

Effects of Sublethal Methylmercury Exposure on Pigment Coloration in a Model
Songbird

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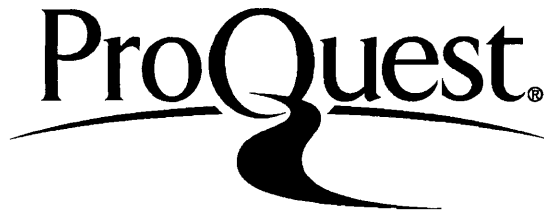
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APPROVAL PAGE

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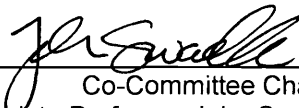
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COMPLIANCE PAGE

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ABSTRACT

Mercury is a globally distributed environmental toxin causing negative impacts on many aspects of physiology. In primarily aquatic environments, inorganic mercury is converted to the bioactive form of methylmercury which readily accumulates in animal tissues and biomagnifies through food webs. While previous research has focused on animals with dietary ties to aquatic systems, recent studies have revealed that terrestrial animals can suffer the same impacts from environmental mercury contamination. Birds can serve as useful bioindicators of mercury contamination. Specifically, their coloration may offer insight into the degree of mercury contamination because coloration is sensitive to environmental stressors. This study aimed to assess effects on pigment-based coloration in Zebra Finches (*Taeniopygia gutatta*) dosed with environmentally relevant dietary concentrations of methylmercury using three experiments. The first and second experiments asked whether the effect of mercury on coloration was moderated by timing of exposure. In the first experiment, birds were dosed through the diet after reaching sexual maturity (adult-exposed) and in the second experiment, birds were the offspring of parents on a mercury diet, and thus exposure began *in ovo* and continued through adult life (lifetime-exposed). Reflectance spectrometry was used to assess red bill coloration, a carotenoid pigment-based sexually-selected trait, and gray back feather coloration, a melanin pigment-based monochromatic trait. A third experiment sought to determine the effect of mercury on the development of adult coloration in male fledglings. Using lifetime-exposed birds, I carried out a time series of measurements of cheek and flank patch coloration and recorded the transition from the characteristic black beaks of fledglings to the red beaks of adults. Results from the first experiment indicate control males were significantly redder than males with lifetime exposure to mercury, but that there was no significant effect on males solely exposed to mercury as adults. There was no noticeable effect of mercury exposure on female bill coloration for either exposure mode. Mercury exposure also did not appear to affect the coloration of gray back feathers in either sex. The third study indicated that mercury exposure did not impact the growth of cheek and flank patches, nor did it slow the maturation of bill color from black to red. Mercury exposure did, however, significantly decrease the redness of the bill coloration when compared to controls. Thus, males with lifetime exposure to mercury from both experiments 2 and 3 had altered red bill coloration, a sexually-selected trait in this species. These results imply that sexually-selected carotenoid-based coloration was more sensitive than the same trait in females or non-carotenoid-based coloration in either sex, but the timing and/or extent of mercury exposure are important factors in altering this trait. Because carotenoids were still present in the diet, it indicates that these pigment molecules were unable to be sequestered into the bill, and may have been used for other functions, like immune support. These results imply there may be negative implications for birds in contaminated environments which use carotenoid-based colors for communication or camouflage.

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This Masters Thesis is dedicated to my parents, Clifford and Annette Spickler,
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Introduction

Environmental Mercury Contamination and Songbirds

Mercury is a global contaminant emitted by anthropogenic sources such as small-scale gold mining, coal-fired plants, municipal incinerators, electrical utilities (Driscoll et al. 2007; Schmidt 2012), and to a lesser extent by pulp and paper mills and chlor-alkali plants (Weiner et al. 2003). Because these sources release inorganic mercury species into the air and water, they can be carried away and deposited far from their sources. Regardless of source, inorganic mercury deposits can be converted to the bioactive form of methylmercury by sulfate-reducing bacteria, which thrive at the interface between oxic and anoxic conditions within aquatic environments. This form of mercury accumulates in animal tissues and subsequently biomagnifies through trophic levels.

Mercury has well documented negative effects on neurological, reproductive, developmental, and immune function in animals (Yates et al. 2005; Weis 2009; Frouin et al. 2012; Basu et al. 2013; Ceccatelli et al. 2013; Chin et al. 2013; Hopkins et al. 2013). Understanding the mechanisms behind the effects of mercury on organisms remains an important goal in ecotoxicology. To achieve this, controlled dosing studies evaluating mercury contamination through dosing at sublethal levels, as opposed to lethal dosing or correlational field studies, are a good tool for answering questions about causal mechanisms of mercury toxication. Birds are useful bioindicators of mercury contaminated environments, and extensive literature exists on the effects of mercury on their biology (Monteiro and Furness 2001; Heath and Frederick 2005; Adams and Frederick

2008; Herring et al. 2010; Brasso et al. 2012; Cristol et al. 2012). Previous work on mercury contamination in birds has focused on species with dietary connections to fish or aquatic invertebrates, but recent research has shown that terrestrial birds also accumulate and suffer the effects of mercury contamination (Rimmer et al. 2005; Cristol et al. 2008; Hawley et al. 2009; Wada et al. 2009; Wada et al. 2009; Edmonds et al. 2010; Hallinger et al. 2010; Jackson et al. 2011a; Jackson et al. 2011b; Lewis et al. 2013; Townsend et al. 2013). Thus, studying the effects of mercury on terrestrial birds remains a research priority (Seewagen 2010). Secondary sexual characteristics, like coloration, are suggested to serve as indicators of environmental contamination (Hill 1995); I therefore chose to study the effects of mercury on coloration in a terrestrial songbird.

Avian Coloration: An Overview of the Importance of Coloration and the Common Pigment Types

Birds rely heavily on coloration, having evolved colorful traits in all integumentary tissues, with each species having distinct shades, patterns, and color types (Hill and McGraw 2006a). Colors are produced through either light scattering from the keratin structure of tissues, pigment deposition into integumentary tissues, or a combination of both mechanisms. Color is critical for camouflage, thermoregulation, species recognition, dominance interactions, and mate attraction. For the purpose of this study, only carotenoid and melanin pigment-based coloration will be discussed. I studied coloration in a model

songbird, the Zebra Finch (*Taeniopygia guttata*), because of the vast information available on their biology, the ease of maintaining them in captivity, their rapid rate of sexual maturation, and the presence of both carotenoid and melanin-based colors in their integument (Zann 1996).

Carotenoids are lipophilic pigment molecules which produce colors ranging from yellow to red. They also serve as antioxidants and might function in immune support through scavenging free radicals (Woodall et al. 1997; Sujak et al. 1999). In birds, these pigments are exclusively acquired through the diet since they cannot be internally synthesized (Brush 1990). Carotenoids are extensively used for ornamentation, predominantly in males, and can either be deposited for pigmentation in the same form as ingested or be metabolized into many other forms to produce different color shades (McGraw and Hill 2001; McGraw et al. 2002a; McGraw et al. 2003b; Saks et al. 2003; McGraw 2004; McGraw and Schuetz 2004; McGraw and Toomey 2010). In the case of females, carotenoids are also deposited into egg yolks where they act with other antioxidants in reducing lipid peroxidation in developing embryos (Blount et al. 2000). The carotenoids astaxanthin, alpha-doradexanthin, adonirubin, and canthaxanthin, are present in the red coloration of male Zebra Finch bills, and females show preference for males with the reddest bills (Burley and Coopersmith 1987; McGraw and Toomey 2010; Simons and Verhulst 2011). While both male and female finches deposit the same four carotenoids in their bills, females accumulate a lesser amount, causing their bills to appear orange rather than red.

There are many hypotheses for explaining the role of carotenoids in the honest signals of sexually-selected ornaments (reviewed in Hill and McGraw 2006b). One widely studied hypothesis is that carotenoid coloration is a sign of foraging ability and nutritional health, since these pigments must be eaten in order to be used for coloration (Hill and Montgomerie 1994; Eeva et al. 1998; McGraw and Hill 2001; Senar et al. 2003; Senar et al. 2008; Eeva et al. 2009). Captive male American Goldfinches (*Carduelis tristis*) and Northern Cardinals (*Cardinalis cardinalis*) molted into significantly duller breeding plumage than wild males when fed a carotenoid limited diet (McGraw and Hill 2001). In the wild, nutritional condition, as measured by daily feather growth bars (ptilochronology), significantly correlated with carotenoid hue in Great Tits (*Parus major*), and similar results were found in House Finches (*Carpodacus mexicanus*) where males with the brightest feathers had the widest growth bars (Hill and Montgomerie 1994; Senar et al. 2003).

Another well studied hypothesis regarding carotenoids as honest signals involves their involvement in immune function. Because of their antioxidant and immune boosting properties, the amount of dietary carotenoids available for use as integumentary pigmentation or egg deposition may be sensitive to stressors, making tissues colored with carotenoids honest signals of individual quality (Burley et al. 1992; Lozano 1994; Brawner et al. 2000; Brawner et al. 2000; McGraw and Hill 2000; McGraw et al. 2003b; McGraw and Ardia 2003; McGraw and Ardia 2004; Peters et al. 2004; Birkhead et al. 2006; McGraw et al. 2006; Pérez-Rodríguez et al. 2010). Many studies support the existence of a trade-off

between use of carotenoids for immune function and ornamental coloration. Carotenoid coloration in Red-legged Partridges (*Alectoris rufa*) was a significant predictor of the ability to cope with oxidative damage inflicted by an immune challenge (Pérez-Rodríguez et al. 2010). In Zebra Finches, testosterone upregulates carotenoid pigmentation, so males with more endogenous testosterone have more saturated bills and more plasma carotenoids (McGraw et al. 2006). However, testosterone may suppress immune function by increasing oxidative stress in this species (Alonso-Alvarez et al. 2007; Roberts et al. 2007). This hypothesis is not without controversy, however, as some studies have found no support for trade-offs between the dual uses of carotenoids for immune function and coloration (Navara and Hill 2003; Fitze et al. 2007; Isaksson et al. 2007).

I believe studies overwhelmingly point to carotenoid-based coloration as honest signals of quality. While no single mechanism, whether it be immune system trade-offs, or dietary acquisition, currently links carotenoid coloration with honest signaling, it is likely that a number of factors interact to make carotenoid colors honest signals in mate choice.

Melanin pigments express a wide variety of colors including black, brown, gray, rufous, tan, and buff. Their versatility as colorants is due to the two molecular forms of melanin, eumelanin and pheomelanin, which are present within tissues in varying ratios to produce these colors (Ito and Wakamatsu 2003; McGraw et al. 2004). These pigments are synthesized within individuals in specialized cells known as melanocytes, located in the epidermis in birds. Within

these melanocytes, the enzyme tyrosinase catalyzes the rate-limiting step in melanogenesis using the amino acid L-tyrosine, meaning that without this enzyme, melanogenesis cannot occur (Ito and Wakamatsu 2003; Yu et al. 2004). In birds, these pigments are largely responsible for basic and cryptic coloration, but there are also many species with ornamental patches of melanin which are used to attract mates or display dominance (Hill and McGraw 2006b). The cheek and flank patches in male Zebra Finches largely consist of phaeomelanin, and while there is still no concrete evidence that these patches are used as social signals, it is possible they are worth looking into (Zann 1996; McGraw and Wakamatsu 2004).

Unlike carotenoid coloration, the literature points toward melanin production being relatively unaffected by nutritional stress and immunocompetence (McGraw and Hill 2000; McGraw 2008). Instead, social influences, environmental factors, such as the amounts of environmental contaminants or dietary minerals, and hormones impact melanin-based ornamentation (Hall 1969; Folstad and Karter 1992; Kimball and Ligon 1999; McGraw et al. 2002b; Jawor and Breitwisch 2003; McGraw 2003; Mennill et al. 2003; Griffith et al. 2006; Alonso-Alvarez et al. 2007; Bókony et al. 2008; Galván and Alonso-Alvarez 2008; McGraw 2008; Roulin et al. 2008). For example, the number of aggressive social interactions with conspecifics and the levels of endogenous testosterone have been linked with the size of melanin-based badges in House Sparrows, with more aggressive birds having higher endogenous testosterone levels and larger melanin badges (*Passer domesticus*;

Evans et al. 2000; McGraw et al. 2003a). In addition, male Zebra Finches fed a calcium enriched diet grew more feathers in their eumelanin chest patch, and these feathers contained seven times more calcium compared to control males (McGraw 2007). This suggests that dietary mineral intake may be a limiting factor controlling melanin badge size. It is surprising that melanin ornamentation is not known to be affected by immunocompetence, since environmental contaminants or hormones, like testosterone, can suppress immune function, thereby providing an avenue for melanin color disruption. However, as far as I know, no evidence has been found that clearly links immune suppression or nutritional condition with melanin coloration.

Melanins have many qualities other than color production. They can function as tissue strengtheners, increasing the abrasion resistance of feathers as well as the hardness of bill keratin (Bonser and Witter 1993; Bonser 1995). Melanins can also deter microbial degradation of feathers, can offer photoprotection from damaging UV light, and can have thermoregulatory properties due to high absorption of sunlight (Wood et al. 1999; Burt and Ichida 2004; Hill and McGraw 2006b; Gunderson et al. 2008; Ruiz-De-Castañeda et al. 2012). Melanins can also serve as cation chelators, since they have cation binding sites. This means they have the ability to bind particles such as positively charged transition metal ions, and draw them into melanocytes where they are no longer a burden on other organs (Riley 1997). White-tailed Eagles (*Haliaeetus albicilla*) have black-and-white patterned feathers, and melanized portions of individual feathers contained significantly higher concentrations of calcium, zinc,

and manganese, suggesting that melanins can also act as chemoprotectants by sequestering excess minerals into dead tissues (Niecke et al. 1999; see also Niecke et al. 2003).

Avian Coloration and Environmental Contaminants

Exposure to environmental contaminants may alter pigment production, accumulation, and incorporation into the integument. Because sexually-selected traits are often costly to produce and maintain, their expression is thought to be relatively sensitive to stressors (Hamilton and Zuk 1982; Folstad and Karter 1992; Lozano 1994; McGraw 2008). Therefore, it has been suggested that the coloration of sexually-selected ornaments can indicate biotic stress caused by environmental chemicals, since contaminant-exposed individuals are likely to have reduced capacity to display ornaments (Hill 1995). To support this notion, there are studies indicating associations between environmental contaminants and the decrease in color quality of avian ornamentation (Møller 1993; Eeva et al. 1998; McCarty and Secord 2000; Bortolotti et al. 2003; Bustnes et al. 2007; Dauwe and Eens 2008; Eeva et al. 2009; Geens et al. 2009).

Melanin and carotenoid traits are differentially affected by pollutants (Dauwe and Eens 2008). Great Tits living along a metal pollution gradient had duller carotenoid coloration, but had wider melanin breast stripes. Since, in this species, carotenoids predominantly signal health and melanins signal dominance, these observations seem conflicting. However, metals can increase circulating testosterone, known to positively influence melanin production (Evans

et al. 2000; Janssens et al. 2003; Bókony et al. 2008). Therefore, the increase in breast stripe size could be a result of endocrine disruption, not necessarily an increase in dominance. This could be especially problematic in species with melanin-based ornaments, like Black-capped Chickadees (*Poecile atricapillus*), where melanin patch darkness and size indicates social status and reproductive success (Mennill et al. 2003; Doucet et al. 2005). In these birds, if melanin patch size is increased due to endocrine disruption by contaminants, it could provide a false signal to conspecifics regarding that individual's condition. This may increase the contaminant-exposed individual's success, but may subsequently decrease the fitness of birds choosing to mate with these contaminant-exposed individuals.

Contaminants may also alter the growth of adult traits in juveniles. Female Tree Swallows captured from sites contaminated with polychlorinated biphenyls (PCBs) developed adult plumage earlier than those from reference collections, with PCB-exposed second-year females having significantly more iridescent feathers than reference skins (McCarty and Secord 2000). This may appear advantageous and could increase breeding success in affected second-year females since, to potential mates, they appear similar to the more experienced after-second-year females. However, this subadult plumage in second-year females reduces the occurrence of aggressive intrasexual interactions, so molting into this plumage early as a result of environmental contaminants may force young females into more aggressive encounters (Coady and Dawson 2013).

Environmental contaminants might also affect coloration without directly affecting the birds. Great Tit nestlings raised along a copper smelter pollution gradient (polluted with high levels of copper, zinc, nickel, and lead) exhibited a decrease in carotenoid coloration with decreasing distance from the pollution source. Correlated with this coloration decrease was the decrease in abundance of carotenoid-rich prey items, meaning that dietary access to this pigment was reduced as the distance to the smelter got smaller (Eeva et al. 1998). This indicates that while individuals can be directly affected by pollutants, there are also possible indirect effects, such as decreased carotenoid-rich prey availability.

Captive American Kestrels (*Falco sparverius*) exposed to PCBs were evaluated for the red coloration in their ceres and lores (Bortolotti et al. 2003). After one month of dosing with PCBs, control males were significantly redder than those dosed with PCBs, while females from both groups did not significantly differ. Interestingly, the effects on males did not last after birds were paired for breeding. Within seven days of being paired (deemed the courtship-phase), PCB-exposed males were visually no different than controls. When assessed over time, control males did not change significantly from pre-experimental to courtship-phase measurements, whereas PCB-exposed males significantly decreased in redness from pre-experimental levels until being paired with a female, when redness increased again. Although not addressed in this study, it is possible pairing the males still stimulated a breeding hormonal profile regardless of PCB exposure, since the total PCB exposure time had only been five weeks.

Not all studies on contaminants have shown color disruption, however. A more recent study on Great Black-backed Gulls (*Larus marinus*) did not find a relationship between the levels of total organochlorides in blood samples and the degree of carotenoid coloration in facial and bill colors (Bustnes et al. 2007). These birds were wild, however, and varied in life history, age, and body condition at the time of blood organochloride and carotenoid color measurement. It is possible any effects of organochlorides on carotenoid coloration may have been masked by the variance in the study population, and the study concluded that body condition was a more reliable predictor of carotenoid coloration than organochloride exposure in this study population. To my knowledge, no studies have specifically examined the effects of dietary mercury exposure on carotenoid coloration.

Mercury negatively affects the copper cofactor binding capacity within a critical enzyme, tyrosinase, involved in the melanin production pathway (Lerner 1952). An *in vitro* study exposed cofactor-free tyrosinase to both copper and mercury ions, resulting in competitive inhibition between the ions. Although this study was done with mammalian tyrosinase, this enzyme is highly conserved across taxa and it is likely avian tyrosinase would be affected in a similar manner (Sato et al. 2001; Camacho-Hübner et al. 2002). We know little about the consequences of such inhibitive binding in melanin production in animals exposed to mercury. Recent work has, however, shown that mercury exposure in a contaminated watershed is associated with increased brightness in the back feathers of Belted Kingfishers (*Megaceryle alcyon*, White 2007). While the

chemical composition of the feathers was not assessed, it is possible these feathers contained less melanin as a result of mercury exposure, causing feathers to appear brighter. Overall, more data, based on experimental manipulation and control treatments, are needed to assess mercury exposure as a mode for melanin-based color disruption.

Because of the importance of coloration in communication and camouflage, if mercury is found to affect carotenoid and melanin pigmentation, numerous fitness implications exist for birds consuming prey from environments with elevated mercury concentrations. Since colors serve as signals to conspecifics, behaviors mediated by coloration, such as those involved in social dominance interactions and reproductive displays, could be altered, leading to reductions in social status or the inability to attract mates. For example, males relying on melanin-based badges for dominance may have these patches artificially increased as a by-product of endocrine disruption by mercury. This could lead to increased social status, which would theoretically increase fitness, but could also increase aggressive interactions with other individuals. While this may seem like an avenue for signal “faking”, those birds with artificially increased badges are likely to be unable to fight off challengers, ultimately decreasing condition further. For Zebra Finches, which can breed in the same season they are hatched in, reduction in the melanin patches of sexually mature birds (i.e. cheeks and flanks) or retention of the melanin in juvenile bills could cause missed breeding opportunities since birds appear juvenile. With regards to carotenoid pigments, species being assessed based on the quality of their

carotenoid-based ornaments, like Zebra Finches, may have decreased fitness since they appear less desirable because carotenoids are not able to be sequestered for ornamentation. In this particular case, while birds heavily relying on carotenoid ornaments may have reduced fitness, they may also benefit from this decreased conspicuousness since they may be better able to avoid predators. However, since both pigments serve many additional functions, including immune support, feather strengthening, photoprotection, and cation chelation, any decrease in these functions is likely to decrease the health and overall condition of birds exposed to mercury, further affecting reproduction and survival.

Experimental Hypotheses and Rationale

I studied the effects of dietary mercury on Zebra Finch coloration in a controlled laboratory setting. I examined both carotenoid-based bill coloration and melanin-based feather coloration to give a multi-pigment view of the effect of mercury on coloration.

Because immune stress commonly decreases carotenoid-based coloration (Bortolotti et al. 2003; McGraw and Ardia 2004; Peters et al. 2004; Pérez-Rodríguez et al. 2010), I hypothesized that mercury, an immune suppressor (Lewis et al. 2013), would decrease the use of dietary carotenoids for integumentary pigmentation. I predicted that color saturation (wavelength purity) would decrease and brightness (total reflectance) would increase, since these are likely related to carotenoid concentration. I also predicted that increasing

exposure to mercury through higher doses would yield greater effects. Mercury disrupts a critical component of the melanin coloration pathway *in vitro* (Lerner 1952), hence I hypothesized that mercury would increase the brightness of feathers in birds exposed to mercury, since fewer melanin molecules should be available for deposition. I predicted the birds with the highest mercury exposure would exhibit the largest increase in feather brightness because of their increased mercury burden.

The first part of my study (Experiments 1 and 2) consisted of two parallel investigations assessing the impact of mercury on adult birds exposed to mercury across different stages of their life: either throughout life or only after reaching sexual maturity. The lifetime-exposed group (LIFE) was bred from parents exposed to mercury, therefore exposure began from zygote formation and continued for the duration of the entire study. The adult-exposed group (ADULT) was exposed through their diet after sexual maturity (birds ranging in age from approximately 6 months to 2 years) and then subsequently throughout the remainder of the study. This experimental design is ecologically useful because the two types of exposure models mimic exposure scenarios of wild birds that are residents on contaminated sites (i.e. lifetime exposure) versus those that are raised on an uncontaminated site but migrate or disperse as adults to contaminated sites (i.e. adult exposure). Organisms are often more sensitive to stressors during earlier developmental stages (Hoffman and Eastin 1981; Weis 2009; Bergeron et al. 2011; Ceccatelli et al. 2013), and also receive more mercury overall, since their exposure starts with development and continues

through life. Therefore, I predicted that lifetime exposure to mercury would cause greater effects on coloration than adult mercury exposure.

The second part of my study (Experiment 3) examined the development of adult coloration in fledglings. In order to assess plumage patch growth and bill color change, birds bred from dietarily-exposed parents were monitored from 25 days after hatching (defined as the age of the oldest chick in the nest) until 130 days after hatching (adult maturity). I hypothesized that mercury would slow the transition from juvenile to adult coloration. Since nutritional stress (which decreases health) can decrease the speed of the post-juvenile molt in Zebra Finches (Naguib and Nemitz 2007), I predicted the plumage ornaments would show similar responses to a pollutant stressor, like mercury, since mercury reduces overall health.

Methods

Animal Care

For Experiments 1 and 2, I used 107 young, but sexually mature wild-type Zebra Finches of both sexes with similar breeding history. They were housed in single-sex indoor cages on a 14:10 light:dark schedule for the duration of this study. For Experiment 3, I used 68 male fledglings beginning at 25 days old, when fledglings still resided with their parents. Once they reached 50 days old, I housed them as I did for Experiments 1 and 2. A timeline of all experiments appears in Figure 1. All birds were provided *ad libitum* access to food, water, vitamins, calcium-rich cuttlefish bone, and a carotenoid supplement.

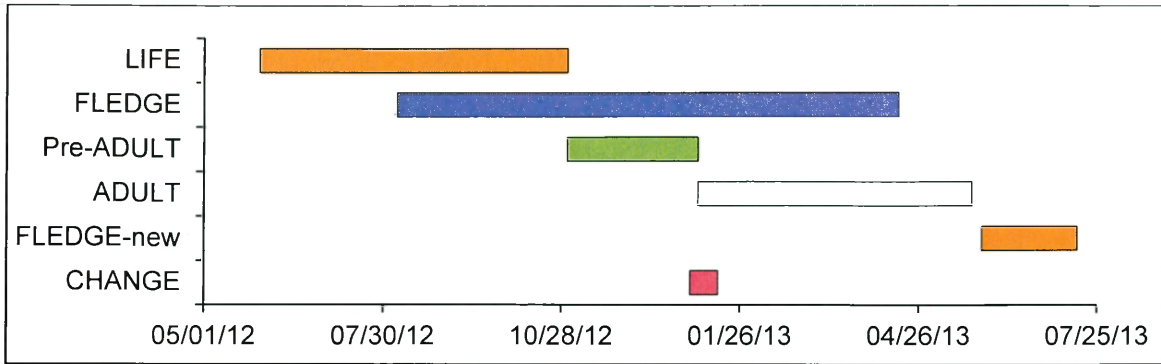


Figure 1. Study timeline outlining the start and end dates for each part of the study in chronological order. Experiment 1 is adult-exposed (ADULT), Experiment 2 is lifetime-exposed (LIFE), and Experiment 3 is the fledgling color change analysis (FLEDGE). Pre-ADULT is the period of acclimation from a seed-based diet to a mercury-dosed pelletized diet for adult exposed birds from Experiment 1. The additional 5 birds photographed for Experiment 3 (FLEDGE-new) and the approximate time frame of the commercial formulation change for the pelletized diet (CHANGE) are also included.

While I used the same brand of pelletized complete grain diet (ZuPreem FruitBlend, Premium Nutrition Products Inc., Shawnee, KS) for the duration of the study, the commercial formulation changed unexpectedly during the study, resulting in the elimination of previously supplemented carotenoids. The original formulation contained 80µg/g additional canthaxanthin, which was removed in the new formulation sometime in January (Figure 1). All of the lifetime-exposed birds in Experiment 1 received the original formulation, and all of the adult-exposed birds in Experiment 2 received the new formulation. For Experiment 3, the formulation change occurred mid-experiment. Since all birds were staggered based on their hatch dates (i.e. not all birds were present in the study at the same time) some birds received only the old formulation, but most birds received a combination of both formulations (Table 1). It is unclear whether the removal of canthaxanthin significantly affected bill coloration in this study, but both

formulations of the pelletized feed contained other natural-source carotenoid-rich ingredients (Dr. Lyn Huffaker, personal communication).

Table 1. Number of Experiment 3 birds in each treatment receiving the old and new formulations of pelletized feed. “Only Orig.” birds received only the original formulation, “Mostly Orig.” birds received one month or less of the new formulation, “Both” birds ate a relatively equal amount of both formulations, and “Mostly New” birds received one month or less of the original formulation.

Treatment	Only Orig.	Mostly Orig.	Both	Mostly New
0.0ppm	3	10	-	-
0.3ppm	3	6	5	1
0.6ppm	3	4	5	1
1.2ppm	2	4	6	1
2.4ppm	-	4	5	5

Because one of the traits I wanted to assess is carotenoid-based (bill coloration), I also provided a carotenoid supplement in the water. While canthaxanthin was listed as an ingredient in the initial formulation of the pelletized food, this is a carotenoid found in aquatic and marine organisms and does not naturally occur in plant materials (Hill and McGraw 2006b). Because Zebra Finches are granivores, it is unlikely they would encounter canthaxanthin in the wild. It has been proposed, however, that they can internally metabolize it from β -carotene consumption, since it is one of the four carotenoids deposited for bill ornamentation (McGraw 2004; Hill and McGraw 2006b). Nonetheless, I mixed a 10% lutein and 1% zeaxanthin (FloraGlo Lutein, Kemin Industries Inc., Des Moines, IA) powder with their water at 7 μ g/mL to provide plant carotenoids in an amount representing approximately 10% of what is found in commercial seed-based Zebra Finch diets (McGraw and Ardia 2004). I intended to provide the

birds with 100% of the carotenoids found in commercial seed-based diets, but during the analysis of collected data, I realized a calculation error was made, resulting in all birds receiving 10% of the total amount of carotenoids intended.

Food Preparation

I dosed food with environmentally-relevant levels of dietary mercury ranging from 0.0 μ g/g to 2.4 μ g/g, hereafter parts per million abbreviated ppm (Cristol et al. 2008). To prepare dosed food, aqueous methylmercury cysteine, representing 10% of the weight of the food, was thoroughly mixed into pelletized food using a tumbler. The control (0.0ppm) dose received 10% water and cysteine alone to account for differences in moisture content. After the addition of the mercury, the prepared food contained 13.9% moisture. To ensure the amount of mercury consumed was within 10% of the intended dose, 10 samples from each prepared batch were analyzed for mercury content.

Blood mercury concentration

I used monthly blood samples to monitor exposure level in birds because it is a reliable indicator of mercury burden (Kahle and Becker 1999). I accomplished this by puncturing the brachial vein with a 30 gauge needle and collecting approximately 50 μ L with a sterile capillary tube. After collection, I froze samples for later analysis on the DMA. I measured all mercury samples in this study on a direct mercury analyzer (DMA-80, Milestone Inc., Shelton, CT). For

every 20 samples, three method blanks and two standards (DORM-3 and DOLT-4, National Research Council of Canada) were run for quality control.

Color Measurements

I measured bill coloration using a USB2000 UV-VIS portable reflectance spectrometer with a PX-2 pulsed xenon lamp (Ocean Optics Inc., Dunedin, FL). This method can be used to determine color quality based on the hue (dominant color wavelength), brightness (total reflectance), and saturation (color purity) reflected from the surface of an object (Butler et al. 2011). On each sampling occasion, I took three measurements of bill color from each bird, aiming the probe at the upper surface of the mandible between the nares. Each measurement was determined to be highly repeatable by taking the average percent difference from the mean (Measurement Repeatability = 99.99%), so all triplicate readings were averaged for analysis.

For Experiments 1 and 2, I also sampled feather coloration. Three weeks prior to initiating sampling, a patch of approximately 45 feathers was removed from the interscapular region of the spinal feather tract. After allowing feathers in this region to regrow (approximately three weeks), 15 new feathers were removed from the center of this regrown patch and stored in glassine envelopes for later mounting and analysis. An additional 15 feathers were removed above and below the sampling site to ensure that with each sampling time point, only new feathers were sampled. I repeated this process to collect a total of five newly grown feather samples. Each sample of 15 feathers was later mounted on black

card stock in a way that mimics how the feathers naturally lay on a bird's back before being measured three times with a portable spectrometer as described above (Measurement Repeatability = 99.92).

All reflectance curves were processed using the Java-based CLR program (supplied by Robert Montgomerie, Queen's University) to generate metrics of bill color variation: hue, saturation, and brightness (Table 1). While another peak in the spectrum was observed between 300-400nm, the UV portion of the spectrum was not important in this study as carotenoids are not predicted to influence UV coloration, and was excluded for all color analyses. Because Zebra Finch back feathers are monochromatic, I only assessed the brightness of feathers.

Table 1. List of equations used to calculate color variables from raw reflectance spectra. Equations A-C were used to analyze all bill color measurements, and only Equation A was used for feather analysis. All variables were calculated by the CLR program (supplied by Robert Montgomerie, Queen's University).

	Color Metric	Equation	Tissue Analyzed
Equation A	Brightness	$*B = \sum_{\lambda R700}^{\lambda R450} Ri$	Bill and Feather
Equation B	Saturation	$S = (\sum_{\lambda 700}^{\lambda 610} Ri) / B$	Bill
Equation C	Hue	$**H = \lambda_{R450-700}$	Bill

*where R_i is the proportion of light reflected at the i^{th} wavelength (λ_i)

**where λ is the wavelength at maximum reflectance in the given range of wavelengths

Experiment 1: Impacts of Adult Mercury Exposure on Adult Coloration

Experiments 1 and 2 consisted of two parallel experiments on of the effects of dietary mercury on coloration. In Experiment 1, I used birds exposed to mercury only as adults (ADULT) and in Experiment 2, I used birds exposed to

mercury *in ovo* through parental dosing and then continuously after hatching (LIFE).

For Experiment 1, male (N = 27) and female (N = 27) Zebra Finches were divided randomly into three treatment groups of 18 individuals (Table 2) and housed in single sex cages in social groups of three birds per cage. I included no more than two related individuals (parent, offspring, or siblings) in each treatment to reduce confounding factors of relatedness. Ten weeks prior to the start of the study, birds were converted from a whole seed diet to a pelletized diet dosed with environmentally relevant levels of mercury (0.0, 0.6, or 1.2ppm). I took an initial blood sample to ensure birds had not been exposed to mercury prior to beginning the experiment. Additional measurements of both bill coloration and mercury blood levels were taken once every two weeks for a total of ten weeks (five pre-samples) in order to assess when bill coloration and blood levels of mercury stabilized (Fig. 1). After this, I collected blood samples monthly as described above. While a whole seed diet may contain more carotenoids than the pellet diet used in this study (McGraw and Ardia 2004), bill hue stabilized over the ten weeks it took to switch diets (Fig. 2).

Table 2. Sample sizes for each experimental analysis. Adult-exposed (ADULT) and lifetime-exposed (LIFE) males and females are from Experiments 1 and 2, respectively, and Experiment 3 birds are only male (FLEDGE). Bill and feather categories indicate spectrophotometric data, and plumage and photo categories represent color maturation data.

Exp. 1 and 2	0.0 ppm		0.6 ppm		1.2 ppm	
	M	F	M	F	M	F
ADULT Bills	9	9	9	9	9	9
ADULT Feathers	8	9	9	7	8	9
LIFE Bills	9	9	9	9	9	8
LIFE Feathers	8	9	8	9	8	8

Exp. 3	0.0ppm	0.3ppm	0.6ppm	1.2ppm	2.4ppm
FLEDGE Bills	13	15	13	13	14
FLEDGE Plumage	13	15	13	13	14
FLEDGE Photos	10	9	7	8	9

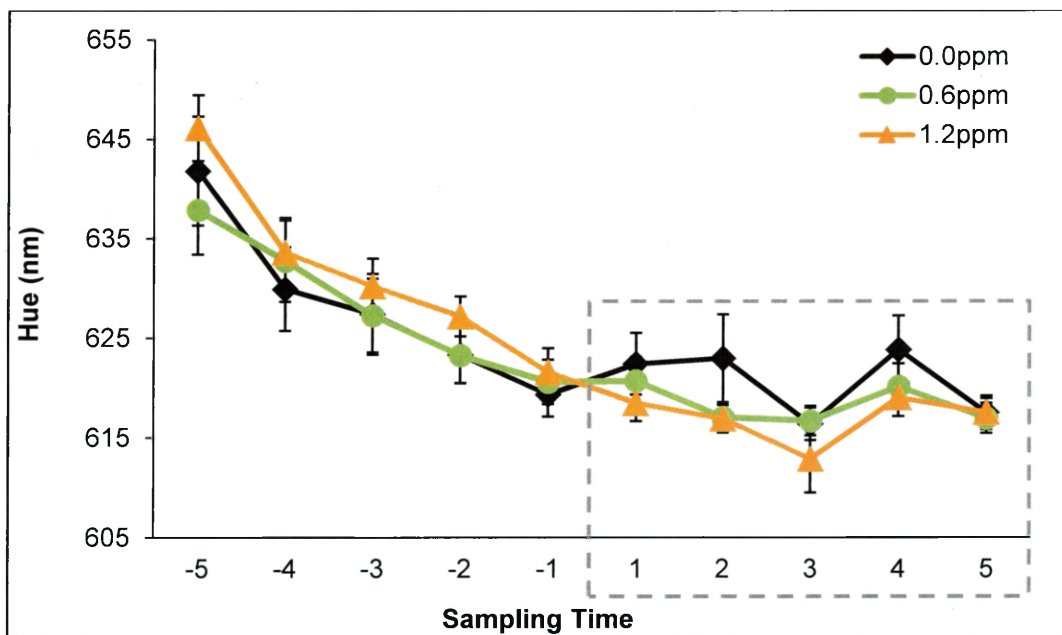


Figure 2. Displayed is the average bill hue of adult-exposed birds during the pre-experimental phase when their diets were switched from a seed-based diet to the pelletized food used throughout this study. The experimental samples are outlined by the dashed box, and show that prior to the experiment, bill hue, or the dominant wavelength, had roughly stabilized onto the new diet.

Experiment 2: Impacts of Lifetime Mercury Exposure on Adult Bill Coloration

Male (N = 27) and female (N = 26) Zebra Finches were bred from parents dosed with 0.0, 0.6, or 1.2ppm mercury. Parents were dosed through the entire breeding process, and the young produced were kept throughout life on the same dosage for use in the current study. To reduce confounding factors due to relatedness, I included no more than two offspring of each sex from a single breeding pair in the study.

Experiment 3: Impact of Mercury Dosing on Part of the Post-juvenal Molt in Juveniles

Male Zebra Finches (N = 68) were bred from parents fed mercury doses of 0.0, 0.3, 0.6, 1.2, or 2.4ppm. In almost all cases, I only used two or fewer related offspring, but in three cases I added a third sibling to increase sample size (Table 2). Fledglings start with a drab gray plumage and a black bill but begin their post-juvenile molt and bill color transition soon after fledging (Zann 1996). Starting at 25 days after hatching (defined as age of oldest chick in the nest) and continuing through 130 days after hatching (postsexual maturity), I began ranking both cheek and flank patches on a scale from 0 to 5 for basic plumage proportion (Humphrey and Parkes 1959). This starting age was selected to ensure that all birds had naturally fledged before the first measurement, and the ending age allowed most of the birds to reach full adult plumage. Zero indicated no adult feathers were present in the patch; 1 = 1-24% adult feathers; 2 = 25-49% adult feathers; 3 = 50-74% adult feathers; 4 = 75-99%; and 5 = 100% adult feathers

(Measurement Repeatability = 93.12%). Feather patch scores were averaged and converted to percentages to assess the percent adult plumage growth per week for a total of 16 weeks (130 days after hatching).

Fifty days after hatching, I took the birds from their parents and housed them in single-sex cages of no more than five individuals per cage. Birds began supplementation with carotenoids at this point (7 μ g/mL in water dish) and after allowing one month for carotenoid accumulation, I measured their bill color weekly for a total of 8 weeks using a portable spectrometer as in Experiments 1 and 2 (Fig. 1).

Beginning at 25 days old, I digitally photographed the left and right profiles of the bills to analyze the latency in transitioning from black fledgling bills to red adult bills. The photographs taken represent the transition from the full black juvenile bills to the full red adult bill and were taken every seven days starting at 25 days after hatching and proceeding until I determined the bill had no more black markings visible to my eyes. I photographed each bird using a digital camera mounted perpendicular to the surface of a 2mm grid sheet by positioning the bill tip at a standard point on the grid and holding the bird flat against the surface of the grid approximately six inches from the camera lens. Camera settings and lighting were standardized for all photographs. I analyzed photographs of the birds at 39, 46, and 53 days after hatching (sampling weeks 3, 4, and 5, respectively) to determine the proportion of black remaining in the bills. I selected these times because they represent a range of transitional points from mostly black to mostly red.

To increase sample size of controls (0.0ppm), I photographed an additional sample of five birds approximately four months after I took the last photograph from Experiment 3. Living and social conditions of the birds were identical to those in Experiment 3, with the only difference being the time lapse (Fig. 1). The mean percent red bill coloration at the midpoint of data collection proved sufficiently similar to the previous five control birds (Student's t-test, $t = 0.803$, $df = 8$, $P = 0.445$) so all birds were subsequently grouped together for analysis.

Image Analysis

Bill photographs from a subsample of Experiment 3 birds ($N = 44$) were analyzed using Geographic Information Systems (ArcGIS 10.1, Esri, Redlands, CA) to assess the latency in reaching the adult red bill coloration. This subsample of birds was selected because I only had a complete series of photos from these birds, since I chose to collect this data after beginning Experiment 3. Although typically a spatial analysis tool, GIS is a useful tool to quantitatively analyze digital images through reclassifying pixel values into sensible categories.

All images were imported as JPG files into GIS and converted to TIFFs, which splits images into three color bands, red, blue, and green. Band 1, the red band, was extracted for all analyses because red coloration was of primary interest.

Forty-four of the 88 photographs representing fully transitioned adult bills were randomly chosen in order to determine the range of pixel values

representative of adult bill coloration. I clipped the images by first hand digitizing individual bill shape in shapefiles from the bill tip to the nares, excluding any bristle feathers (Fig. 3). I used extracted pixel values from each image to develop a frequency distribution of pixel values for all 44 photographs. Pixel values, ranging from 88-230, were normally distributed. I removed the lower 2.5% of the values to reduce the noise in the analysis due to minor overlapping of adult and juvenile bill colors. This effectively set the cutoff value for what was considered an adult bill to a minimum pixel value of 123.

For the color transition analysis, I clipped each photograph as shown in Figure 3. I reclassified images where a value of 1 was assigned to cells with pixel values ≤ 123 , which represents juvenile coloration, and a value of 2 assigned to cells with pixel values > 123 , representing adult coloration. The percent of adult coloration at each time point was used for analyses.

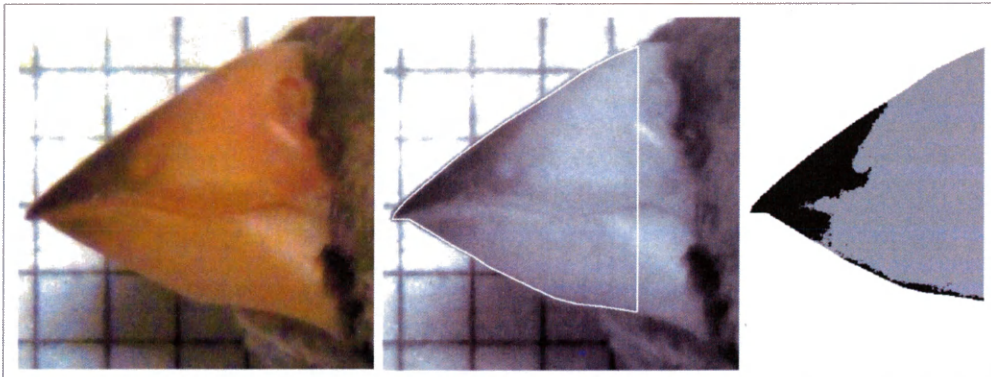


Figure 3. Schematic showing the conversion of a bill photograph to a binary image for analysis. The JPG image (A) taken by the camera is converted into a TIFF file, from which the red color band (B) can be extracted. The white line shows where the image was clipped. The raster calculator then reclassifies the photograph into a binary image (C), representing adult coloration in gray and juvenile coloration in black.

Statistics

Because the three color metrics (brightness, saturation, and hue) were correlated, I used a Principle Components Analysis to reduce the dataset. Males and females were analyzed separately because I expected them to be different due to the Zebra Finch bill's function as a signal of male quality. Because Experiments 1 and 2 are so closely related and because I wanted to be able to directly compare the statistical output, I combined the dataset from both experiments before running the PCA.

In Experiments 1 and 2, for males, all three variables loaded heavily onto component 1 (PC1), accounting for 67.3% of the total variation (Table 3). As PC1 increased, saturation and hue increased and brightness decreased. In other words, PC1 indicated bills that were more red-saturated, were more concentrated in the red portion of the spectrum, and were less bright across the entire spectrum. Female bill variables loaded onto two components, with PC1 accounting for 52.9% of the variation and PC2 accounting for a further 34.1%, for a combined explained variance of 87%. An increase in PC1 indicated an increase in brightness and decrease in saturation. This means that for females, PC1 is an inverse measure of "redness". In addition, an increase in PC2 indicated an increase in red hue, or the wavelength at which the peak reflectance occurs. However, because hue is a less accurate measure of the concentration of carotenoids, PC1 most accurately depicts what is occurring to carotenoid concentration in females. Experiment 3 variables loaded most heavily onto one component, accounting for 57.2% of the variation. An increase in PC1 indicated

an increase in saturation and hue and a decrease in brightness, thus indicating that bills become darker and redder as PC1 increases.

Table 3. Principle component loading factors for bill coloration variables in all three experiments. Experiments 1 and 2 have been combined so adult-exposed and lifetime-exposed birds are part of the same analysis, however the sexes were analyzed separately (M and F).

	Experiments 1 and 2			Experiment 3
	M-PC1	F-PC1	F-PC2	M-PC1
Brightness	-0.816	0.892*	0.135	-0.845
Saturation	0.951*	-0.890	0.145	0.917*
Hue	0.669	0.009	0.993*	0.402

*Indicates most important predictor in each loading

In order to determine if mercury decreased the quality of red bill or gray feather coloration, PC scores for beaks and brightness values for feathers were analyzed using a Linear Mixed Model (LMM) where treatment was included as a fixed effect, family and individual were included as random effects to account for any differences due to relatedness, and sampling time was included as both a repeated and random effect to account for the lack of independence between repeated measures of individuals. Parameter estimates were calculated using Restricted Maximum Likelihood because of the small sample sizes.

Because bill color variables loaded onto two PC factors for females from Experiments 1 and 2, I analyzed both PC1 and PC2 separately using LMM. To determine if mercury alters the acquisition of adult bill coloration, I analyzed the three transition time points for bill photographs, including the approximate midpoint of transition (week 4) and the weeks on either side of this midpoint

(weeks 3 and 5). To determine the same for feather patch completion, I analyzed both the approximate midpoint of patch completion (week 8) and the degree of completion at sexual maturity (week 12). All statistics were conducted using SPSS software (IBM, Inc., Armonk, New York) employing two-tailed tests of probability throughout where significance was determined by $P \leq 0.05$.

Results

Experiment 1: Impacts of Adult Mercury Exposure on Adult Coloration

Experimental exposure to dietary mercury, either throughout life (LIFE) or as adults only (ADULT), created non-overlapping treatment groups with mercury levels across a wide range of blood mercury concentrations (Table 4).

Feather brightness did not differ between treatments for adult-exposed birds (Fig. 4; $F = 1.38$, $df_{2,43.26}$, $P = 0.26$). Bill coloration was also not significantly affected by mercury exposure for either adult-exposed females (Fig. 5A; PC1, $F = 0.86$, $df_{2,25.63}$, $P = 0.44$; Fig. 5B, PC2, $F = 1.12$, $df_{2,24.44}$, $P = 0.34$) or males (Fig. 6; PC1, $F = 0.75$, $df_{2,23.41}$, $P = 0.93$).

Table 4. Average blood mercury levels for adult-exposed (ADULT) birds from Experiment 1, lifetime-exposed (LIFE) birds from Experiment, and fledglings (FLEDGE) from Experiment 3. All blood levels are presented in $\mu\text{g/g}$ wet weight with subscripted standard errors.

Treatment	0.0 ppm	0.3ppm	0.6 ppm	1.2 ppm	2.4ppm
ADULT	0.01 _{0.002}	---	8.32 _{0.237}	16.50 _{0.407}	---
LIFE	0.01 _{0.001}	---	10.65 _{0.231}	18.62 _{0.467}	---
FLEDGE	0.07 _{0.022}	4.14 _{0.219}	7.95 _{0.280}	14.02 _{0.547}	30.03 _{1.038}

Experiment 2: Impacts of Lifetime Mercury Exposure on Adult Coloration

Feather brightness did not differ between treatments for lifetime-exposed birds (Fig. 4; $F = 0.71$, $df_{2,44.32}$, $P = 0.50$). Female bill coloration also was not significantly affected by mercury, for either component score (Fig. 5A, PC1, $F = 0.76$, $df_{2,26.47}$, $P = 0.48$; Fig. 5B, PC2, $F = 1.28$, $df_{2,22.85}$, $P = 0.30$). However, in contrast to adult-exposed males from Experiment 1, bill coloration was significantly affected by treatment in lifetime-exposed males (Fig. 6; $F = 3.49$, $df_{2,24.52}$, $P = 0.05$). PC1 showed that with increasing mercury exposure, bills shift to lower hue values, become less red saturated, and are overall brighter than control bills. Post hoc analysis revealed that among lifetime-exposed males, only the 1.2ppm treatment group males significantly differed from control males ($t = -2.64$, $df_{0,24.57}$, $P = 0.01$).

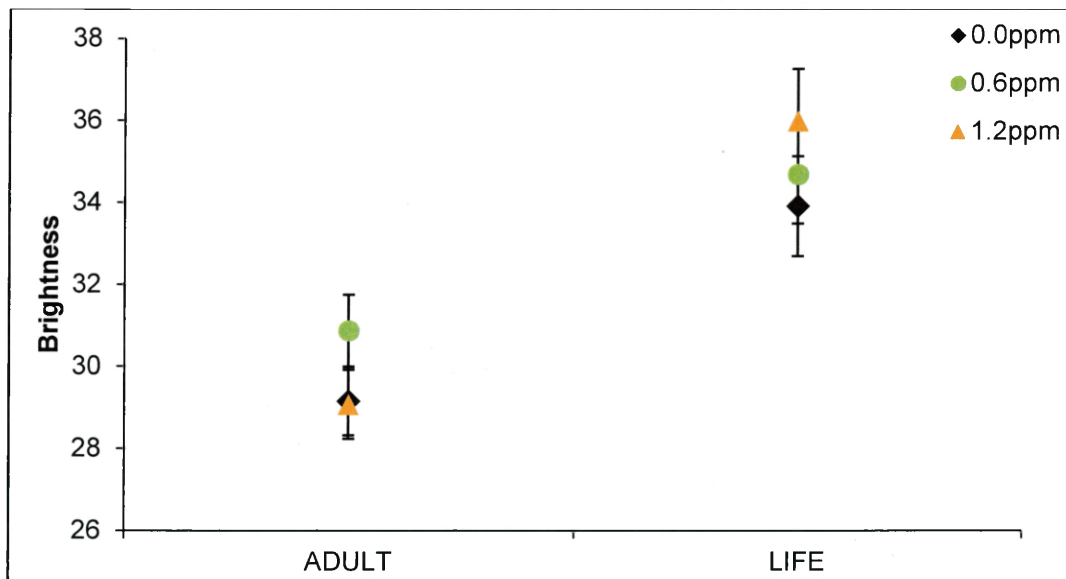


Figure 4. Back feather brightness did not differ for either adult-exposed (ADULT) or lifetime-exposed (LIFE) Zebra Finches in response to two modes of mercury treatment. Displayed are the treatment estimated marginal means with standard error bars.

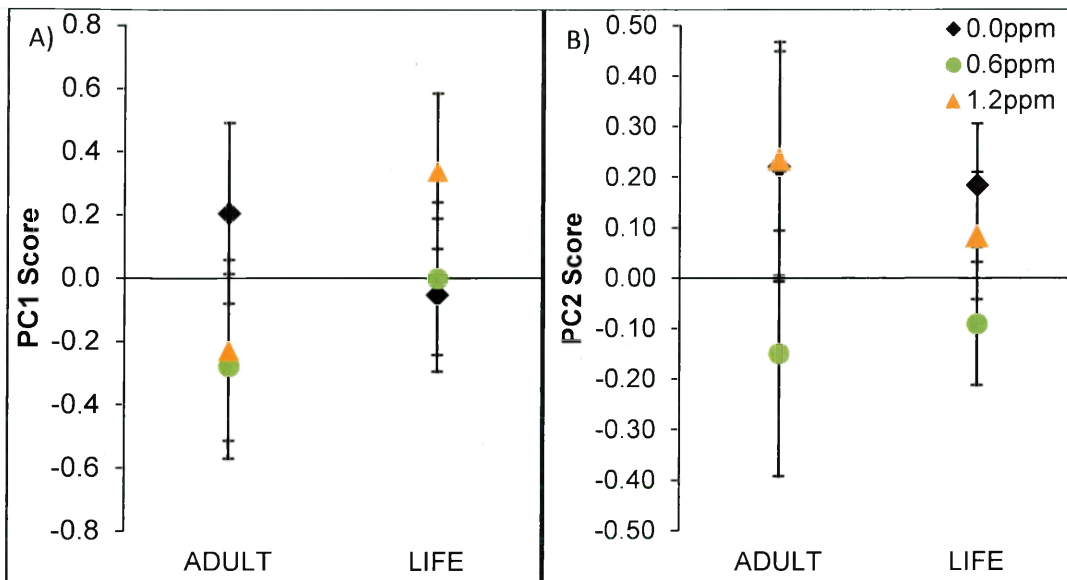


Figure 5. Female bill coloration did not differ in response to mercury treatment for either adult-exposed (ADULT) or lifetime-exposed (LIFE) birds using either Principle Component 1 or 2. PC1 (A) accounts for saturation and brightness while PC2 (B) accounts for hue. Displayed are the treatment estimated marginal means with standard error bars.

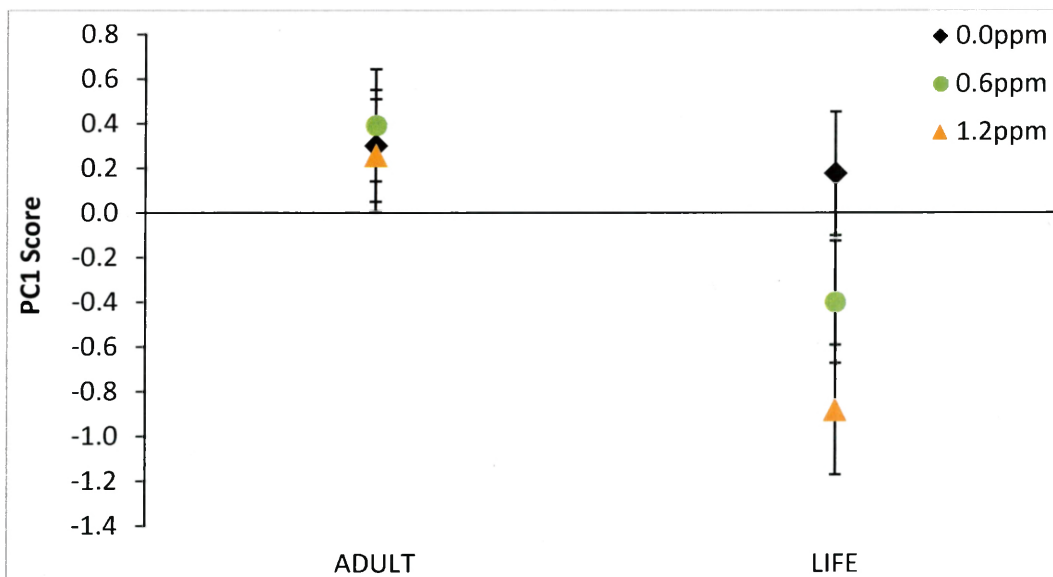


Figure 6. Adult-exposed Zebra Finch male bill coloration (ADULT) was not significantly affected by mercury treatment. However, lifetime-exposed males (LIFE) did differ significantly in response to mercury, with control males having redder, darker bills. Post hoc analysis revealed only the 1.2ppm treatment lifetime-exposed birds differed significantly from controls. Displayed are the treatment estimated marginal means with standard error bars.

Experiment 3: Impact of Mercury Dosing on Part of the Post-juvenal Molt in Juveniles

In juveniles transitioning toward sexual maturity, the emergence of adult red bill coloration revealed a significant overall effect of mercury exposure (Fig. 7; $F = 3.76$, $df_{4,58.90}$, $P = 0.01$), such that higher mercury concentrations resulted in lower PC1 scores. Higher PC1 scores were associated with higher saturation and hue and lower brightness. Upon further investigation, post hoc contrasts of PC1 revealed that only the 2.4ppm treatment group differed significantly from controls ($t = -2.33$, $df_{0,58.68}$, $P = 0.02$).

A separate measure of bill color development, the percent of the bill that had turned red by a specific date, did not significantly differ between mercury treatment groups (Fig. 8; $F = 1.01$, $df_{4,35.15}$, $P=0.41$).

Cheek and flank plumage growth was assessed at the midpoint of molt (week 8), where the largest differences between treatments were observed, and at sexual maturity (week 12). Analysis revealed no significant differences at the midpoint (Fig. 9; $F = 1.21$, $df_{4,35.45}$, $P = 0.33$) or at sexual maturity in these birds ($F = 1.12$, $df_{4,25.55}$, $P = 0.37$).

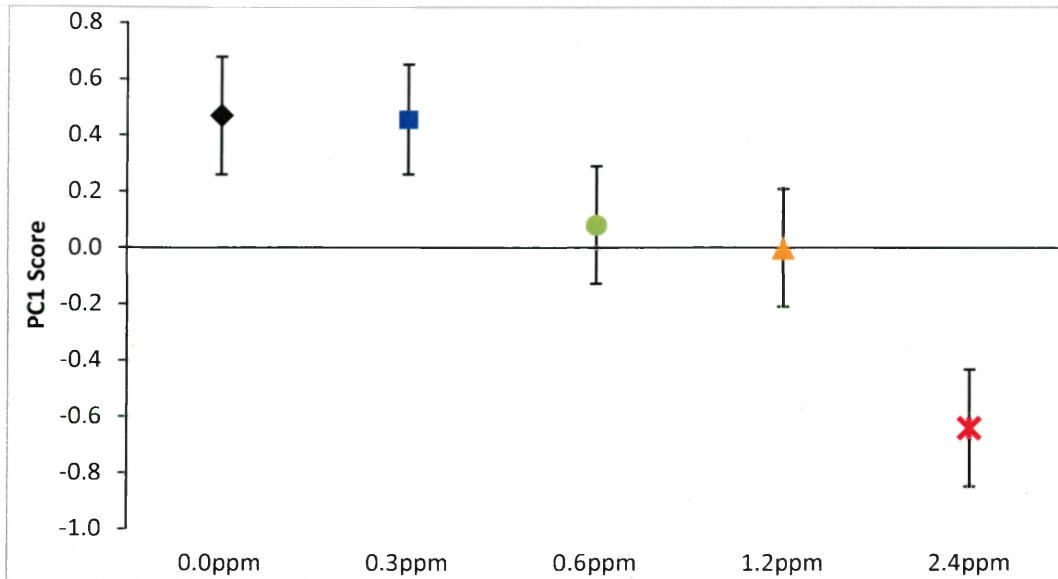


Figure 7. A comparison of PC1 scores for lifetime-exposed juvenile Zebra Finches indicated that mercury exposure significantly affected bill coloration, with mercury-exposed birds having brighter and less red saturated bills. Post hoc analysis revealed that only the 2.4ppm treatment group differed significantly from controls. Displayed are the estimated marginal means with standard error bars.

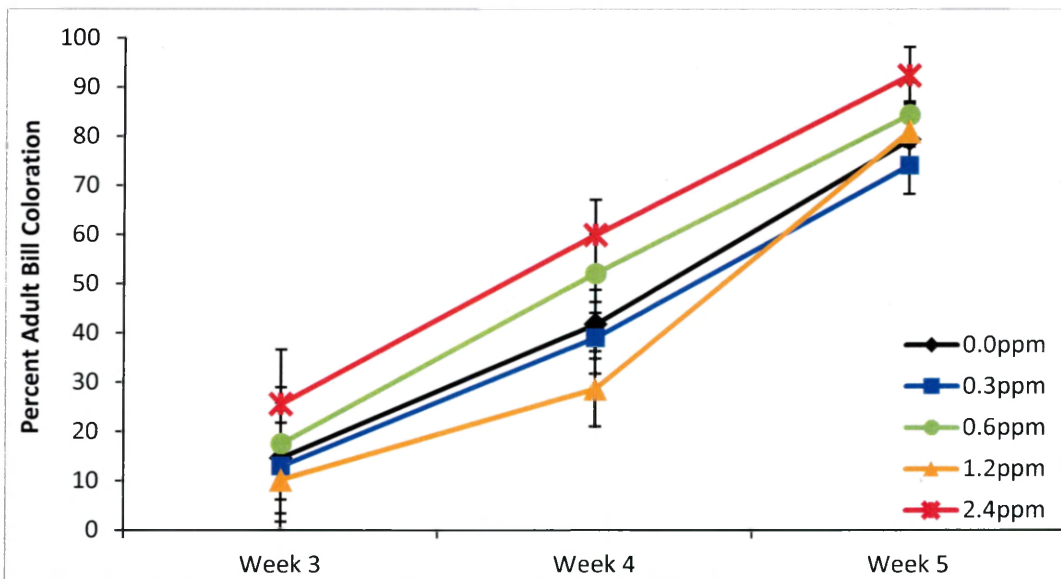


Figure 8. Image analysis of juvenile bill coloration as the birds matured into adults indicated that there was no significant difference among mercury-exposed treatments in the timing of the transition from black to red bill coloration. Displayed are the weekly treatment means with standard error bars.

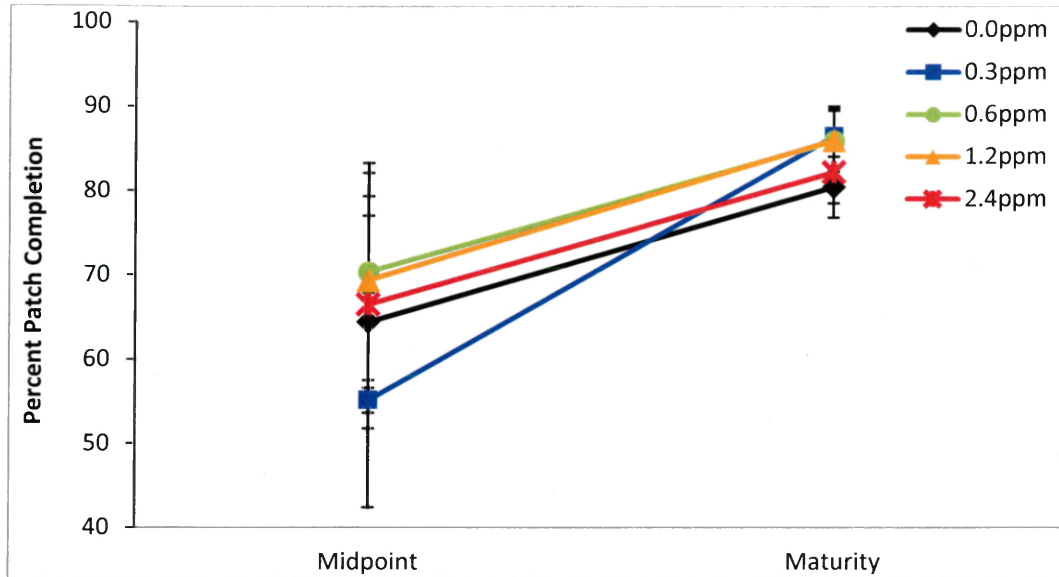


Figure 9. The growth of basic plumage patches in juveniles was not significantly affected by mercury treatment at the approximate midpoint of patch completion (week 8) or at sexual maturity (week 12). Displayed are the treatment estimated marginal means with standard error bars.

Discussion

Zebra Finches from Experiments 2 and 3, exposed to mercury *in ovo* and throughout life, exhibited the expected decrease in bill coloration as indicated by brighter and less red-saturated bills (Figs. 6 and 7). Plasma carotenoid concentration correlates well with expression of bill coloration in Zebra Finches (Blount et al. 2003a; McGraw et al. 2003b). Thus, the reduction in color saturation is consistent with the hypothesis that male finches stressed by mercury were using carotenoids for other functions, such as the scavenging of free radicals, and were not able to sequester them for use in bill ornamentation. Less intense bill coloration would have severe implications in a species such as the Zebra Finch where red bill ornamentation is a preferred trait in mate selection

by females (Burley and Coopersmith 1987; McGraw and Toomey 2010; Simons and Verhulst 2011). Males with lifetime exposure to mercury may not have been able to breed if they had been competing for mates with unexposed males. The severity with which mercury impacts male Zebra Finch bill coloration can be further emphasized by observing the effects on the young juveniles from Experiment 3. Throughout this study, these birds were in a constant state of feather growth, which is known to be a mode of mercury excretion in birds (Condon and Cristol 2009). Despite constant feather growth and mercury excretion, environmentally relevant levels of dietary mercury were able to cause significant decreases in the red bill coloration of these males.

Male Zebra Finches exposed to mercury after sexual maturity did not show the same decreases in the redness and saturation of their bill coloration as those exposed *in ovo* and throughout life (Fig. 6). These observations are initially surprising, given this species has decreased immune function when exposed to sublethal levels of mercury (Lewis et al. 2013) and that other studies have shown that Zebra Finches challenged as adults through immune or nutritional stressors showed measureable decreases in bill coloration (McGraw and Ardia 2003; Blount et al. 2003b; McGraw et al. 2003b; McGraw and Ardia 2004; Naguib and Nemitz 2007). Because adult exposed birds from Experiment 1 were only exposed to mercury for approximately seven months in this study, it is plausible that mercury had not accumulated for long enough to cause coloration effects. However, there may be a more fundamental difference between adult and lifetime exposure, for example organizational effects of hormones on the

development of regulatory mechanisms. Endocrine disruption during embryonic development can permanently modify the organization of body systems, leading to lifelong effects on traits, possibly including those mediating sexual coloration (Guillette et al. 1995; Weiss 2012). While previous studies have focused on endocrine-disrupting contaminants like phthalates and estrogen mimics, mercury is also known to cause similar endocrine disruption (Tan et al. 2009). Because hormones are critical in many vertebrate lifecycle events, implications for mercury changing the organization of endocrine systems during development have consequences that likely reach far beyond coloration.

One caveat in these conclusions is the fact that birds in my study may have been carotenoid limited. Without testing the carotenoid content of the pelletized grain diet, there is no way of knowing how many carotenoids they received. While I did provide all birds with 7 μ g/mL supplemented 10% lutein and 1% zeaxanthin their water, this only represents 10% of what is typically found in commercial seed blends (McGraw and Ardia 2004). While this may not have affected the outcome, it is possible in the wild, where foraging opportunities for carotenoid-rich foods may be more (or less) abundant, differences in coloration would not be observed. On the other hand, since the pelletized feed also contained other carotenoid-rich ingredients, it is possible that has 100% of the intended carotenoids been supplemented, the ample carotenoids might have hidden any effects of mercury exposure in my study.

While no bill coloration effects were observed in female Zebra Finches (Fig. 5), there is still a mode by which mercury could affect their carotenoid

allocation. Since Zebra Finches are sexually dimorphic, and yet consume the same types and amounts of carotenoids, there is a difference in the allocation of these pigment molecules between the sexes (McGraw et al. 2003b; McGraw and Toomey 2010). The ability of carotenoids to scavenge free-radicals in adults is maternally transferred to the egg yolk (Blount et al. 2000). Females breeding at mercury contaminated sites might invest fewer carotenoids into the egg if they are using the carotenoids to combat effects of mercury, thereby increasing the oxidative stress on developing embryos since fewer carotenoid molecules are invested for reproduction. Several studies indicate that development and neonatal condition have lifelong effects on birds (Blount et al. 2003a; Blount et al. 2006; Naguib and Nemitz 2007; Criscuolo et al. 2008), thus a decrease in maternal investment is likely to decrease the survivorship and/or fitness of the offspring produced at contaminated sites.

The transition of juvenile birds into adult plumage and bill coloration was not affected by mercury exposure (Figs. 8 and 9). While some studies link social and nutritional stressors to a slower transition into the adult coloration (Leader and Nottebohm 2006; Naguib and Nemitz 2007), this does not appear to be the case despite mercury's function as an immune stressor (Lewis et al. 2013). It is likely that the transitions into adult plumage and bill coloration are less plastic than a sexually-selected trait like bill coloration, which was affected in these birds (Fig. 7).

Body feather melanization, as assessed by spectrometry did not differ across mercury treatments, regardless of the point in the life cycle at which Zebra

Finches were exposed (Fig. 4). Other studies linking melanin coloration changes with environmental contaminants have assessed melanized ornamental patches and have measured patch size rather than color (McGraw et al. 2002b; Niecke et al. 2003; Senar et al. 2003; Bókony et al. 2008; Dauwe and Eens 2008; McGraw 2008; Senar et al. 2008; Eeva et al. 2009; Galván 2010). Sexually dichromatic melanin patches in the Zebra Finch, such as the rufous cheeks, chestnut flanks, or black breast patch, may be affected by mercury because they are believed to act as signals to females, although no concrete evidence for this is yet available (Zann 1996; McGraw and Wakamatsu 2004). It is possible that no effects of mercury were found on melanization of back feathers because there are no honesty-reinforcing mechanisms regulating them. Thus, although the original hypothesis of the present study was that mercury would affect melanin, the trait may not have provided an appropriate test of the hypothesis that is generalizable to sexually-selected colors as well.

Future Directions

Future work should determine if the coloration effects on lifetime-exposed birds can be remediated through removal of mercury from the diet after reaching sexual maturity, thereby simulating a wild bird that hatched at a contaminated site but dispersed to a non-contaminated site. If they cannot recover due to permanent damage caused by mercury, potential consequences can become severe for populations residing at contaminated sites. By controlling for variables in a dosing study, one can see in detail what traits are able to recover from

previous mercury exposure by conducting parallel investigations of birds initially dosed with mercury before being switched to a mercury free diet versus those kept on their initial mercury dosing regimen.

Plasma assessment of carotenoid levels would have been helpful in directly assessing the effects of mercury on total carotenoid allocation. Early neonatal nutritional stress has been shown to limit the ability of Zebra Finches to circulate carotenoids in their plasma, although there were no effects on adult bill coloration upon reaching sexual maturity (Blount et al. 2003a). Because contaminants are also stressors, adult-exposed birds from Experiments 1, which showed no changes in bill coloration, may have been circulating fewer plasma carotenoids as a result of using them to repair oxidative damage. This cannot be deduced from this study, however, since no direct measurements of carotenoids were made.

Conclusions

Based on the results of this study, I conclude that in Zebra Finches, mercury has negative effects on coloration in birds exposed to mercury beginning with *in ovo* deposition and continuing through life, and that carotenoid-based bill color was affected whereas melanized feather color was not. The development of adult coloration in lifetime-exposed juveniles was also not affected by mercury exposure. These results suggest that populations of non-migratory residents living near a point source contamination site would suffer greater effects on coloration than would immigrants arriving as adults, and that effects might be

limited to sexually-selected ornaments. Adult Zebra Finches exposed to mercury for a total of seven months, similar to the time migratory birds spend at any one site, showed no effects of mercury on coloration. It is therefore plausible that in the wild, birds exposed to mercury for only a single season may not show changes in coloration. Caution must be exercised in extrapolating these results to wild populations, as not only will different species vary in their response to mercury, but wild birds are exposed to many additional stressors to which captive birds in this study were not exposed. Wild birds may also have greater access to dietary carotenoids than those in this study. What can be said, however, is that mercury has the potential for far reaching effects in avian species which rely on carotenoid-based coloration for communication or camouflage.

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