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A Novel Test of the Immunocompetence Handicap Hypothesis

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COMPLIANCE PAGE

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ABSTRACT

The immunocompetence handicap hypothesis (ICHH) provides an explanation for the display of honest secondary sex characters as influenced by androgen levels, the immune system and its response, and parasite infection and burden. According to this hypothesis, these three variables, along with the secondary sex character, are involved in a feedback loop in which their interactions produce an honest signal of genetic resistance to parasites. The ICHH has received a lot of attention since its introduction, with many studies focusing on single variables of the hypothesis. I performed a novel test of the ICHH, in which all variables were examined, by exposing a model songbird, the zebra finch (Taeniopygia guttata), to low levels of a known toxin, methylmercury, to manipulate phenotypic quality. I factorially manipulated parasitic infection in both treatment groups by experimentally infecting one group with a parasite known as the coccidian. I assessed the expression of bill color in male zebra finches, a sexually selected trait. According to the ICHH, males that had the dual challenge of parasitic infection and immune suppression due to methylmercury would have the least red bills, demonstrating their lower quality. I did not find a difference between immunosuppressed males infected with coccidians and all other birds. This does not provide support for the ICHH, however it lays important groundwork for the thorough study of the ICHH in future research.

TABLE OF CONTENTS

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This Master's is dedicated to Joseph Smith and my family who have helped me countless times along the way…

LIST OF TABLES

LIST OF FIGURES

Introduction

Charles Darwin introduced the theory of sexual selection in 1871, after observing that some members of a species appeared to be chosen as mates while others were not. He hypothesized that exaggerated secondary sex characters evolved as a result of certain individuals having an advantage over others in gaining access to mates. Darwin concluded that female mate choice could be a selective force for these exaggerated characters or behaviors, but had no explanation for why they had evolved. Since its introduction, sexual selection has been a major focus in research, spawning many hypotheses that provide explanations for the evolution of exaggerated characteristics including the handicap hypothesis (Zahavi 1975), parasite-mediated sexual selection (Hamilton and Zuk 1982), and the immunocompetence handicap hypothesis (Folstad and Karter 1992).

Handicap Hypothesis

The handicap hypothesis proposes an explanation for the evolution of exaggerated secondary sex characters. According to this hypothesis, exaggerated secondary sex characters signal specific information about an individual's quality to potential mates - the more extravagant the display, the better quality the male (Zahavi 1975). These extravagant displays are considered handicaps because they are costly to maintain. Males have to take risks and invest time and energy to produce quality displays. Females, therefore, should prefer males who can bear the costs of higher handicaps because they

demonstrate the male's ability to survive despite the presence of the handicap. The cost of the handicap serves as an honest signal of the quality of the individual. Only a higher quality individual would be able to develop the greater expression of the handicap. In turn, this can also imply high quality in other areas, such as the ability to provide food and avoid predators (Krebs and Davies 1993).

For a signal to be reliable and honest, "cheating" should not occur. The handicaps result in the signaler (the individual expressing the characteristic) having to invest more in the signal than it would gain by conveying unreliable information. As a result, cheating is prevented because faking the signal would be unprofitable to the cheater, and females are ensured a quality mate (Zahavi and Zahavi 1997). Examples of handicaps can be seen in a variety of displays and behaviors. For example, male songbirds that invest most of their days in singing rather than foraging demonstrate their superior quality and ability to properly feed themselves while still producing an impressive repertoire of songs (Zahavi and Zahavi 1997). Male barn swallows with experimentally elongated tails had decreased foraging and survival, demonstrating the cost of long tails (Møller and Delope 1994). The results could be interpreted, however, as demonstrating the cost of cheating, rather than the cost of the handicap. Bright, colorful plumage has also been suggested to serve as a handicap because of its potential to attract rivals or predators and only a high-quality male would be able to take that risk (Zahavi and Zahavi 1997).

While some studies have provided examples of secondary sex characters as handicaps, the hypothesis has its limitations. It is important to consider the signal the handicap is sending. An assumption of this hypothesis is that the signal is consistent across signalers and that each individual receiving the signal perceives it in the same way. This may not always be the case. Signals from individuals can vary in size, intensity, and frequency (Bullock 2012) and may not be perceived the same by each individual, thus reducing the validity of the honest signal. In addition, the exaggerated trait serving as the handicap must in fact be preferred by females in order for selection to act, regardless of its representation of quality. The handicap hypothesis and its general ideas prompted the further development of sexual selection hypotheses, some an expansion of the original hypothesis.

Parasite-Mediated Sexual Selection

Parasite-mediated sexual selection expands the handicap hypothesis by stating that the degree of development of secondary sex characters indicated an individual's health and quality, but more specifically their ability to resist parasites and pass on this resistance (Hamilton and Zuk 1982). According to this hypothesis, good health and freedom from parasites are demonstrated by bright plumage and pelage, or vigor conveyed in fighting or athletic performances. Females are able to distinguish healthy males by greater development of these ornaments or behaviors, and in turn prefer these males (Zuk et al. 1990). According to this hypothesis, exaggerated secondary sex characters are still

considered honest signals because an unhealthy male may be prone to even more disease later if it expends too much energy on ornament development, rather than using those resources to combat infection (John 1997). Based on their observations of North American passerines and their blood parasites, Hamilton and Zuk (1982) went so far as to propose that on an interspecific level, the brighter, more showy bird species are most subject to parasitic infection, have a higher diversity of parasites, and thus undergo stronger selection for resistance. However the surveys used to develop this prediction were limited in their locality and may not be widely applicable (John 1997). In addition these surveys examined the variety of blood parasites present, and did not examine parasites in other parts of the body. Blood parasites may be pathogenically different from other parasite species, thus affecting the host and the host's immune system differently. Therefore, it is important to consider parasite ecology in edition to host ecology (Clayton and Moore 1997).

This hypothesis makes three assumptions: 1) the development of the secondary sex characters is related to the intensity of parasitic infection; 2) females base their mate choice on the secondary sex character that is related to parasitism; and 3) resistance to the parasite is heritable (Brawner et al. 2000). By mating with less-parasitized males, females gain direct and indirect benefits – the indirect benefit of passing parasite resistance genes onto their offspring and the direct benefit of avoiding contamination during mating (John 1997, Fedorka and Mousseau 2007).

Studies involving parasite-mediated sexual selection have provided mixed support for the hypothesis. A review by Zuk (1992) indicated that the best support for this hypothesis occurred in systems where the ornament is carotenoid derived or mediated by testosterone. When red jungle fowl (*Gallus gallus*) were experimentally infected with roundworms, parasitized males had shorter tail feathers and combs, and duller feather and comb colors (Zuk et al. 1990). Non-sexually selected characters, such as tarsus length and bill length and width, however, did not differ between parasitized and unparasitized males. When mate choice trials were performed, females preferred the unparasitized males to the parasitized males. Horn size of male African buffalo (*Syncerus caffer*) is a positive predictor of body condition and infection with both coccidian and nematode parasites. More specifically, males with larger horns are less likely to be infected with both parasites (Ezenwa and Jolles 2008). In two species of Lake Victoria cichlid fish (genus *Pundamilia*), brighter blue males and males with more red (sexually selected characters) were infected with fewer species of parasites (Maan et al. 2006, 2008). Further support for parasite-mediated sexual selection has been observed in house finches (*Haemorhous mexicanus*, Brawner et al. 2000), black grouse (*Tetrao tetrix*, Hoglund et al. 1992), and ring-necked pheasants (*Phasianus colchicus*, Hillgarth 1990). It should be noted, however, that many of these studies did not include mate choice trials, and therefore lack in evidence for one of the main assumptions of the hypothesis. In addition, the use of a controlled experimental infection is not commonly used and instead, unaltered, naturally occurring parasite loads are examined. This opens the

possibility that other variables such as the environment, different parasite species, and previous parasite infections may be influencing both the secondary sex characters and the parasite load.

Immunocompetence Handicap Hypothesis

The immunocompetence handicap hypothesis (ICHH: Folstad and Karter 1992) is an application of the handicap principle to parasite-mediated sexual selection in which the development of exaggerated secondary sex characters are influenced not only by parasite burden, but also its interaction with several additional physiological variables, or hidden costs. There are three variables that are responsible for the expression of an honest signal in the form of a secondary sex character: androgen levels, the immune system and its response, and parasite infection and burden. These three variables, along with the secondary sex character, are involved in a feedback loop in which the variables interact with one another to produce an honest signal of genetic resistance to parasite infection. Folstad and Karter (1992) based this prediction on the observation that these variables have been shown to interact with one another individually. It is generally accepted that testosterone levels positively influence secondary sex characters; specifically, an increase in testosterone leads to an increase in the expression of many secondary sex characters (Fusani 2008). In contrast, testosterone can negatively affect the immune system by suppressing cell production and proliferation (Folstad et al. 1989, Zuk et al. 1995, Evans et al. 2000, Roberts et al. 2004, Deviche and Cortez 2005, Kurtz et al. 2007, Gil and

Culver 2011, Kindt et al. 2007 but see Fuxjager et al. 2011, Martinez-Padilla et al. 2010, Roberts et al. 2007, and Hasselquist et al. 1999). In addition, reduced immune performance has been observed during the mating season in vertebrates, presumably when secondary sex characters are at their greatest expression. This indicates that males may face a trade-off between high expression of secondary sex characters or a strong immune response to potential disease and infection, and that testosterone levels may serve as a handicap in secondary sex character expression. Parasites will elicit an immune response from the host, but in addition they have been shown to negatively influence the development of secondary sex characters and behaviors (Zuk et al. 1990, Bucholz 1995, Enzenwa and Jolles 2008).

The aforementioned variables and their interactions create a negative-feedback connection between signal intensity of the secondary sex characteristic and the interactions of parasitic infection with the immune system and testosterone (Figure 1). A parasitic infection may become more severe in response to an increase in testosterone (Arrow F), which elicits an immune response from the host (Arrow D). The increase in testosterone may result in an increase in expression of secondary sex characters (Arrow A) but will also suppress the immune system and presumably the response to the parasitic infection (Arrow B and C). Finally, this increase in parasitic infection may be simultaneously suppressing secondary sex character expression (Arrow E). The expression of the secondary sex characters is a plastic response that is adjusted according to the cost of infection and immunosuppression compared to the

benefit of increased reproductive success, which is mediated by the degree of expression of the secondary sex characters (Folstad and Karter 1992). In addition, the immunocompetence handicap allows for honest signaling by suggesting that only an individual with "good genes" for parasite resistance would be able to allot less energy into fighting a parasitic infection, resulting in a lower cost, relative to an individual with low genetic resistance, for a high-quality signal. Ultimately, males with showy and highly developed secondary sex characters are demonstrating their ability to resist the effects of a parasitic infection even with a compromised immune system (Zuk 1992).

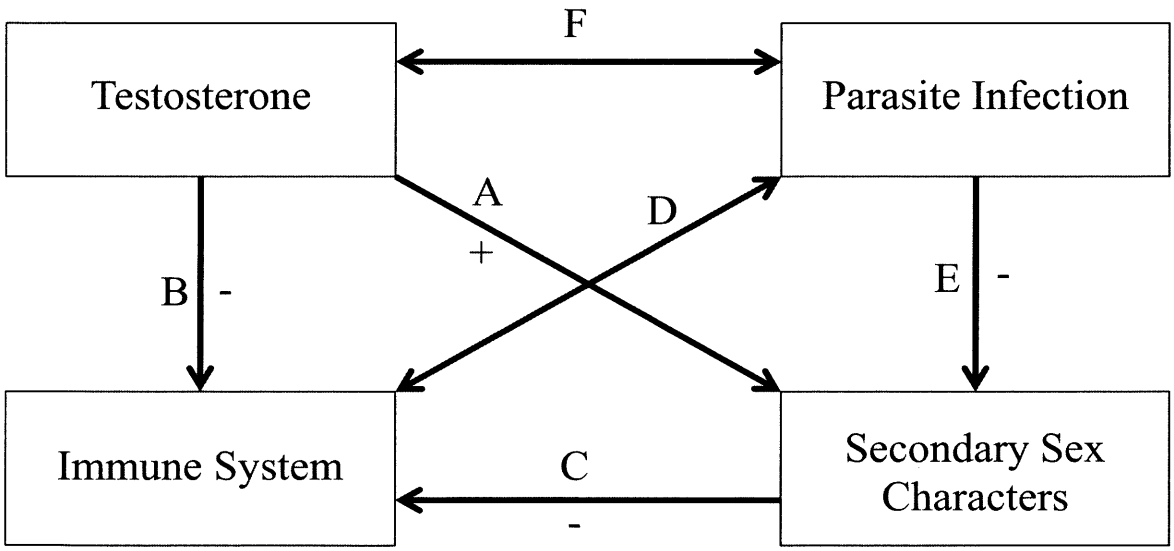

Figure 1: Diagram describing the variables of the immuncompetence handicap hypothesis and the interaction of each variable with one another.

In mammals, birds, lizards, and fish, the expression of many secondary sex characters - including aggressive and display behavior, vocalizations, pheromones, social status, and even fertility – are dependent on androgen levels and are often sensitive to variation in testosterone levels in particular (Zuk 1992,

Kurtz et al. 2007). Male vertebrates have demonstrated a rise in testosterone during mating season and prior to sexual activity (Roberts et al. 2004, Kurtz et al. 2007, Fusani 2008, Fuxjager et al. 2011), indicating that, though there may be exaggerated expression of secondary sex characters, male immune systems may simultaneously be suppressed, exposing males to higher risk of infection. While a rise in testosterone tends to have a positive effect on the expression of secondary sex characters, experimental manipulation of testosterone levels has shown that a high level of testosterone has a negative effect on the immune system, increasing parasite load and decreasing cell-mediated and humoral immunity (Folstad et al. 1989, Zuk et al. 1995, Evans et al. 2000, Roberts et al. 2004, Deviche and Cortez 2005, Kurtz et al. 2007, Kindt et al. 2007, Gil and Culver 2011). It should be noted however, that other research has challenged this idea by showing that males with artificially high levels of testosterone had similar immune responses to males with normal or reduced levels of testosterone (Fuxjager et al. 2011, Martinez-Padilla et al. 2010, Roberts et al. 2007, Hasselquist et al. 1999). Parasitic infections elicit an immune response in an infected individual, but they can also influence the development of secondary sex characters (Zuk et al. 1990, Mougeot et al. 2007), for example by decreasing plumage coloration (Brawner et al. 2000, Costa and Macedo 2005, Horak et al. 2004, McGraw and Hill 2000). Some studies have shown that parasite development, behavior, and reproduction are often correlated with the host's reproductive events - while high testosterone levels may be decreasing immunity,

parasite fecundity could be increasing (Folstad and Karter 1992, Poiani et al. 2000, Fuxjager et al. 2011).

Since its introduction, the ICHH and its components have received a lot of attention, with much research focusing on the mechanisms and assumptions of the ICHH. In domestic fowl (*Gallus domesticus*) artificially selected for either low antibody or high antibody response to sheep red blood cell antigen, male fowl from the low antibody line had significantly higher levels of testosterone in their blood, and larger combs (a sexually selected character) than those from the high antibody line (Verhulst et al. 1999). These results demonstrate a trade-off between testosterone and immune suppression, indicating that males with suppressed immune systems were expressing higher levels of testosterone; however, this study failed to provide a parasitic infection to demonstrate immune suppression in response to an antigen. Male red grouse (*Lagopus lagopus scotica*) with testosterone implants had reduced immunocompetence and condition, and higher prevalence of coccidian infection (Mougeot et al. 2004). In addition, in a separate experiment, male red grouse that were experimentally infected with a nematode parasite had reduced carotenoid-based coloration of their combs, while males with experimentally increased levels of testosterone increased the red coloration of their combs (Mougeot et al. 2007). However, the nematode infection and testosterone manipulation experiments were performed separately from one another and thus do not demonstrate an interaction between parasitic infection and testosterone levels. Barn swallows (*Hirundo rustica*) with higher testosterone levels had higher ectoparasite loads, providing some support

for the suppression of the immune system by the testosterone mechanism of the ICHH (Saino et al. 1995). In contrast to the ICHH, however, barn swallows that had elevated testosterone levels also possessed an increase in eosinophil white blood cells, which could indicate that the males were not paying an immune cost for high testosterone levels. Tail length in barn swallows is a sexually selected character influenced, at least in part, by testosterone, and barn swallows that had their tails experimentally elongated had a suppressed response to sheep red blood cells. Barn swallows whose tails were not experimentally manipulated did not show a suppressed response to sheep red blood cells (Saino and Møller 1996). These findings indicate that tail length is a costly secondary sex character to maintain and males may dedicate more resources to maintenance of this character than other areas, such as immune response.

While the studies listed above provide partial support for the ICHH, many other studies have failed to find evidence for the ICHH, with some providing evidence against key mechanisms in the hypothesis. In red grouse that were experimentally manipulated with testosterone and parasitic infection, no interaction between testosterone and parasitic infection on ornament size or coloration was observed (Martinez-Padilla et al. 2010). This result provides evidence against testosterone acting as an immunosuppressant, but it is possible that comb coloration may not be heavily influenced by the specific parasitic infection. In a study that looked at the genetic component of the ICHH in scorpionflies (*Panorpa vulgaris*), half-sibling families were bred with males that had high expression of a condition-dependent ornament trait (saliva secretion)

and males that had low expression of the trait (Kurtz and Sauer 1999). There was no difference in immune traits between the male half-siblings, which fails to support the "good genes" effect on offspring as predicted by the ICHH. Mountain spiny lizards (*Sceloporus jarrovi*) with experimentally increased testosterone levels had a decrease in the number of gut-dwelling parasites, which is contrary to the predications of the ICHH in which an increase in testosterone would suppress the immune system and result in an increase in parasite number (Fuxjager et al. 2011). This experiment, however, examined natural parasite presence and number, with no manipulation of infection, and thus could have been influenced by random factors such as previous infection and immunity to certain parasites. Similarly, in red-winged blackbirds (*Agelaius phoeniceus*) with experimentally increased levels of testosterone, antibody response levels to an injected antigen were not related to levels of testosterone, indicating no relationship between testosterone levels and the immune system (Hasselquist et al. 1999).

The studies to date have provided mixed results regarding the ICHH, and earlier studies that have provided support should be treated with caution, particularly because of the more recent evidence against the suppression of the immune system by testosterone (Roberts et al. 2004). Many studies, including those listed above, have examined specific components of the ICHH (i.e. immune suppression due to testosterone, secondary sex character expression due to testosterone, parasitic influence on expression of secondary sex characters), and not the hypothesis as a whole, providing, at best, partial

support. While most studies have focused on manipulation of testosterone, few have manipulated parasite infection, and even fewer have experimentally infected organisms with a known number of parasites.

In this experiment I examined all components of the ICHH except testosterone using a model songbird, the zebra finch (*Taeniopygia guttata*). Because there is increasing evidence that testosterone is not a consistent immunosuppressant, I used methylmercury, an ecotoxin that has been shown to suppress the immune system (Kenow et al. 2007, Fallacara, et al. 2010, Lewis et al. 2013), to compromise the immune system. I also challenged the birds with a standardized parasitic infection using a coccidian parasite, resulting in four treatment groups: (i) immunocompetent and uninfected, (ii) immunocompromised and uninfected, (iii) immunocompetent and infected, and (iv) immunocompromised and infected (Figure 2). I measured an important sexually selected and heritable trait of the zebra finch, the bill color (Zann 1996, Blount et al. 2003, Simons and Verhulst 2011), to determine if a change in the production of this secondary sex character occurred in response to the immune suppression and parasite challenge, as predicted by the ICHH. The following paragraphs further describe the four components used in my novel test of the ICHH (Figure 2).

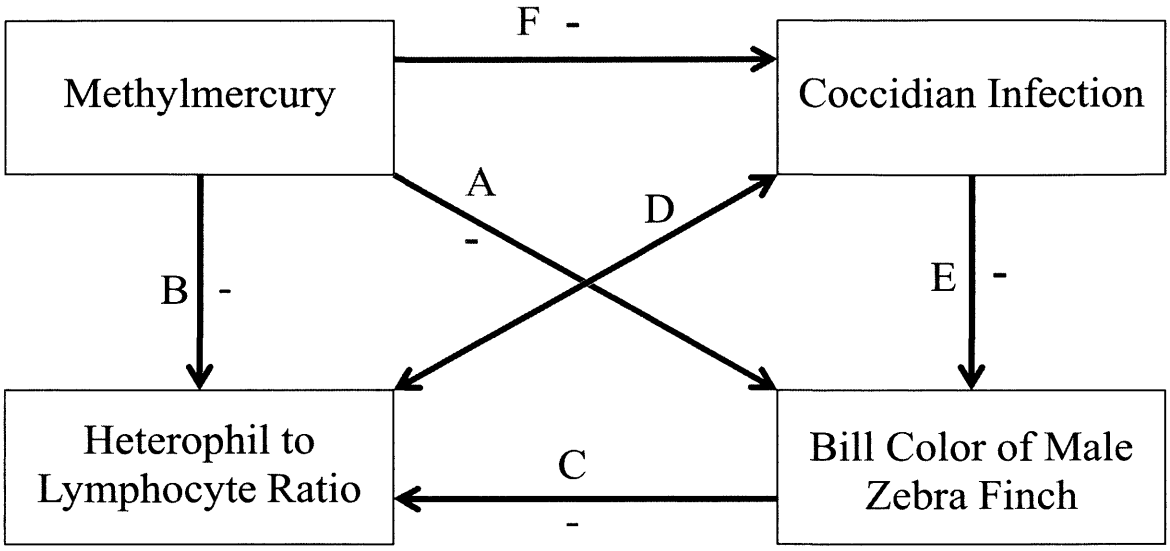

Figure 2: Diagram describing the variables in my experimental test of the ICHH and their known (in black) and predicted (in red) interactions with one another.

The Avian Immune System

The ICHH describes many variables contributing to the feedback loop responsible for secondary sex character expression. An important component is the immune system and its function, which I measured using heterophil to lymphocyte ratios (Figure 2, arrows B, C, D). The immune system is an organism's primary defense against invading foreign substances, such as pathogens, parasites, foreign proteins, and anything considered "nonself". The immune system has the ability to communicate throughout the body, remember previous encounters, and react to an invasion of foreign pathogens (Fairbrother et al. 2004, Kindt et al. 2007). All types of immune function require resources that the host might have otherwise used for another function. The use and allocation of these resources may influence the immune system directly or indirectly. For

example, the energetic cost of an immune response, like a fever, may be substantial and thus a direct influence on the immune system. If an organism has poor nutrition or condition, the immune response may be indirectly influenced due to the necessity to allocate resources elsewhere (Sheldon and Verhulst 1996, Norris and Evans 2000).

The immune system is comprised of two parts: innate (nonspecific) immunity and acquired (specific) immunity. Innate immunity is the first line of defense against invading pathogens, and involves the use of phagocytic macrophages and the inflammatory response (Kindt et al. 2007). Acquired immunity is specific to particular antigens and has the ability to recognize, eliminate, and remember pathogens (Kindt et al. 2007). Acquired immunity is further divided into two parts: humoral immunity, which involves the production of antibodies by B cells, and cell-mediated immunity, which involves the development and proliferation of T cells (Kindt et al. 2007). In birds, the main immune organs in which many immune cells are produced are the thymus and the bursa of Fabricius, which are the sites of T cell and B cell maturation, respectively (Grasman 2002, Fairbrother et al. 2004). T cells produce cytokines, chemicals that act as intercellular messengers and can enhance the immune response of other T cells, B cells, or macrophages (Fairbrother et al. 2004). Avian B cells produce three classes of antibodies, immunoglobulin M (primary antibody), immunoglobulin G (secondary response) and immunoglobulin A (mucosal). Antibodies will neutralize viruses, coat bacteria for phagocytosis, bind to T cells, and activate the complement system of the innate immunity

(Fairbrother et al. 2004). In general, acquired immunity is crucial in controlling pathogens such as viruses, intra- and extra-cellular parasites and ectoparasites, while innate immunity plays a role in the control of many infections at the initial stages, and is the primary means of controlling bacterial infections (Norris and Evans 2000).

There are two main techniques for measuring immunocompetence: monitoring or challenge (Norris and Evans 2000). Monitoring techniques provide a measure of an individual's health status at the time they were sampled. Monitoring techniques include counting leukocytes, calculating heterophil to lymphocyte (H:L) ratios, or measuring plasma and serum proteins and immune organs (Norris and Evans 2000, Grasman 2002). Challenge techniques involve exposing a component of the immune system to a novel antigen and quantifying the immune response. Challenge techniques are useful because the pathogen challenge is standardized, so any variation between individuals' strength of response is a measure of their immunocompetence (Norris and Evans 2000). Examples of challenge techniques include the use of phytohemagglutinin (PHA) to stimulate T cell proliferation and differentiation, or immunizing an organism with sheep red blood cells (SRBC) to measure antibody titers in response to the antigen (Grasman 2002).

In this experiment, I used monitoring techniques to determine the status of the immune system. Specifically, I determined the H:L ratio (heterophils to lymphocytes) prior to parasitic infection and again after infection with the parasite. Heterophils are granulated phagocytic cells from the innate immune

system (Norris and Evans 2000) that are early modulators of the inflammatory response and are known to increase in response to physiological perturbations. Lymphocytes are the circulating T and B cells of the acquired immune system (Norris and Evans 2000) and will increase in response to an infection. H:L ratios are often used to assess stress levels in organisms because they have been shown to increase in response to stress (Grasman 2002). These ratios have also been shown to increase in response to infections (Davis et al. 2004). Rose et al. (1979) examined white blood cell changes in response to a coccidian infection in rats and chickens and found an increase in lymphocytes in infected individuals.

Methylmercury

The ICHH emphasizes the suppressive effect that testosterone has on the immune system, however, in this study, I suppressed the immune system with an ecotoxin, methylmercury (Figure 2, arrows A, B, F). Mercury is a globally distributed and persistent environmental contaminant that poses a health risk to a wide variety of taxa, including wildlife, domesticated animals, and humans (Weiner et al. 2002). Methylated mercury has the ability to biomagnify up the food web, increasing in concentration with each trophic level (Weiner et al. 2002). Exposure to low levels of methylmercury can have substantial biological effects. Traditionally, mercury has been considered a threat mainly to aquatic wildlife and piscivorous birds; however, recent research by our lab and collaborators has shown that methylmercury poses a substantial risk to terrestrial organisms as well, including small songbirds (Cristol et al. 2008).

In birds, methylmercury can suppress the immune system, which, as described above, plays a pivotal role in defense against pathogens and parasites. Specifically, exposure to environmentally relevant levels of methylmercury delayed the proliferation of B-lymphocytes in zebra finches (Lewis et al. 2013) presumably resulting in compromised immune functioning. Additional research has indicated immunosuppression by methylmercury in free-living wild birds (Fallacara et al. 2010, Kenow et al. 2007, Hawley et al. 2009).

Coccidians

Coccidian parasites are intestinal parasites that infect the epithelial cells of the intestine in birds, mammals, reptiles, amphibians, and fish (Roberts and Janovy 2005, Figure 2, arrows D, E, F). Coccidia are found in a wide variety of avian species, with the genus *Eimeria* infecting primarily poultry and game species and the genus *Isospora* common in songbirds (Horak et al. 2004). Organisms become infected with coccidians when they ingest sporulated oocysts. Once the oocysts are ingested, the oocyst releases sporozoites into the epithelial cells of the intestine. Here, the sporozoites begin to reproduce asexually and enlarge, forming meronts (Roberts and Janovy 2005). Eventually, the meronts separate into merozoites, which break out of the cells into the cecum lumen, destroying the host cells, and invading new epithelial cells. The asexual reproduction described above is repeated, but some of the merozoites form gametocytes. The gametocytes transform into gametes, fuse, and form a cyst (oocyst) around the zygote. The oocyst is released from the host's cells and is

passed out of the body in the feces (Roberts and Janovy 2005). The asexual cycle is genetically programmed to only occur a specific number of times (3, 4, 5, etc.). Once these cycles no longer occur, only gamonts are produced (Duszynski pers. comm.). In general, oocysts appear in the feces within six days of infection (Roberts and Janovy 2005), but have been observed in passerine species within three days or with a delay of up to nine days after infection (Filipiak et al. 2009). Generally, infection lasts about a month, but can range from two weeks to three months (Hill and Brawner 1998, Filipiak et al. 2009).

Coccidian parasites have been shown to inhibit the uptake of essential dietary components, such as carotenoids, and to reduce carotenoid-based pigmentation in both poultry and passerines (Tyczkowski et al. 1991, Brawner et al. 2000, McGraw and Hill 2000, Horak et al. 2004). Carotenoids are molecules that play an important and diverse role in integument and plumage coloration (reds, yellows, and oranges) and can serve as antioxidants, protecting the body from free radicals by accepting unpaired electrons from singlet oxygen and other free radicals (Hill and McGraw 2006). Carotenoids cannot be manufactured in the body and therefore must be obtained through diet. Once an animal has digested carotenoids, it can metabolize these molecules into different forms and for different uses (Hill and McGraw 2006). Once the carotenoids are retrieved from the food they are absorbed and incorporated into lipoproteins and circulated in the blood. It is believed that coccidia disrupt the absorption and transport of carotenoids throughout the body. When the coccidian encyst in the lining of the intestine it causes a thickening of the epithelium, which can inhibit the absorption

of carotenoids. The encysting of coccidians also disrupts the production of the lipoproteins that transport the carotenoids throughout the body, thus preventing deposition of carotenoids in the integument and potentially affecting secondary sex character displays (Hill and McGraw 2006).

In addition to affecting carotenoids, coccidians have also been shown to cause significant changes in the physiology of infected birds, including a decrease in serum albumin, triglyceride, and vitamin E, and in the mass and size of secondary sex characters in infected individuals (Bucholz 1995, Horak et al. 2004, Costa and Macedo 2005). Male blue-black grassquits (*Volatinia jacarina*) that are heavily infected with coccidian parasites tend to leap, a sexually selected display behavior, less frequently than less infected males (Costa and Macedo 2005). In addition, coccidian infections have been shown to negatively affect feather growth during molt in house sparrows (*Passer domesticus*, Pap et al. 2011). Coccidians are excellent parasites for testing the ICHH due to their abundance in many passerine species, and their ability to affect carotenoid-based secondary sex characteristics, as well as some behaviors and the size of some secondary sex characters.

Immune Response to Parasites

The ability of a host to regulate parasite infection depends on the ability of a host to generate immune responses that can detrimentally affect parasite growth, reproduction, or survival (Figure 2, arrow D). The type of immune response can depend on the type of parasite involved (Clayton and Moore 1997).

Protozoans, for example, are small and can be taken up by phagocytic cells. The most successful protozoan parasites, however, tend to "hide" in host cells, where they can evade immune cells and recognition is difficult (Clayton and Moore 1997). With coccidians, the sporozoites are engulfed by macrophages and carried to the epithelial lining of the intestine. Once in the epithelial lining, they are able to escape the macrophages and enter the epithelial cells (Roberts and Janovy 2005). Some host cells, when invaded, will produce novel antigens on their surface, which will recruit T cells or natural killer cells (Clayton and Moore 1997). Parasites living in tissues can also be subjected to antibodies, such as immunoglobulin A, which are resilient in the environment of the gut (Clayton and Moore 1997). In coccidian infections, once the sporozoites have multiplied into meronts and merozoites, macrophages begin to engulf and digest the merozoites. In addition, T cells residing in the gut associated lymphoid tissues (GALT) begin fighting the infection (Allen and Fetterer 2002, Roberts and Janovy 2005).

The immune response to coccidians has been studied intensely in poultry, but little is known about the immune response of wild birds to coccidians. In poultry, the GALT is the first line of defense against coccidian infections, using gastric secretions, peristalsis, and competition by normal flora to combat cell invasion. In addition, the GALT contains more than half the lymphocyte pool of the whole mucosal immune system (Lillehoj and Lillehoj 2000). Research in poultry has shown that cell-mediated immunity (T cells) is primarily responsible for fighting a coccidian infection. When poultry are experimentally depleted of T

cells prior to an infection with coccidians, they are more susceptible to infections (Lillehoj 1998). In wild birds, experimentally infected greenfinches (*Carduelis chloris*) had an increase in cell-mediated immunity response compared to non-infected birds (Saks et al. 2006). The same was found in Eurasian kestrels (*Falco tinnunculus*, Lemus et al. 2010). Experimentally infected house sparrows had a decrease in antibody response compared to control birds, but an overall increase of white blood cells (Pap et al. 2009, Pap et al. 2011).

Zebra Finch Sexual Selection

The final component of the ICHH involves the expression of secondary sex characters (Figure 2, arrows A, C, E). Zebra finches are sexually dimorphic finches in the family/subfamily Estrildidae. Males have deep-red bills while females tend to have paler orange bills. The bill color of male zebra finches is a sexually selected character and females prefer males with redder and darker bills (Zann 1996, Blount et al. 2003, Simons and Verhulst 2011). Bill color in zebra finches is carotenoid-based and an indicator of the quality of the bird. Bill color is affected by physical condition and environmental influences, such as breeding state, but it also has a heritable component (Zann 1996).

Based on the research mentioned above and represented in Figure 2, I predict that birds exposed to methylmercury will demonstrate a suppressed or lower immune response, as represented by the heterophil to lymphocyte ratio (Grasman 2002). In addition, infection with coccidians will elicit an immune response from the host, as well as challenge a host by preventing the uptake of

essential vitamins and nutrients (Tyczkowski et al. 1991, Lillehoj 1998, Horak et al. 2004), therefore I predict that birds infected with coccidian parasites will demonstrate an increase in H:L ratios compared to birds that are not infected with the parasite. Based on the predictions of the ICHH, I predict that birds with a suppressed immune system will have lower expression of the secondary sex character, or duller red bills. In addition, I predict that birds infected with the coccidian parasite will have duller red bills than uninfected birds. Finally, I predict that birds that are infected with coccidians and have additional immune suppression due to the methylmercury will have the dullest beaks due to the dual challenge they are experiencing.

Methods

I investigated the interaction of mercury-induced immunosuppression and experimental coccidial infection on bill color expression, using a factorial design with captive-bred zebra finches.

Animal Husbandry

All finches were housed individually indoors in small (12" l X 9" w x 16" h) cages on a 14:10-hour light:dark cycle at 20˚C. Birds were provided with ad libitum pelletized food daily, which contained the carotenoid canthaxanthin. In addition, birds received vitamin supplements and a limited amount (10% of a full dietary amount) of lutein carotenoid (FloraGLO Lutein, Kemin Industries Inc.) supplement in their water, plus limited oyster shell grit for digestion. The experimental room was divided in half with a sheet of plastic extending from the ceiling to the floor to separate the uninfected and infected treatment groups. Separate doors were constructed at the entrance to the room allowing for separate entrance into the uninfected side and infected sides. I changed gloves, lab coats, and foot coverings while entering each side to minimize contamination risk. On any one day, I performed all feeding, cleaning, and sampling on the uninfected side first, to reduce further the chance of infection with coccidians from the other treatment. Both sides of the room had separate trashcans, food and water dishes, water sources, and stored food.

Mercury Treatment

The four treatment groups were (i) immunocompetent (0.0ppm MeHg) plus uninfected; (ii) immunocompromised (1.2ppm MeHg) plus uninfected; (iii) immunocompetent plus infected; and (iv) immunocompromised plus infected.

To produce immunocompromised individuals, I exposed 31 adult zebra finches to methylmercury (MeHg) by mixing aqueous MeHgCysteine into their artificial complete pelletized (Zupreem) diet to a concentration of 1.2 μg/g. The control group (immunocompetent, N=30) received the same pelletized diet with water and cysteine added. The mercury (Hg) concentration used is an environmentally relevant sublethal level of exposure that birds could experience at a highly contaminated site (Cristol 2008) and has been shown to delay B-cell proliferation in captive zebra finches (Lewis et al. 2013). All Hg-dosed birds were developmentally exposed to MeHg, from egg through adulthood by dosing their parents prior to and through breeding and then continuing offspring on the same dose after they reached independence. Before and after experimentally infecting the birds with coccidians (see below), I collected a blood sample to determine the average blood mercury level (μg/g) for each bird. I analyzed the blood samples using a Direct Mercury Analyzer (DMA-80, Milestone, Inc.).

Parasite Treatment

Coccidian oocysts were collected from a naturally infected zebra finch in our outdoor zebra finch colony and identified using a compound microscope based on presence of outer oocyst wall and the presence of two or four

sporocysts inside the oocyst wall (Duszynski and Wilber 1997). All oocysts were maintained in 2% potassium dichromate (see below) at room temperature for 7 - 10 days to allow for sporulation and at 4˚C thereafter. By placing the fecal samples in the 2% potassium dichromate, I very likely killed any additional pathogens that may have been found in the feces (Duszynski, pers. comm.). To prepare the inoculation solution, I washed oocysts in distilled water. I placed 1 - 2 ml of the fecal-dichromate solution in test tubes and filled the remaining space with 8 - 9 ml of distilled water. The test tubes were centrifuged at 3000 rpm for 10 minutes and then the supernatant was removed until 1 ml of water and the pellet remained. I then added 8- 9 ml of fresh distilled water and centrifuged the test tubes at 3000 rpm for 10 minutes. This process was repeated five times until all potassium dichromate had been removed from the solution.

Prior to inoculating each bird, I orally medicated them with 0.375 milligrams per 100 µl sulfadimethoxine (Albon) for 10 days to clear any preexisting coccidian infection. After 7 days I took fecal samples to confirm that all birds were clear of a coccidian infection prior to the experimental infection, following the procedure below. Any birds (N = 12) that were not free of infection after 10 days of medication were removed from the experiment. For the remaining birds (N=32), I waited a further week after the successful course of medication and then orally inoculated half of the birds in each treatment (N = 16 in 0.0ppm group and N = 16 in 1.2ppm MeHg group) with a constant solution of sporulated *Isospora* oocysts. A 20-gauge metal animal-feeding needle attached to a 1 ml syringe was used to administer approximately 100 oocysts in 100 µl of

distilled water directly into the crop of the bird. I cleaned the feeding needle and syringe in warm, soapy water and rinsed with clean water between each inoculation. I inoculated birds in the uninfected treatment group with 100 µl of distilled water at the same time as the infected treatment group.

Quantifying Infection

To determine the start of the infection and the parasite load throughout the infection, I collected fecal samples from each bird for the first five days after inoculation, and then weekly for the next six weeks. Fecal samples were collected between 1800 and 2000 hours, when the majority of coccidian oocysts are shed (pers. obs., Brawner and Hill 1999, Brown et al. 2001, Misof 2004, Filipiak et al. 2009). I placed aluminum foil at the bottom of the cages to allow for easy collection of feces. At 2000 hours I removed the aluminum foil from the cage and collected any feces using a P1000 pipetteman and vials containing 5 ml of potassium dichromate. A layer of air was left between the top of the vial and the feces-dichromate mixture to allow the oocysts atmospheric oxygen to aid in sporulation. The fecal-dichromate mixture was maintained as described above.

To determine the presence and number of oocysts in a fecal sample I aliquoted 1 ml of homogenized fecal-dichromate mixture into a 15 ml glass centrifuge tube. I filled the remainder of the centrifuge tube with 14 ml of Sheather's sugar flotation solution (500 grams sucrose, 350 ml tap water, 5 ml phenol) until a reverse meniscus formed at the top. I placed a number one, 18 mm^2 cover slip on top of the centrifuge tube and centrifuged the tube at 3,000

rpm for 10 minutes. After centrifugation, I removed the centrifuge tube and placed the cover slip, onto which the oocysts should have adhered, onto a glass microscope slide where it was allowed to dry for 5 - 10 minutes before counting.

To count the total number of oocysts per slide, I used a compound microscope at 100X magnification. I systematically scanned each slide and used a hand counter to tally each oocyst (a method with 7.31% mean percent error, Duszynski and Wilber 1997). To count the number of oocysts per gram of feces, I determined the dry weight of the feces using a centrifugal evaporator (Savant Speed Vac, Thermo Scientific). I pre-weighed a 1.5 ml eppendorf tube, and then placed 1 ml of homogenized fecal-dichromate solution in the tube. The samples were spun in the vacuum for three hours at high heat until all liquid had evaporated. I divided the oocyst number by the mass of the dried fecal sample to determine the number of oocysts per gram of feces. In some cases, oocysts were too numerous to count. To accommodate such cases, I made new slides, following the procedure above, with smaller aliquots, which ranged from 20 µl to 500 µl, and then I corrected for the smaller quantity of feces when calculating oocysts per gram.

Immunocompetence

To assess the state of the immune system I measured the ratio of heterophils to lymphocytes and the leukocyte profile (eosinophil and monocyte counts) from blood smears. To make blood smears I collected a small amount of blood from each bird, on the same days that I collected fecal samples, by

pricking the brachial vein of the wing with a 30-gauge needle. Using a capillary tube I transferred a drop of blood from the bird to a microscope slide and smeared the blood across the slide with a second microscope slide. After drying for 10 - 15 minutes, I fixed and stained the blood cells using DipQuick stain (Jorgensen Laboratories, Inc). Once the stained slide had dried I observed the cells using oil immersion on a compound microscope (Houwen 2000). I identified heterophil, eosinophil, monocyte, and lymphocyte white blood cells and counted the number of each in the first 100 cells (a method with 27.9% mean error overall, 11.9% mean error for heterophils, 4.47% mean error for lymphocytes). From this information I determined the heterophil to lymphocyte ratio.

Bill Color

Because coccidians have been shown to influence coloration of carotenoid-based sexually selected traits in birds (McGraw and Hill 2000, Brawner et al. 2000, Horak et al. 2004), I measured the bill color of each bird prior to and after the infection. Carotenoids play an important and diverse role in integument and plumage coloration but can also serve as antioxidants, protecting the body from free radicals (Hill and McGraw 2006). Bill color in zebra finches is influenced by carotenoid concentration and has been indicated as a signal for female mate choice with females preferring males with redder bills (Simon and Verhulst 2011). I measured the bills using a USB2000 UV-VIS Miniature Fiber Optic Spectrometer with OOIbase32 software (Ocean Optics, Inc.) and the color program CLR 1.05 (Montgomerie 2008). I recorded measurements of brightness,

red saturation, and red hue (Hill and McGraw 2006). Hue indicates the wavelength that is contributing most to the color we perceive on the visible spectrum. Saturation measures the degree to which a color appears to be pure, or is composed of a single wavelength. Brightness refers to the total amount of light that is coming from a unit area of a surface at a particular angle (Hill and McGraw 2006). Colors that are higher on the brightness scale tend to appear more washed out.

Statistical Analysis

To determine the effect of immunosuppression due to methylmercury and parasite challenge on bill color expression, I used the following statistics. To determine if mercury had suppressed the immune system, I averaged the values of all time points into an average H:L ratio for each uninfected bird. I used a one-way ANOVA to compare immunocompetent and immunocompromised treatments in the absence of a parasite infection. Because mercury has been shown to suppress the response of the immune system, I also collapsed all time points into an average H:L ratio for each infected bird. I used a one-way ANOVA to compare immune response in the infected birds across the immunocompromised and immunocompetent treatments. To determine if the coccidians elicited an immune response, I collapsed all time points into an average H:L ratio for all birds on control diets. I used a one-way ANOVA to compare uninfected to infected immunocompetent birds.

To examine the effect of mercury on parasite infection, I first normalized the response variable using a box-cox transformation (y(t)=(((y^lambda)-1)/lambda)) where lambda = 0.020 (Swaddle et al. 1994). To account for the presence of zeros in the data (days when infected birds were not releasing oocysts in their feces), I added 0.001 to all values. To analyze the data, I used a linear mixed model with time as a continuous variable, dose as a fixed effect, and family and date as random effects. Coccidian parasites have a genetically limited number of asexual reproductive cycles, which most likely occur early on in the infection. During this time, newly formed merozoites are destroying old epithelial cells and invading new cells and the immune system is most active. Macrophages are engulfing freed merozoites while T cells are being recruited to the invaded cells. I was interested in the immune response to coccidians in the presence of mercury; thus it is useful to examine the parasite load during the time that the highest level of immune activity is most likely occurring. Therefore, as an alternate analysis, I shortened the time window for analysis of the parasitism data to 21 days to focus on the early part of infection where parasite load was the greatest. I used a linear mixed model to analyze the shortened time points with the same fixed and random effects as described above. I performed a new box cox transformation on the shortened window values and calculated new transformed values where lambda = 0.061. I focused only on the part of the infection after the initial asexual reproductive cycle, from 7 to 21 days, where immune response is most likely at its highest.

To help describe the bill color, I reduced three of the five color variables (hue of red, saturation of red, and brightness) into a single, composite redness color score using Principal Component Analysis separately for males and females. The principal component analysis of male zebra finches resulted in a single principal component (PC1) that explained 70.0% of the variance (Table 8). All three variables loaded strongly, with red saturation (S1R) and red hue (H1R) increasing as the PC1 value increased and brightness (B1) decreasing as the PC1 value increased. This indicates that males with higher PC1 values had redder and less bright bills. The principal component analysis of female zebra finches resulted in a single principal component (PC1) that explained 58.5% of the variance (Table 9). Just as with the males, red saturation (S1R) and red hue (H1R) increased as the PC1 value increased and brightness (B1) decreased as the PC1 value increased. This indicates that females with higher PC1 values had redder and less bright bills.

I then compared the slopes of the birds that were both infected and dosed with mercury (immunocompromised/infected) to all other treatment groups. I added an additional variable, setting immunocompromised/infected birds equal to 2 and all other birds equal to 1. I ran a repeated measures ANOVA with the PC scores before and after infection as within-subjects variables and the new "treatment" variable as a between-subject variable. I repeated this process to compare immunocompetent/uninfected to immunocompromised treatments and to compare the immunocompetent/uninfected treatment to all infected birds. To check for floor and ceiling effects I ran a univariate linear model comparing the

PC1 values after the infection of the immunocompromised/infected treatment to all other treatment groups.

All analyses were performed using SPSS Statistical Software and R Version 2.13.2 (R Core Team 2013).

Results

The average blood mercury level for the immunocompetent (0.0ppm Hg)/uninfected treatment was 0.006 µg/g (SEM = 0.001). The average blood mercury level for the immunocompromised (1.2ppm Hg)/uninfected birds was 20.4 µg/g (SEM = 0.55). The average blood mercury level for the immunocompetent/infected birds was 0.006 µg/g (SEM = 0.001). The average blood mercury level for the immunocompromised/infected birds was 22.2 µg/g (SEM = 1.4). Hence, the mercury dosing was highly effective in creating the intended blood mercury differences.

The average H:L ratios of mercury-dosed (immunocompromised) and non mercury dosed (immunocompetent) birds were not statistically different ($F_{1, 23}$ = 0.372, p = 0.547) however the mercury-dosed birds had a higher mean H:L ratio, which is in the predicted direction (Table 1, Figure 1). The average H:L ratios of immunocompromised/infected and all immunocompetent birds were also not statistically different ($F_{1,30}$ = 0.493, p = 0.488) however the immunocompromised/infected treatment birds had a higher mean H:L ratio, which was also in the predicted direction (Table 2, Figure 2). The average H:L ratios of uninfected and infected birds were not statistically different ($F_{1,26}$ = 1.26, p = 0.298) however the infected birds had a higher mean H:L ratio, which is in the predicted direction (Table 3, Figure 3). The average H:L ratios of uninfected and infected birds during the heaviest part of the infection were not statistically different ($F_{1,25}$ = 2.128, p = 0.156) however there was a trend in the predicted direction of infected birds having higher H:L ratios (Table 4, Figure 4).

All birds orally inoculated with oocysts became infected with coccidians. All but two birds started releasing oocysts in their feces three days after inoculation. The remaining two birds began releasing oocysts earlier, one to two days after inoculation. The infection lasted six weeks, with only six birds still releasing oocysts at the end of the six weeks (Figure 5).

Parasite load (oocyst/gram of feces) was not significantly different throughout the infection (3-42 days) between immunocompetent and immunocompromised birds ($F_{1,57} = 0.631, p = 0.430$, Table 5, Figure 5). The box-cox transformed parasite load in the shortened time window (3-21 days) was not significantly different throughout the infection between control and mercury dosed birds ($F_{1,35} = 0.490$, $p = 0.489$, Table 6, Figure 6). Between 7 days and 21 days, a separation appeared between immunocompromised and immunocompetent birds, which although non-significant, suggests the possibility that mercury-dosed birds were maintaining higher parasite loads ($F_{1,33} = 2.325$, $p = 0.137$, Table 7, Figure 7).

For males, all four treatment groups had PC1 values that decreased after the date of the coccidian infection. The immunocompromised/infected birds' bill color was not significantly different than the other three treatment groups combined ($F_{1,32} = 2.44$, $p = 0.128$). However, there is a trend that indicates that overall, the bill color of birds in the immunocompromised/infected treatment was the least red when compared to the other three treatments. The slope of the immunocompromised/infected treatment was not significantly different than the

other three treatment groups ($F_{1,32}$ = 1.50, p = 0.230, Figure 8), indicating the change in bill color did not differ among treatment groups.

The bill color of the immunocompromised/uninfected birds was not different than the bill color of the immunocompetent/uninfected birds ($F_{1,15}$ = 0.626, p = 0.441). The bill color of the immuncompetent/infected birds was not different than the bill color of the immunocomptent/uninfected birds ($F_{1,17}$ = 0.015, p = 0.905, Figure 8). When I compared the post-incoulation PC1 values, there was no difference between the immunocompetent (mercury-dosed) and infected birds and all other treatment groups ($F_{1,33}$ = 1.42, p = 0.242).

For females, all four treatment groups had PC1 values that decreased after the date of the coccidian infection. The immunocompromised/infected birds' bill color was not significantly different than the bill color of the other three treatment groups ($F_{1,25}$ = 0.005, p = 0.529). The slope of the immunocompromised (mercury-dosed) and infected birds was not significantly different than the other three treatment groups ($F_{1,25}$ = 0.409, p = 0.230). However, the immunocompromised/uninfected birds had the least red mean bill color (Figure 9).

Immunocompromised (mercury-dosed) birds had less red bills than control birds ($F_{1,10}$ = 4.24, p = 0.066). The bill color of the immunocompetent/infected birds was not different than the bill color of the immunocompetent/uninfected birds ($F_{1,9}$ = 0.014, p = 0.908). When I compared the post-inoculation values, there was no difference between the immuncompromised/infected birds and all other treatment groups ($F_{1,26}$ = 0.110, p = 0.743).

Discussion

Previous studies of the ICHH have provided mixed results, and support for the hypothesis should be treated with caution. I performed the first test of the ICHH in which all components of the hypothesis were examined, while previous studies observed one or two of the components at a time. In addition, recent research has indicated that high levels of testosterone may not be causing immunosuppression, further weakening previous support for the ICHH. To account for this new research, I used the ecotoxin, methylmercury, to suppress the immune system. I measured bill color of the zebra finch, a sexually selected character, to determine if a change in the production of this secondary sex character occurred in response to immune suppression and a parasitic challenge.

The analyses of the color data indicated that the bill color of all of the zebra finches became more orange and brighter after the date of the coccidian infection. The pelletized food I used to dose the birds with mercury does not contain all of the nutrients found in the natural diet of zebra finches. To account for this, I provided additional vitamins and carotenoids in the water. However, in the middle of the experiment, the proprietary commercial formula of the pelletized food was changed with a reduction in the amount of the carotenoid canthaxanthin, which is one of four carotenoids primarily found in the bills of zebra finches (McGraw and Toomey 2010). The lack of canthaxanthin in the new food formula may have contributed to the decrease in redness observed in all of the zebra finches.

For my first prediction, I expected birds with a compromised immune system (mercury-dosed) to have a lower expression of the secondary sex character, or duller red bills. In both males and females, there was no difference in bill color between immunocompetent (control-dosed) birds and immunocompromised (mercury-dosed) birds. This is contrary to what was predicted. Carotenoids have been shown to be immunomodulators, and an organism may face a trade off between allocating carotenoids towards coloration versus immune system function. The results of this study indicate that perhaps carotenoids are not serving as immunomodulators in zebra finches, and those with suppressed immune systems are not experiencing a trade off. The lack of significance could also be due to the small sample size.

For my second prediction, I expected birds infected with the coccidian parasite to have duller red bills than uninfected birds. In both males and females, there was no difference in bill color between infected and uninfected birds (Figure 2 of introduction, arrow E). This is contrary to what was predicted. Coccidia infections have consistently been shown to block the uptake of carotenoid molecules in the digestive system, presumably lowering the amount of carotenoids circulating in the blood that is available for deposition throughout the body. The results of this experiment demonstrate that, in zebra finches, coccidians may not block the uptake of carotenoids in the gut. It is also possible that, because the zebra finches had access to food *ad libitum*, the coccidians did not entirely block the absorption of the carotenoids. It should be noted, however,

that the food provided to the zebra finches is low in carotenoids and may not have been providing sufficient carotenoids in the first place.

Another explanation for these findings could be due to the timing of the bill color measurement. The bill color was measured six weeks after the start of the infection, with the heaviest part of the coccidian infection in the first few weeks. I did not measure the bill color halfway through the infection. By the end of six weeks, almost all birds had completely cleared the infection; therefore it is possible that the infected birds were able to begin depositing more carotenoids into the bill before I took the final measurement. It should be noted, however, that previous research has demonstrated that bill color change in zebra finches tends to be gradual (taking up to four weeks) (Birkhead et al. 1998, Bloundt et al. 2003).

The final prediction of my research, centered on the ICHH, predicted that the birds with the dual challenge of coccidian infection and immune suppression due to methylmercury would have the dullest beaks. The infected and immunocompromised (mercury-dosed) male zebra finches' bill color was not statistically different than the other three treatment groups, however their bills were the least red bills out of all four treatment groups, which is in the predicted direction. Interestingly, the bills of the infected and mercury-dosed female birds were not the least red of the four treatment groups. These findings do not provide support for the ICHH, which predicts that a sexually selected character will honestly display the quality of the individual. The birds with the least red bills had suppressed immune systems due to methylmercury and were being challenged

with a parasitic infection; but the lack of significance prevents the conclusion that they were in fact of lowest quality. The lack of a significant result may be due to the small sample size. It is also possible that the true indication of the quality of the male can be seen during the peak of the infection, and measuring the bill color throughout the coccidian infection would provide a better indicator of male quality.

Before testing the predictions of the ICHH, I examined some assumptions of the experiment. The first assumption was that methylmercury would suppress the immune system. Recent research from our lab has indicated that methylmercury delays the proliferation of B cells in zebra finches, presumably suppressing the immune system (Lewis et al. 2013). In addition, studies from the field have demonstrated a suppressed response of cell mediated immunity and T cell proliferation (Fallacara, et al. 2011, Jorissen et al. 2013, and Frouin et al. 2012). To determine if methylmercury had suppressed the immune system (Figure 2 of introduction, arrow B) of the zebra finches I compared the heterophil to lymphocyte ratios of control (immunocompetent) and mercury-dosed (immunocompromised) birds that were not infected with a coccidian, and control and mercury-dosed birds that were infected with a coccidian. Heterophil to lymphocyte ratios are used to examine the state of the immune system at the time it was sampled, providing information about the number and presence of white blood cells circulating in the blood. I did not find a significant difference in H:L ratios between control and mercury dosed birds, however mercury dosed birds consistently maintained higher H:L ratios than control birds. It is generally

accepted that H:L ratios increase in response to stress (Cirule et al. 2012, Vleck et al. 2000) with heterophil counts increasing and lymphocyte counts decreasing. In addition to mercury dosed birds maintaining higher H:L ratios than control birds, they also consistently had slightly higher heterophil counts and slightly lower lymphocyte counts. Though the differences were not significant, this is indicative of a stressed immune system in the mercury-dosed birds. In addition, the lower lymphocyte counts are consistent with previous work from our lab that demonstrated a suppressed proliferation of B cells (Lewis et al. 2013).

Previous research on mercury and the immune system has indicated that the response of the immune system is suppressed due to the presence of mercury (i.e. less proliferation and recruitment of white blood cells) (Lewis et al. 2013, Fallacara et al. 2010, Kenow et al. 2007, Hawley et al. 2009). Because H:L ratios provide a snapshot of the immune system at the time it was sampled, I also compared the H:L ratios of control and mercury-dosed birds that were infected with coccidians. The infection with coccidian parasites is a controlled challenge to the zebra finch immune system and therefore can provide information on how mercury can affect the immune response. The H:L ratios of the infected mercury-dosed birds were not different than the infected control birds. However, the mercury-dosed birds infected with the coccidian parasites maintained higher average H:L ratios than the birds that were not dosed with mercury but infected with the coccidian. In addition, infected plus mercury-dosed birds had lower lymphocyte counts throughout the experiment compared to infected control birds.

The lack of significant difference in H:L ratios between the control and mercury dosed birds could be explained in several ways. First, the sample sizes were small (N = 14 and N = 15), which could explain the lack of significance in the face of a consistent trend. It is also possible that the monitoring technique of heterophil to lymphocyte ratios is not the best technique for measuring the immune system. Heterophil to lymphocyte ratios are generally used when examining the effects of stress on the immune system (Grasman 2002, Johnstone et al. 2012) and may not be the best indicator of the effects of pollutants or mercury in particular. While heterophil to lymphocyte ratios provide information about the immune system at the time the sample is taken, it is often suggested that additional tests of immune function should be used as a follow up to better characterize potential immunological effects (Grasman 2002).

A second assumption of the ICHH involves the presence of a parasitic infection and its ability to challenge the immune system (Figure 2 of introduction, arrow D). Coccidian parasites infect the epithelial cells of the intestine of passerines and will elicit both a cell-mediated and macrophage immune response (Clayton and Moore 1997, Allen and Fetterer 2002). The oral inoculation I performed on the zebra finches was successful in establishing a coccidian infection in all birds. There was no significant difference in H:L ratios between uninfected and infected birds, however the infected birds consistently maintained higher H:L ratios than the uninfected birds. Heterophil to lymphocyte ratios will increase in response to infection (Davis et al. 2004) and the results suggest that the coccidian infection did in fact elicit an immune response.

I found no significant difference in parasite load between control and mercury dosed birds (Figure 2 in introduction, arrow F). However, when I looked specifically at the time during the infection when immune response is presumably at its highest (7-21 days), a non-significant trend suggests that mercury dosed birds were maintaining higher parasite loads. This finding cannot be confirmed, however, due to the lack of significant results.

The results of this study did not provide support for the ICHH, however it is still successful as a novel experiment. The factorial design of this experiment provided a unique and practical set up for the testing of the ICHH. By controlling two of the mechanisms predicted by the ICHH to influence secondary sex characters, I was able to examine their individual and interacting influences on the secondary characters. Previous studies focused on specific mechanisms involved in the ICHH, but fell short in testing the interaction between the variables of the ICHH. The factorial design allows for all mechanisms and interactions to be tested. In addition, in this experiment I was able to control the parasitic infection, where previous experiments would examine natural or existing infections. Not only did I provide a standardized inoculation of parasites, but also performed the experiment in an aviary setting and thus provided additional control over other environmental factors that may have influenced the outcome of this experiment, such as additional parasitic infections. The design of this experiment provides the groundwork for future studies involving the ICHH. Overall, however, the results of the study do not provide support for the ICHH.

Appendix

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min	Max
					Lower Bound	Upper Bound		
0.0	14	.374	.152	.041	.287	.461	.168	.691
1.2	15	.421	.245	.063	.285	.557	.206	1.24
Total	29	.398	.203	.038	.321	.476	.168	1.24

Table 1: Mean H:L ratios of uninfected control (0.0ppm) and mercury (1.2ppm) dosed birds.

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min	Max
					Lower Bound	Upper Bound		
0.0	16	.449	.225	.056	.329	.569	.142	.934
1.2	16	.548	.517	.129	.273	.823	.078	1.99
Total	32	.498	.395	.070	.356	.641	.078	1.99

Table 2: Mean H:L ratios of infected control (0.0ppm) and mercury (1.2ppm) dosed birds.

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min	Max
					Lower Bound	Upper Bound		
U	14	.375	.142	.038	.293	.457	.158	.624
I	16	.449	.225	.056	.329	.569	.142	.934
Total	30	.414	.191	.035	.343	.486	.142	.934

Table 3: Mean H:L ratios of control, uninfected (U) and control, infected (I) birds.

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min	Max
					Lower Bound	Upper Bound		
U	14	.419	.197	.0523	.305	.533	.186	.892
I	16	.566	.330	.082	.390	.742	.187	1.14
Total	30	.497	.282	.051	.392	.603	.186	1.14

Table 4: Mean H:L ratios of control, uninfected (U) and infected (I) birds during the heaviest part of the infection (7-21 days).

Dose	Mean	Std. Error	df	95% Confidence Interval	
				Lower Bound	Upper Bound
Control	9.37	1.26	57.0	6.84	11.9
Mercury	10.8	1.26	57.0	8.26	13.3

Table 5: Estimated Marginal Means of the average parasite load (oocyst/gram of feces) between control and mercury dosed birds throughout the course of coccidian infection.

Dose	Mean	Std. Error	df	95% Confidence Interval	
				Lower Bound	Upper Bound
Control	10.6	2.00	32.5	6.56	14.7
Mercury	12.6	2.00	32.5	8.54	16.7

Table 6: Estimated Marginal Means of the average parasite load (oocyst/gram of feces) between control and mercury dosed birds from 3 to 21 days, the heaviest part of the infection.

Dose	Mean	Std. Error	df	95% Confidence Interval	
				Lower Bound	Upper Bound
Control	2.62	1.52	33.97	-.479	5.71
Mercury	5.90	1.52	33.97	2.81	8.998

Table 7: Estimated Marginal Means of the average parasite load (oocyst/gram of feces) between control and mercury dosed birds from 7 to 21 days.

	Component
	1
S1R	.918
B1	-.832
H1R	.730

Table 8: Principal Component 1 loadings for red saturation, red hue, and brightness of male bill color.

	Component
	1
B1	-.677
S1R	.885
H1R	.717

Table 9: Principal component 1 loadings for red saturation, red hue, and brightness of female bill color.

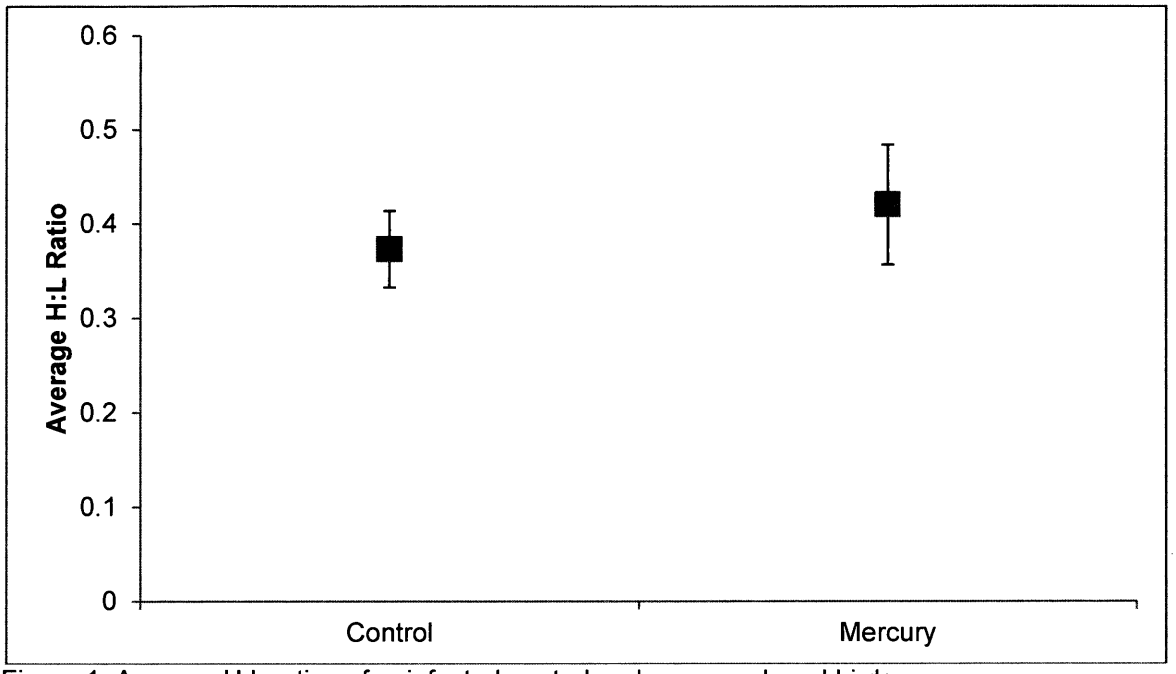

Figure 1: Average H:L ratios of uninfected control and mercury dosed birds.

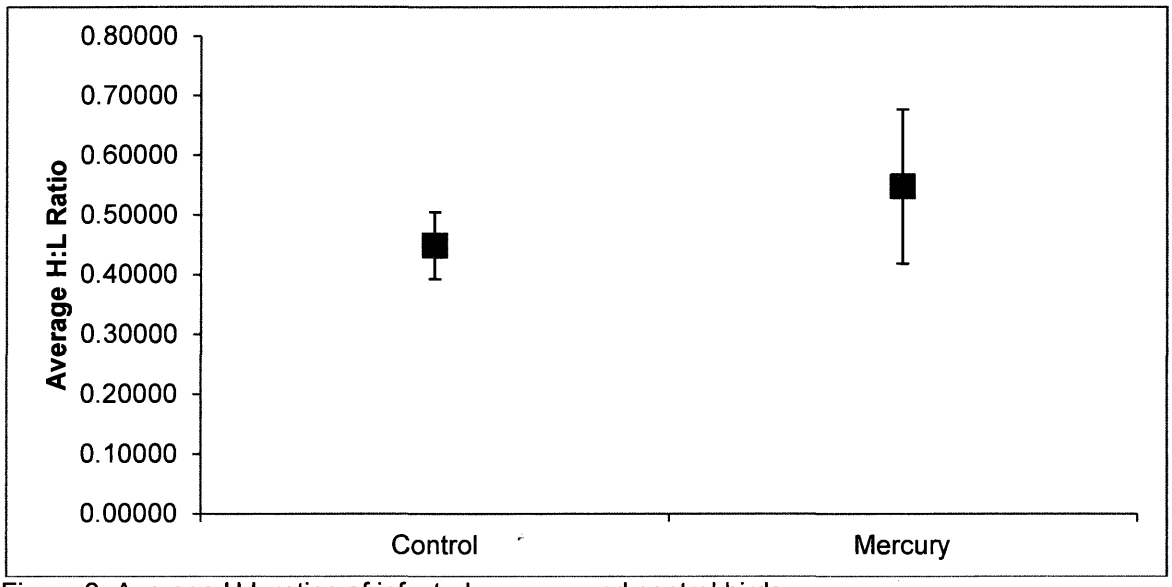

Figure 2: Average H:L ratios of infected mercury and control birds.

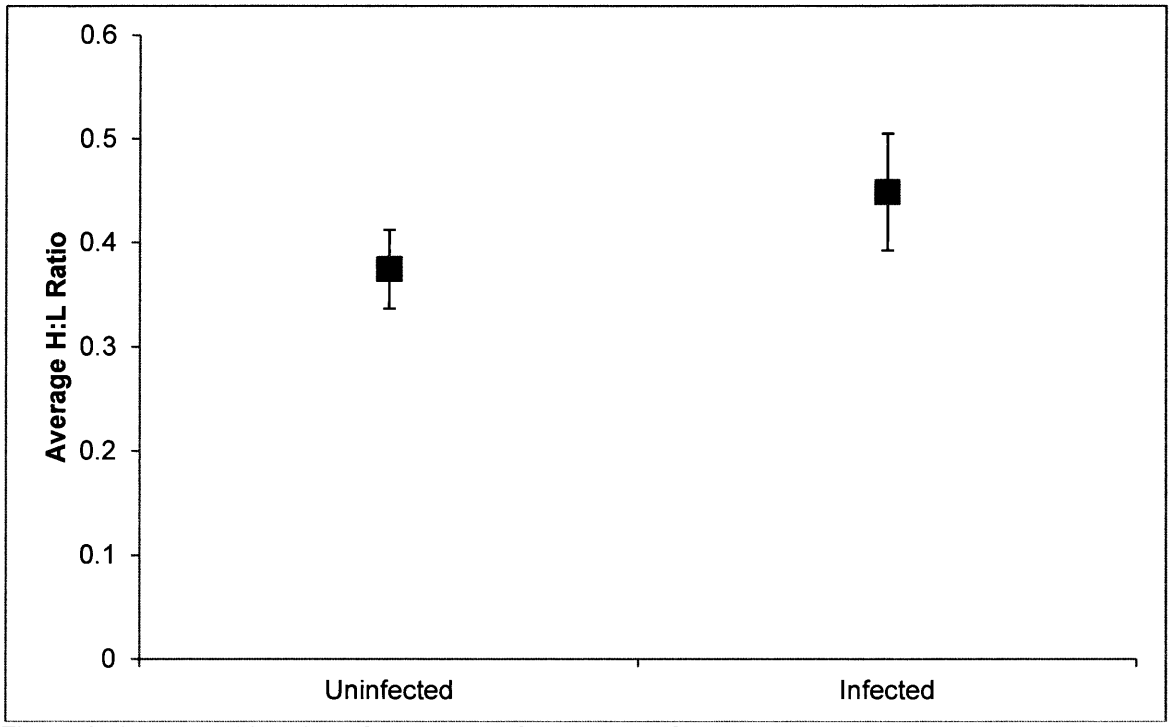

Figure 3: Average H:L ratios of control uninfected and infected birds.

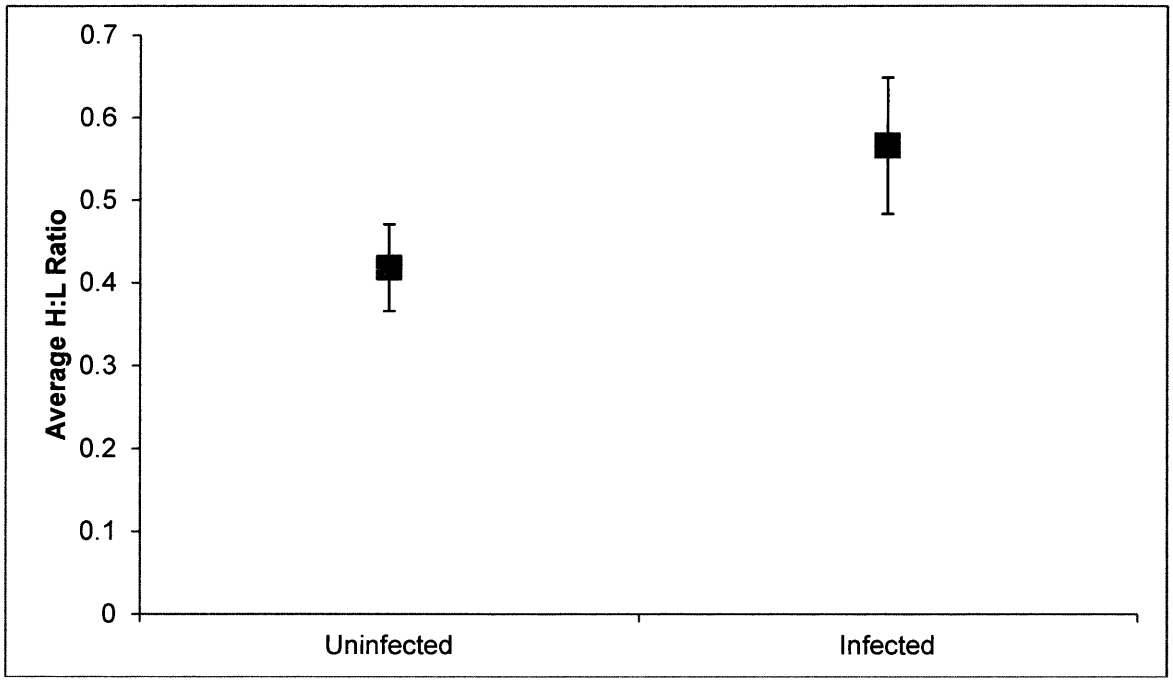

Figure 4: Average H:L ratios of control uninfected and infected birds during the heaviest part of the infection (7-21 days)

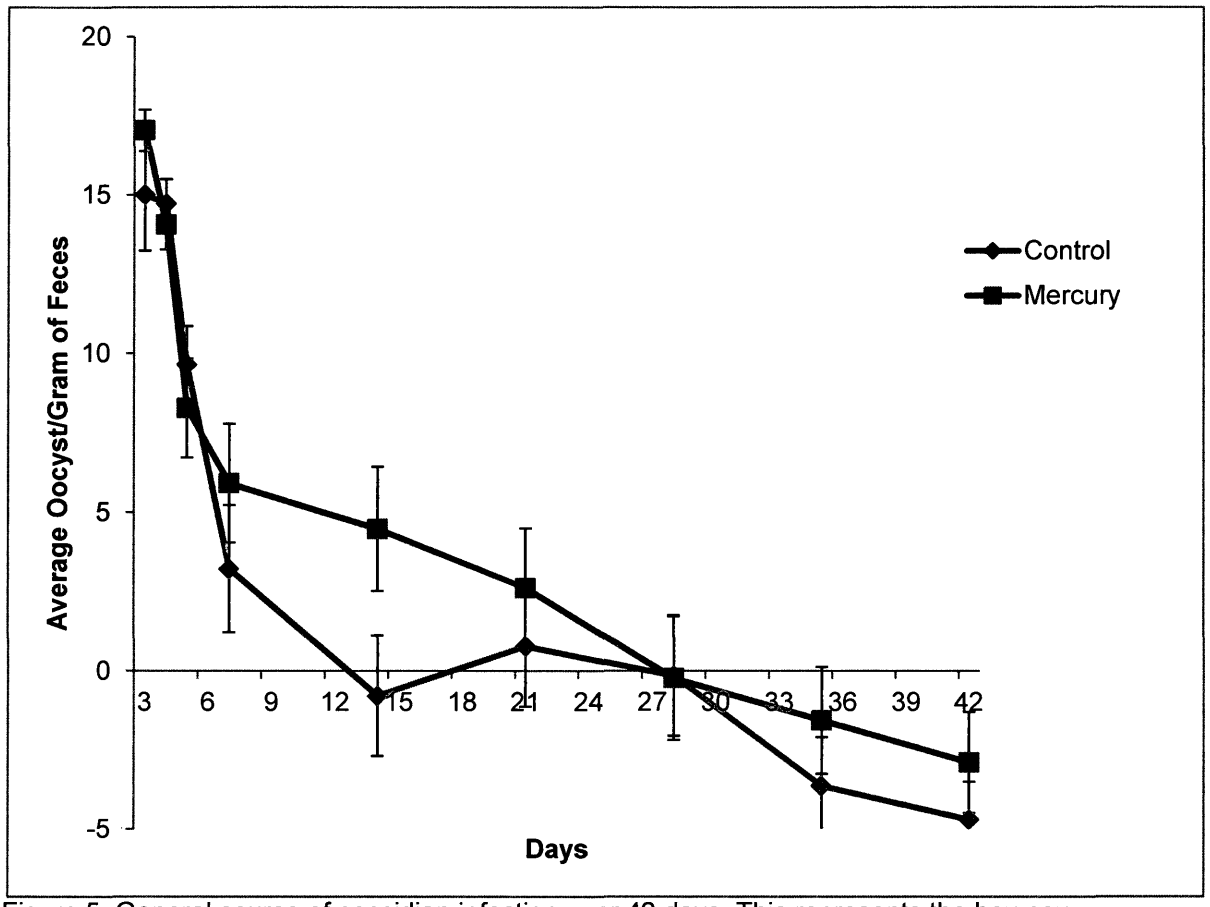

Figure 5: General course of coccidian infection over 42 days. This represents the box-cox transformed data, where the equation results in negative numbers.

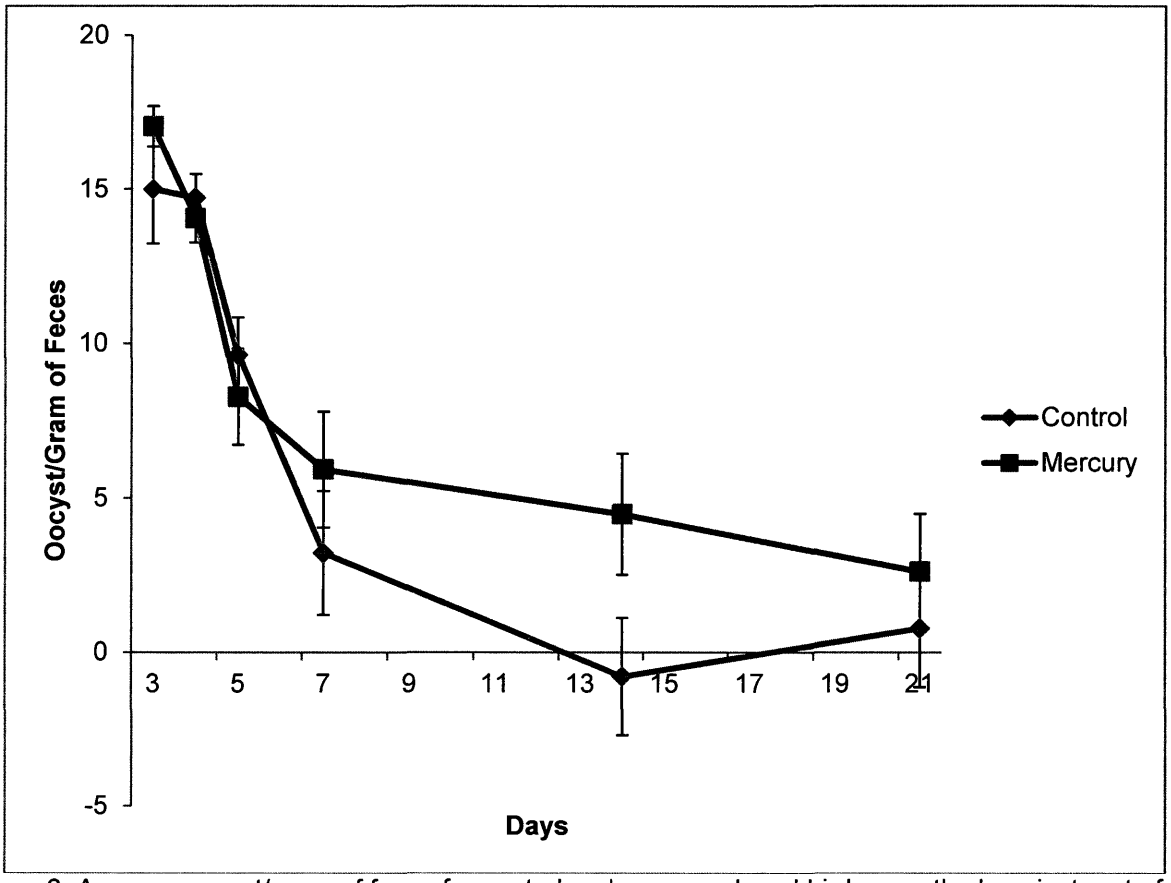

Figure 6: Average oocyst/gram of feces for control and mercury dosed birds over the heaviest part of the infection. This represents the box-cox transformed data, where the equation results in negative numbers.

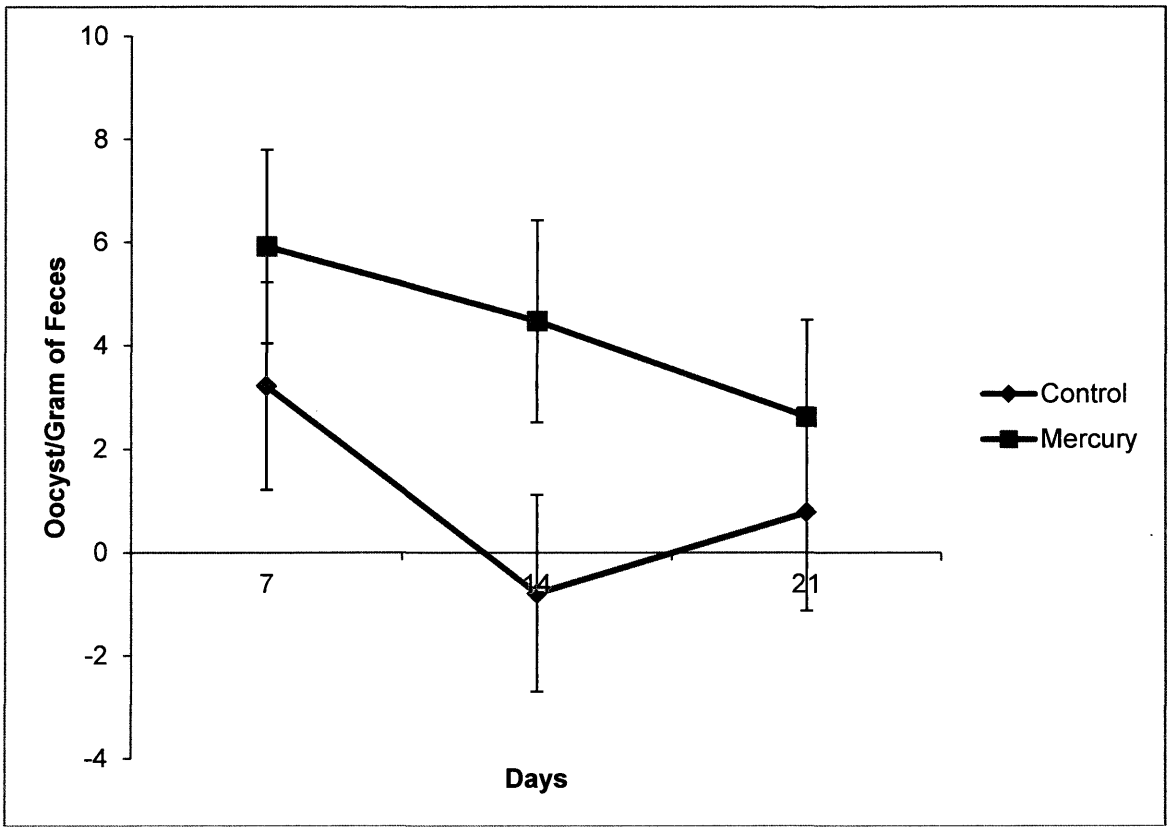

Figure 7: Average oocyst/gram of feces for control and mercury dosed birds from day 7 to day 21. This represents the box-cox transformed data, where the equation results in negative numbers.

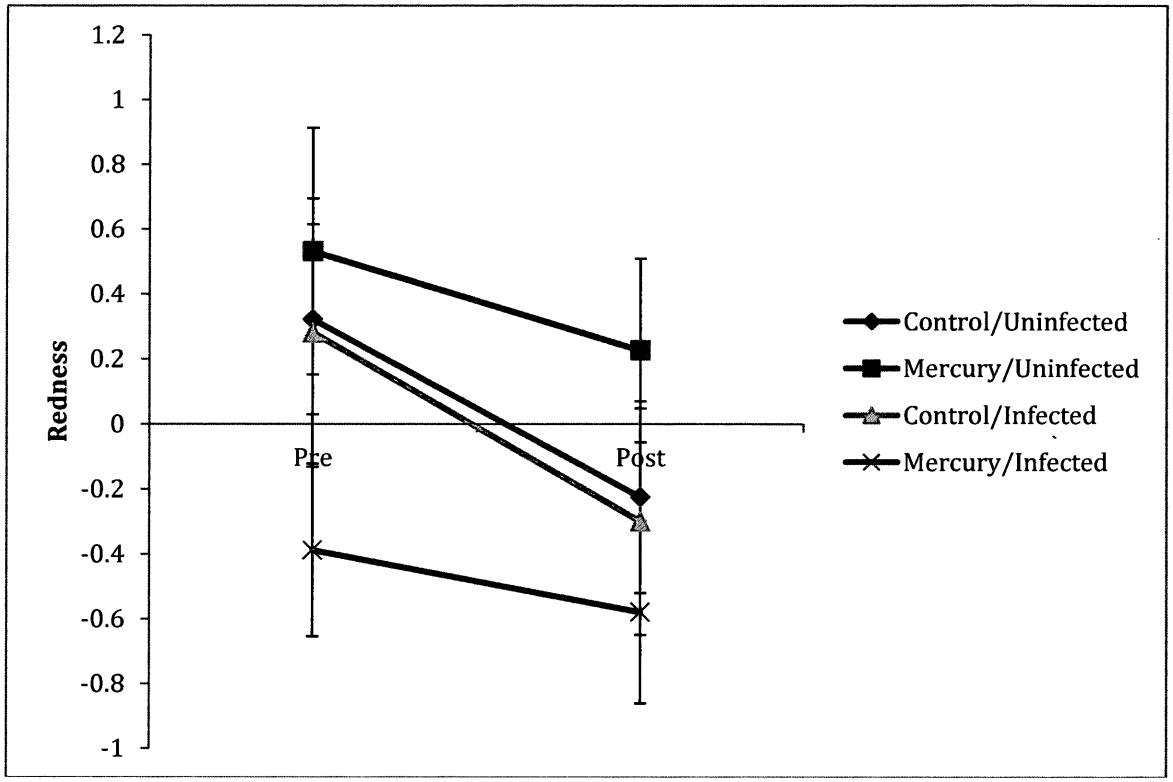

Figure 8: Average PC1 values of male bill color before coccidian infection (pre) and after coccidian infection (post) for all four treatment groups.

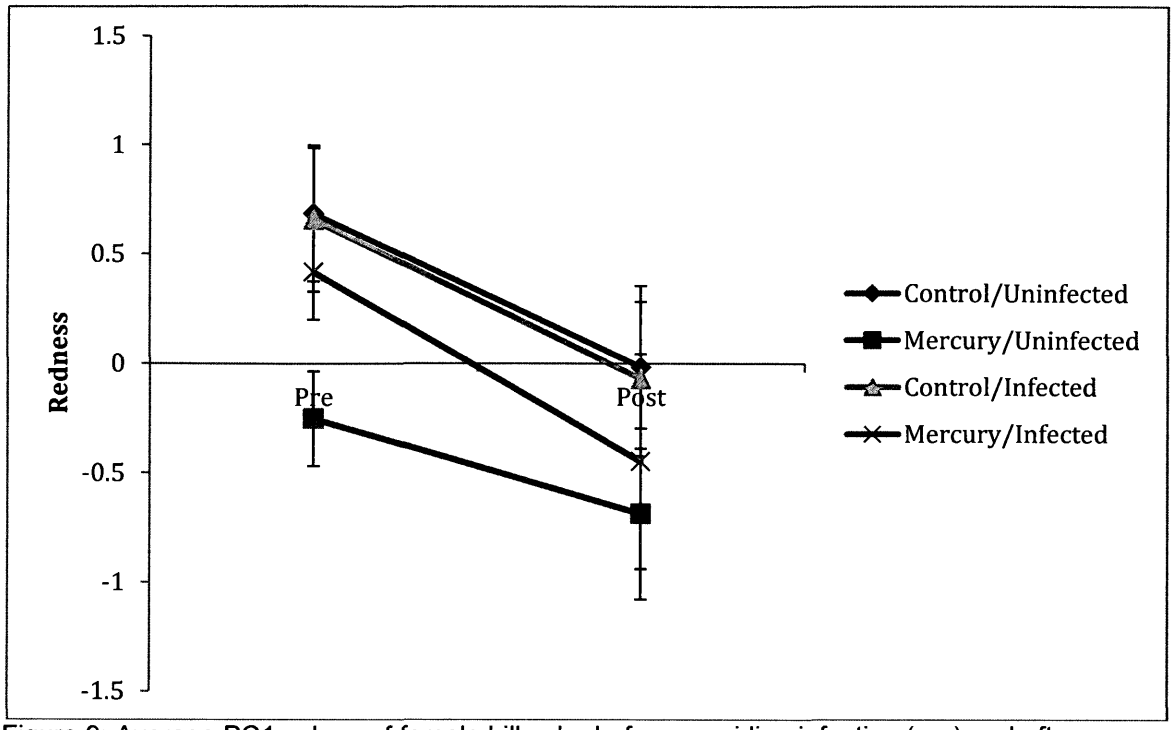

Figure 9: Average PC1 values of female bill color before coccidian infection (pre) and after coccidian infection (post) for all four treatment groups.

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