The effects of fluctuating embryonic incubation temperatures and nighttime light exposure on the physiology of zebra finches (*Taeniopygia guttata*)

by

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Abstract

Anthropogenic effects, such as fluctuating incubation temperatures and light pollution, can have both behavioral and physiological impacts on an organism, particularly within avian species. Specifically, when parents leave the nest to forage, eggs are susceptible to temperature fluctuations, which may have downstream effects on physiology later in life. Additionally, light pollution is also of major concern regarding increased urbanization, which has been found to disrupt circadian rhythms and increase collisions with artificially lit structures in birds. However, little is known about the influences these environmental stressors have on avian species long-term. To address this gap in the literature, we utilized zebra finches (*Taeniopygia guttata*) as a model to understand the impacts of these environmental stressors. In Chapter 2, we exposed zebra finch eggs to one of three incubation regimes: (1) a Control incubator, which held a constant temperature of 37.4°C, (2) a Low incubator, which held a constant temperature of 36.4°C, or (3) a Periodic incubator, which exposed eggs to five periodic cooling events daily with an average temperature of the Low incubator. Beak photographs were taken at 45-, 60-, 75- and 95-days post-hatch (DPH) to examine the long-term effects of fluctuating incubation temperatures on the development of beak color. Additionally, we exposed these individuals to a repeated capture and restraint protocol to examine the short-term effects of handling on beak color maintenance. Overall, we found that there are age- and sex-specific effects of incubation treatment on beak hue, where females from periodically cooled eggs had decreased beak hues (redder) in adulthood. We also found that eggs laid later in a clutch had decreased beak saturation levels throughout life, regardless of incubation treatment. Females were found with lower beak hues and saturation following a capture and restraint stressor, while males showed increased beak saturation. Males that were subjected to the Low incubation treatment exhibited

higher activity levels than those in the Control group. These findings suggest that fluctuating incubation temperatures combined with repeated, short-term stressors can have significant sex-specific effects on sexual ornamentation and behavior. In Chapter 3, we exposed female zebra finches to constant light at night, or nighttime light exposure (NLE) for 23 days to examine the impacts of light pollution on body mass, beak coloration, bacterial killing ability against *Escherichia coli*, and gut microbial communities. We did not find a main effect of NLE (~160 lux) on body mass, beak color, or bacterial killing ability against *E. coli*. However, we found that light-treated individuals had significantly lower body masses than control birds at the conclusion of the experiment. Additionally, we found that individuals on the third day of the experiment had higher beak hues (less red) than at the start of the experiment. Birds exposed to constant light tended toward higher bacterial killing abilities on both the third and twenty-third day of the experiment. These findings suggest that the timing and length of NLE play a major role in the physiological effects of an organism, particularly in avian species.

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List of Abbreviations

ALAN	Artificial light at night
BKA	Bacterial killing assay
CORT	Corticosterone/cortisol
DPH	Days post-hatch
GC	Glucocorticoid
HBT	Handling bag test
LAN	Light at night
Lx	Lux
NLE	Nighttime light exposure
QIIME2	Quantitative Insights into Microbial Ecology 2
ZEFI	Zebra finch

Chapter 1: An introduction to the impacts of climate change-induced fluctuating temperatures and impacts of light pollution

Many anthropogenic environmental perturbations have been found to have negative impacts on global biodiversity. While many animal and plant species have adapted to new stresses, food sources, predators, and threats (Hunter, 2007), there are still a number of organisms that face extinction as a result of human-induced environmental changes. Two ecologically relevant anthropogenic changes currently affect biodiversity – rising temperatures due to climate change and light pollution. Constantly increasing (and fluctuating) temperatures because of climate change have been shown to affect a vast number of species globally, forcing them to adapt to relatively higher temperatures. Increasing temperatures force animals to either relocate or adapt to the new environment, which can be a major source of stress on an organism, regardless of the outcome. Light pollution, a result of industrial civilization, is another cause for concern. The World Atlas of Artificial Night Sky Brightness (2016) revealed that 99% of the United States and Europe do not experience natural light, as much outdoor lighting used at night is often overly bright, poorly targeted, improperly shielded, and in many cases, unnecessary (International Dark Sky Association, 2022). It is crucial to both identify and understand the impacts of anthropogenic effects across species, as the effects of rising temperatures due to climate change and light pollution have repeatedly shown either direct or indirect negative impacts on many organisms.

The study of physiological and biochemical mechanisms that set limits for environmental tolerance across organisms has become increasingly popular in the current era of climate change (Farrell, 2009), as species survival depends on keeping pace with changing climates (Loarie et al., 2009). According to NASA, atmospheric CO_2 has increased dramatically

since the Industrial Revolution compared to previous climate measurements over the past 800,000 years, and humans are the primary driver of these climatic changes. As a result, the planet's average surface temperature has risen approximately 2 degrees Fahrenheit (1 degrees Celsius) since the late 19th century, and most of atmospheric warming has occurred during the past 40 years (NASA, 2022). There are multiple components of climate change that are anticipated to affect all levels of biodiversity, from organism to biome levels (Bellard et al., 2012), and many recent studies have focused on physiological impacts of rising temperatures on birds. Climate change is of extreme relevance across many organisms, as fluctuating temperatures experienced during development can later affect biological rates such as photosynthesis or reproduction (Bernhardt et al., 2020). Organisms experience the same environment differently (Bernhardt et al., 2020), as some may be more or less sensitive to environmental cues/signals than others. Temperature fluctuations experienced during development are extensively studied in the context of temperature-dependent sex determination (TSD), particularly in ectothermic vertebrates who are highly vulnerable to the potentially detrimental effects of a changing climate (Bowden and Paitz, 2018). Many TSD studies utilize constant incubation temperatures to understand resulting sex ratios, however, it is undeniable that constant temperatures are not biologically realistic for most situations (Bowden et al., 2014). A study in pond sliders (*Trachemys scripta*) found that embryos from all populations were extremely sensitive to brief exposures to female-producing temperatures, suggesting that more emphasis should be placed on how organisms responds to realistic temperatures, particularly those who rely on temperature changes as a signal for varying phenotypes (Carter et al., 2018). While fluctuating temperatures pose a threat to organisms with TSD, daily temperature fluctuations are generally challenging for ectotherms, as these organisms must match metabolic

demands for cell maintenance, which are increased at high temperatures (Kern et al., 2015). Fluctuating temperatures have also been found to affect endotherms (mammals and birds). A study done across a taxonomically diverse species of birds spanning the U.S. and southern Canada revealed that various species alter their clutch sizes, laying dates, and breeding periods according to increasing temperatures (Torti and Dunn, 2005). As climate change is predicted to bring more frequent changes in temperatures, it is crucial to consider under which circumstances developmental thermal conditions predispose or constrain animals' capacity to deal with temperature variation (Nord and Giroud, 2020). These findings suggest that avian species can detect small-scale temperature fluctuations in the environment, which can have downstream effects on overall fitness.

Light pollution, or the excessive or inappropriate use of outdoor artificial light, is affecting human health and wildlife behavior (National Geographic, 2022) and is considered a threat for biodiversity (Dominoni, 2015). Nocturnal light can disrupt the circadian rhythm across many species, which perturbs appropriate melatonin secretion when the animal should be asleep. Sleep is an extremely important animal behavior that is widespread across the animal kingdom, which allows animals to recover from daily stress (Raap et al., 2015). Melatonin, a pineal hormone, serves as a chemical signal to the animal indicating day length, which can be crucial for seasonal adaptations, breeding, and reproduction across a wide array of species. In great tits (*Parus major*), artificial lighting caused experimental birds to wake up earlier, slept less, and spent less time in nest boxes (Raap et al., 2015), and a case report in zebra finches (*Taeniopygia guttata*) suggests that sleep deprivation from exposure to constant light led to increased mortality rates (Snyder et al., 2013). Another study in adult great tits revealed that individuals exposed to white light at night increased nighttime activity levels and were more susceptible to malaria

infection (Ouyang et al., 2017). Additionally, 3-week old king quail (*Excalfactoria chinensis*) exposed to constant light at night for 6 weeks showed that individuals exposed to constant light had significantly increased bactericidal activity against *Escherichia coli*, but this response varied between males and females (Saini et al., 2019). Put together, these findings suggest that light pollution (in the form of constant light at night) has extreme, and possibly sex-specific, physiological effects on an organism, particularly in avian species.

The zebra finch (*Taeniopygia guttata*) is an altricial passerine native to arid regions of Australia. Zebra finches are an excellent model for understanding the effects of global fluctuating temperatures during development and light pollution on host physiology for several reasons. There is a vast amount of literature utilizing zebra finches to understand a variety of biological processes, including song development and learning (Slater et al., 1988; MacDougall-Shackleton and Ball, 1999; MacDougall-Shackleton and Spencer, 2012), sexual behaviors (Adkins-Regan and Wade, 2001; Adkins-Regan, 2005; McGraw et al., 2006), plumage coloration (McGraw et al., 2011; Merrill et al., 2016), and responses to increasing temperatures (Cooper et al., 2020a, 2020b). The zebra finch was the second avian species to have its genome sequenced (Warren et al., 2010), which has implications for genome editing for understanding human health and disease. Also, zebra finches are opportunistic breeders when given proper resources in captivity, allowing for sufficient sample sizes for experimental purposes, and this species is also relatively easy to care for in captivity. Overall, utilizing zebra finches as a model for understanding physiological changes induced by anthropogenic changes in the environment can provide insight for making better choices for the environment – both for humans and for surrounding biodiversity. Because developmental stress has been shown to have effects on phenotypic traits (DuRant et al., 2013), offspring body composition (Wada et al., 2018), immune

function (Wada et al., 2015), and behavior (White and Kinney, 1974) later in life, we exposed zebra finch embryos to various incubation regimes and monitored beak coloration and behavior later in life to understand how developmental stress, in the form of fluctuating temperatures, can affect secondary sex characteristic development and behavioral stress responses later in life (Chapter 2). Artificial light at night has also been shown to have effects on body mass (Fonken et al., 2010), immune function (Moldofsky et al., 1989; Bedrosian et al., 2011; Fonken et al., 2012; Ouyang et al., 2017; Ziegler et al., 2021; Walker et al., 2022), and gut microbiota (Benedict et al., 2016; Poroyko et al., 2016; Jiang et al., 2020; Wei et al., 2020). Therefore, in Chapter 3, we exposed adult female zebra finches to 23d of constant light and monitored changes in body mass, bactericidal abilities of whole blood, and cloacal microbiota to elucidate the effects of nighttime light exposure on zebra finch physiology.

Chapter 2: Suboptimal embryonic incubation temperature has long-term, sex-specific consequences on beak coloration and the behavioral stress response in zebra finches

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Introduction

Anthropogenic disturbances to the environment such as climate change, habitat destruction, and introduction of environmental toxicants and pathogens pose novel threats to an organism's health and can act as stressors (stimuli that have the potential to inflict damage at a molecular, cellular, organ, or organismal level (Wada, 2019)). It is crucial to identify and understand the impacts of such stressors in order to relieve and conserve at-risk species (Carey, 2009). One major concern of biologists is climate change, as global annual temperature has increased, on average, at a rate of 0.08°C per decade since 1880 (NOAA, 2022). Furthermore, climate models predict that not only absolute temperature, but temperature variability will increase in the future (Bathiany et al., 2018), which may expose various species to temperature ranges that they have not previously encountered. Yet, many published laboratory studies that investigate the effects of temperature use absolute temperature changes (*i.e.*, static increases/decreases in mean temperature), which may not give an accurate depiction of temperature fluctuations that occur in nature. One major developmental perturbation for avian species is temperature variation/fluctuations experienced during the incubation period. Parents of most avian species sit on their eggs and use their bodies to transfer heat to developing embryos (Bertin et al., 2018), ranging from temperatures of 36°C to 40.5°C (Lundy, 1969; Conway, 2000), with 37°C to 38°C being the optimal temperature range for normal development of most avian species (French, 2009). When parents leave the nest to forage, the egg is often susceptible

to periodic cooling events until the parents return to continue incubating. Although it is difficult to mimic these fluctuations in the laboratory (Valenzuela et al., 2019), studies that account for fluctuating temperatures can shed light on the conditions that force parents to leave the nest more often, therefore resulting in high incubation temperature variability, which can influence development, survival, and reproductive abilities and attractiveness of the offspring.

In birds, secondary sex characteristics such as plumage, beak color, and song, can strongly influence reproductive success. These ornamental traits can serve as signals to a potential mate to indicate overall quality of the contender and have also been shown to reflect an individual's developmental history (Merrill et al., 2016). For instance, male zebra finches (*Taeniopygia guttata*) who received high quality food as young had larger cheek patches and were preferred by females than ones received a control diet (Naguib and Nemitz, 2007). Although secondary sex characteristics fully develop by sexual maturity in birds, some also change in relation to an individual's condition (Rosenthal et al., 2012) and can indicate current condition of a mate. For example, unlike plumage, vascularized beaks are particularly susceptible to environmental perturbations over time (Schull et al., 2016). At a molecular level, beak color is derived from carotenoids (Mcgraw, 2006), which are lipid-soluble pigments that are synthesized by plants, algae, bacteria, and fungi. These carotenoid pigments that are solely obtained from diet, which in turn, circulate in the bloodstream and deposit into the highly vascularized beak tissue (Pérez-Rodríguez et al., 2010). Male beak color of zebra finches has been correlated with defense against parasites (Hamilton and Zuk, 1982; McGraw and Hill, 2000; Van Oort and Dawson, 2005) and resistance to oxidative damage (Pérez-Rodríguez et al., 2010). Beak color can change rapidly, as carotenoids are quickly deposited into the living tissue in the beak (Ardia et al., 2010a; Rosenthal et al., 2012; Merrill et al., 2016), which makes beak

color a well-suited indicator of an individual's current condition to a potential mate. Additionally, individuals that maintain their beak color in the face of a stressor are deemed resistant to that specific stressor. This aforementioned stress resistance is defined as an organism's ability to elicit anti-damage mechanisms (*e.g.,* behavioral, physiological, or cellular responses to avoid persistent damage), so that they are more resilient to perturbations and overall performance is unaffected (Wada, 2019; Wada and Coutts, 2021). Therefore, if males can maintain a red and bright beak color despite facing a particular stressor, such as decreased food availability/quality, disturbances in environmental temperatures, and/or repeated handling or human interference, then they are considered resistant to that stressor, meaning better at regulating carotenoid distribution throughout the beak to attract a mate. Thus, male beak color can signal stress tolerance as well as developmental history, where a female can choose males with an attractive phenotype and/or genes to further pass on to offspring.

While stress resistance can be observed in beak color development and maintenance at the expense of attracting a suitable mate, stress tolerance can also be observed by the magnitude of a stress response an individual displays and its consequences. In response to a stressor, vertebrates elicit stress responses to escape or cope with that stressor, and these responses vary depending on the severity and persistence of the stressor in the environment. These include sympathetic nervous system and adrenocortical responses, prompting release of catecholamines (epinephrine and norepinephrine), and glucocorticoids (cortisol and corticosterone) into the bloodstream. Persistent physiological stress elicited during development have been shown to have long-lasting effects in avian species, including effects on body mass (Wada et al., 2015; Zito et al., 2017) and song learning (Lindström, 1999; Buchanan et al., 2003; Spencer et al., 2005; Wada and Coutts, 2021). In addition to the adrenocortical responses traditionally examined

in relation to developmental stress, organisms display behavioral stress responses (*e.g.,* anxiety-, fear-, and depression-related behaviors) due to variations in hormone signaling (Adkins-Regan, 2005; Myers et al., 2017). Although there are more studies that examined the effects of developmental nutritional stress and incubation temperature on corticosterone (CORT) levels, relatively little is known about how a developmental stressor affects behavioral stress responses later in life (Wada and Coutts, 2021) and how these short-term behavioral stress responses can impact secondary sex characteristics.

To address knowledge gaps related to stress cross-tolerance and effects of developmental conditions on secondary sex characteristics in adulthood when fitness-related traits like beak color would be most important, we explored the long-term consequences of fluctuating and low embryonic incubation temperatures on the maturation of beak color and sensitivity to a capture and restraint stressor in zebra finches. Specifically, we assessed three aims to elucidate the relationships between developmental stress, secondary sex characteristics, and the behavioral stress response: 1) the effects of suboptimal embryonic incubation temperatures on the development of beak hue, saturation, and value from juvenile stages to sexual maturity, 2) the short-term changes of beak coloration following a repeated capture and restraint (Cockrem et al., 2008; McGraw et al., 2011) associated with a behavioral response test (a 'handling bag test' adapted from Martin and Réale, 2008) on beak color maintenance, and 3) the consequences of suboptimal embryonic incubation temperatures on responses to a repeated handling stressor. While the impacts of developmental stress may appear negative, there is increasing evidence for an adaptive role of low-level developmental stress in shaping animal phenotype throughout life (Crino and Breuner, 2015; Briga et al., 2017; Krause et al., 2017). Considering a fluctuating incubation temperature as a mild developmental stressor as opposed to constantly low incubation

temperature, we hypothesized that fluctuating incubation temperatures induces cross-stress resistance (*i.e.,* further resistant to future stressors) later in life when individuals are exposed to a capture and restraint stressor, as stress in early life has been shown to condition an individual to future stressors in adulthood (Hoffman et al., 2018). Because developmental stress has been shown to have sex-specific effects on offspring phenotype in zebra finches where females are more negatively affected (Wada et al., 2008; Zito et al., 2017), we predicted that females incubated in the consistently low and periodic cooling incubation treatments would exhibit a more red beak color (increased hues/less attractive) across time, and short-term, repeated capture and restraint would cause a temporary decrease in female beak hue. Ultimately, the correlations between a developmental stressor and the impacts on an individual's behavioral stress response is largely unknown aside from bird song (Buchanan et al., 2003; MacDougall-Shackleton and Spencer, 2012). Thus, the purpose of this study is to correlate a developmental stress, in the form of fluctuating embryonic incubation temperatures, experienced in early life to beak color maturation and behavioral stress response profiles of individuals later in life.

Materials and Methods

Animal Models and Husbandry

The animal husbandry and thermal manipulation protocols in this study have been extensively described in (Rubin et al., 2021). Briefly, fertile eggs from 23 pairs (average of 3.7 eggs per nest) of zebra finches (*Taeniopygia guttata*) housed in at Auburn University, AL, USA were collected from April 2018 to December 2018. All breeding pairs had *ad libitum* access to seed (Kaytee Supreme (Finch) Chilton, WI), water, grit, and cuttlefish bone pieces. Breeding pairs were given a tablespoon (9.50 ± 0.39 g) of egg food (boiled egg, bread, and cornmeal mixture) daily. Once a week, they were provided with spinach and a probiotic supplement (Bene-

Bac, PetAg) mixed into egg food. Each breeding pair was provided an external nest box (19.5 cm x 14.5 cm x 14.5 cm) and nesting materials of shredded paper and irradiated hay (Rubin et al., 2021). To stimulate breeding, the birds were spritzed with water until egg laying began, as zebra finches use rainfall as a cue to initiate breeding (Zann et al., 1995). Each nest box was checked daily between 1000h and 1200h for freshly laid eggs. Once collected, eggs were labeled with a non-toxic marker to reflect nest origin and order, and eggs were randomly assigned to one of three incubators (Brinsea Octagon EX incubators, Brinsea Products Inc. Titusville, FL, USA): 1) an incubator with a constant temperature of 37.4°C further referred to as "Control," 2) an incubator that periodically cooled eggs by powering off 5 times a day for 30 minutes every 2 hours further referred to as "Periodic," and 3) an incubator with a constant low temperature of 36.4°C, which was the average temperature of the Periodic treatment, further referred to as "Low." There was one incubator per experimental group. Each incubator was programmed to hold a constant humidity reading of 55%. Temperature readings on the incubators were confirmed using thermometers (Thermco, 25°C/45°C ACCI310) for the Low and Control incubators. The lowest average temperature that the Periodic incubator reached during periodic cooling events was 29°C. After hatch, hatchlings were marked with unique feather patterns and haphazardly placed in nests, as breeding pairs were in different stages of laying and incubation (Rubin et al., 2021). Parents were removed from the nests once the youngest offspring in a nest gained nutritional independence, which occurs at ~45 dph. Additionally, birds were visually sexed and separated into isolation cages according to their sex between 55-60 dph, where they continued to receive *ad libitum* access to seed, water, grit, and cuttlefish bone pieces and weekly servings of spinach and egg food with probiotic mixture (Hoffman et al., 2018; Rubin et al., 2021). Each isolation cage held no more than three individuals. After individuals reached sexual

maturity (~95 dph), they were returned to communal flight cages. When individuals were re-captured in adulthood for this study, they were placed back into isolation cages (two individuals per cage, sex separated) for a two-week acclimation period. Individuals were only disturbed for daily animal care, where they continued to receive *ad libitum* seed, acidified water, egg food mixture, and cuttlefish bone. This experiment utilizes the 22 individuals assigned to the Control incubator (12 female; 10 male), 22 assigned to the Low incubator (11 female; 11 male), and 28 individuals assigned to the Periodic incubator (13 female; 15 male; total of 72 individuals). All procedures listed above were completed at and approved by Auburn University, AL, USA under IACUC #2018-3274.

Beak Coloration Measurements

Beak photographs were taken using an iPhone 8 Plus camera set to standard settings (flash, HDR, Live Mode, Timers, and Filters disabled) within a table-top photography studio with two light sources on either side of the photography arena. The light sources were marked using lab tape to ensure that light source positioning was consistent across all photos. The camera was secured on a tripod within the photography arena to maintain consistent distances between the bird and the iPhone. Red, blue, and green (RBG) coloration was quantified using ImageJ (Schneider et al., 2012). RGB values were then converted to hue, saturation, and value for each image using an online tool available from RapidTables (https://www.rapidtables.com). Beak color measures were cross-examined and confirmed with Adobe Photoshop CC using the Magic Wand, Histogram, and Color Picker tools with tolerance set to 60 for each photo. To explore the effects of suboptimal incubation temperatures on beak color maturation, measures for hue, saturation, and value were obtained for each bird at 45 dph, 60 dph, 75 dph, and 95 dph (**Figure 1**). Beak coloration was again measured as adults (average age 385.82 ± 32.32 dph)

prior to and after a ‘handling bag test’ (described below) to test whether incubation temperature has a long-term effect on beak coloration, to examine a change in beak coloration due to repeated handling, and whether the degree of change is influenced by varying embryonic incubation temperatures. Photographs, ImageJ analyses, and Photoshop analyses were completed by the same individual blind to treatments.

To determine the effect of suboptimal incubation temperature on development and maintenance of beak coloration through an acute stressor, we examined three aspects of color: hue, saturation, and value (HSV). Hue refers to the actual color type, such as red, green, or blue and is measured in degrees on a color wheel, with 0° (and 360°) being red. Birds with lower hues have ‘redder’ beaks, and birds with higher hues have lighter, more orange beaks. Saturation is the intensity (richness) of a color and is measured by a percent, with 0% meaning “no color” and 100% meaning “intense color.” Finally, value is the brightness of the color and is also measured by a percent, with 0% being black and 100% being white.

Handling Bag Test

To assess a behavioral stress response to a capture and restraint, we conducted a “handling bag test” adapted from Martin and Réale, (2008), illustrated in **Figure 2**. To test the effect of incubation temperatures on behavioral stress responses and maintenance of beak coloration after repeated handling, 72 individuals (36 of each sex) exposed to the Control (n=12 females; n=10 males), Low (n=11 females; n=10 males), or Periodic (n=13 females; n=16 males) incubation regimes were used. Birds were placed in sex-specific cages with two birds per cage (temperature treatments randomized) for a period of two weeks, during this time they were not handled and only disturbed to perform daily care. Following this acclimation period, individuals were photographed to document a baseline beak coloration prior to the handling bag test and

were then analyzed for their behavioral response to handling stress within one minute of capture. Individual birds were caught and placed in an opaque brown paper bag suspended in the air with the bird inside for 60 seconds (Martin and Réale, 2008). During this time, each bird's activity, described as any visible movement or rustling (visible shaking) of the bag during the measurement period, was recorded, and the proportion of time spent moving was calculated. The handling bag test occurred in a separate room from where conspecifics were housed to eliminate potential behavioral responses to chirps and song. An opaque brown paper bag was chosen instead of a mesh bag (as described in Martin and Réale (2008)) to (1) allow for disposal after each individual to prevent olfactory signals between individuals, (2) prevent visual cues while restrained in the opaque bag, and (3) the opaque bag is normally used in our field as a capture and restraint stress protocol. Bag tests were conducted in triplicate for each bird every other day over a period of 5 days, giving birds a day between replicates in which they were not handled and allowed to recover from the stress of capture and restraint. The order of birds that participated in these behavior trials was randomized daily. If two birds shared the same isolation cage, they were not tested in the same day to allow recovery of the individual that was not susceptible to the handling bag test in that day. As behavioral responses can vary amongst each trial, three replicates across a period of five days were used to generate a "temperament" profile for each bird. In order to measure repeatability of these behavioral responses, we calculated an intra-class coefficient (ICC) to quantify the reliability of phenotypic variation as a fraction of variance among individuals and variance within individuals over time (Hayes and Jenkins, 1997; Bell et al., 2009; Nakagawa and Schielzeth, 2010; Wolak et al., 2012), listed in **Table 3**. The ICC values obtained in this study describe how similar the "time spent moving" replicates are to each other for each individual. Specifically, a value less than 0.5 indicates poor reliability, and a

value greater than 0.9 indicates excellent reliability of the data. All handling bag tests, and beak measurements occurred between 08:00 and 09:30 am to ensure that baseline measures were obtained prior to disturbance for daily care.

Statistical Analyses

All statistical analyses were performed using RStudio version 4.0.5 “Ghost Orchid” (RStudio Team, 2021) using the lme4 (Bates et al., 2015), tidyverse (Wickham et al., 2019), and lmerTest (Kuznetsova et al., 2017) packages. All error metrics are reported as standard error unless otherwise noted. Aim 1 (beak color changes due to incubation temperature over time) was analyzed using linear mixed effects (LMER) models with a three-way interaction (along with corresponding two-way interactions) between age at measurement, incubation treatment, and sex, with numerical variables being scaled within the model. Additionally, all models for Aim 1 included an independent fixed effect of egg laying order and a random nested effect of nest of origin and individual to account for both genetic and individual differences. When interactions were significant in the global model ($P < 0.05$), those variables were analyzed separately. Hue, saturation, and value were used as measures of beak color. We used principal component analysis (PCA) to reduce collinearity between these variables. PCA showed that one component had an eigenvalue of 2.01, representing 67% of the variance in the data. When alternative models were performed, consistent results were obtained. Therefore, we are presenting hue, saturation, and value parameters separately.

Aim 2 (observing short-term changes in beak color from the handling bag test) was analyzed using LMER with a three-way interaction (along with corresponding two-way interactions) between sex, treatment, and timepoint (before/after the handling bag test), and a random effect of individual.

For Aim 3 (determining if thermal stress alters future responses to a stressor), we used a general linear model (lm) with fixed effects of treatment and sex and an interaction between the two variables. The independent variable in this case was the proportion of time spent moving while restrained in the bag. To verify the reliability of these repeated behavioral measures in Aim 3, we calculated an intra-class coefficient (ICC) using a generalized linear mixed effects model (binomial) with ID as a random effect using the rptR package (Stoffel et al., 2017). Statistical summaries are reported in **Tables 1-4.**

Results

Aim 1: Effects of suboptimal incubation temperature on beak coloration throughout development

All statistical measures for Aim 1 can be found in **Table 1.** We found that individuals in the Periodic group had significantly lower beak hues than those in the Control group (1.61º ± 0.734; $t(371.00) = 2.19$; $P = 0.029$). Males also exhibited significantly lower beak hues than females (7.59º ± 0.825; $t(329.14) = 9.20$; $P < 0.001$). We found a significant interaction between sex and age (-2.70 ± 0.753; $F(1, 348.65) = 19.77$; $P < 0.001$). When sexes were analyzed separately, we found that male hue decreased as they aged (3.84º ± 0.64; $t(1932.00) = 6.00$; $P < 0.001$, **Figure 4**). In females, we found that female hue decreased with age (1.19º ± 0.379; $t(145.15) = 3.14$; $P = 0.002$, **Figure 4**).

In contrast to hue, we found no significant interaction between sex and age on beak saturation. However, age significantly affected saturation, where beak saturation (intensity of color) increased by 2.77% ± 1.28 ($t(355.43) = 2.17$; $P = 0.031$) in both sexes (**Figure 4**) and plateaued around 75 dph. We did not find a significant effect of incubation treatment on neither male nor female beak saturation. Interestingly, we found that as egg laying order increased, saturation levels decreased (1.22% ± 0.552; $t(31.97) = 2.22$; $P = 0.0339$).

Males exhibited significantly lower beak values than females (5.92% ± 1.98; t(372.00) = 2.99; $P = 0.003$). There was a significant interaction between sex and age on beak value ($F(1, 372) = 6.67$; $P = 0.010$), so sexes were analyzed separately. There was no significance with regard to males. However, we found that female beak saturation increased (3.84% ± 1.43; $t(179.00) = 2.69$; $P = 0.0078$) with age.

Aim 2: Effects of repeated handling and influence of suboptimal incubation temperatures on beak coloration

Generally, we found that hues in all individuals were higher prior to the initial handling bag test compared to after the final bag test in the global model (1.0833º ± 0.3754; $t(65.71) = 2.89$; $P = 0.0053$), but this is dependent on sex ($F(1, 66) = 17.20$; $P < 0.001$). We also found a sex x treatment interaction ($F(2, 76) = 3.46$; $P = 0.036$). When sexes were analyzed separately, we found that males in the Periodic treatment had significantly higher hues (1.55º ± 0.77; $t(43.52) = 2.02$; $P = 0.0496$) than Control males. We found a significant interaction between sex x timepoint ($P < 0.001$). Females had significantly higher hues (1.08º ± 0.40; $t(33.00) = 2.70$; $P = 0.011$) before the handling bag test regardless of the incubation treatment, meaning that female beak color became redder after repeated handling. In contrast, male beak hue did not change with repeated handling ($t(32.82) = 1.58$; $P = 0.124$) (**Figure 5A**). We feel that it is important to mention that individuals in both the Periodic and Low treatments tended lower hues than Control birds (Periodic: $t(73.32) = 1.91$; $P = 0.064$; Low: $t(73.32) = 1.67$; $P = 0.099$, respectively).

We found a statistically significant sex x timepoint interaction ($F(1, 66) = 50.12$; $P < 0.001$) when analyzing beak saturation. When sexes are analyzed separately, we found that males had significantly increased beak saturation following the last handling bag test (3.88% ± 1.19; $t(32.95) = 3.26$; $P = 0.003$; **Figure 5B**). On the other hand, females had significantly decreased

beak saturation following the last handling bag test (3.62% ± 0.95; $t(33.00) = 3.80$; $P = 0.001$; **Figure 5B**).

We found that males had significantly lower beak value (intensity) when compared to females in the global model (13.25% ± 1.46; $t(100.40) = 9.06$; $P = 1.1 \times 10^{-14}$; **Figure 5C**). Additionally, a sex x treatment interaction was observed ($F(2, 69) = 8.36$; $P < 0.001$), so sexes were analyzed separately. Compared to Control males, males in the Low group tended toward higher beak values ($t(57.89) = 1.92$; $P = 0.060$), and males in the Periodic group exhibited significantly higher beak values (3.54% ± 1.45; $t(57.32) = 2.44$; $P = 0.018$). Females in the Periodic group exhibited significantly lower beak values (2.74% ± 1.30; $t(42.77) = 2.10$; $P = 0.041$) than females in the Control group. All statistical measures mentioned above can be found in **Table 2**.

Aim 3: Fluctuating incubation temperature alters future responses to a handling stressor

We estimated an ICC of 0.301 (95% CI: 0.080 to 0.432) for whether an animal moves during the one-minute stress test, indicating that the data is poorly reliable in quantifying an overall behavioral profile for each individual when both sexes and all treatments are considered. This ICC was calculated based on a binomial model, meaning that the data was coded by specifying whether the animal moved during the restraint period. ICCs were then calculated for each sex and treatment, which are listed in **Table 3**. We found that males tended to elicit less activity (proportion of time spent moving) than females when restrained during the handling bag test ($t(66.00) = 1.98$; $P = 0.051$). We did find a sex x treatment interaction trending toward statistical significance ($F(2, 66) = 2.84$; $P = 0.066$), so we analyzed sexes separately due to biological significance. Males in the Low treatment group showed relatively higher activity levels than males in the Control group (0.26 ± 0.10; $t(33.00) = 2.48$; $P = 0.018$, **Figure 6**), but

there was no effect of the Periodic group on activity levels in males ($t(33.00) = 1.10$; $P = 0.28$). The incubation temperature treatment had no effect on activity levels in females. Statistical measures are summarized in **Table 4**.

Discussion

Our study sheds light on the impacts of suboptimal embryonic incubation temperatures on beak coloration and the behavioral stress response in zebra finches throughout post-hatch development and into sexual maturity. Because fluctuating incubation temperatures are commonly experienced by avian eggs, we sought to disentangle the effects of temperature variation from absolute temperature and to illuminate their downstream effects on beak color development using zebra finches as a model. We predicted that females in the Low and Periodic groups would have decreased beak hue later in life, as females have been shown to be the most susceptible to environmental stressors. Because of this, we also predicted that females would exhibit higher activity levels during capture and restraint while birds from the Periodic group show improved stress tolerance due to frequent exposure to a mild stressor during development. In sum, we did not find an overall effect of incubation temperature on beak color development, but we found that periodically cooled females had decreased hues at 386 dph compared to Control females at that age. We also found that eggs laid later within a clutch had lower beak saturation than earlier laid eggs. When these individuals were subjected to repeated capture and restraint, females had lower beak hue and saturation following the final handling bag test. Conversely, we found that males had increased beak saturation following the final handling bag test. Lastly, we found that males within the Low incubation treatment had relatively higher activity levels than Control males when restrained.

Embryonic incubation temperature had a stronger effect on female beak color maturation than in males

Many studies have explored the environmental, anthropogenic, and pathogenic effects of beak color in avian species. Beak color in goldfinches (*Spinus tristis*) is similarly regulated by carotenoid pigments and is impacted by repeated handling stress and immunostimulatory lipopolysaccharide (LPS) injection, as yellow saturation decreased within 6.5h of repeated capture, and upon addition of LPS injection, beak hue and luminance drastically decreased (Rosenthal et al., 2012). Additionally, beak color in birds has been extensively experimentally modified via eliciting a stress response, modifying diet, or immune activity over a time period of weeks (Blount, 2003; Ardia et al., 2010a; Rosenthal et al., 2012). Thus, it is apparent that maintaining beak color through the context of carotenoid distribution is a major tradeoff at the expense of reproduction (Bertrand et al., 2006a). It is important to note that many studies that analyze beak color mainly use hue as a parameter, as hue is the main component of defining a particular color. However, data is lacking for other aspects of beak color such as saturation and value and so the consequences of changes in these traits on sexual ornamentation and reproduction is not well understood.

First, we found that females at 386 dph had lower beak hues than Control females. This finding aligns with our prediction that females would be more affected than males, but the 'attractiveness' of a redder beak in a female remains unclear without conducting mating trials. Specifically, females prefer males with the reddest, brightest beak color (Blount, 2003; Burley and Coopersmith, 2010; Merrill et al., 2016). Conversely, males prefer females whose beaks are not too red or too yellow but more orange with less intense coloration (Zann, 1996; Mcgraw, 2006). A previous study found that male and female zebra finches subjected to daily, 10-minute

handling treatments for four weeks displayed deeper orange/red beak coloration than control animals (McGraw et al., 2011). Although ’attractiveness’ of a redder beak on a female to a male beak is relatively unknown, a small set of evidence shows that males who experience no known stressor during development prefer females with orange beaks (higher hues) (Zann, 1996; Mcgraw, 2006). If a males’ preference is not influenced by a developmental stressor, high carotenoid allocation resulting in lower beak hue in a female may not be beneficial, as males may interpret this as a more ‘masculine’ phenotype. Previous studies have shown that exogenous testosterone administration increases beak redness (lower hue) in female zebra finches (Mcgraw, 2006). Since testosterone increases redness in beaks but also suppresses immune functions, testosterone can mediate a trade-off between attractiveness and immune function. To fully understand these dynamics, future studies should evaluate how consistently low and fluctuating incubation temperature affects sex steroid levels, attractiveness via mate choice trails, and immunocompetence. It is possible that consistently low or periodic cooling events that occur during embryonic incubation of the parental generation may increase testosterone levels within the yolk of subsequent offspring (Gil et al., 2004). McGraw et al. (2006) proposes that testosterone-induced immunosuppression in females with naturally high testosterone levels may divert carotenoids away from the beak and thus fading in beak color.

In contrast to females, we found that there was no significant effect of suboptimal embryonic incubation temperatures on male beak hue maturation. It is possible that males were able to maintain their beak hue despite embryonic perturbation as their post-hatch environment contained *ab libitum* access to food and nutrients. While there is strong evidence that an individual’s secondary sex characteristics can reflect developmental history (Merrill et al., 2016) specifically in birdsong (Buchanan et al., 2003; Spencer et al., 2005), beak hue does not appear

to be a strong indicator of developmental history with regard to male zebra finches. It has been shown that carotenoid-derived beak color in males is sex-steroid-dependent (*e.g.,* androgens) (Nelson, 2005), where maternally-deposited androgens within the yolk increase offspring competitiveness and development (Schwabl, 1996). Female red-legged partridges (*Alectoris rufa*) have been found to mate with redder males and produce more eggs which could favor the survival of later hatchlings by increasing androgen allocation to eggs (Alonso-Alvarez et al., 2012). The next step would be to quantify circulating androgen levels to determine whether an ability to secrete comparable levels of androgen would explain how males from eggs incubated at Low or Periodic regime can maintain beak hue later in life.

We did not observe an effect of suboptimal incubation temperature on beak saturation in males nor females. However, we did confirm that beak saturation (color richness) increases with age in both sexes. Interestingly, we found that eggs laid later in a clutch had decreased beak saturation compared to earlier-laid eggs. It has previously been discovered that yolk testosterone levels increase as laying order increases in canaries and zebra finches (Schwabl, 1993), which could have implications of yolk androgen levels and its impacts on beak saturation later in life. This pattern can vary depending on other factors, including diet. When mothers were fed a low-quality diet, their yolk testosterone levels decreased with egg laying order (Sandell et al., 2007). Therefore, it is possible that the eggs utilized in this experiment that were laid later in the clutch had higher levels of testosterone by the mother, resulting in decreased beak saturation later in life. This is a loose speculation, as the parents of eggs from this experiment were not experimentally monitored nor exposed to any known stressors to cause variations in testosterone supplementation into the yolk.

Repeated capture and restraint has sex-specific effects on beak hue, saturation, and value

We found that females decreased beak hue and saturation while males increased beak saturation following the handling bag test. These findings are similar to a previous study where zebra finches of both sexes were subjected to daily handling stress over 4 weeks, and stressed males lost body mass and marginally decreased in circulating carotenoid concentrations (McGraw et al., 2011). However, our results in females differed from the aforementioned study, where stressed females maintained their orange beak color. Both McGraw et al. (2011) and the current study show a sex-specific effect on handling stress on beak coloration in zebra finches, suggesting that males and females experience differential prioritization of beak color. It has been shown that carotenoids can be allocated to various parts of the body, such as within the integument, retina, and liver tissue (Rowe et al., 2012). Specifically, carotenoids can be stored within liver tissue until needed during periods of molt or migration (Negro et al., 2001). It is possible that, because of repeated capture and restraint, females mobilized carotenoid pigments, whereas males pulled carotenoid pigments from their beak tissue. The current study exposed finches to a handling stressor every other day for 5 days. Since this is much shorter and less frequent exposure than daily handling over 4 weeks used in McGraw et al. (2011), the data in the current study demonstrate that beak coloration can change with relatively mild handling stress. It is known that beak hue can change rapidly in response to a short-term stressor, as glucocorticoid-related stress is known to impair various condition-dependent traits (Buchanan, 2000). Generally, color saturation describes the vividness, richness, or intensity of a color. In this context, females had ‘less vivid’ beaks, whereas males had ‘more vivid’ beaks because of the handling bag test. Males subjected to suboptimal incubation temperatures (both Low and Periodic groups) exhibited significantly higher beak values (brighter) compared to Control males following the capture and restraint protocol. It has been found that repeated handling significantly decreases

body mass and depletes circulating carotenoid levels during the course of handling, but not long-term (McGraw et al., 2011). It is possible that short-term perturbation of carotenoids within beak tissue may make the beak itself less pigmented, thus affecting beak value, but our results show that individuals can potentially recover beak coloration by re-depositing carotenoid pigments within the beak. Unfortunately, we did not collect body mass/condition measures at or around the time of behavioral testing. However, our post-hoc testing of our previous study revealed that 10dph individuals in the Periodic group were 14.2% lighter than individuals in the Low and Control groups (Rubin et al., 2021). Therefore, these results may be snapshots of the short-term changes in beak saturation because of repeated handling showing the movement of physiological and energetic resources away from secondary sex characteristics.

Embryonic incubation treatment alters future responses to a capture and restraint stressor

Overall, we found that males exhibited lower activity levels than females when restrained during the handling bag test. This is to be expected, as females generally elicit a more heightened stress response than males (Martins, 2004; Verhulst et al., 2006; Spencer et al., 2010; Marasco et al., 2012), which has been observed in rats in response to alcohol (Rivier, 1993), but females have been found to show a better response to a chronic stressor over time (Dalla et al., 2005). However, females do not always respond greater than males, as a previous study found that there is no sex differences in the CORT response to handling stress in 16 dph or as adults (Wada et al., 2008). However, we did find that males in the Low incubation group exhibited higher activity levels than males in the Control group. While the embryonic environment on the behavioral development of birds remains relatively unexplored, a recent study has shown that chronic exposure to suboptimal temperatures of 27.2°C for 1 hour twice a day resulted in experimental chicks with elevated neophobic responses in novel food and novel environment tests, and these

experimental chicks also showed higher corticotropin-releasing factor in the nuclei of the amygdala, which is involved in regulating fear-related behaviors (Bertin et al., 2018). Chronic exposure to low incubation temperatures has been shown to cause oxidative damage and changes in antioxidant pathways (Loyau et al., 2014; Bertin et al., 2018), which may have impacted the developmental programming of behavioral responses observed in males during the handling bag test. In fact, a study utilizing the same cohort of animals used in the present study found that individuals in the Periodic and Control groups were able to habituate, in terms of their adrenocortical response, to a repeated capture and restraint stress, whereas the individuals exposed to the Low incubation treatment were not (Rubin et al., 2021). This lack of the ability to habituate to a repeated stressor in the Low group viewed in conjunction with higher activity levels during restraint from the present study suggest a possible neuroendocrine link between heightened activity and elevated CORT levels. This inability to habituate to a subsequent stressor can lead to higher circulating CORT levels throughout life, which negatively impacts fitness-related traits, such as immunocompetence, growth, and survival (Sapolsky et al., 2000; Wada et al., 2015; Jimeno et al., 2018; Rubin et al., 2021). These hormonal responses are dependent on neuroendocrine pathways that are mostly established during early life. Lastly, there were no long-term effects of incubation temperature observed in females. Overall, these data suggest that incubation temperature, specifically consistent temperatures under the thermal neutral zones of zebra finches, may contribute to the stress responses mounted later in life, which can have downstream effects on beak coloration and reproductive efforts.

Chapter 3: The effects of nighttime light exposure on the gut microbiota, immunocompetency, and stress physiology in zebra finches

Introduction

Birds are globally near-ubiquitous and contribute to vast ecosystems that either directly or indirectly benefit humanity by serving as pollinators, seed-dispersers, ecosystem engineers, scavengers, and predators to maintain biodiversity (Lees et al., 2022). It is estimated that bird populations in the United States have decreased by 29% since the 1970s (Brennan and Kuvlesky, 2005), and about 48% of worldwide extant bird species are known or suspected to be undergoing population declines (Lees et al., 2022). There are many interacting and largely anthropogenic causes that result in declines in bird populations. These include, but not limited to, increased use of pesticides in agricultural production, habitat loss due to continued conversion of grassland acres, and climate change (Li et al., 2020). Another contribution to the drastic decline in avian populations is light pollution, as artificial lighting has transformed the global nighttime environment (Ouyang et al., 2017) correlated with increased urbanization efforts, particularly in cities. Light pollution (often synonymous with artificial light at night [ALAN] or generally, nighttime light exposure [NLE]), is an increasing worldwide anthropogenic environmental pollutant (Falchi et al., 2016) that is defined as any alteration of night natural lighting levels caused by anthropogenic sources of light (Cinzano et al., 2000). ALAN has been found to impact the ability of migrating birds to detect navigational and orientational cues (Lees et al., 2022). ALAN often refers to "dim" light at night (commonly associated with urbanized cities). Here, we refer to nighttime light exposure (NLE) as constant light exposure during which an organism is exposed to constant light intensity levels for a set period. ALAN is an extensively studied stressor, as it is an indispensable tool for human safety and function but has been shown to be

disruptive for many organisms (Alaasam et al., 2018), particularly in birds. ALAN has been shown to interrupt many physiological processes in avian species (Saini et al., 2019; Jiang et al., 2020; Moaraf et al., 2020), including impacting the response of the endogenous circadian clock that is entrained by cyclic environmental signals (Dominoni, 2015). ALAN illumination levels often exceed real needs (*e.g.,* lighting of monuments or advertisements for aesthetic purposes) and follows untouched natural areas (Falcón et al., 2020). This nocturnal light disrupts circadian rhythms, which thereby decreases melatonin production at night when the animal should be asleep. Melatonin is a hormone that is released into blood circulation at night, which provides the organism with an accurate depiction of day length and allows the organism to tell whether day length is increasing or decreasing. Ultimately, this cyclic release of melatonin allows birds to synchronize their behavior and physiology for hatching, feeding, and migration (Gwinner, 1996; Gwinner et al., 1997; Bentley, 2001). This disruption in the organism's natural circadian clock can cause downstream effects on physiological processes, which rely on precise light information, such as timing of daily rhythms and seasonal adaptations (Bedrosian et al., 2011). While birds can sleep in the light (as sleep is a necessity for survival), the duration of sleep is likely shorter due to interruptions associated with the movement of conspecifics and frequent noise, which could lead to downregulation of repair mechanisms that naturally occur during sleep.

While ALAN often implies relatively 'low' light intensity levels ("dim" light), utilizing NLE in the laboratory can elucidate the severe impacts constant light can have on avian physiology and reproduction. Light illuminance (measured in lux [lx]) varies depending on the distance from the light source and reflectance from the environment itself; for example, car headlights can range from 0.00001 to 10,000 lx, daylight from 1 – 100,000 lx, moonlight from

0.01 – 1 lx, and light from a 7-foot lamp post can emit around 190 lx (Bennie et al., 2016). With this drastic range in illuminance levels, it is imperative that we identify ranges of light intensity that may negatively impact surrounding ecosystems while also balancing the need for nighttime light for human use. NLE has been shown to have immunological, behavioral, and molecular effects across both mammalian and avian species. A study in Siberian hamsters (*Phodopus sungorus*) revealed that four weeks of constant low-intensity light (5 lx) ALAN was sufficient to alter both cell-mediated immunity and bactericidal capacity, showing that this chronic NLE is sufficient to alter physiology and behavior (Bedrosian et al., 2011). House sparrows (*Passer domesticus*) exposed to 5 lx ALAN maintained higher West Nile Virus titers longer than control individuals (Kernbach et al., 2019) and that exposure to this low-intensity ALAN significantly suppressed melatonin levels throughout the night (Kernbach et al., 2020), indicating that their circadian rhythm was disrupted, which may have contributed to a delayed response to a viral infection. American robins (*Turdus migratorius*) exposed to large amounts of ALAN frequently began singing their morning songs during true night (Miller, 2006). Interestingly, a case study reported a drastic increase in mortality in a colony of zebra finches (*Taeniopygia guttata*) that were unknowingly exposed to constant light for a period of 30 days, and four out of six individuals analyzed in postmortem histological examinations were diagnosed with megabacteriosis (Snyder et al., 2013). There are no studies to date, however, on the effects of NLE on secondary sex characteristics in avian species, such as beak coloration development throughout life (described in Chapter 2). It has been previously shown that beak color in zebra finches can rapidly change in response to stress (Ardia et al., 2010a), and there is a major knowledge gap in understanding the ecological impacts of NLE on beak color maintenance at the expense of attracting a mate and reproduction. Put together, these findings suggest that major

cases of NLE and the resulting sleep disruption can impact the organism across multiple biological levels, including cognitive, metabolic, hormonal, and immunological impairments, that are thought to be detrimental to fitness (Faraut et al., 2012; Cooper et al., 2019).

When an individual is exposed to constant NLE, immune function is impacted via a combination of oxidative, neural, metabolic, and/or endocrine pathways (Navara and Nelson, 2007). It has been extensively shown that melatonin, a hormone secreted by the pineal gland in correlation with night length, has immense functional versatility, including antioxidant, oncostatic, anti-aging, and immunomodulatory properties (Reiter, 2003; Macchi and Bruce, 2004; Carrillo-Vico et al., 2005). Exposure to light at night is accompanied by a significant decrease in melatonin levels, which in turn, can elicit changes in immune function (Navara and Nelson, 2007). In birds, melatonin mediates the entrainment of circadian activity rhythms, and thus helps to time egg hatching and facilitate migration (Bentley, 2001). NLE and subsequent decreases in melatonin have been repeatedly shown to disrupt various metabolic processes, including causing weight gain in broiler chickens (Robbins et al., 1984) and modulating gut efficiency in mammalian species (Nelson and Drazen, 1999). It has also been shown that 40h of wakefulness in humans resulted in a significant decrease in natural killer cell activity (Moldofsky et al., 1989). NLE can also promote oxidative stress. Melatonin levels have been shown to correlate with total antioxidant capacity of blood in humans (Benot et al., 1999), and glutathione peroxidase activity levels have been shown to decrease in rats reared in constant light (Baydaş et al., 2001). Lastly, sleep deprivation as a result of NLE activates the hypothalamic-adrenal-pituitary axis (HPA) in rats, which affects their subsequent responses to stress (Meerlo et al., 2002). Taken together, melatonin, and ultimately NLE, have been shown to be a crucial part of

an integrative system to coordinate immunologic and physiological processes to cope with energetic stressors (Nelson and Drazen, 1999).

While the effects of light pollution on an organism's immune function have been extensively explored across species, very little is known about its impacts on gut microbiota composition across species. The 'microbiota' is the entire collection of microorganisms (*e.g.,* viruses, bacteria, fungi, archaea) present within a specific environment, such as the human gut (Berg et al., 2020). Recently, there has been a drastic increase in interest in the gut-brain axis (GBA), which describes the bidirectional communication between the central and the enteric nervous systems, which links emotional and cognitive centers of the brain with peripheral intestinal functions (Carabotti et al., 2015). Light pollution is a source of stress on organisms that rely on light signals for various physiological purposes. Stress, for the sake of this study, is defined as any disruption in homeostasis at any biological level. Because birds are sensitive to environmental changes and are often regarded as ideal indicators for environmental perturbations (Jiang et al., 2020), it is imperative to understand how light pollution, an ecologically relevant stressor to avian species, can impact the composition of their gut microbiota, which can have implications on diet, immune response, and disease susceptibility. A study in Eurasian tree sparrows (*Passer montanus*) concluded that ALAN not only affected circadian rhythms and suppressed melatonin release, but also affected taxonomic compositions, species diversity, and communal structure of intestinal microbiota in this species. Specifically, experimental birds that were set to a 12L:12h dim light cycle exhibited lower species diversity and species richness (Jiang et al., 2020). This loss in species diversity may attribute to decreased ability to respond to pathogens and decreased metabolic abilities in terms of nutrient assimilation (Le Chatelier et al., 2013). With the recent revelation in and increased accessibility to 16S rRNA gene sequencing,

there are more studies being done describing how environmental perturbations can impact avian gut microbial communities. The gastrointestinal (GI) tract of avian species is dominated by members of *Firmicutes*, *Actinobacteria, Bacteroidetes,* and *Proteobacteria* (Waite and Taylor, 2015). Interestingly, it has been shown that the ratio between *Firmicutes* and *Bacteroidetes* (F/B ratio) is an important marker of gut dysbiosis in humans, with higher F/B ratios correlated with obesity as *Firmicutes* have been shown to be more effective at extracting nutrients from food, leading to subsequent weight gain (Magne et al., 2020). There is increasing interest in manipulating this F/B ratio via diet in potentially treating, or 'reversing,' obesity. Thus, utilizing birds as a model in understanding these microbial community perturbations following a light pollution stressor could shed light on how vertebrates can plastically respond to their environment at the expense of survival and overall physiological maintenance. Therefore, the present study utilizes zebra finches (*Taeniopygia guttata*), a diurnal and altricial species, as a model to explore the effects of light pollution on body mass, beak color maintenance, bacterial killing abilities against *Escherichia coli*, and gut microbiota communities to elucidate how light pollution can affect avian physiology at the expense of anthropogenic urbanization. We hypothesized that individuals exposed to constant light at night for a period of two weeks would exhibit significantly lower bacterial killing abilities against *E. coli* and have significantly decreased species richness and diversity within their GI tracts. Ultimately, the goal of this study is to shed light on the negative impacts of light pollution on avian physiology to bring awareness to the consequences of anthropogenic urbanization globally.

Materials and Methods

Animal Model and Experimental Design

Adult female zebra finches (n = 32) obtained from Rockefeller University were housed at Avian Research Laboratory 2, Auburn University, AL, USA and used for this experiment. All procedures utilized in this experiment were completed at and approved by Auburn University, AL, USA under IACUC #2020-3805. All individuals had *ad libitum* access to seed (Kaytee Supreme (Finch), Chilton, WI), water, grit, and cuttlefish bone. Individuals responsible for daily care wore gloves, gowns, and shoe covers to prevent contamination across individuals entering the aviary. Prior to the experiment, birds were haphazardly assigned to either Control (n = 16) or Light (n = 16) treatment groups, while also ensuring body masses were comparable between groups at the time of the treatment assignment (13.69g ± 0.12 std. error, Control; 13.88g ± 0.15, Light). This experiment was divided into two phases, where 8 individuals from both Control and Light groups (16 birds total) were in the first phase and the other half of the birds were in the second phase. To account for any light differences in the rooms, we switched rooms following the first phase. Specifically, Control birds were housed in room A and Light birds were housed in room B for Round 1, whereas Control birds were housed in room B and Light birds were housed in room A in the second round. Between rounds, both rooms were thoroughly inspected, cleaned, and disinfected to ensure standardization throughout the entire experiment. All individuals were treated with Nystatin (an anti-fungal agent) solution mixed into their water bottles daily for 23d for both rounds to prevent gastrointestinal issues that are often common in communal aviaries and to also minimize any interference with the future microbial sampling within this study. After Nystatin treatment, both Rounds 1 and 2 birds underwent a "recovery" period of 58d and 72d, respectively. Round 2 birds had a longer Nystatin recovery, as these individuals needed two weeks to acclimate to their new tower cages prior to experimental start. Tower cages were covered in white tarp to allow for even distribution of light throughout individuals, as birds

toward the top of the tower cage had more light exposure than the individuals towards the bottom of the tower. Sterile blood samples, cloacal swabs, and body mass measurements were collected 11d prior to experimental start to serve as a baseline measurement. The experimental treatment started on Day 0 (D0), where the Control group (n = 8 for each round) remained under the normal 14L:10D photoperiod in room A, whereas the Light group (n = 8 for each round) were exposed to constant light (*i.e.,* 24L:0D photoperiod) for 23 days in room B. Experimental birds were exposed to 172.84 lux (SD = 18.02) and 165.93 lux (SD = 16.79) in rooms A and B, respectively. To determine the effects of nighttime light exposure, sterile blood samples, cloacal swab samples, body mass measurements, and beak color photographs were collected on D3, D23, and D35. Following D23 of treatment, all birds had a 13-day recovery period where individuals were exposed to the standard 14L:10D photoperiod. On D35, we collected all aforementioned samples, and the following day (D36), birds were humanely euthanized using isoflurane vapors and dissected for tissue analyses for another study. The timeline for this experiment is thoroughly described in **Figure 1**.

Sterile blood collection

We used a sterile technique to collect blood samples for use in bacterial killing assays (BKA) at baseline (D-11), D3, D23, and D35 of the experiment. At each sampling, approximately 15µL of whole blood was collected into a heparinized capillary tube via a small puncture of the brachial vein using a 26-gauge needle. After collection, the capillary tube was placed on a sterile field. Sterile blood samples were quickly transferred to a 0.5mL Safe-Lock Eppendorf tube and flash frozen in liquid nitrogen. The samples were stored in a -80°C freezer no longer than a week.

Bacterial killing assays

Bacterial killing assays were performed on sterile blood samples collected on Baseline (D-11), D3, D23, and D35, and all samples were analyzed within a week of collection after storage in a -80°C freezer. This protocol was adapted from a previous methodology study (Millet et al., 2007) and optimized for zebra finches (Wada et al., unpublished data). No more than 16 samples were analyzed at a time, and all samples were kept on ice throughout the duration of the assay. One day before the BKA, we placed 40mL 1X sterile phosphate-buffered saline (PBS) into a sterile Falcon tube and allowed it to incubate at 37°C for 30min. Sterile tweezers were used to remove one *Escherichia coli* ATCC #8739 pellet (VWR catalog #043E7, Microbiologics ®) from its vial and placed into a new, sterile Falcon tube and was allowed to equilibrate to room temperature for 30 minutes. The *E. coli* pellet was then transferred to the tube containing PBS, and this tube was incubated at 37°C for another 30 minutes (further referred to as the 'stock solution'). Once incubation was complete, the stock solution was thoroughly vortexed to ensure all solid pieces of the pellet dissolved. We made a working solution that contained 6mL sterile PBS and 105µL of the stock solution. To validate the stock solution prior to each assay, 15uL of the stock solution was added to 150µL sterile PBS. From this, we aliquoted 45µL onto a Tryptic Soy Agar (TSA) plate in triplicate. These plates were incubated for 24hr at 37°C, and CFUs were counted the following day. The stock solution made the day prior to the BKA was stored in a 4°C refrigerator for use on the following day.

Once we ensured proper CFU counts, a BKA with the experimental samples commenced utilizing a modified protocol (Ardia et al., 2010b). Using the stock solution, a new working solution was prepared for that day that contained approximately 110 CFUs. Briefly, we prepared time 0 (T0), media control (MC), and sample tubes for this assay. T0 tubes contained 150µL sterile PBS and 15µL working solution, MC tubes contained 15µL sterile PBS, 135µL sterile

tryptic soy broth (TSB, Hardy Diagnostics), and 15µL working solution. Sample tubes contained 135µL sterile TSB, 15µL working solution, and 15µL sterile whole blood. All T0, MC, and sample tubes were kept on ice until the incubation step. MC and sample tubes were briefly vortexed and transferred to a 41°C shaker incubator for 30 minutes, while T0 tubes were kept on ice. Once the incubation step was complete, we placed all tubes back onto ice, and we aliquoted 45µL of T0, MC, and sample solutions onto TSA in triplicate. For each BKA, we had three negative controls: one at the beginning of plating, one in the middle of plating the samples, and one at the very end of plating samples. The aliquots were spread onto TSA using a sterile L-shaped spreader. Plates were incubated agar side up at 37 °C for 24hr, and CFUs were counted the following day. CFUs were averaged amongst the triplicates, and killing ability was calculated using **Formula 1**. If a plate had CFUs that were too numerous to count, that plate was omitted from the average.

$$\%\ killing\ ability = \left(\frac{MC\ Avg. - Replicate\ Avg.}{MC\ Avg. - Neg.\ Control}\right) * 100$$

Formula 1. Percent killing ability of *E. coli* used for calculations in BKAs.

Cloacal sampling

To investigate the effect of nighttime exposure to light on the avian gut microbiota, cloacal swab samples were collected at Baseline (D-11), D23, and D35. An alcohol pad (VWR catalog #15648-916, PDI ®) was used to move feathers away from the cloaca and to sterilize the cloacal opening. A sterile swab (Puritan ® SKU #25-3318-U) was gently and completely inserted into the cloaca and rotated five times. The swab was gently removed and placed into a cryotube pre-filled with 0.5mL RNAlater (Thermo Fisher Scientific), which serves as a sample preservative (Berlow et al., 2020). Samples were flash-frozen in liquid nitrogen and stored at -80 °C.

DNA extractions

Samples were thawed on ice then bacterial DNA was extracted from these samples using the Quick-DNA™ Fecal/Soil Microbe MiniPrep Kit (ZYMO Research) and its corresponding protocol. DNA quality and concentration was confirmed using a NanoDrop 2000C (Thermo Fisher Scientific). Raw DNA was stored at -20°C until DNA was extracted from all samples. 16S Amplicon Metagenomics Sequencing were performed by Novogene where the V3 and V4 regions of the 16S rRNA gene were amplified, along with QIIME2 (Quantitative Insights into Microbial Ecology 2) microbial pipeline analyses.

Statistical Analyses

All statistical analyses were performed using RStudio version 4.0.5 "Ghost Orchid" (RStudio Team, 2021) using the lme4 (Bates et al., 2015, 4), tidyverse (Wickham et al., 2019), and lmerTest (Kuznetsova et al., 2017) packages. All error metrics are reported as standard error unless otherwise noted.

When exploring the effects of NLE on body mass, beak color, and bacterial killing ability, a linear mixed effects model (LMER) was utilized with an interaction term of treatment*day, along with a random effect of ID nested within round. Statistical significance is noted as $P \leq 0.05$. When interactions were statistically significant, those variables were analyzed separately. Novogene performed 16S amplicon sequencing utilizing the QIIME2 (Quantitative Insights into Microbial Ecology 2) pipeline for this dataset. Statistical analyses were performed in R, and we selected bacterial ribotypes with at least 1% relative abundance within cloacal samples. A total of 10,960,629 sequences were obtained, with a median of 128,444 and a mean of 124,553 sequences per sample. The lowest number of reads in a sample was 30,448, and the highest was 139,727. Sampling depth was rarefied to 1,000 reads (Momozawa et al., 2011). Out

of a total of 88 cloacal swab samples, we identified a total of 8,070 unique features, or amplicon sequence variants (ASVs).

Results

Effects of NLE on body mass

We did not find a main effect of NLE on body mass ($P = 0.216$); however, a significant treatment*day interaction was observed ($P < 0.001$) in the global model. We found that light-treated individuals at day 35 had significantly lower body mass (0.739g ± 0.334) than control birds ($P = 0.0354$) while control individuals' body mass stayed relatively constant throughout the duration of the experiment, regardless of sampling timepoint. We also found that light-treated individuals had significantly lower body mass at baseline (1.04g ± 0.255; $P < 0.001$), day 3 (0.711g ± 0.255; $P = 0.00645$), day 35 (1.32g ± 0.255; $P < 0.001$) when compared to day 23 (last day of experimental treatment). A statistical summary of model output is reported in **Table 5.**

Effects of NLE on beak coloration

We did not find a main effect of NLE on beak hue ($P = 0.2837$), but sampling day had a significant effect on hue ($P = 0.0399$) where individuals on day 3, regardless of treatment, had significantly higher beak hues (1.06° ± 0.496; $P = 0.0351$) than at baseline sampling. We did not find a main effect of NLE on saturation ($P = 0.726$), but sampling day had a significant effect on saturation ($P = 0.00292$) where individuals had significantly increased beak saturation on day 3 (6.81% ± 2.37; $P = 0.005$), day 23 (5.00% ± 2.41; $P = 0.041$), and day 35 (7.93% ± 2.41; $P = 0.0014$) when compared to baseline. We did not find a main effect of NLE on value ($P = 0.928$), nor with regard to sampling day ($P = 0.1808$). A statistical summary of model output is reported in **Table 7.**

Effects of NLE on bacterial killing ability

We did not find a main effect of NLE on bacterial killing abilities against *E. coli* in ZEFI whole blood ($P = 0.9557$). However, we did find that individuals in the light-treated groups at days 3 and 23 tended toward higher bacterial killing abilities ($P = 0.0640$, $P = 0.0632$, respectively), even though there was not a significant treatment*day interaction ($P = 0.102$). Because we feel that these results are biologically significant, we split the model regarding sampling day. We found that light-treated individuals on day 3 trended toward higher bacterial killing abilities than control birds ($P = 0.0563$). We also want to note that data could not be obtained on day 23 for a subset of individuals, as there were contamination issues with TSA plates, preventing CFU counting. A statistical summary of model output is reported in **Table 8.**

Effects of NLE on avian gut microbiota

Campylobacteria was the most dominant class present across all individuals (ranged from 28.27 – 49.14% across all five sampling groups). The next dominant class was *Gammaproteobacteria* (ranged from 15.85 – 24.96%). The third dominant class was *Alphaproteobacteria* (ranged from 10.11 – 27.59%). A summary of percent frequencies describing class distribution is listed in **Table 9.** We confirmed that we sufficiently sampled across timepoints, as confirmed in an alpha rarefaction curve (**Figure 8**). A Kruskal-Wallis (one-way ANOVA) revealed that there was not a significant effect of sampling timepoint ($P = 0.517$) on species diversity within samples. We also found no significant differences in Chao1 or Shannon indices (**Figure 11** and **Figure 12**). A permutational MANOVA (adonis) revealed that there were no significant differences across sampling groups (unweighted UniFrac $P = 0.196$; weighted UniFrac $P = 0.737$), represented in **Figure 13** using a Bray Curtis Emperor plot.

Discussion

Our goal was to determine the effects of nighttime light exposure on various physiological factors in a vertebrate model, the zebra finch. We know that artificial light at night affects circadian and circannual biology, but the consequences on an individual's physiological parameters under actual conditions remains unknown (Ouyang et al., 2017). Furthermore, whether physiological changes due to light exposure at night are permanent is underexplored. We exposed female zebra finches to constant light (~160 lux) for 23d and measured various physiological parameters, including changes in body mass, beak coloration, bacterial killing ability of whole blood, and gut microbiota after the light exposure as well as after a two-week recovery period (35d).

Overall, we found that light-treated birds had higher body masses on the last day of experimental treatment (before the recovery period began, at D23) when compared to all other timepoints. In contrast, Control birds' body masses stayed relatively constant throughout the duration of the experiment. These findings point out that constant light altered body mass and that body mass did not return to the baseline after the recovery period. Body mass was similar 3 days after the treatment, indicating that it was likely not be enough time of exposure to constant light to elicit a change in body mass. This is consistent with previous work where ecologically relevant levels artificial light at night (0.5, 1.5, and 5 lx) suppresses nocturnal melatonin production in a dose-dependent manner in zebra finches, where percentage body mass relative to Day0 during ALAN was higher in the 1.5 lx group when compared to the Control group, but only during the first week of ALAN exposure (Moaraf et al., 2020). Another study hypothesized that light at night exposure (~150 lux) affects metabolic parameters in Swiss-Webster mice; it was found that individuals exposed to 24h of continuous lighting for 8 weeks experienced a

significant increase in body mass by shifting the time of food intake (Fonken et al., 2010). This study found that nocturnal rodents eat more food at night, whereas mice exposed to 8h dim light at 5 lx consume 55.5% of their food during the light phase when compared to 36.5% in exposed to a standard light/dark cycle. Although we did not measure food consumption in this study, it is possible that individuals exposed to constant light consumed more than Control birds, as they did not have a particular signal or cue for feeding, or they simply had longer access to food. During sleep in vertebrates, a 'rest and recover' process occurs where metabolism slows, and melatonin is secreted (under optimal conditions). Subjects exposed to constant light at night may experience delayed or decreased melatonin secretion, which may indirectly cause subsequent weight gain in zebra finches over time, as elevated endogenous melatonin production and exogenously administered melatonin has repeatedly been shown to lower body weight (Reiter et al., 2012). Therefore, we speculate that longer treatments of constant light at night would result in continued increases in body mass as an indirect result of delayed melatonin secretion.

To date, there have been no studies exploring the effects of nighttime light exposure on beak coloration in birds. Our study found that nighttime light exposure did not have a direct impact on beak hue. This is not too surprising given that a study in adult male strawberry finches (*Estrilda amandava*) administered melatonin intramuscularly over 30 days found that melatonin had no effect on luteinizing hormone (LH)-dependent beak coloration (Gupta and Thapliyal, 1986). At the same time, our study found that beak hue changed through time regardless of the light treatment. Individuals on D3 had increased beak hues (less red) compared to baseline, regardless of treatment. Beak color in zebra finches is modulated by carotenoid pigments, which are red and yellow pigments that animals cannot synthesize *de novo* and ultimately must obtain through their diet (Blount, 2003). Carotenoid-derived sexual colors are proposed to signal overall

condition of males because of specific antioxidant and immunostimulatory activity (McGraw and Ardia, 2003), and this indicates that carotenoids can be quickly deposited and pulled from the beak tissue when an animal is under stress. Our findings suggest that while NLE did not have a direct effect on beak color, the short-term stress associated with handling at the beginning of the experiment may play a role in perturbing carotenoid levels implanted within beak tissue. Because we did not directly measure carotenoid levels throughout the experiment, future studies are needed to elucidate the effects of NLE on carotenoid circulation and implantation within beak tissue. Recent hypotheses have proposed that carotenoid-based sexual traits signal the availability of other non-pigmentary antioxidant molecules (*e.g.,* melatonin) that might protect carotenoids from free radical attacks and make them available for sexual advertisements (Bertrand et al., 2006b). One study found that melatonin supplementation within the drinking water of zebra finches resulted in redder bill color at the end of the experiment, but did not find a significant interaction between carotenoid and melatonin supplementation, which suggests that both treatments have an additive effect on the expression of beak color (Bertrand et al., 2006b). This indicates that melatonin not only has an antioxidant role but may also be involved in sexual selection and reproduction in zebra finches in conjunction with carotenoid implantation.

We also investigated the effects of NLE on bacterial killing abilities of zebra finch whole blood against *Escherichia coli*. We again did not find a main effect of NLE on bacterial killing abilities, but we found that light-treated birds at both the beginning (D3) and end (D23) of the experiment tended toward increased bacterial killing abilities when compared to Control birds. This finding suggests that NLE stimulates the immune response at not only the first exposure to NLE, but also at the conclusion of NLE treatment. It would be useful to have yet another sampling timepoint in between D3 and D23, but we were limited in terms of the amount of

whole blood that could be taken from the animal within a given time frame. Another study observed a similar result, where king quail exposed to experimental ALAN had significantly increased bactericidal capacity of plasma for both males and females, following 4 and 6 weeks of treatment, respectively (Saini et al., 2019). They also found that this effect was short-term, as bactericidal capabilities in females decreased after the 4-week period. Therefore, individuals invest early in immunity (Netea et al., 2011), which may lead to more effective innate immune responses later in life when exposed to other stressors. Additionally, wild great tit (*Parus major*) nestlings exposed to 3 lx for seven consecutive nights experienced decreased haptoglobin and increased nitric oxide levels (two key inflammatory markers) after LPS-injections compared with dark-night nestlings (Ziegler et al., 2021). This effect is possibly a result of reduced melatonin, as ALAN-treated individuals had around 49% lower melatonin concentrations than dark-night birds, as it has been demonstrated on multiple occasions that ALAN can suppress an organism's ability to respond to an immune challenge (Walker et al., 2022). Specifically, melatonin has been found to antagonize the depression of antibody production induced by acute restraint stress or CORT treatments in mice (Maestroni et al., 1986). Ultimately, there are several interacting factors when considering effects of NLE on bactericidal abilities in birds, but it is clear that NLE impacts immune function through various biochemical and physiological pathways.

Lastly, we explored the effects of NLE on gut microbial communities utilizing cloacal microbiota as a proxy. Our study reveals that 23d of constant light exposure does not have an effect on gut microbial communities sampled from the cloacal/large intestinal region of zebra finches, which may indicate that avian gut microbial communities are 'resistant' to short-term NLE. The avian gut microbiota is predominantly composed of *Firmicutes*, *Actinobacteria, Bacteroidetes,* and *Proteobacteria*. Our study revealed that *Campylobacteria* was the most

dominant class present across all individuals used in our experiment, along with *Gammaproteobacteria* and *Alphaproteobacteria*. We did not find a significant effect of sampling timepoint on species diversity within or across our samples. We also did not find a significant difference of microbial composition across sampling groups. These findings suggest that NLE does not have an overall effect on gut microbial communities sampled from inside the cloacal opening of female zebra finches. However, a study in 40 male Eurasian tree sparrows (*Passer montanus*) showed that ALAN affects taxonomic compositions, species diversity and community structure of intestinal microbiota over a period of 3 weeks. This study physically collected intestinal tissue from these individuals, resulting in immediate death of the individual, thus not allowing for sampling at multiple timepoints (Jiang et al., 2020). Another study compared the gut microbiome across five sample types in zebra finches (proventriculus, small intestine, large intestine, cloacal swabs, and feces) and found that cloacal swabs and feces were generally indistinguishable from large intestinal samples, indicating that non-lethal samples may be useful proxies for large intestinal bacterial communities (Berlow et al., 2020). We feel that more research is needed on the long-term effects of NLE on bird gut microbiota, as non-lethal sampling provides similar, if not identical, results to samples that require dissection of the individual.

Chapter 4: Conclusions and implications for fluctuating temperatures and light pollution

This Thesis describes two separate studies where we sought to understand the ecological impacts of climate change (in the context of fluctuating temperatures) and light pollution using zebra finches as a model. First, we exposed zebra finch eggs to varying incubation regimes (Rubin et al., 2021) and monitored their beak coloration and behavior over time in order to understand the long-term impacts of fluctuating temperatures on secondary sex characteristics and behavior. Specifically, we found that periodically cooled females had decreased beak hues later in adulthood, and eggs laid later in a clutch had decreased beak saturation throughout life, regardless of incubation treatment. Also, we found that females had lower beak hue and saturation after participating in a capture and restraint protocol, whereas males showed increased beak saturation. Lastly, we found that males reared in the consistently low incubator had relatively higher activity level during the capture and restraint protocol than control males. Overall, we found that fluctuating embryonic incubation temperatures have long-term, sex-specific effects on beak coloration and the behavioral stress response in zebra finches. These findings have implications for birds (and generally, organisms with carotenoid-regulated sexual traits) in the context of consistently rising global temperatures. There is strong evidence that secondary sexual characteristics can reflect an individual's developmental history (Merrill et al., 2016), and our findings suggest that female zebra finches exposed to fluctuating temperatures have more red beaks later in adulthood, which may not be as attractive to a male. Males generally have darker red beaks, and females have lighter orange beaks (Zann, 1996), and it seems that incubation temperature experienced in development modulates the implantation of carotenoid pigments in beak tissue later in life. However, we do not know the implications of 'attractiveness' of the females from this experiment, as we did not perform mating trials. This

leaves room for further research on the impacts of temperature fluctuations on secondary sex characteristics (*i.e.,* beak color) at the expense of reproduction later in life. The National Oceanic and Atmospheric Association reports that annual temperature has increased, on average, at a rate of 0.08°C per decade since 1880. However, temperature changes are not simply gradual increases or decreases over time – one must consider the mean climate and whether and how climate variability will change (Bathiany et al., 2018) over time. At the expense of climate change in wild plants and animals, climate-induced extinctions, distributional and phenological changes, and species range shifts are being documented at an alarming rate (Easterling et al., 2000). It is therefore of utmost importance to understand the ecological impacts that fluctuating (and extreme) temperatures have on organisms that contribute to Earth's biodiversity, as these stressors have not only been shown to affect metabolism and timing of migration, but also secondary sex characteristics in zebra finches, which can further impact reproduction in this species. It has been repeatedly shown that the developmental environment has strong and pervasive effects on animal phenotype, and exposure to stress during development is an environmental cue that can have strong effects on morphology, physiology, and behavior (Crino and Breuner, 2015). We also found that there were sex-specific effects of beak color changes in response to a capture and restraint stressor, which confirms that beak color (*i.e.,* carotenoid implantation in beak tissue) can change quickly and indicates the individuals' current condition. Another study showed similar results, where male and female zebra finches exposed to daily, 10-min handling treatments for 4 weeks displayed deeper orange/red beak coloration compared to control individuals (McGraw et al., 2011). Harsh and unpredictable events, such as climatic shifts can rapidly elevate CORT levels and subsequently prioritize critical bodily processes, such as glucose mobilization, to allow the animal to survive the event (Nelson, 2005; McGraw et al.,

2011). Therefore, we show that the handling bag test (or generally, a capture and restraint stressor) is sufficient to elicit a short-term change in beak color parameters, which may affect further reproductive strategies in avian species.

Lastly, we exposed adult female zebra finches for constant light at night for a 23d period to understand its impacts on various physiological parameters, including body mass, bactericidal abilities, and gut microbiota. Briefly, we found 23d of exposure to constant light at night caused subsequent increases in body mass, and individuals on D3 and D23 tended toward higher bactericidal abilities than control birds. There was no main effect of NLE on beak color or gut microbiota. We predicted that light-treated individuals would have decreased body masses and decreased bactericidal abilities, and we were surprised to see opposing results. We predicted that light-treated individuals would lose weight throughout the duration of the experiment, as birds would not have a specific cue of when they should be eating. However, that was not the case – the *lack* of an environmental cue (in this case, light) caused light-treated individuals to eat more (Fonken et al., 2010). Additionally, we proposed that light-treated individuals would exhibit decreased bactericidal abilities, as they would overall have 'less energy' to dedicate to killing off pathogens, viruses, or fungal infections. We were surprised to see that gut microbial communities were not altered because of NLE, but this shows that individuals were able to maintain these communities, likely for essential metabolic processes. Many microbial species within the vertebrate gut assure key functions that are necessary for health and well-being of the host, including colonic fermentation, strengthening resistance to colonization of opportunistic pathogens, and maturation of the intestinal epithelium and immune system (Mondot et al., 2013).

Table 1. Statistical summary of effects of incubation temperature on beak color before and after sexual maturity (Aim 1) using a global LMER model. Statistical significance is indicated by asterisk(s) (*) where Pr ≤ 0.05. Hue is measured in degrees (º), and both saturation and value are measured as percentages (%). Numerical variables (*i.e.,* Age and Egg Order) were scaled within the model. Interactions are specified within the "Fixed Effect" column by a single asterisk (*) between variables.

Independent Variable	Variance due to EggID (random)	Residual variance	Fixed Effect	*df*	*F* value	*P* value
Hue (º)	1.178; SD = 1.085	16.40; SD = 4.05	Treatment	2	0.1623	0.8502
			Sex (Male)	**1**	**201.9**	**< 0.001 *** **
			Age	**1**	**181.6**	**< 0.001 *** **
			Lay Order	1	0.2158	0.6446
			Treatment*Sex	**2**	**3.158**	**0.0438 ***
			Treatment*Age	2	0.5964	0.5514
			Sex*Age	**1**	**19.77**	**< 0.001 *** **
			Treatment*Sex*Age	2	1.273	0.2813
Saturation (%)	0.6689; SD = 0.8179	102.68; SD = 10.13	Treatment	2	0.0273	0.9731
			Sex	1	0.1841	0.6682
			Age	**1**	**14.65**	**< 0.001 *** **
			Lay Order	**1**	**4.912**	**0.0339 ***
			Treatment*Sex	2	0.1813	0.8343
			Treatment*Age	2	0.1548	0.8567
			Sex*Age	1	1.733	0.1888
			Treatment*Sex*Age	2	0.6211	0.5379
Value (%)	0.00; SD = 0.00	109.4; SD = 10.46	Treatment	2	0.2037	0.8158
			Sex	**1**	**19.56**	**< 0.001 *** **
			Age	**1**	**12.12**	**< 0.001 *** **
			Lay Order	1	2.416	0.1209
			Treatment*Sex	2	1.636	0.1963
			Treatment*Age	2	0.1295	0.8786
			Sex*Age	**1**	**6.671**	**0.0102 ***
			Treatment*Sex*Age	2	0.6189	0.5391

Table 2. Statistical summary of the short-term changes in beak color due to repeated handling and incubation temperature (Aim 2) using a global LME. Statistical significance is indicated by asterisk(s) (*) where Pr ≤ 0.05. Hue is measured in degrees (º), and both saturation and value are measured as percentages (%). Interactions are specified within the "Fixed Effect" column by a single asterisk (*) between variables.

Independent Variable	Variance due to Bird ID (random)	Residual variance	Fixed Effect	*df*	*F* value	*P* value
Hue (º)	7.890; SD = 2.809	0.846; SD = 0.920	**Sex**	**1**	**191.7**	**< 0.001 *** **
			Treatment	2	0.8195	0.4445
			Timepoint	1	0.0777	0.7813
			Sex*Treatment	**2**	**3.463**	**0.0363 ***
			Sex*Timepoint	**1**	**17.20**	**< 0.001 *** **
			Treatment*Timepoint	2	1.648	0.2003
			Sex*Treatment*Timepoint	2	0.5186	0.5977
Saturation (%)	10.22; SD = 3.197	6.26; SD = 2.50	Sex	1	0.1690	0.6824
			Treatment	2	0.2723	0.7624
			Timepoint	1	0.2983	0.5868
			Sex*Treatment	2	1.510	0.2279
			Sex*Timepoint	**1**	**50.12**	**< 0.001 *** **
			Treatment*Timepoint	2	0.0410	0.9600
			Sex*Treatment*Timepoint	2	2.7820	0.06915
Value (%)	6.553; SD = 2.56	5.11; SD = 2.26	**Sex**	**1**	**194.9**	**< 0.001 *** **
			Treatment	2	0.1339	0.8749
			Timepoint	**1**	**4.415**	**0.0394 ***
			Sex*Treatment	**2**	**8.362**	**< 0.001 *** **
			Sex*Timepoint	1	0.4459	0.5066
			Treatment*Timepoint	2	0.9785	0.3812
			Sex*Treatment*Timepoint	2	0.2600	0.7719

Table 3. ICC calculations for repeated capture and restraint of zebra finches. An ICC value of 0.5 indicates poor reliability of the data, 0.5-0.75 moderate reliability, 0.75-0.9 good reliability, and >0.9 excellent reliability. These ICC values were calculated using a binomial LMER.

	ICC	**Reliability**
All (sexes and treatments combined)	0.3010	Poor
Females (all treatments)	0.6028	Moderate
Males (all treatments)	0.5561	Moderate
Females (Control)	0.7031	Moderate
Males (Control)	0.3727	Poor
Females (Periodic)	0.6596	Moderate
Males (Periodic)	0.6729	Moderate
Females (Low)	0.3396	Poor
Males (Low)	0.4106	Poor

Table 4. Statistical summary elucidating the effects of suboptimal incubation temperature on activity levels (Aim 3) using a global LM. Statistical significance is indicated by asterisk(s) (*) where $P \leq 0.05$. P-values trending toward statistical significance are indicated by a dagger symbol (†). The independent variable is "AvgProp," which is the average proportion of time spent moving while restrained during the handling bag test.

Independent Variable	**Fixed Effect**	***df***	***F* value**	***P* value**
AvgProp	Treatment	2	0.5123	0.6015
	Sex	1	0.1183	0.7320
	Treatment*Sex	2	2.8405	0.0656 †

Table 5. Statistical summary of results from linear mixed-effect models on body mass of zebra finches (*Taeniopygia guttata*) when exposed to NLE. When a variable is statistically significant, it is marked with asterisks ($P < 0.001$ ***, $P < 0.01$ **, $P < 0.05$ *). In the global model, 'Baseline' is the reference variable for sampling day, and 'Control' is the reference variable for treatment.

Response	**Factor**		**Estimate ± SE**	***P***
Body mass (g)	Treatment		-0.461 ± 0.367	0.216
	Day 3		-0.00375 ± 0.180	0.983
	Day 23		-0.0138 ± 0.180	0.939
	Day 35		0.0294 ± 0.18	0.871
	Treatment*Day			< 0.001 ***
		Baseline	-0.461 ± 0.353	0.202
		Day 3	-0.129 ± 0.404	0.752
		Day 23	0.583 ± 0.370	0.126
		Day 35	-0.739 ± 0.335	0.035 *

Table 6. Summary of PCA loadings in determining which beak color parameters contribute the most to understanding the effect of NLE on beak color. The loadings that contribute the most to the principal component (PC) are in bold font.

	PC1	PC2	PC3
Hue	**0.708**		**0.706**
Value	**0.699**	-0.165	**-0.696**
Saturation	0.101	**0.986**	-0.132

Table 7. Statistical summary of results from linear mixed-effect models on beak hue and value of zebra finches (*Taeniopygia guttata*) when exposed to NLE. When a variable is statistically significant, it is marked with asterisks ($P < 0.001$ ***, $P < 0.01$ **, $P < 0.05$ *). In the global model, 'Baseline' is the reference variable for sampling day, and 'Control' is the reference variable for treatment.

Response	Factor	Estimate ± SE	*P*
Hue (°)	Treatment	-1.25 ± 1.15	0.284
	Day 3	1.06 ± 0.496	0.035 *
	Day 23	0.353 ± 0.508	0.489
	Day 35	-0.114 ± 0.508	0.823
	Treatment*Day		0.752
Saturation (%)	Treatment	0.875 ± 2.49	0.726
	Day 3	6.81 ± 2.37	0.00502 **
	Day 23	5.00 ± 2.41	0.041 *
	Day 35	7.93 ± 2.41	0.0014 **
	Treatment*Day		0.654
Value (%)	Treatment	-0.125 ± 1.37	0.928
	Day 3	1.19 ± 1.12	0.291
	Day 23	0.480 ± 1.14	0.675
	Day 35	1.88 ± 1.14	0.103
	Treatment*Day		0.106

Table 8. Statistical summary of results from linear mixed-effect models on bacterial killing abilities against *E. coli* of zebra finches (*Taeniopygia guttata*) when exposed to NLE. When a variable is statistically significant, it is marked with asterisks ($P < 0.001$ ***, $P < 0.01$ **, $P < 0.05$ *). If the variable is not statistically significant but biological significant, it is indicated with a dagger (†). In the global model, 'Baseline' is the reference variable for sampling day, and 'Control' is the reference variable for treatment.

Response	**Factor**	**Estimate ± SE**	***P***
BKA (%)	Treatment	-0.548 ± 9.83	0.956
	Day 3	-0.545 ± 7.75	0.944
	Day 23	-10.42 ± 9.73	0.288
	Day 35	-0.571 ± 7.92	0.943
	Treatment*Day		0.102
		Baseline	0.966
		Day 3	0.056 †
		Day 23	NA
		Day 35	0.982

Table 9. Relative frequencies describing class distribution across five sampling groups in NLE experiment.

	Campylobacteria	*Gammaproteobacteria*	*Alphaproteobacteria*	*Bacilli*	*Actinobacteria*
Baseline Control	43.24%	15.85%	10.11%	16.44%	3.02%
D23 Control	47.42%	18.58%	11.33%	8.11%	9.55%
D35 Control	28.27%	24.96%	21.55%	13.57%	2.92%
Baseline Light	49.14%	20.23%	13.39%	10.28%	1.45%
D23 Light	35.31%	19.39%	27.59%	11.86%	2.48%
D35 Light	44.80%	18.11%	13.00%	14.96%	1.59%

Figure 1. Beak color measurement and RGB/HSV conversion protocols. (1) Beak photos were taken using a standardized photography arena, with lights placed on either side of the arena and a tripod calibrated to a set distance to hold the iPhone. **(2)** The bird was laid flat onto the surface of the photography arena, ensuring visibility of the beak. Beak color/carotenoid accumulation is visualized in **A-D**, which represents a male zebra finch at 45 **(A)**, 60 **(B)**, 75 **(C)**, and 95 **(D)** dph. **(3)** Beak photos were then quantified for red, green, and blue (RGB) measurements using ImageJ, **(4)** those RGB values were converted into hue, saturation, and value (HSV) correlates and those HSV measurements were cross-examined using Adobe Photoshop.

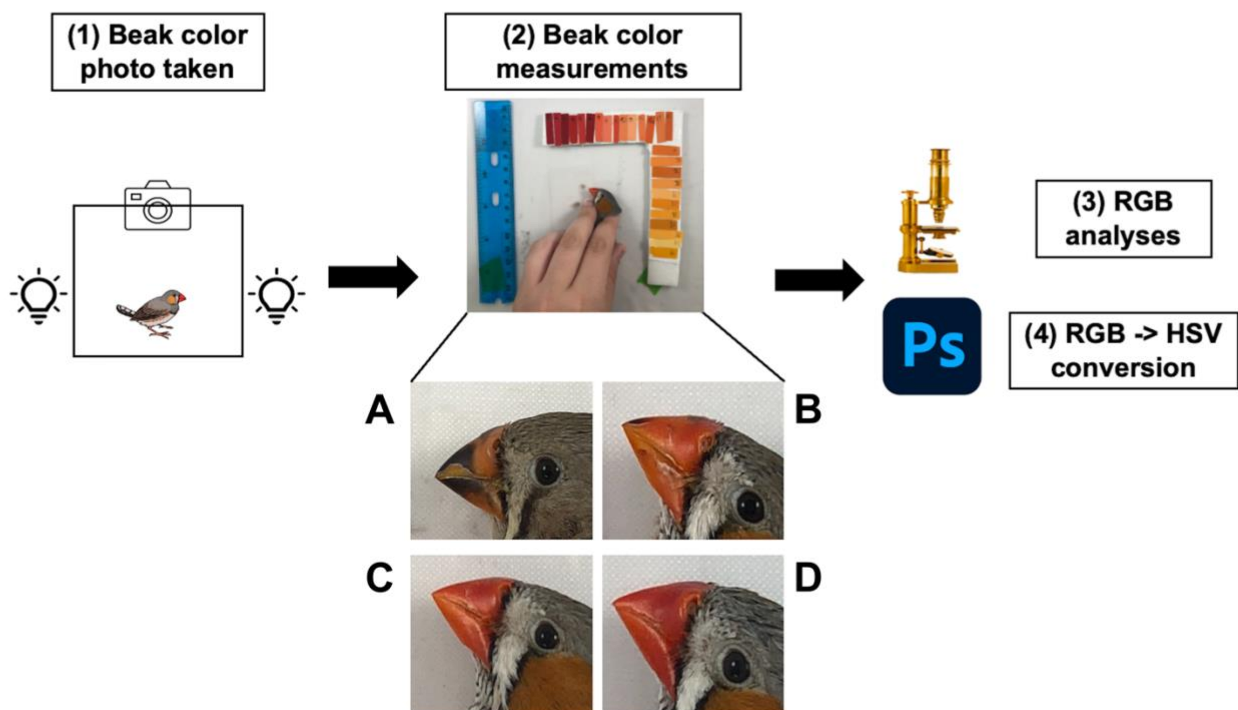


Figure 2. Handling bag test experimental design. Zebra finches at an average age of 386 dph participated in a handling bag test. Prior to the first handling bag test, beak color photos of each bird were taken. Then, each bird was placed in a brown, opaque paper bag and restrained for one minute, and the time spent moving ('activity') was recorded. The handling bag test was conducted in triplicate every other day (over span of 5d) for each bird to ensure for a recovery period. At the conclusion of the third handling bag test, another beak photo was taken.

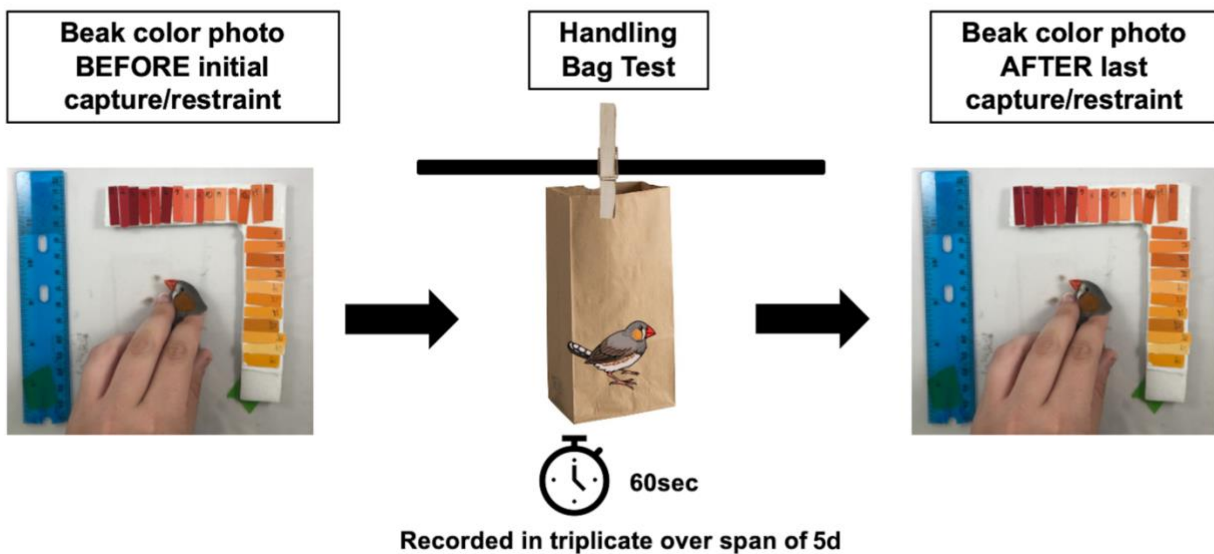


Figure 3. Suboptimal embryonic incubation temperatures do not impact beak color (hue, saturation, value) development. Control, Low, and Periodic treatments are shown in black, yellow, and blue, respectively. The changes in hue, saturation, and value are measured on the y-axis, measured in degrees and percentage, respectively. Sex is indicated on the x-axis.

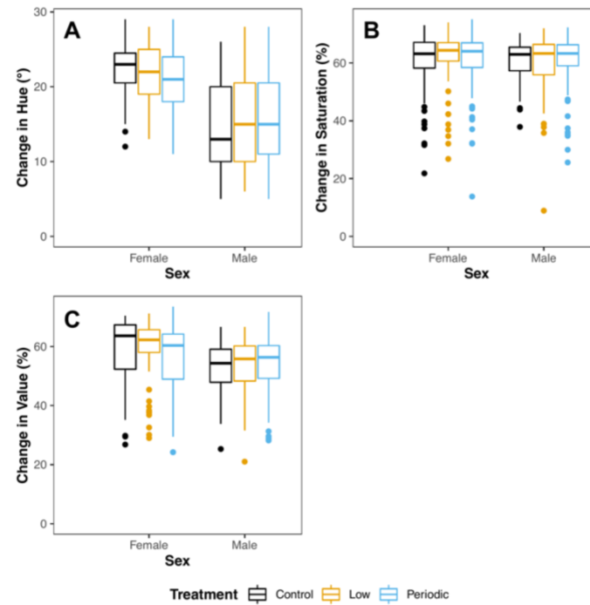


Figure 4. Male and female hue decreases over time, and saturation and value plateau on 60 dph and 75 dph, respectively. Females are shown in the left panel, and males are shown in the right panel. Control, Low, and Periodic treatments are shown in black, yellow, and blue, respectively. The changes in hue, saturation, and value are measured on the y-axis, measured in degrees and percentage, respectively. Age is indicated on the x-axis, including 45, 60, 75, 95, and 386 dph, respectively.

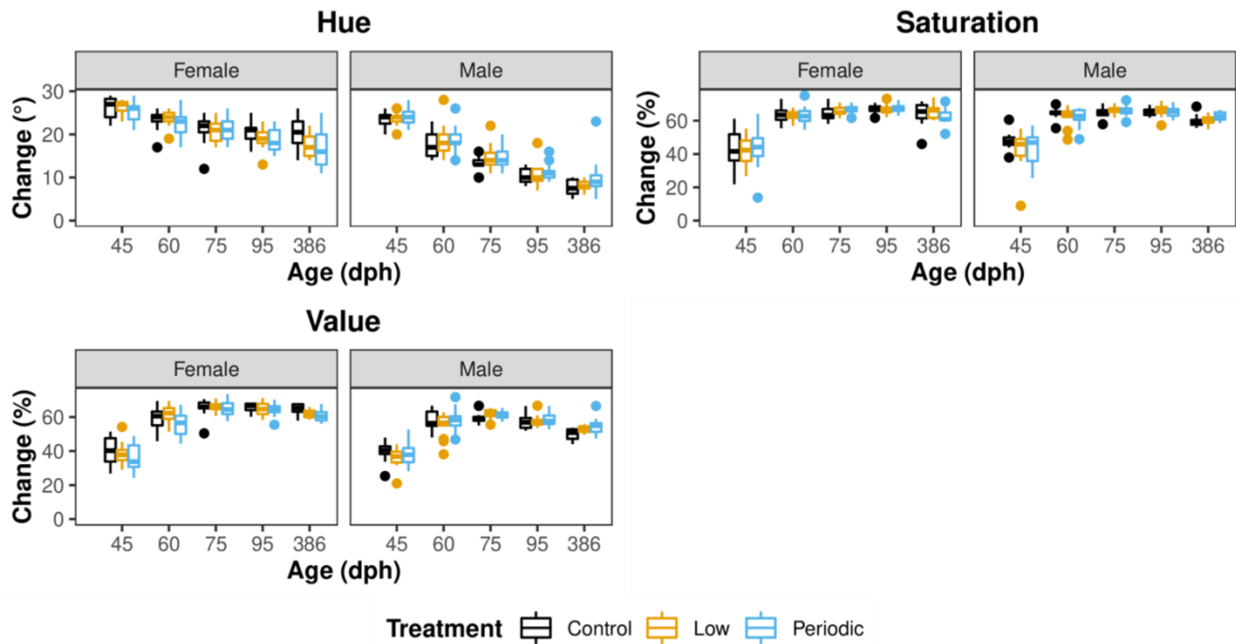


Figure 5. Beak hue and saturation are impacted by repeated capture and restraint. The change in the beak color parameter (hue, saturation, or value) is presented on the y-axis, and sex is shown on the x-axis. Data indicated in red boxplots are indicative of "Before" the handling bag test, whereas blue boxplots are indicative of "After" the handling bag test. **(A)** Female beak hue significantly decreases following the initial handling bag test. **(B)** Both male and female beak saturation are inversely affected following the initial handling bag test. **(C)** There are no effects of the handling bag test on beak value. Three asterisks (***) indicate *P* < 0.005.

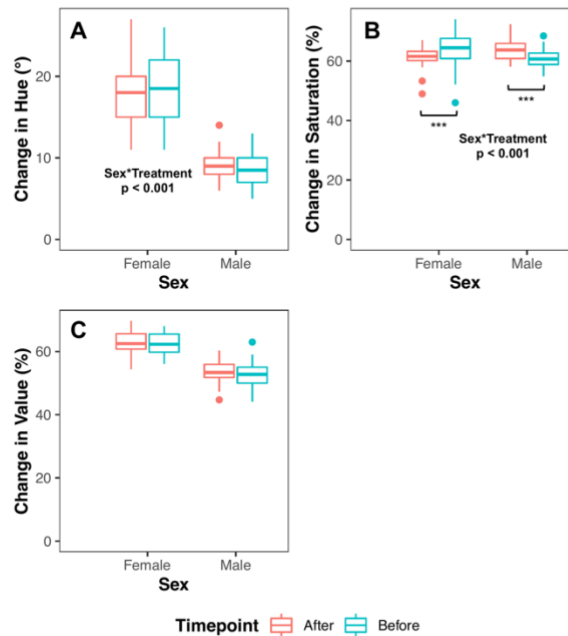


Figure 6. Males in the Low incubation treatment had higher activity levels than those in the Control treatment. The average proportion of time (seconds) spent moving during the handling bag test is recorded on the y-axis. The three incubation treatment groups are shown on the x-axis (Control, Low, and Periodic). Females are indicated by pink boxplots, and males are indicated by blue boxplots. Two asterisks (**) indicate $P < 0.05$.

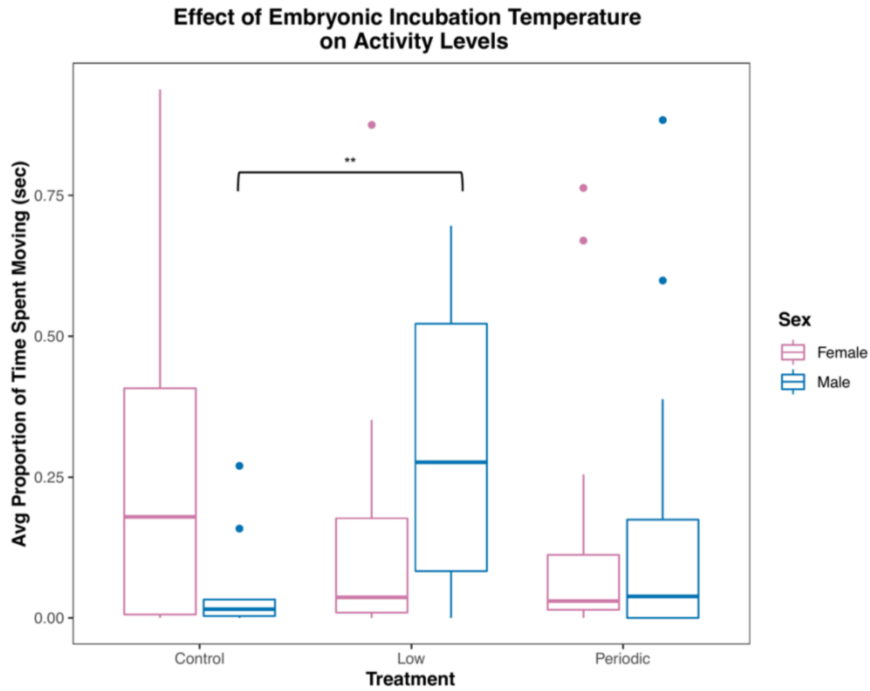


Figure 7. NLE experimental timeline. The Control group is shown on the top half, and the Light group is shown on the bottom half of the figure. Green arrows indicate the experimental period of 23 days. Continuous light (*i.e.,* 24L:0D photoperiod) is shown by a yellow sun icon. Body mass measurements, cloacal swabs, and sterile blood samples were collected on day (D) -11, D3, D23, and D35 (denoted with three asterisks). Birds were euthanized and tissue samples were collected on D36 (the pancreas icon). Days are listed at the bottom of each timeline. Both Control and Light (n = 16) groups were exposed to an acclimation period of 11 days prior to the experiment. During the acclimation period and for controls, birds were on a 'normal' 14L:10D photoperiod. All individuals underwent a recovery period where they were on a 14L:10D photoperiod and were humanely euthanized on D36 for tissue collection and analyses.

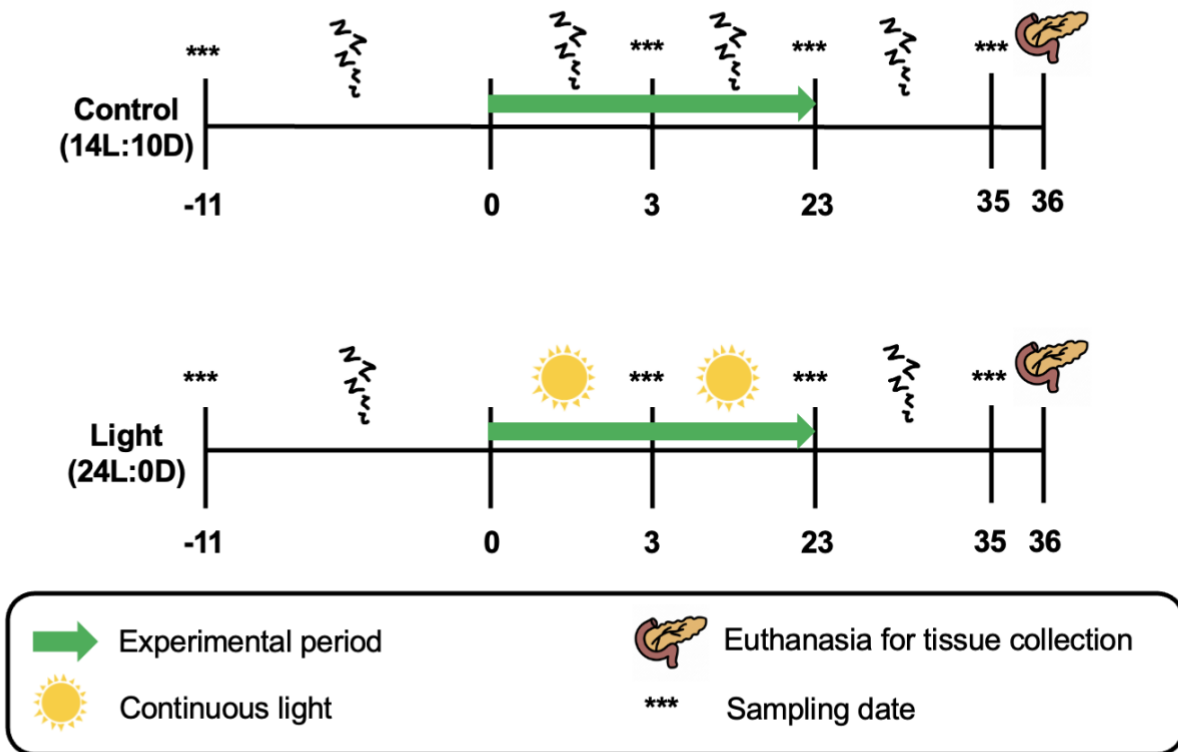


Figure 8. Change in body mass because of NLE. Days are reflected on the x-axis, and body mass is reflected on the y-axis. A green arrow represents when the experiment occurred. Control and Light-treated individuals are represented by navy and orange lines, respectively. Statistical significance is represented by three asterisks (***) when $P < 0.001$, and two asterisks (**) when $P < 0.01$.

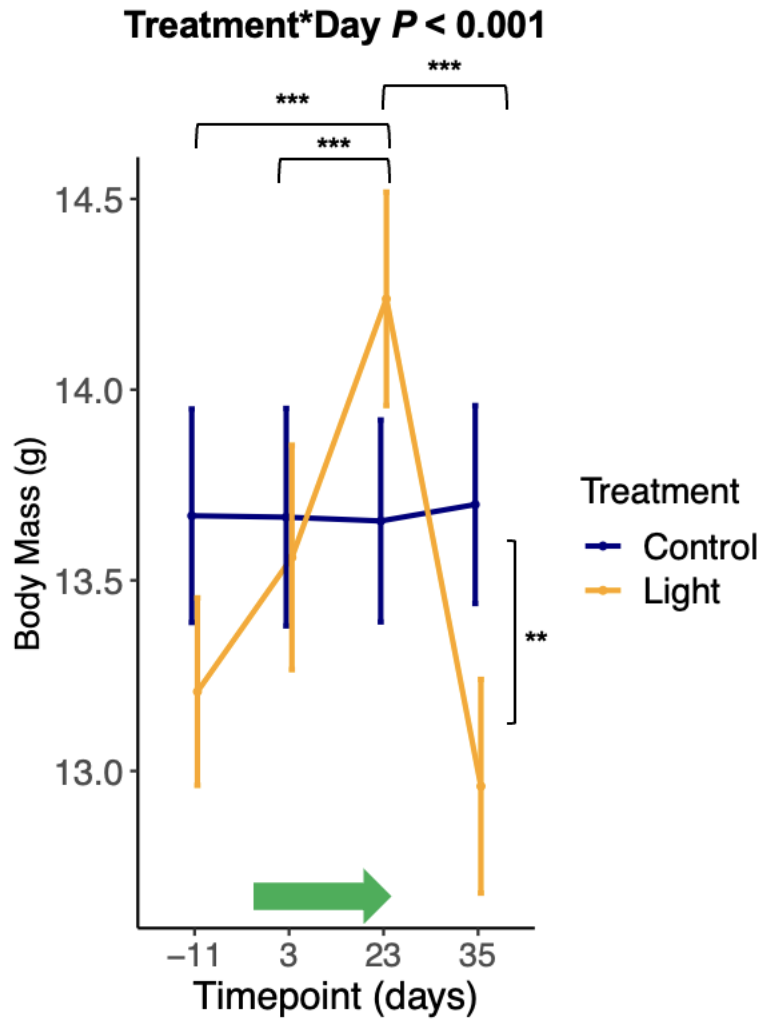


Figure 9. Change in beak hue throughout NLE experiment. Timepoint in days is reflected on the x-axis, and beak hue is reflected on the y-axis. A green arrow represents when the experiment occurred. Control and Light-treated groups are represented by navy and orange lines, respectively. Statistical significance is indicated by two asterisks (**) where $P < 0.05$.

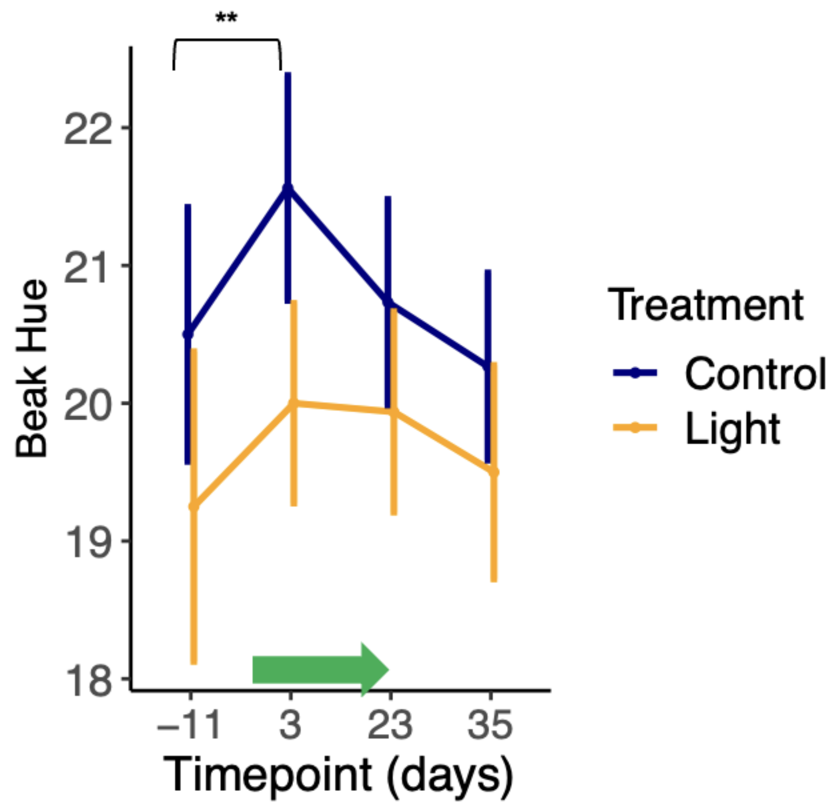


Figure 10. Change in beak value throughout NLE experiment. Timepoint in days is reflected on the x-axis, and beak value is reflected on the y-axis. A green arrow represents when the experiment occurred. Control and Light-treated groups are represented by navy and orange lines, respectively.

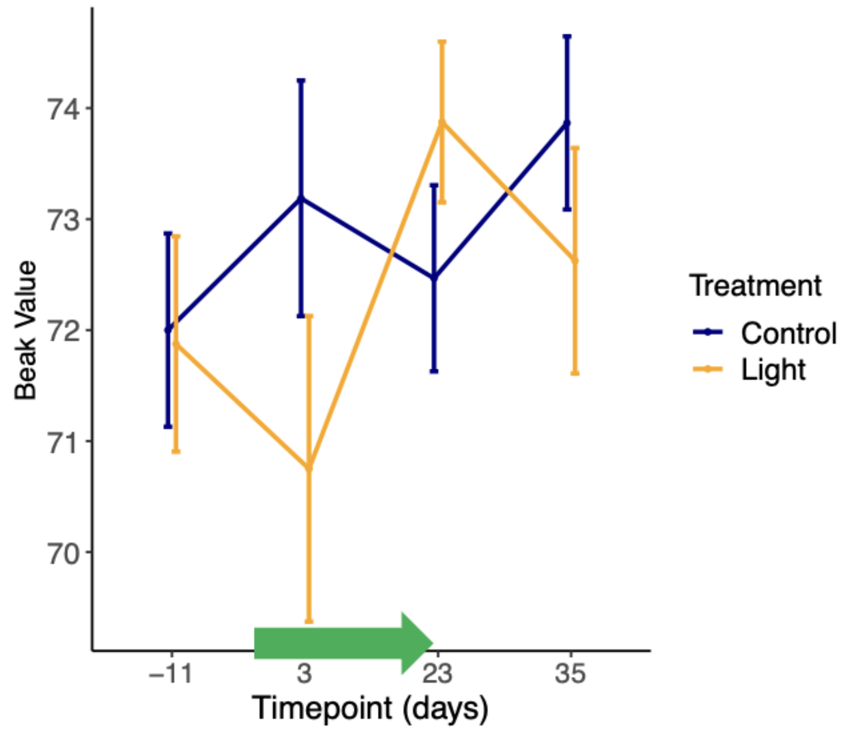


Figure 11. Bacterial killing abilities against *E. coli* in whole blood of zebra finches.

Timepoint (sampling day) is represented on the x-axis, and percent killing against *E. coli* is on the y-axis. A green arrow represents when the experiment occurred. Control and Light-treated groups are represented by navy and orange lines, respectively. Comparisons that were not statistically significant but remain biologically significant are represented by a diamond.

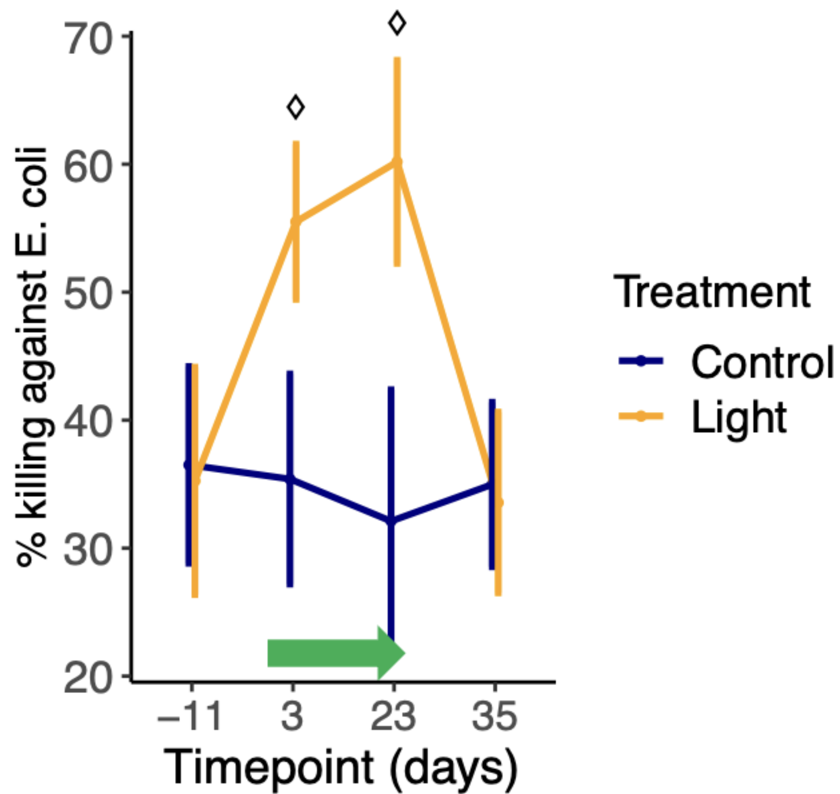


Figure 12. Alpha rarefaction curve describing sequencing depths of cloacal microbiota in NLE experiment. Sequencing depth is shown on the x-axis, and the number of observed OTUs is on the y-axis. Baseline Control and Light groups are dark blue and light blue lines, respectively. Experimental (D23) Control and Light groups are dark orange and light orange lines, respectively. Recovery (D35) Control and Light groups are dark green and light green lines, respectively.

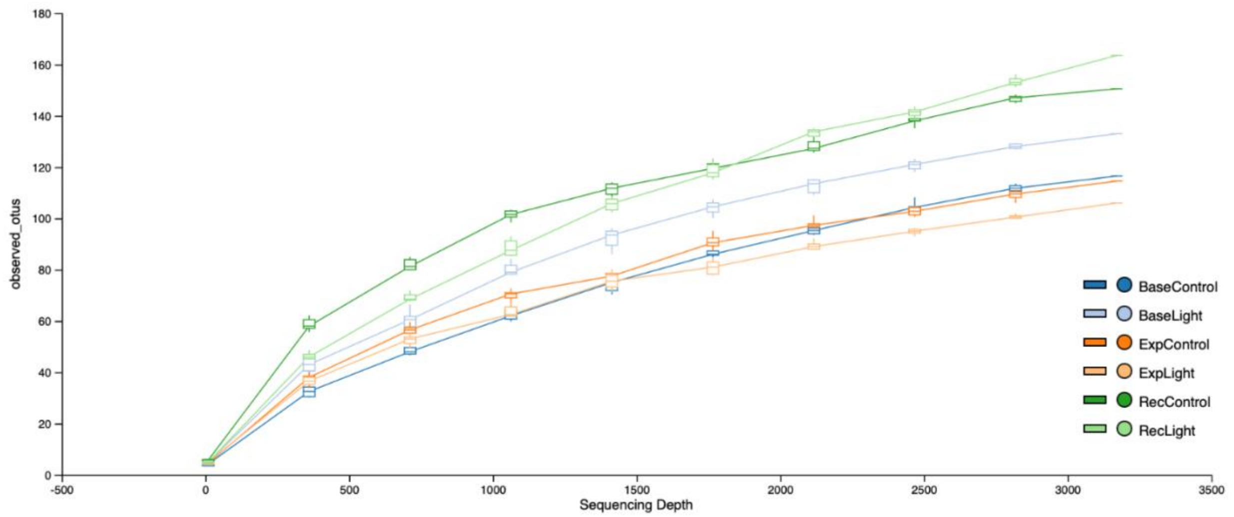


Figure 13. Relative abundances of bacterial classes by sampling timepoint in NLE experiment. Sampling timepoints include Control and Light treatments sampled at Baseline, Experiment (D23), and Recovery (D35). Relative abundances of classes *Campylobacteria, Gammaproteobacteria,* and *Alphaproteobacteria, Bacilli, Actinobacteria*, and *Clostridia* are shown in red, pink, dark green, light green, dark blue, and light blue bars, respectively.

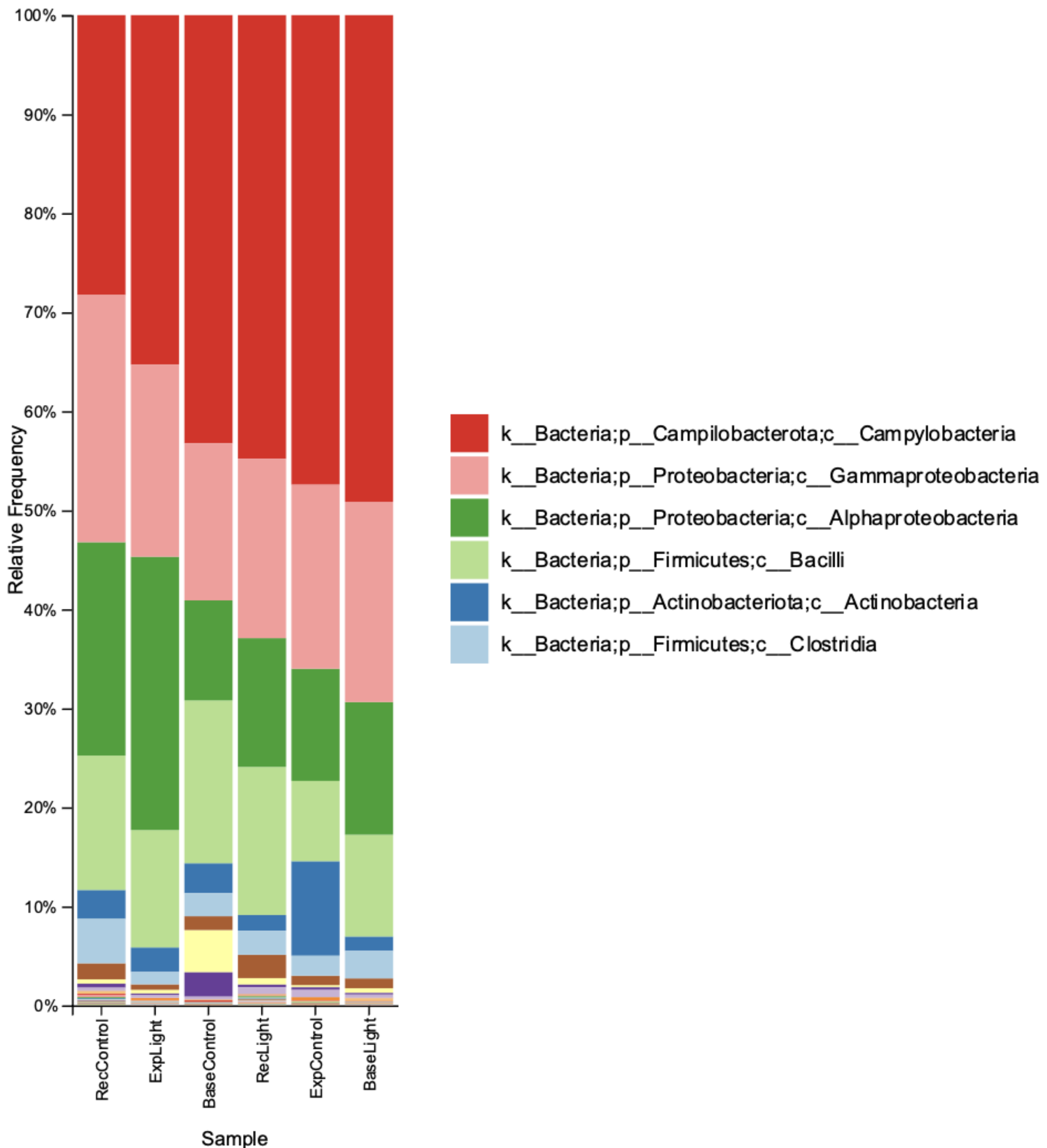


Figure 14. Alpha diversity index utilizing Shannon index of gut microbiota samples from NLE experiment. Each sampling group is shown on the x-axis, and Shannon indices are reflected on the y-axis.

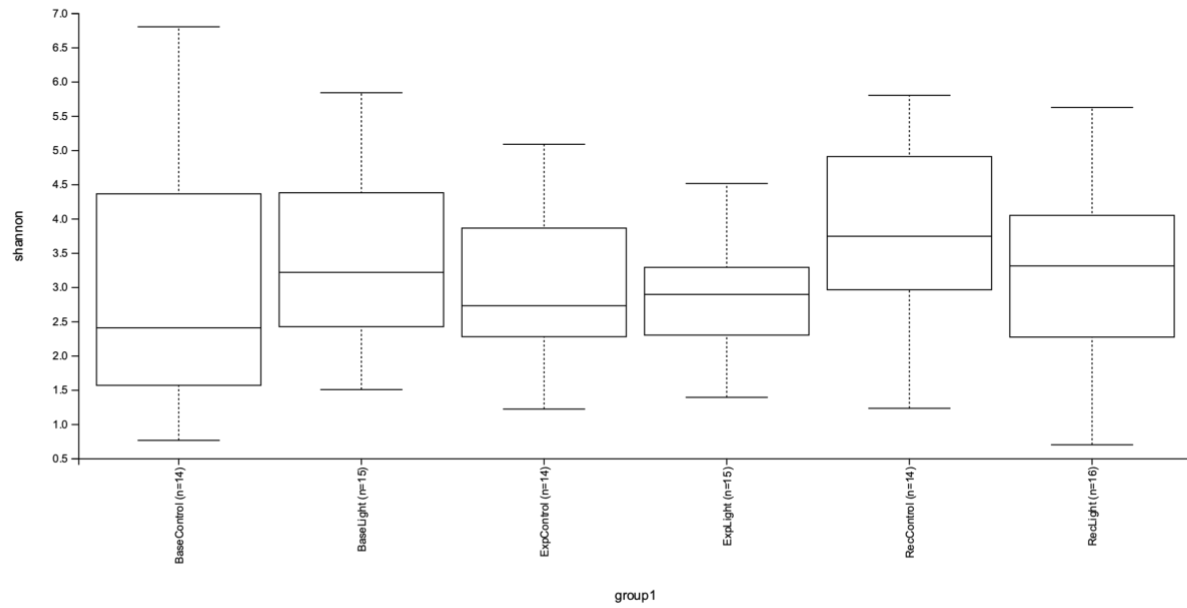


Figure 15. Alpha diversity index utilizing Chao1 of gut microbiota samples from NLE experiment. Each sampling group is shown on the x-axis, and Chao1 indices are reflected on the y-axis.

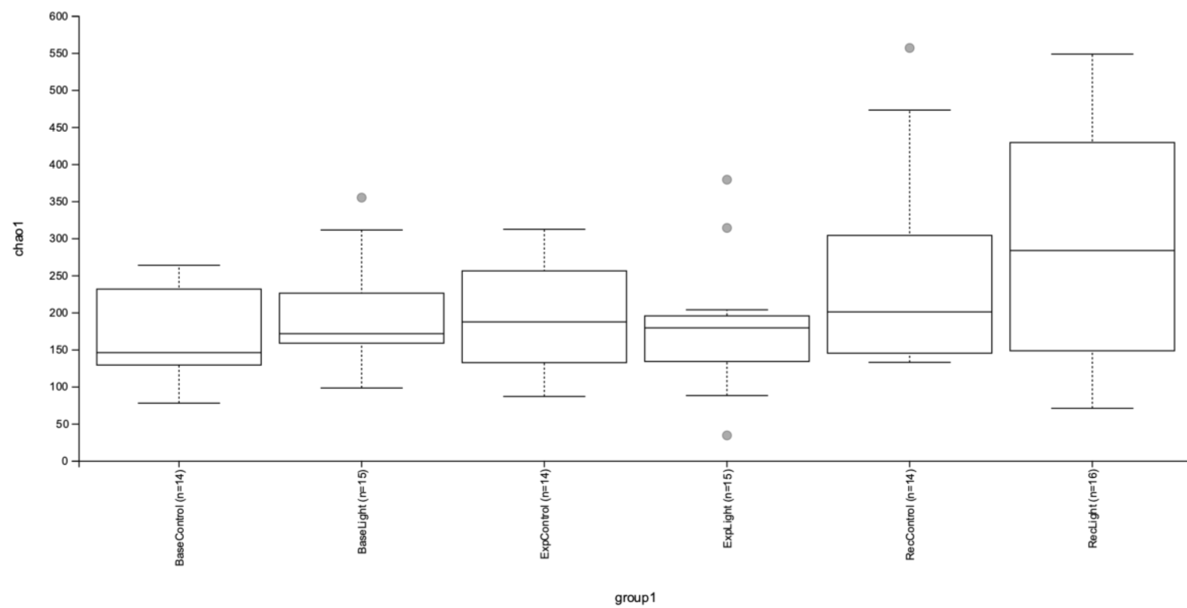
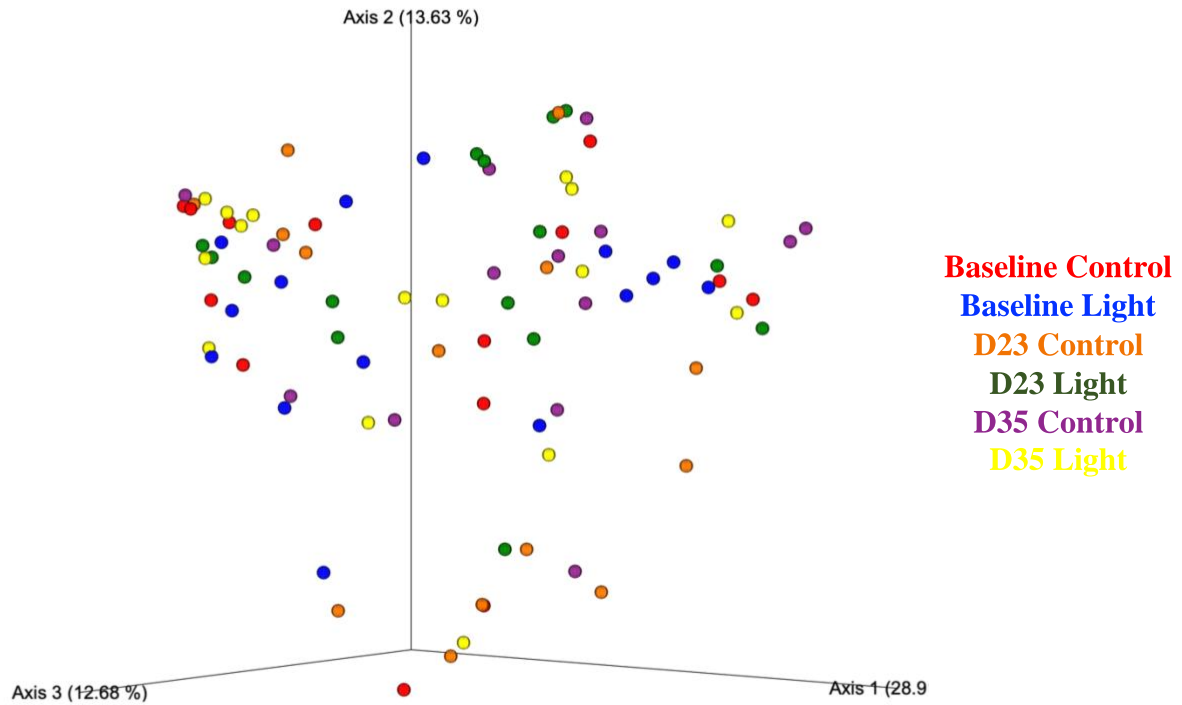


Figure 16. Bray-Curtis emperor ordination as a beta diversity metric for cloacal microbiota of zebra finches. Overall, we did not find any differences across sampling groups.



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