treatment groups ($F = 0.732$, $p = 0.396$). On average, mercury treated birds decreased their stress-induced corticosterone levels from 16.86 ± 1.83 ng/mL to 14.53 ± 2.79 ng/mL and control birds decreased their levels from 21.74 ± 3.93 ng/mL to 14.83 ± 3.02 ng/mL.

Effects of dietary mercury and translocation on factor increase in corticosterone levels

When treatment groups were examined separately, only the control birds decreased their stress response (i.e., factor increase in corticosterone in response to capture and handling stress) after translocation ($p = 0.013$) while mercury treated birds did not change their stress response with regards to translocation ($p = 0.388$). On average, mercury treated birds decreased their stress response from 7.23 ± 1.21 times baseline before translocation to 5.68 ±1.51 after translocation. In contrast, control birds decreased from 8.65 ±1.71 times baseline before translocation to 3.52 ±0.49 after translocation. When both groups were examined together, lifetime mercury treatment did not alter the magnitude of the stress response ($F = 0.071$, $p = 0.791$). Similar to the results of the stress-induced levels of corticosterone, stress responses of finches were reduced after the translocation compared to before ($F = 6.217$, $p = 0.016$; Fig. 2.3). Main effect of treatment and location interaction was not significant ($F = 1.782$, $p = 0.187$), suggesting that variation within groups was high compared to the sample size to detect the treatment by location interaction.

Discussion

Previous studies have shown equivocal results in regard to the effect of dietary mercury on plasma corticosterone levels in birds. In tree swallows, late-stage nestlings in a methylmercury-contaminated field site had double baseline CORT levels and showed lower stress-induced levels in response to handling stress compared to nestlings in the reference sites (Wada et al. 2009). In contrast, baseline CORT levels are not impacted by methylmercury exposure in captive zebra finches (Maddux et al. 2014). We investigated whether two stressors, dietary mercury and translocation stress, have an additive or synergistic effect on plasma corticosterone levels. Our study showed that birds experienced increased baseline CORT levels due to translocation. When the treatment groups were examined separately, only methylmercury-

exposed birds appeared to experience an increase in baseline CORT. This raises a possibility that mercury treated birds are more sensitive to translocation stress than control birds. However, when the treatment groups were examined together, treatment by translocation interaction was not statistically significant, likely due to high variation in the mercury group. Results from this study warrants a further test of the hypothesis that dietary mercury increases sensitivity of the hypothalamic-pituitary-adrenal axis to an additional stressor.

Translocation stress encompasses a number of stressors. Some stressors are novel, such as street lights, sounds of cars moving at high speeds, radio broadcasts, and starting and stopping at unpredictable intervals. Birds also experienced the psychological stress of being handled several times. Lastly, birds experienced potential nutritional stress, as while they were provided with Zupreem during transport, the movement of the van, darkness and novel environment may have discouraged them from eating. Thus, translocation stress is not a singular phenomenon, but rather a combination of multiple types of stressors on an animal. As a result, the process of being transported can be considered energetically demanding, which may explain the increase in baseline CORT levels. For example, it is known that food removal can result in a baseline CORT level that is double that of normal (Lynn et al. 2003). Additionally, increases in baseline CORT facilitate energetically demanding activities, such as reproduction (Apfelbeck et al. 2017). It is possible that transportation stress may have masked the effect of methylmercury rather than having additive effect.

All birds had significantly lower stress-induced CORT levels after translocation, with no difference in treatment groups. This is consistent with the previous study that showed a reduction in the ability to mount a stress response due to translocation stress in chukar (Dickens et al. 2009). High levels of baseline CORT in combination with reduced stress-induced CORT indicate

that transportation stress disrupted the negative feedback loop. Nutritional stress, exposure to novel environment, and disturbance of transportation impeded the ability of the HPA axis to return the CORT levels to baseline. As a result, the birds were unable to mount an increased stress response.

In summary, translocation stress increased baseline corticosterone levels and suppressed magnitude of the stress responses, indicating altered negative feedback loop. Dietary methylmercury had a smaller effect, if any, on the corticosterone levels compared to transportation stress. Analyses of baseline and stress responses within treatment groups leads to a hypothesis that dietary mercury increases sensitivity of the animals to transportation stress. Further studies with larger sample size will be needed to test this hypothesis.

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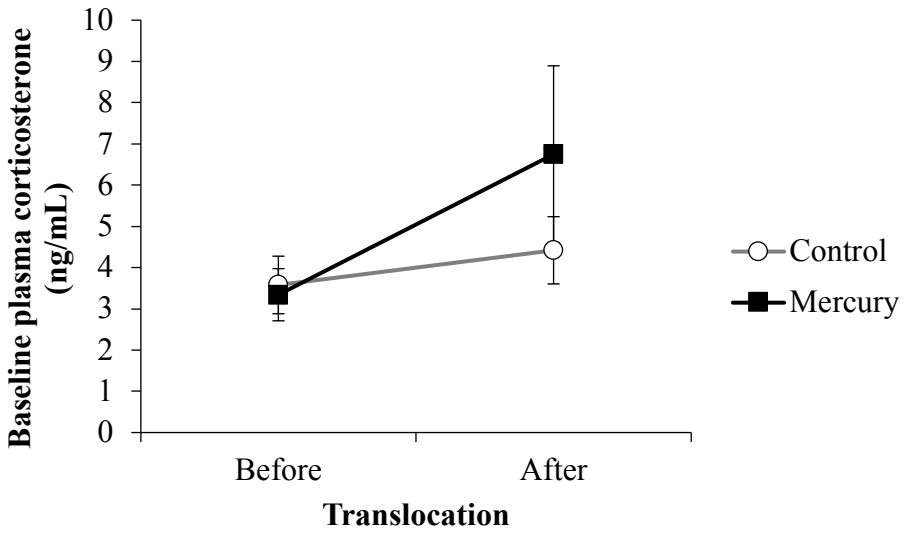

Figure 2.1: Effect of translocation on baseline plasma CORT. Birds were transported overnight (10:00pm EST to 7:00am CST) from Williamsburg, VA to Auburn, AL with approximately 8 birds per cage with cages covered with tarp to prevent outside light from disturbing them. Birds were provided with Zupreem pellets during transportation.

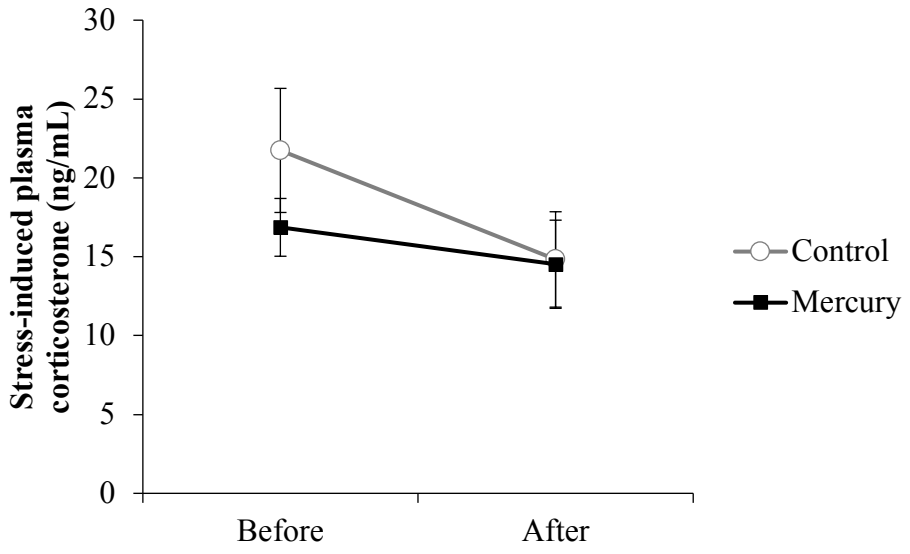

Figure 2.2: Stress-induced plasma CORT before and after translocation.

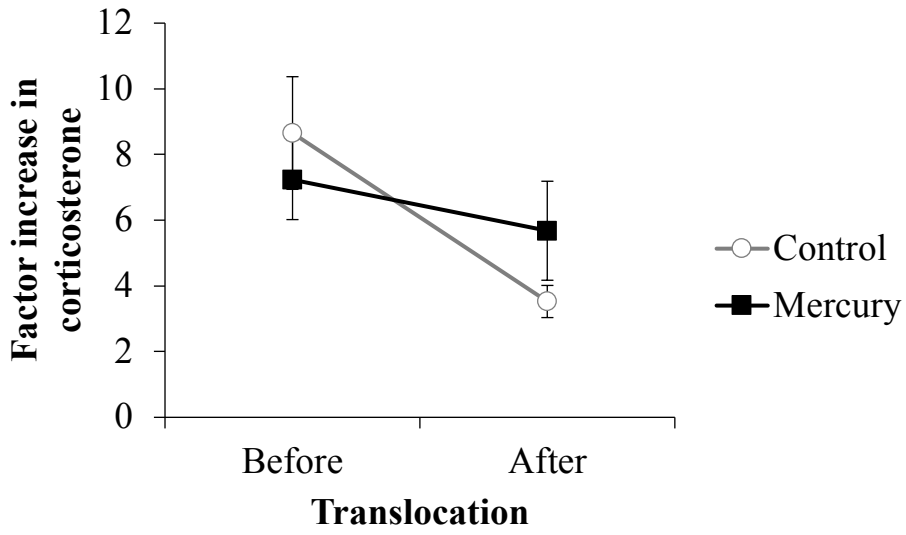

Figure 2.3: Factor increase in CORT before and after translocation. Stress responses of the finches were reduced after the translocation compared to before.

Chapter 3: Adrenocortical responses and spatial memory: how dietary mercury influences this relationship

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Keywords: zebra finch, methylmercury, HPA, cognition, memory

Abstract

Dietary methylmercury has been shown to suppress spatial learning and memory in birds and rodents. However, the mechanism behind this suppression is not well understood. To investigate whether corticosterone plays a role in mercury-associated cognitive impairment, we measured circulating corticosterone levels before and after transportation stress and examined whether the baseline or adrenocortical response to transportation stress correlated with cognitive performance in captive zebra finches. We found that a higher magnitude of stress response was positively correlated with an increase in total number of trials to pass criterion for shaping (i.e., learning to approach one of four blocks). In general, birds that failed Phase 3, which consisted of trials where the bird had to remember the location of the food reward, had a higher magnitude of stress response due to translocation.

Introduction

Spatial memory refers to an organism's ability to remember information regarding its environment and orientation, e.g., where food has been cached or where a nest is located. Areas of the brain that process spatial memory include the hippocampus as well as the medial temporal lobes. Neurons within the hippocampus, place cells, are characterized by their spatial firing patterns (Moser et al. 2015). When an organism explores an environment, place cells fire in a particular pattern that allows the organism to map out the environment. This allows the organism to approximate its location based on its surroundings. Additionally, remapping can occur with the addition of associated sensory stimuli, such as colors and textures.

Spatial memory has representations in working, short-term, and long-term memories. Spatial working memory involves immediate retention of visuospatial information and keeping that information active for a short period of time, and it includes two systems: a 'phonological loop' and a 'visuospatial sketchpad.' As such, both auditory and visual information are kept active in the spatial working memory (van Asselen et al. 2006). Short-term spatial memory allows an organism to temporarily store information necessary to complete a cognitive task. Short-term spatial memory is utilized when an organism needs to remember different locations and the relationship between the locations (Banta Lavenex et al. 2014). Lastly, long-term spatial memory includes recollection of spatial details, including environmental boundaries and layout (Morris and Mayes 2004). Long-term spatial memory is considered to be more permanent and can persist in the presence of external stimuli that divert attention elsewhere. Thus, performing a long-term spatial memory task requires retrieving stored spatial information and using that information to perform a task (Parslow et al. 2004).

Stress exposure influences memory, typically through an increase in glucocorticoids levels; further, glucocorticoids have several effects on memory, with the majority of them being

seen through the impact of stress on emotional memory and long-term memory (Albrecht et al. 2013). The amygdala and hippocampus, sites which regulate the hypothalamic-pituitary-adrenal response, are involved in conditioning. During exposure to a stressor, animals begin to associate an external stimulus, such as environment or an auditory tone, with the onset of stress. In the end, the stimulus alone can cause the stress response in the absence of the actual stressor itself (Ulrich-Lai and Herman 2009). Glucocorticoid receptors modulate spatial memory, and acute stress induces a rapid rise in corticosterone that is associated with recollection deficits (De Quervain et al. 2009, Chauveau et al. 2010). Further, increased levels of corticosterone have been associated with increased memory-retrieval deficits (Dorey et al. 2012).

Methylmercury is a neurotoxin and it can suppress neural development and memory. For instance, rats exposed to methylmercury during development experience acute hippocampal apoptosis, reduced neurogenesis, and deficiencies in learning while in puberty; specifically, rats were deficient in their ability to perform a spatial navigation task (Falluel-Morel et al. 2007). While it is known that methylmercury also affects spatial cognition in zebra finches, the underlying mechanism is still unclear (Swaddle et al. 2017). It is possible that glucocorticoids play a role in mercury-associated memory deficit. Lifetime exposure to methylmercury has been shown to disrupt the stress response in zebra finches. Specifically, a finch's ability to mount a stress response is debilitated with increasing methylmercury exposure (Moore et al. 2014). Rats exposed to methylmercury in utero show significant learning impairments and higher levels of corticosterone compared to their un-exposed counterparts (Carratù et al. 2008). Thus, our goal of this study was to quantify the degree to which methylmercury negatively impacts spatial memory, what parts of spatial memory are most vulnerable, and if glucocorticoids play a role in this negative impact.

Our experiment consists of five distinct phases: shaping (Phase 1), bias assessment (Phase 2), recall testing (Phase 3), validation of the absence of olfactory stimuli (Phase 4), and recollection after a 24-hour period (Phase 5). As such, the type of spatial memory—working, short-term, or long-term—that is most vulnerable to methylmercury neurotoxicity can be visualized and estimated based on performance on each phase of the experiment. Phase 1 consists of shaping (some closeness to working memory), Phases 2, 3, and 5 test long-term memory, and Phases 3 and 4 test short-term memory. We hypothesized that the magnitude of stress response due to translocation is a predictor of performance on spatial learning and memory tests and that birds with higher CORT have poorer performance on these tests. Further, chronic exposure to dietary mercury increased corticosterone levels and birds with higher corticosterone levels have suppressed spatial cognition.

Methods

Spatial memory trials were conducted on the captive colony of zebra finches. Birds were fed either control or mercury diet (1.2 ppm) throughout their lives (beginning in ovo through parental diet). After reaching nutritional independence, experimental birds were kept on the same diet as their parents had been. Two days prior to testing, birds were moved from their home cages to a separate room in the aviary for behavioral trials. Each bird was placed in a cage (0.6 x 0.41 x 0.41 m) by themselves with access to the same diet from their home cage, water, and grit. Cages were placed on large shelving with two cages on top and two on the bottom. The testing room contained two of these shelving arrangements for a total of 8 cages. Silence was

maintained during trials. The birds were able to see the observers but did not display any signs of distraction or phobia as a result.

Birds were food deprived by removing their food dishes the evening before the morning behavioral trials. Birds went an average of 13 hours without access to food in order to induce food motivation (however much of this was during a time when they were asleep and would not have been feeding). Behavioral trials began at 8:00am every morning, with Phase 1 beginning on Monday. Each trial lasted 2 minutes with 8 minutes of rest; birds underwent no more than 10 trials in one day. During the trials, a divider was placed between cages so that birds were not able to see each other. The divider remained in place until trials were completed for the day. After completion of trials each day, dividers were removed, and birds were allowed to see each other, so as not to be socially isolated, until the evening when the food dish was removed.

Trial cages consisted of wooden blocks, paper discs, and control Zupreem diet. The wooden blocks were square (9 x 9 x 4 cm high), each with a central cylindrical food well (approximately 2 cm diameter) drilled into the center of the top surface in the middle to allow for food to be placed and covered with a paper disc. Blocks were intended to be indistinguishable in appearance and were painted white.

Phase 1

In Phase 1, birds were exposed to the blocks and learned to associate them with food. Birds were presented with two blocks placed in the center of the cage. The blocks were baited with food, and the paper disc was placed on the block so that it did not cover any part of the food well. A trial was considered a pass if the bird landed and fed from either block within 2 minutes. A trial was considered a fail if the bird did not approach the block or feed from it within 2

minutes. The time to land on the block and the time the bird ate from the block were both recorded.

Phase 1 was divided into 3 steps. In step 1, the food blocks were placed in the center of the cage with the paper disc on the block so that it did not cover the food well. After 3 successful passes, the disc was moved so that it covered half of the well for Step 2. Upon passing 3 consecutive trials of Step 2, the disc was moved so that it entirely covered the food well for Step 3. To pass a trial of Step 3, the bird had to move the disc to successfully feed from the well. A bird passed Step 3, and thus Phase 1, upon successfully feeding from the covered block 5 consecutive times.

Phase 2

In Phase 2, four blocks were placed in the cage, one in each corner, with each block baited with food. Each food well was completely covered by a paper disc. This phase was primarily for bias assessment. Birds were allowed to feed from any of the four blocks. Blocks that were landed on and fed from were recorded. To pass Phase 2, a bird had to pass 10 consecutive trials by feeding from any block. For bias assessment, the bird's favorite and least favorite blocks were noted. The block to be baited for Phase 3 was chosen between the remaining two blocks by flipping a coin.

Phase 3

Phase 3 tested the spatial memory of the bird. Four blocks were placed in the cage, but only one block was baited with food. All food wells were covered with the paper discs. A trial was considered a pass if the bird successfully landed on and fed from the baited block without

visiting other unbaited wells. Omissions were counted as failures. To pass Phase 3, a bird had to successfully feed from the correct block in 5 of 6 consecutive trials.

Phase 4

Ten minutes after the completion of Phase 3, a bird underwent Phase 4. In this phase, four blocks were placed in the cage, one in each corner, but this time the baited block was replaced with a clean, empty block for the purpose of demonstrating that the bird was not using olfaction to remember the location of the food. Phase 4 consisted of only one trial and was considered a pass if the bird successfully landed on or approached the previously baited block.

Phase 5

Twenty-four hours after the completion of Phase 4, birds underwent Phase 5. Four blocks were placed in the cage with each food well covered by the paper disc with none of the blocks baited. Phase 5 was considered a pass if the bird approached the block that had been previously baited the day before.

Statistical analyses

All statistical analyses were performed using SPSS 21. We tested whether baseline, adrenocortical stress response, or change in corticosterone secretion after translocation, predicted birds' spatial learning and memory using stepwise linear regression and generalized mixed model. The following parameters indicated birds' ability to learn: total number of trials a bird took to complete phase 1, 2, or 3. Latency to approach the wooden block in phase 1, 2, or 3 indicated neophobia and exploration tendencies. These parameters were regressed against

baseline and stress-induced corticosterone levels before and after translocation, magnitude of change in baseline or stress-induced corticosterone levels due to translocation (calculated by dividing baseline levels after translocation by baseline levels before translocation), and magnitude of stress response before and after translocation (calculated by dividing stress-induced levels by baseline levels). Whether a bird passed or failed phase 3 also indicated its learning abilities. In comparison, whether a bird passed or failed in phase 4 and 5 indicated spatial memory of the subject. These three parameters were analyzed using logistic regression, using subject ID as a random effect and corticosterone values mentioned above and treatment (control or mercury) as fixed factors. Values outside of 4 standard deviations away from the mean were considered outliers, thus were removed from the statistical analysis. This excluded values from one control male whose baseline and stress-induced levels were 5 to 8 times higher than the average values of respective corticosterone levels. Final sample sizes for phase 1, 2, 3, 4, and 5 were 17, 21, 20, 17, and 20, respectively. Interaction terms were removed when $p > 0.15$. Means ± standard errors are presented in the text and the figures.

Results

Birds' ability to learn and corticosterone levels

In general, the higher the corticosterone levels, the longer it took the bird to learn the procedure (removing the paper disc) and to learn the location of food. Baseline corticosterone level before translocation was positively correlated with total number of trials in phase 1 ($r^2 = 0.234$, $F = 4.581$, $p = 0.049$; Fig. 3.1). Magnitude of the stress response after translocation was also positively correlated with total number of trials in phase 2 ($r^2 = 0.499$, $F = 18.944$, $p < 0.001$; Fig. 3.2). None of the corticosterone parameters predicted the total number of trials to complete phase 3 ($p > 0.05$). In contrast, when corticosterone levels were compared among birds which

passed and failed phase 3, birds that failed phase 3 had a significantly higher magnitude of change in baseline corticosterone levels due to translocation ($F = 5.216$, $p = 0.033$; Fig. 3.3). Birds which failed phase 3 also tended to have higher baseline corticosterone levels ($F = 3.066$, $p = 0.095$) and magnitude of adrenocortical response ($F = 3.212$, $p = 0.090$; Fig. 3.4) before translocation compared to ones that passed. Mercury treatment did not alter the chance of passing phase 3.

Latency to approach and corticosterone levels

Similar to the relationship between a bird's ability to complete a spatial task and corticosterone level, latency to approach a food block was positively correlated with corticosterone levels. More specifically, latency in phase 1 was positively correlated with stress-induced levels of corticosterone before birds were translocation from Virginia to Alabama ($r^2 = 0.311$, $F = 6.765$, $p = 0.02$; Fig. 3.5). In addition, latency in phase 2 was positively correlated with the magnitude of stress response after translocation ($r^2 = 0.200$, $F = 4.741$, $p = 0.042$). Latency in phase 3 was not significantly correlated with corticosterone parameters ($p > 0.05$).

Birds' spatial memory and corticosterone levels

We could not analyze the relationship between birds' chances of passing phase 4 and corticosterone levels as all the birds with corticosterone values passed phase 4. Corticosterone levels of birds that failed were similar to those of ones that passed ($p > 0.05$). The same occurred for Phase 5. This is because all birds with CORT data passed Phases 4 and 5.

Discussion

A higher magnitude of stress response was positively correlated with total number of trials in Phase 1, the shaping phase. Phase 1 consisted of associating the wooden blocks with food and learning to move the paper disc to expose the food. The same occurred for Phase 2: a higher magnitude of stress response due to translocation correlated with a higher total number of trials to complete Phase 2. Phase 2 served to assess spatial bias in the zebra finches and to provide them with practice at the test procedure using four covered blocks. Thus, birds that experienced a higher magnitude of stress response due to translocation generally took longer to learn the procedure of removing the paper disc to feed from the wooden block than those that did not. Similarly, latency in Phase 1 was positively correlated with stress-induced levels of CORT before birds were translocated from Virginia to Alabama, and latency in Phase 2 was positively correlated with the magnitude of stress response after translocation. This indicates that birds that are more sensitive to the translocation stressor were more neophobic and took longer to learn the tasks than birds that were less sensitive to the translocation stressor.

Birds that failed Phase 3, remembering the location of the food, experienced a higher magnitude of change in baseline CORT levels due to translocation. This again indicates that birds which are more sensitive to one stressor had lower ability to learn spatial information compared to birds which were more tolerant to the stressor. Additionally, birds that failed Phase 3 tended to have a higher baseline CORT level and higher stress-induced levels before translocation. In general, we have shown that translocation increases baseline CORT levels and suppresses stress-induced levels of CORT in zebra finches (Chapter 2). The fact that we did not observe any significant or positive trend between baseline CORT levels, or stress-induced levels of CORT, and failing of Phase 3 indicates that a change in CORT due to translocation may have disrupted a potential relationship between CORT levels and spatial learning.

It is well documented that stress exerts potent effects on learning and memory in a U-shaped dose-effect curve (Sandi and Pinelo-Nava 2007). It is possible that the addition of the translocation stress moved the birds from the peak of the U-shape to lower on the curve, with the impacts of methylmercury not being as obvious. Thus, sensitivity to one stressor could serve as a predictor to how an organism will perform cognitively.

Contrary to our prediction, corticosterone levels were not related to whether a bird remembered the location of the food (Phase 3). Reported elsewhere, we also showed that mercury only suppressed spatial learning (Phases 1 and 2) but did not affect spatial memory (Phase 3). This is contradictory to previous findings from Swaddle et al. which found that methylmercury had a negative impact on spatial memory (Swaddle et al. 2017). Nonetheless, the fact we only observed a significant relationship between CORT levels and spatial learning whose phases were affected by dietary mercury suggests that CORT level may play a role in the mercury-associated suppression of spatial learning.

Birds with a higher magnitude of stress response due to translocation took longer to learn the procedure and pass Phases 1 and 2, and they were more likely to fail Phase 3. Increased CORT is associated with memory retrieval and recollection deficits (Chauveau et al. 2010, De Quervain et al. 2009, Dorey et al. 2012). Our results are consistent with the literature in that birds with a higher magnitude of stress response due to translocation took longer to remember the procedure for moving the disc to expose the food reward; further, they were more likely to fail Phase 3. Phase 3 served as our test of spatial memory as it was the phase that required birds to remember the location of the food reward and approach the appropriate block as a result.

Our experiment found that translocation stress and subsequent increased baseline CORT before translocation that did not return to normal, negatively impacted a bird's performance on

spatial memory testing. Chronically high CORT circulating through the bird's body for an extended period of time likely combined with the lifelong methylmercury exposure is the likely culprit for the increased number of trials to pass Phases 1 and 2 and the inability to pass Phase 3 (recalling the location of the food reward). More studies are needed to know if this is unique to the translocation stress event or if another type of acute stressor would produce the same effects.

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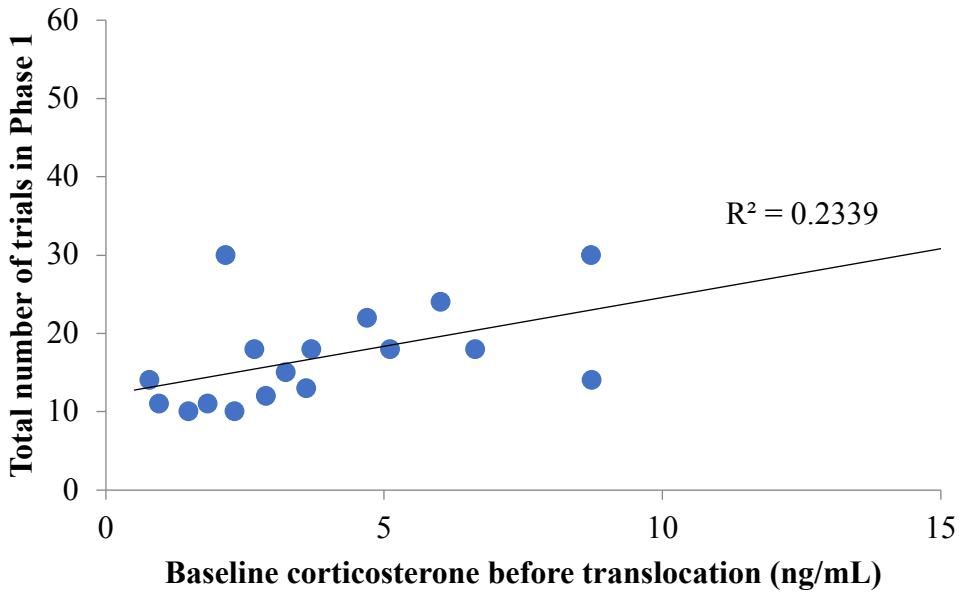

Figure 3.1: Effect of baseline CORT before translocation on total number of trials to complete Phase 1, which consisted of shaping and learning to move the paper discs to expose the food reward.

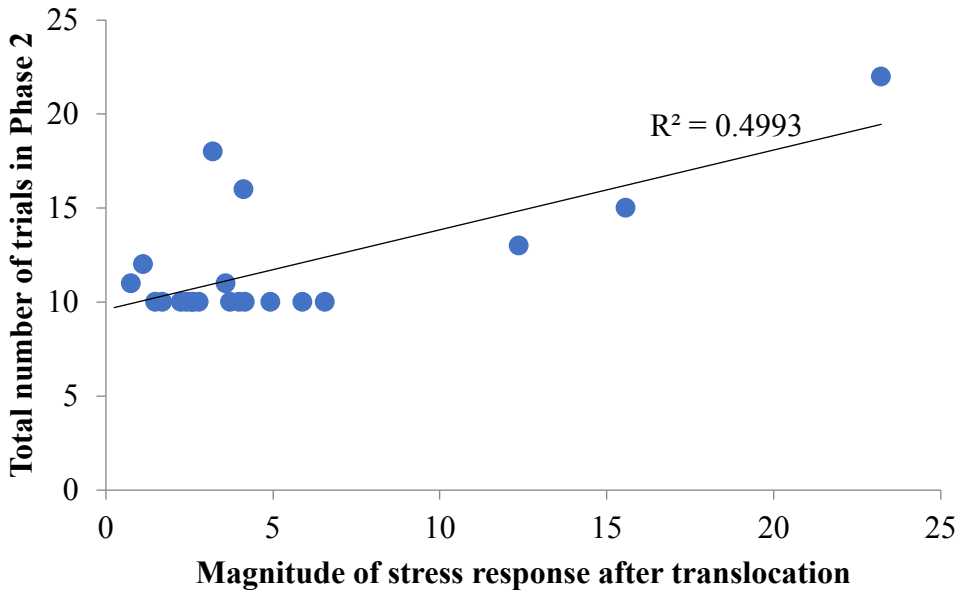

Figure 3.2: Effect of stress response after translocation on total number of trials to complete Phase 2, which consisted of bias assessment and learning to approach 1 of 4 blocks in a trial for a food reward.

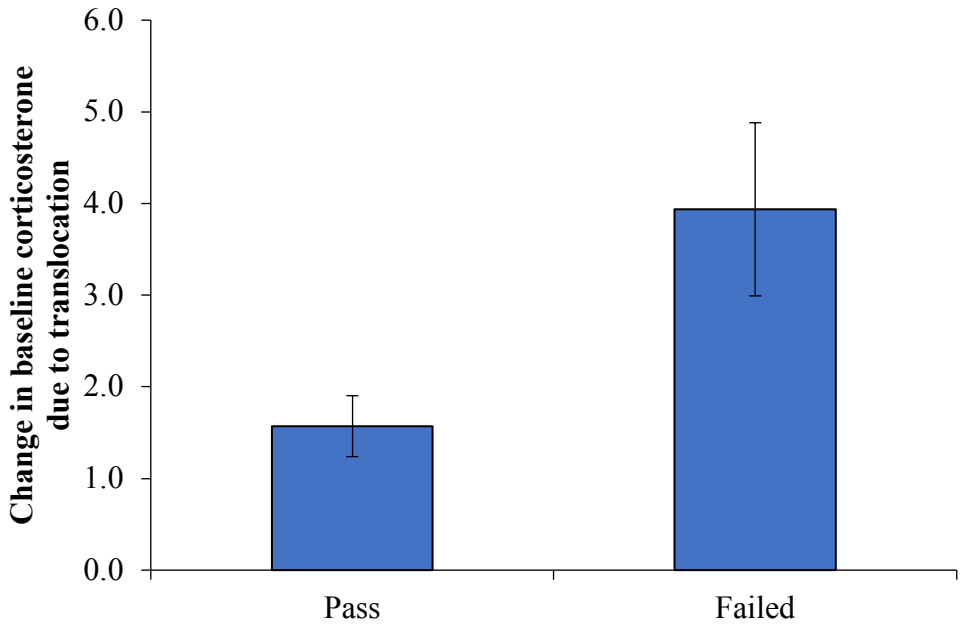

Figure 3.3: Change in baseline CORT due to translocation in birds which passed Phase 3 and in birds which failed Phase 3. Birds with a higher change in baseline CORT due to translocation were more likely to fail Phase 3, which consisted of recalling the location of the food reward.

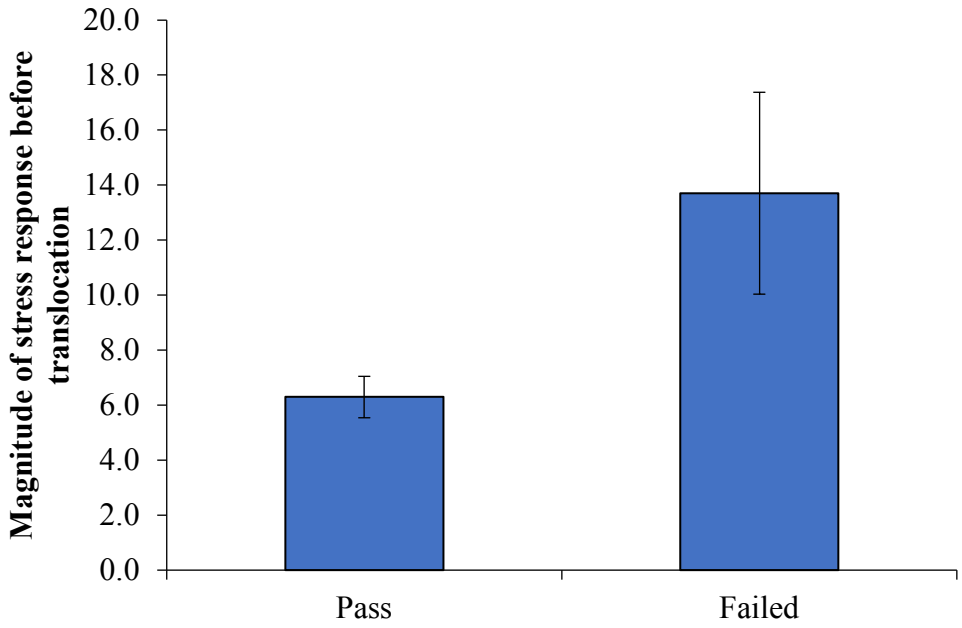

Figure 3.4: Magnitude of stress response before translocation compared to number of passes and fails in Phase 3, which consisted of recalling the location of the food reward. Birds that failed Phase 3 had more of a stress response before translocation than those birds that passed.

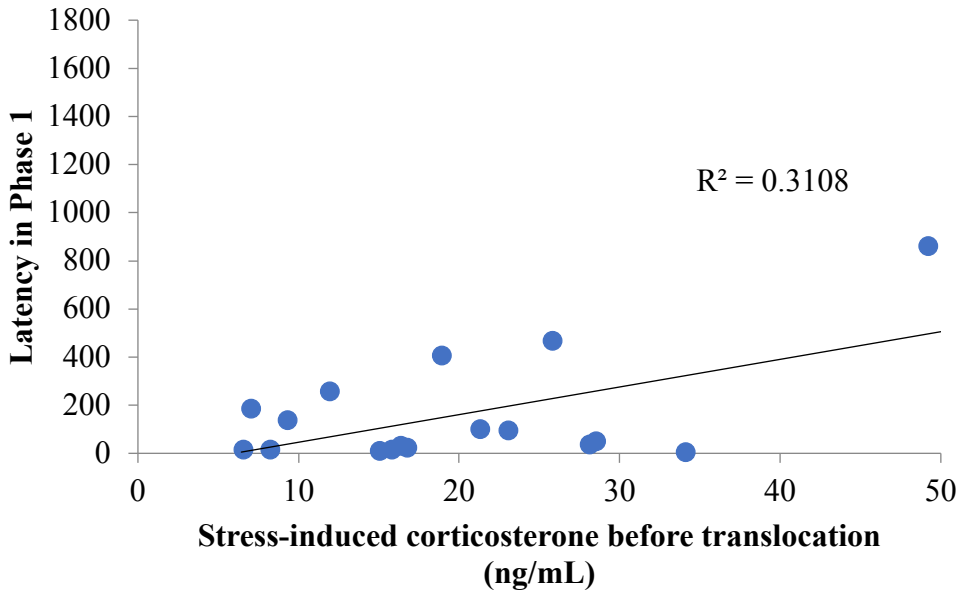

Figure 3.5: Effect of stress-induced CORT before translocation on latency in Phase 1, which consisted of shaping and learning to move the paper disc to expose the food reward. Latency in Phase 1 is positively correlated with stress-induced levels of CORT before translocation.