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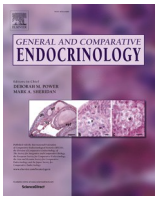

Zebra finches (*Taeniopygia castanotis)* display varying degrees of stress resilience in response to constant light☆

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ABSTRACT

The ability for traits to recover after exposure to stress varies depending on the magnitude, duration, or type of stressor. One such stressor is circadian rhythm disruption stemming from exposure to light at night. Circadian rhythm disruption may lead to long-term physiological consequences; however, the capacity in which individuals recover and display stress resilience is not known. Here, we exposed zebra finches (*Taeniopygia castanotis*) to constant light (24L:0D) or a regular light/dark cycle (14L:10D) for 23 days, followed by a recovery period for 12 days. We measured body mass, corticosterone, and glucose levels at multiple timepoints, and relative protein expression of glucocorticoid receptors at euthanasia. Body mass significantly increased over time in light-exposed birds compared to controls, but a 12-day recovery period reversed this increase. Baseline levels of circulating glucose decreased in light-exposed birds compared to controls, but returned to pretreatment levels after the 12-day recovery period. In contrast, the glucose stress response did not show a similar recovery trend, suggesting longer recovery is needed or that this is a persistent effect in light-exposed birds. Surprisingly, we did not detect any differences in baseline corticosterone or reactivity of the hypothalamic-pituitiary-adrenal (HPA) axis between groups throughout the experiment. Moreover, we did not detect differences between relative protein expression of glucocorticoid receptors or a relationship with HPA axis reactivity. Yet, we found a positive relationship between glucocorticoid receptors and the glucose stress response, but only in the light group. Our results indicate that physiological and morphological traits differ in their ability to recover in response to constant light and warrants further investigation on the mechanisms driving stress resilience under a disrupted circadian rhythm.

1. Introduction

With the advent of artificial lighting sources, the nighttime environment has become permanently altered with nearly 83% of the world's population living under some form of artificial light at night (ALAN) (Falchi et al., 2016). Across vertebrates and invertebrates, ecologically relevant levels of ALAN (<35 lx) has been shown to alter a myriad of physiological and behavioral parameters including hormone levels (Ouyang et al., 2015; Secondi et al., 2021), metabolism (Yadav et al., 2022), immune function (Gastón et al., 2019), body condition (Grunst et al., 2020) and behavioral patterns (Levy et al., 2021; Mardones et al., 2023). These physiological and behavioral alterations are thought to be a result of circadian rhythm disruption. Circadian rhythms are 24-hour internal biological clocks that synchronize biological processes (*e.g.,* hormonal release or behavior) to external light/dark cues, such that the timing of these processes coincides with changes in the environment (Gnocchi and Bruscalupi, 2017). A properly functioning circadian system is fundamental to maintain homeostasis. Thus, it is unsurprising to see that circadian rhythm disruption has such a diverse array of biological effects in both nocturnal and diurnal species (Potter et. al., 2016). Indeed, circadian disruption through perturbed light/dark environments is associated with irregular hormone production, metabolic impairments, fat accumulation, weight gain, and disease (Arble et al., 2010; Bedrosian et al., 2016).

The hypothalamic–pituitary–adrenal (HPA) axis is one neuroendocrine system that is subject to circadian rhythm disruption through altered light/dark environments (*e.g.,* constant light or ALAN) (Ouyang et al., 2018). This is because the HPA axis and circadian system have a

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bidirectional relationship that orchestrates rhythmic secretion of glucocorticoids and hormone precursors (Rao & Androulakis, 2019). Glucocorticoids are metabolic hormones released by the HPA axis that prime organism physiology and behavior in anticipation to the switch from inactivity to activity (Spencer et al., 2018; Oster et al., 2017). In birds, the main glucocorticoid is corticosterone (hereby referred to as Cort), which exerts its effects by binding to mineralocorticoid (MR) and glucocorticoid receptors (GR) in target tissues throughout the body (Kuo et al., 2018; Lattin et al., 2012). Once bound, these receptors play an important role in activating or supressing genes associated with metabolic, immune, and oxidative pathways through binding of glucocorticoid response elements (GREs) upstream (Kuo et al., 2018; Bekhbat et al., 2017). Thus, up- and/or downregulation of corticosteroid receptors may be an adaptive response that modulates or optimizes the efficiency of the adrenocortical response and downstream physiology to environmental challenges (Jimeno and Rubalcaba, 2024; Jimeno and Zimmer, 2022; Zimmer et al., 2022). However, whether Cort has stimulatory or suppressive effects depends on the duration, magnitude, and type of stressor (Sapolsky et al., 2000).

During immediate or sudden threats to homeostasis (such as in a predator attack), the adrenal medulla releases catecholamines (epinephrine and norepinephrine), signaling for the breakdown of glycogen stores in the liver and upregulation of lipolysis in adipocytes, increasing circulating glucose levels and providing immediate energy to cells for escape and survival (Romero and Butler, 2007). Once released by the HPA axis and bound to intracellular receptors, glucocorticoids redirect glucose to target tissues, such as the brain, by internalizing glucose transports and inhibiting glucose uptake in other tissue to maximize function (Romero and Butler, 2007; Braun and Sweazea, 2008). Furthermore, glucocorticoids readjust and replenish energy stores through GR-mediated transcription of enzyme genes along the gluconeogenesis pathway (Kuo et al., 2018). In doing so, glucocorticoids maintain sufficient energy levels while reallocating resources towards physiological or behavioral processes necessary to maximize survival and fitness (Rivers et al., 2012; Wada and Breuner, 2008). However, under prolonged stressors, continuous activation of the HPA axis can have deleterious, pathological effects on the body (Sapolsky et al., 2000). HPA axis dysregulation is associated with the manifestation of metabolic syndromes such as insulin resistance, obesity, or diabetes and behavioral deficits such as in mental health disorders (Herman et al., 2016; Vegiopoulos and Herzig, 2007). In fact, 16 weeks of unpredictable stressors (forced restraint/water maze) increased plasma Cort and fasted glucose levels in Swiss albino mice and led to increased glycated hemoglobin levels (Raghav et al., 2019). Although it has long been thought that glucocorticoids facilitate an organism's ability to cope with stressors by initiating gene transcription and maintaining energy homeostasis, how glucocorticoids and downstream effects vary after recovery from chronic stress is not well understood. Thus, several theoretical models have been proposed to answer fundamental questions regarding stress tolerance, robustness, and resilience and understanding how they interact to maintain function (Davis et al., 2021; Romero et al., 2009; Wada, 2019).

Here, stress resilience was defined as the ability to restore physiological, morphological, or behavioral traits back to pretreatment or baseline levels after a temporary decline or change (Wada, 2019; Wada and Coutts, 2021). To test this, one experimental approach is to sample before, during, and after the exposure to a stressor (*i.e.,* introduce a recovery period). If posttreatment levels of a parameter after recovery differ from that of pretreatment, or if there is no response to recovery, we would conclude that there is a persistent effect of the stressor (*i.e.,* failure to recover) resulting in a lack of stress resilience. For example, one study found that female mice (*Mus musculus)* had higher baseline Cort levels than control mice after 10 days of persistent psychological stress, but a 10-day recovery period reversed this rise in Cort (West et al., 2022). However, voiding frequency, a behavioral phenotype associated with bladder dysfunction, did not return to values comparable to the control group after the stress regime ended, suggesting a persistent effect from the chronic stressor or that more time is needed to recover (West et al., 2022). Although this study implemented a recovery period and mice showed differences in the capacity to recover both physiologically and behaviorally, stress resilience cannot be determined because pretreatment levels of Cort and voiding frequency were not measured and compared to posttreatment levels. In contrast, another study measured pretreatment levels of a behavioral phenotype (climbing behavior) in fruit flies (*Drosophila melanogaster)* before administering a cold stressor to the treatment group. They found that an acute 12-hour exposure to cold stress significantly decreased climbing behavior in both sexes of Drosophila. Although climbing performance improved after 72-hours of recovery, neither sex returned to pretreatment levels, indicating a persistent effect of the cold stressor on a behavioral phenotype and a lack of stress resilience (Garcia and Teets, 2019). It is possible that climbing behavior may have returned to pretreatment levels if the recovery period was extended; however, determining the length of time necessary to mitigate physiological costs or behavioral deficits associated with stress has not been discussed thoroughly in the literature, and may vary depending on the the level of damaged incurred by the stressor and the organism's repair capacity (Wada, 2019).

To better understand the physiological costs of light exposure and how organisms recover from stress, we exposed zebra finches to high intensity constant light as a means to disrupt their circadian rhythm and examined how their physiological phenotype changed during treatment and after recovery from the stressor. Specifically, we tested whether zebra finches displayed stress resilience by exposing them to constant light for 23 days and allowing a recovery period of 12 days. We hypothesized that chronic high intensity constant light would lead to dysregulation of the HPA axis, downstream physiology, and morphology, but that recovery would be able to reverse these effects. In particular, we predicted that birds exposed to constant light would 1) have higher levels of baseline Cort and an attenuated Cort response to capture and restraint; 2) have higher baseline blood glucose levels and a blunted glucose stress response; and 3) have higher body mass, but would all return to pretreatment levels after recovery. Lastly, we predicted that birds exposed to constant light would have higher protein expression of GR.

2. Materials and methods

2.1. Animal Husbandry

Adult female zebra finches (*Taeniopygia castanotis)* (N = 32) were obtained from Rockefeller University, NY and housed at the Avian Research Laboratory 2, Auburn University, Auburn, AL, USA under a 14L:10D photoperiod commonly used in zebra finch studies and a standard in our laboratory. We chose to only include female zebra finches in this study because they have been shown to be more sensitive to stressors. All individuals were housed separately in cages (38.10 cm width × 45.72 cm depth × 45.72 cm height) provided with a white tarp as a background and were allowed to acclimatize to their new cage for at least 2 months. Within their respective housing rooms, birds were able to see and hear each other. While acclimatizing, all birds were treated with Nystatin, an anti-fungal treatment, to eliminate potential underlying infections for three weeks and further allowed to repopulate fungal microbiome for the remainder of the acclimation period. All individuals had *ad libitum* access to seed (Kaytee Supreme (Finch), Chilton, WI), water, and cuttlefish bone throughout the entire duration of this study. All procedures done in this experiment were approved by the Institution of Animal Care and use Committee at Auburn University (IACUC) (protocol #2020–3805).

2.2. Experimental design

To maximize sample size (N = 32), this experiment was done in two

phases with 16 birds in each phase following the same experimental protocol and timeline. In phase one (n = 16), birds were randomly assigned to two light regimes, constant light (24L:0D; n = 8) located in Room A or control light/dark cycle (14L:10D; n = 8) located in Room B (Fig. 1). Eleven days prior to the start of treatment, blood samples and body mass were collected from all individuals to serve as a Pretreatment collection timepoint. Treatment began on Day 0 in which birds were exposed to constant light (24L:0D) while the control group remained under a 14L:10D light cycle. We collected blood samples and body mass after 3 and 23 days of exposure to the light treatment (D3 and D23, respectively). After 23 days of treatment, a 12-day recovery period began for the constant light group by returning to the original 14L:10D light cycle. We collected blood samples and body mass on Day 35 to serve as a Posttreatment collection timepoint. Birds were humanely euthanized the next day, Day 36, via overdose of isoflurane vapors. Liver, pectoralis muscle, pancreas, brain, ovaries, and spleen were collected, and flash frozen in liquid nitrogen for future analyses, concluding phase one. After the conclusion of phase one, each room was thoroughly inspected, cleaned, and disinfected to ensure standardization between phases. To account for any room effect, light treatment of each room from phase one were switched for phase two (indicated by blue arrows; Fig. 1). In phase two (n = 16), birds were randomly assigned to a control light/dark cycle (14:10D; n = 8) now in Room A or a constant light (24L:0D; n = 8) now in Room B. Phase two followed the same experimental timeline as phase one for a total sample size of 16 birds per treatment.

All room lighting was provided by 17-Watt MaxLite® light-emitting diodes (LED) at a color temperature of 5000 K (model #L17T8DE450-CG120-277 V), as this has been shown to affect glucocorticoid levels when birds are exposed at a low light intensity (Alaasam et al., 2018) as well as from a supplementary lamp at the same color temperature that reflected on the white background for even distribution of light. We measured light intensity (lx) at perch level (middle of the cage) in triplicate at pretreatment, D23, and D35 during both phases of the experiment using a Digi-Sense light meter (± 3% of reading). Light measurements for their respective rooms were then averaged. The light level was 172.84 lx (± 18.02 SD) in Room A, while it was 165.93 lx (± 16.79 SD) in room B throughout the entirety of the experiment.

Standard personal and protective equipment (shoe covers, disposable gowns, gloves, and masks) was worn during all sampling procedures. All blood samples were taken via the brachial vein using a 26-gauge needle and a sterile bleeding technique in which the feathers were wiped with 70% ethanol, allowed to air dry for 15 s without touching or blowing on the area, and repeated a second time after 15 s had passed. We followed this collection method to prevent contaminating blood samples drawn for a bacterial killing assay (Lazenby et al., in prep). All baseline blood samples were taken within 3 min of entering the room (Romero and Reed, 2005) in heparinized microhematocrit tubes and immediately placed in a 4°C refrigerator. Individuals were then restrained in opaque brown paper bags for 30 min as a standardized inducer of the adrenocortical response (Wada et al., 2007). Afterwards, a stress-induced blood sample was taken and placed in a 4°C refrigerator. Blood samples were centrifuged at 14,800 xg for 10 min within 2 h of collection. Plasma and red blood cells were separated and collected in 0.5 mL Safe-Lock Eppendorf tubes. Red blood cells were immediately flash frozen in liquid nitrogen and plasma was stored at 4°C until all samples had been processed. Following sample processing, plasma and red blood cells were transported to the lab and stored at −80°C. All sampling occurred between 0800-hours and 1000-hours.

2.3. Glucose Point-of-Care (POC) device validation

Point-of-Care (POC) devices are a promising alternative for quantifying blood metabolites cheaply and efficiently using a small drop of whole blood (<1.5 μL) (Beattie et al., 2022; Morales et al., 2020). We validated the use of the POC device in our study by comparing the

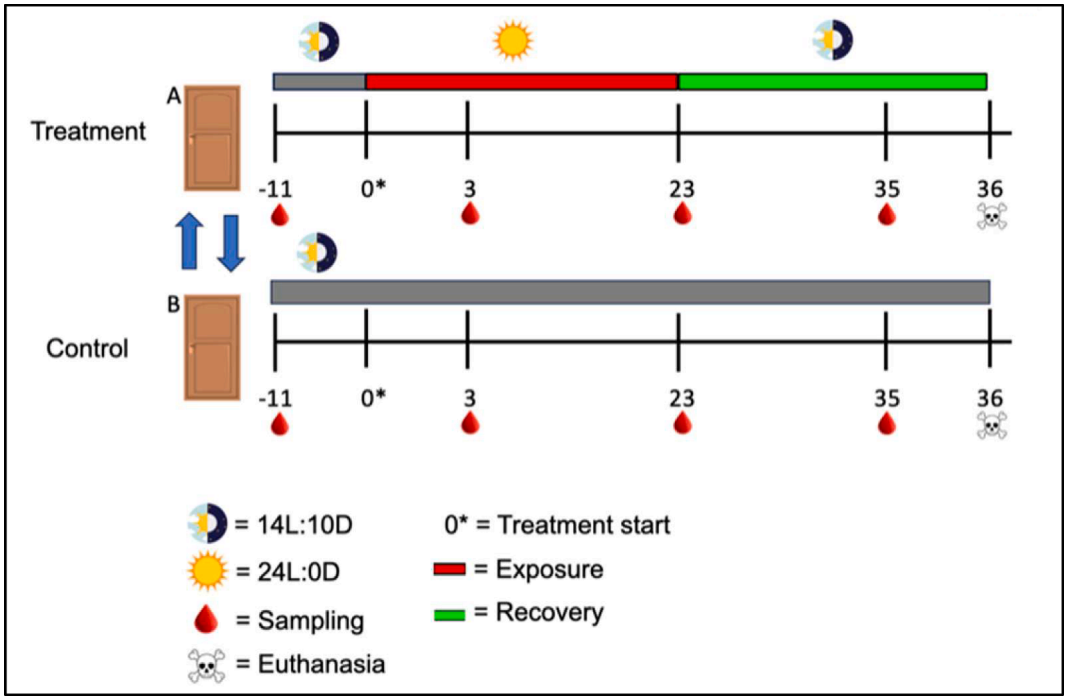

Fig. 1. Illustration of the experimental timeline. Both treatment and control individuals were exposed to the same experimental timeline simultaneously. On the figure lines, −11, 3, 23, and 35, correspond to sampling collection timepoints (Pretreatment, D3, D23, and Posttreatment, respectively) with red blood drops indicating blood and body mass data collection. The "0″ corresponds to when experimental treatment began when birds were exposed to 24-hours of constant light. Euthanasia is indicated by a skull and crossbones icon. 24L:0D and 14L:10D photoperiods illustrated by a full sun icon and a sun/moon icon, respectively. At the conclusion of phase one, rooms were thoroughly cleaned and inspected in preparation for phase two (where treatment and control rooms were switched); as indicated by the blue arrows).

coefficient of determination (R^2) across multiple POC devices (ReliOn™ Prime Glucose Monitoring System, Precision Xtra® Ketone and Glucose Monitoring System, and Contour® Next Blood Glucose Monitoring System) to a lab-based enzyme-linked immunosorbent assay (EIA) Cayman Chemicals Glucose assay kit (Cat #10009582). For our study, we selected the ReliOn™ Prime Glucose Monitoring System. (see supplementary materials for detailed analysis).

2.4. Hormone and Metabolite measures

Plasma Cort levels were measured with Enzo Life Sciences Corticosterone ELISA kits (Cat #ADI-900–097). Baseline (within 3 min of disturbance) and stress-induced (after 30 min in an opaque brown paper bag) plasma samples were prepared at a 1:20 dilution with 2.5% steroid displacement buffer and run in duplicate (Rubin et al., 2021). All samples across multiple collection timepoints (pretreatment, D23, and posttreatment) for each unique individual were run on the same plate and the positions of samples within the plate were randomized. Due to blood volume limitations, we did not measure Cort on D3 of light exposure. Intraplate and interplate coefficients of variation were 2.4% and 11.56%, respectively. Hypothalamic-pituitary-adrenal (HPA) axis reactivity, or the absolute change in Cort, was calculated by subtracting the stress-induced Cort and baseline level per individual at each collection timepoint.

Before centrifugation, baseline and stress-induced whole blood samples collected from each individual were used to quantify blood glucose levels in duplicate using the ReliOn™ POC glucose monitor (Walmart, AR). If blood glucose levels were deemed "too high" by the POC device, a ceiling value of 600 mg/dL was assigned. Out of all blood glucose readings, only 5 replicates of stress-induced blood samples exceeded the meter's capacity and were replaced with the ceiling value. Specifically, only two individuals had 600 mg/dL assigned as their average stress-induced glucose level throughout the experiment. The glucose stress response, or the absolute change in glucose, was calculated by subtracting the stress-induced blood glucose and baseline level per individual at each sampling timepoint.

2.5. Western blot

Western blots were conducted on liver tissue collected at death, 13 days after the last exposure period to constant light. We followed methods described in (Parry et al., 2021) with slight modifications. Briefly, approximately 50–100 mg of raw liver tissue was homogenized in a lysis buffer (5 mM Tris HCL, 5 mM EDTA) with a protease inhibitor cocktail (VWR Cat #97063–970) consisting of AEBSF, aprotinin, E-64, bestatin, and leupeptin. Samples were centrifuged at 1500x*g* for 10 min at 4°C and the homogenate was collected. Total protein concentration of the homogenate was obtained against a linear standard curve via Bradford assay (VWR Cat# 97065–020). Homogenates were standardized for western blotting by adding equal parts sample homogenate with 2x Laemmli buffer and raised to a total volume of 200 μL for a final protein concentration of 1.5 μg/μL across all samples. Proteins were separated by polyacrylamide gel electrophoresis using 4–15% Criterion TGX precast gels for ~1 h at 200 V (Bio-Rad, Hercules, CA). After gel electrophoresis, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane using the wet sandwich method and run at 200 mA in cold transfer buffer for 2 h via Bio-Rad Transfer box (Bio-Rad, Hercules, CA). The membrane's non-specific sites were blocked for 2 h at room temperature while rocking in 5% nonfat milk containing tris-buffered saline with tween (TSB-T). After 3 washes of TBS-T, the membrane was rocked and incubated in a custom polyclonal anti-glucocorticoid receptor primary antibody (LifeTein, raised in rabbits against a portion of the zebra finch GR: C-KVMDSKELLNPLDQDETRK) prepared at a 1:500 concentration with 5% Bovine Serum Albumin with tween overnight in a 4°C fridge. Membranes were washed 3 more times with TBS-T then rocked and incubated with an anti-rabbit horseradish peroxidase secondary antibody (Cell Signaling Cat# 7074) at a 1:1000 concentration at room temperature for 1 h. Protein bands were visualized under UV chemiluminescence using ECL Prime (VWR Cat# 89238–012) and images were captured using ImageQuant LAS 4000. Images were all analyzed using Bio-Rad Image Lab (Bio-Rad, Hercules, CA). Ponceau staining was used as a normalizing and transfer control. Western blot images showed two bands between 70 and 100 kDa, corresponding to the two isoforms of zebra finch GR at ~84 kDa and ~88 kDa. We also ran a protein gel of zebra finch brain lysate, stained it with Coomassie blue, excised a portion of the gel in the region where bands were seen (~70–100 kDa), and sent it to the LSU Health Science Center Proteomics Core Facility for analysis via liquid chromatography mass spectrometry. This gel region contained a protein that corresponded to zebra finch GR.

2.6. Statistical analysis

All statistical analyses were performed using R (Version 4.3.1 "Beagle Scouts") using packages "lme4", "lmerTest", "emmeans", "car", and "nlme"(Bates et al., 2015; Kuznetsova et al., 2017). All plots were generated using "ggplot2". Statistical significance was determined using an alpha value of ($P \leq 0.05$). To test hypotheses related to the duration and effect of experimental treatment on our response variables (body mass, baseline Cort, absolute change in Cort, baseline glucose, absolute change in glucose), global linear mixed effects models were generated with Treatment (2 level factor) and Timepoint (4 level factor) as fixed effects, an interaction term of Treatment and Timepoint (Treatment: Timepoint), and body mass as a continuous covariate for Cort and glucose response variables. To account for repeated measures (non-independence) and the hierarchal nature of the experimental design, we used a random nested structure of Bird.ID/Room/Phase in all global and final models. Non-significant ($P > 0.15$) continuous covariates were removed from all final models. Assumptions of linear models (linearity and homoscedasticity) were tested using histograms and residual plots. Grubb's test was used to identify any significant outliers (± 2 standard deviations from the mean; GraphPad) and were removed from the final analysis. Post-hoc tests followed after finding statistically significant or nearly significant interaction terms ($P \leq 0.15$) utilizing the "emmeans" package with Tukey's post-hoc correction method for pairwise comparisons of timepoints within treatment groups to control a type I statistical error. Furthermore, comparisons were made between treatment groups at each separate timepoint using the same statistical package.

For the POC device validation, we ran three separate linear regression models with the glucose values obtained from each of the three POC devices compared to lab-based EIA. The R^2 value obtained from each model was used to determine the correlation between the POC device and lab-based EIA, and the ReliOn POC device was used to determine glucose levels for the actual experiment (supplementary materials).

For relative protein abundance of GR, we log-transformed the raw data in order to meet model assumptions to investigate differences between treatment groups. To investigate whether GR could explain variation in Cort responses, we ran two linear regressions between log transformed absolute change in Cort (HPA axis reactivity) and absolute change in glucose (glucose stress response) as the response variable and relative abundance of GR as a continuous covariate.

2.7. Body mass

To investigate the effect of constant light on body mass throughout the experiment, we ran a linear mixed effects model (LMM). We then ran a type III ANOVA using the "car" package to determine model significance. Tukey's Post-hoc tests followed using the "emmeans" package to make pairwise comparisons between timepoints within treatment groups and between treatment groups within separate timepoints. The final model included Treatment and Timepoint as fixed effects, an interaction term of Treatment:Timepoint, and a random nested structure

of Bird.ID/Room/Phase. No values were excluded from analysis.

2.8. Cort

To investigate the effect of constant light on baseline Cort (within 3 min of disturbance) and HPA axis reactivity (stress-induced Cort subtracted from baseline value) throughout the experiment, we ran a LMM. In the global model, we initially included body mass as a fixed covariate, but it was later removed as it was not a significant covariate ($P > 0.15$). We then ran a type III ANOVA to determine model significance. The final model chosen was the simpler model which excluded body mass and included Treatment and Timepoint as fixed effects, an interaction term of Treatment:Timepoint, and random nested structure of Bird.ID/Room/Phase. No values were excluded from analysis.

2.9. Glucose

To investigate the effect of constant light on baseline glucose levels (within 3 min of disturbance) and the glucose stress response (stress-induced glucose level subtracted from baseline value) throughout the experiment, we ran a LMM. We included body mass as a fixed covariate in the global model; however, it was removed from the final model as it was not a significant covariate ($P > 0.15$). We ran a type III ANOVA to determine model significance. Tukey's Post-hoc tests followed using the "emmeans" package to make pairwise comparisons between timepoints within treatment groups and between treatment groups within separate timepoints. Out of all baseline glucose levels, one statistical outlier was excluded from analysis (value of 449 mg/dL), resulting in one glucose stress response measure also excluded from analysis as there was no baseline value to subtract from the stress-induced value. Removal of these values were negligible and did not change the main findings of the manuscript (supplementary materials). The final model chosen was the simpler model which excluded body mass and included Treatment and Timepoint as fixed effects, an interaction term of Treatment:Timepoint, and random nested structure of Bird.ID/Room/Phase.

2.10. Glucocorticoid receptors (GR) and relationship with HPA axis reactivity and the glucose stress response

To investigate the effect of constant light on the relative protein expression of GR, we ran a LMM with Treatment as a fixed effect and a random intercept of gel ID on log transformed data. To investigate the relationship between GR and HPA axis reactivity, we ran a LMM between log transformed protein expression of GR and the absolute change in Cort with Timepoint as a fixed factor, separated by treatment groups, and a random nested structure of Bird.ID/Room/Phase. Furthermore, we ran this model with the glucose stress response as the response variable. One statistical outlier for GR (4.91 arbitrary units) was removed from the control group via Grubb's test (Graphpad). Removal of this outlier did not affect the main result (supplementary information). Five samples (three control and two treatment) were excluded from the final analysis due to lack of clear binding.

3. Results

3.1. Body mass

We detected a significant interaction between Treatment and Timepoint on body mass such that the effect of constant light depended on the length of the exposure period ($F_{3,90} = 10.05$, $P < 0.0001$). Within the light group, post-hoc analysis revealed that while body mass did not change after 3 days of treatment ($P = 0.21$), by the time birds were exposed to constant light for 23 days, body mass was significantly higher compared to pretreatment body mass ($\beta = 1.03$; 0.56, 1.5 ± 95% CI; $t = 5.715$; $P < 0.0001$; Fig. 2). Furthermore, after cessation of the light treatment, we found strong evidence that individuals in the light group

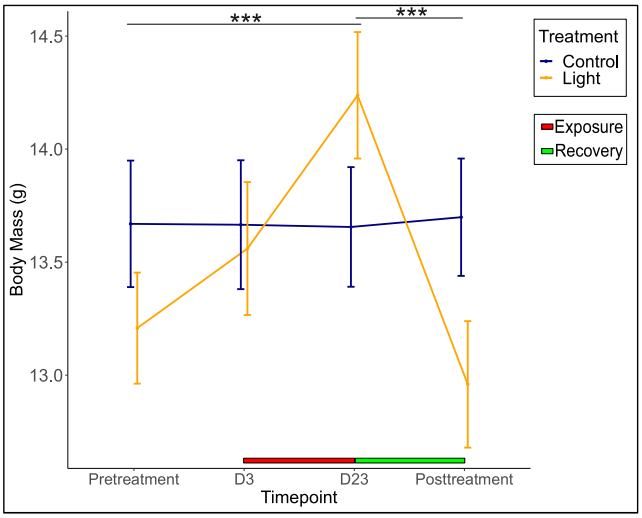

Fig. 2. Body mass. Treatment birds (n = 16) are indicated by the orange line while control birds (n = 16) are indicated by the navy line. The solid red bar represents the exposure period of the light treatment (23 days total), while the green bar represents the recovery period (12 days total). Average body mass (g) of both groups is plotted across timepoints using raw data with error bars indicating ± the standard error of the mean (SEM). Statistical significance within the light group (light-exposed birds) is shown with a solid black line with asterisks denoting associated p values (post hoc analysis; *$P \leq 0.05$; ** $P \leq 0.01$, *** $P \leq 0.001$). See supplementary materials for entire statistical output. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

decreased body mass (β = -1.28; −1.8, −0.81 ± 95% CI; t = -7.095; $P < 0.0001$; Fig. 2) when comparing D23 and posttreatment timepoints. In light-exposed birds, there were no differences between pretreatment and posttreatment body mass ($P = 0.51$). We found no statistically significant differences in body mass between both groups at the pretreatment, D3 or D23 timepoints ($P \geq 0.14$); however, there was weak evidence showing that the light group had lower body mass when compared to controls at the posttreatment timepoint (β = -0.74; −1.5, 0.04 ± 95% CI; t = -1.908; $P = 0.0632$; Fig. 2).

3.2. Cort

We did not detect a Treatment ($F_{3,90} = 0.0003$, $P = 0.99$), Timepoint ($F_{2,58} = 0.30$, $P = 0.74$), or Treatment:Timepoint ($F_{2,58} = 0.33$, $P = 0.72$) effect on baseline Cort during the length of the experiment (Fig. 3, Supplementary materials). Further, we did not detect such effects on the absolute change in Cort (Fig. 4, Supplementary materials).

3.3. Glucose

We detected a significant interaction between Treatment and Timepoint ($F_{3,87} = 2.69$; $P < 0.0511$) on baseline blood glucose levels. Within the light group, we found no statistically significant differences in baseline blood glucose levels after 3 or 23 days of exposure when compared to pretreatment levels ($P \geq 0.26$). However, after cessation of the light treatment, there was a statistically significant increase in baseline blood glucose levels when comparing D23 and posttreatment timepoints in the light group (β = 37.11; 6.52, 67.71 ± 95% CI; t = 3.177; $P = 0.0109$; Fig. 5), with no differences between pretreatment and posttreatment baseline glucose levels ($P = 0.5055$). We detected weak evidence of the light group having lower baseline blood glucose levels when compared to controls after 3 days of exposure (β = -24.96; −53.9, 4.02 ± 95% CI; t = -1.711; $P = 0.0905$), but this difference was larger and became statistically significant after 23 days of exposure (β = -40.08; −69.1, −11.11 ± 95% CI; t = -2.748; $P = 0.0072$; Fig. 5). We

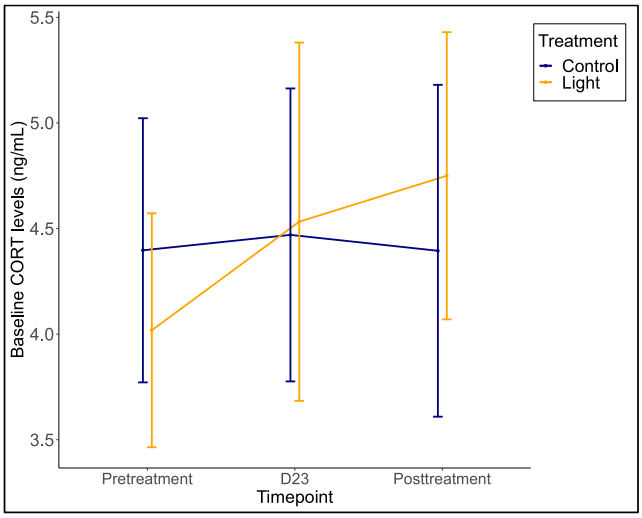

Fig. 3. Baseline plasma Cort levels within 3 min of disturbance. Treatment birds (n = 16) are indicated by the orange line while control birds (n = 16) are indicated by the navy line. Average baseline Cort levels (ng/mL) is plotted across timepoints using raw data (not log transformed) with error bars indicating ± the standard error of the mean (SEM). Due to blood volume limitations, we did not quantify Cort on D3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

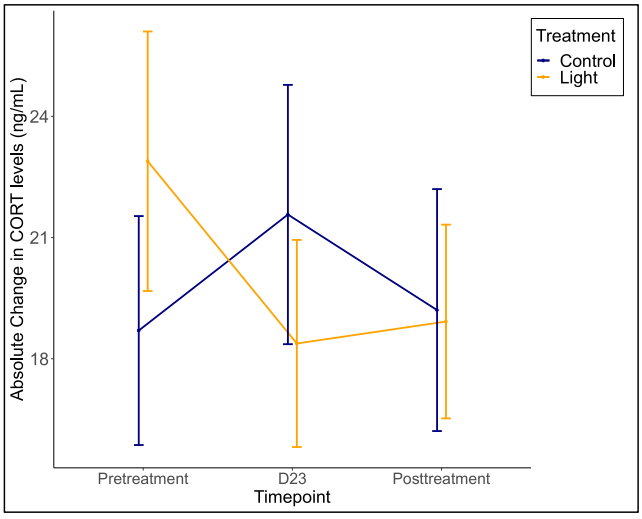

Fig. 4. Absolute change in corticosterone (Cort) levels within 30 min of capture and restraint stress. Treatment birds (n = 16) are indicated by the orange line while control birds (n = 16) are indicated by the navy line. Average absolute change in Cort levels (ng/mL) is plotted across timepoints using raw data (not log transformed) with error bars indicating ± the standard error of the mean (SEM). Due to blood volume limitations, we did not quantify Cort on D3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

detected a nearly significant Treatment:Timepoint interaction ($F_{3,86}$ = 2.08; P = 0.1091) on the absolute change in blood glucose levels, thus we continued the analysis. Post-hoc analysis revealed that the light group had no statistically significant differences in absolute change in glucose throughout exposure to constant light ($P \geq 0.0995$). However, there was moderate evidence showing that the light group had a lower absolute change in glucose after 23 days of exposure when compared to controls (β = -64.55; −120.4, −8.69 ± 95% CI; t = -2.293; P = 0.0240; Fig. 6). Interestingly, there was no statistically significant increase in absolute change in glucose when comparing D23 and posttreatment

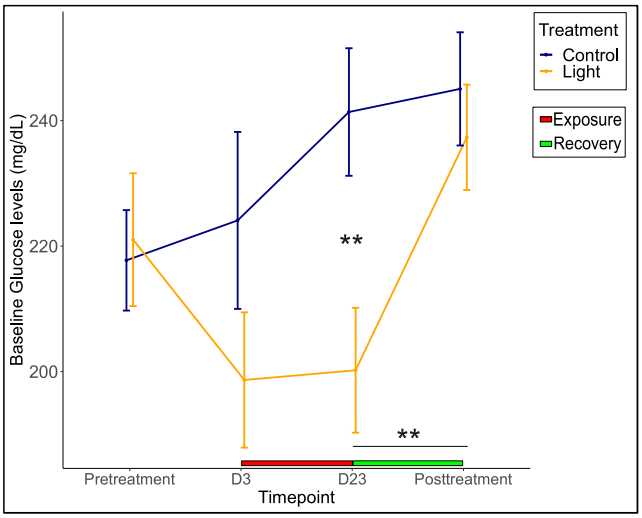

Fig. 5. Baseline glucose level. Treatment birds (n = 16) are indicated by the orange line while control birds (n = 16) are indicated by the navy line. The solid red bar represents the exposure period of the light treatment (23 days total), while the green bar represents the recovery period (12 days total). Average baseline glucose levels of both groups are plotted across timepoints using raw data with error bars indicating ± the standard error of the mean (SEM). Statistical significance within the light group (light-exposed birds) is shown with a solid black line with asterisks denoting associated p values (post hoc analysis; *$P \leq 0.05$; ** $P \leq 0.01$, *** $P \leq 0.001$). Statistical differences between groups at a particular timepoint are shown with just asterisks. See supplementary materials for entire statistical output. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

timepoint (P = 0.8586).

3.4. Glucocorticoid receptors (GR) and relationship with HPA axis reactivity and the glucose stress response

We did not detect a significant main effect of treatment on relative protein expression of GR ($F_{1,23}$ = 0.24; P = 0.63; Fig. 7). Additionally, we did not find a significant relationship between GR and HPA axis reactivity separated by treatment groups (Control: $F_{1,10}$ = 0.04; P = 0.84; Treatment: $F_{1,12}$ = 0.12; P = 0.73; supplementary materials). Yet, we found a positive relationship between GR and the glucose stress response within the light group ($F_{1,12}$ = 9.92; marginal R^2 = 0.24; conditional R^2 = 0.30; P = 0.008), but no relationship within controls ($F_{1,11}$ = 0.78; P = 0.40; Fig. 8).

4. Discussion

In this study, we sought to quantify stress resilience in the diurnal zebra finch by exposing them to high intensity constant light and allowing them to recover from this stressor. Contrary to our predictions, we did not detect any changes in baseline Cort or the reactivity of the HPA axis throughout the experiment but found that baseline glucose levels in light-exposed birds were lower than control birds. Furthermore, light-exposed birds had a blunted glucose stress response compared to controls. We found that 23 days of constant light exposure was necessary to increase body mass, while 3 days of exposure was not sufficient. However, a 12-day recovery period ameliorated the effects of constant light on both body mass and baseline glucose levels, indicating stress resilience in those traits, but the glucose stress response remained blunted, indicating a persistent effect. We found no differences between groups in relative protein expression of GR in the liver and there was no relationship between GR and HPA axis reactivity. Yet, we found a significant positive relationship between GR and the glucose stress

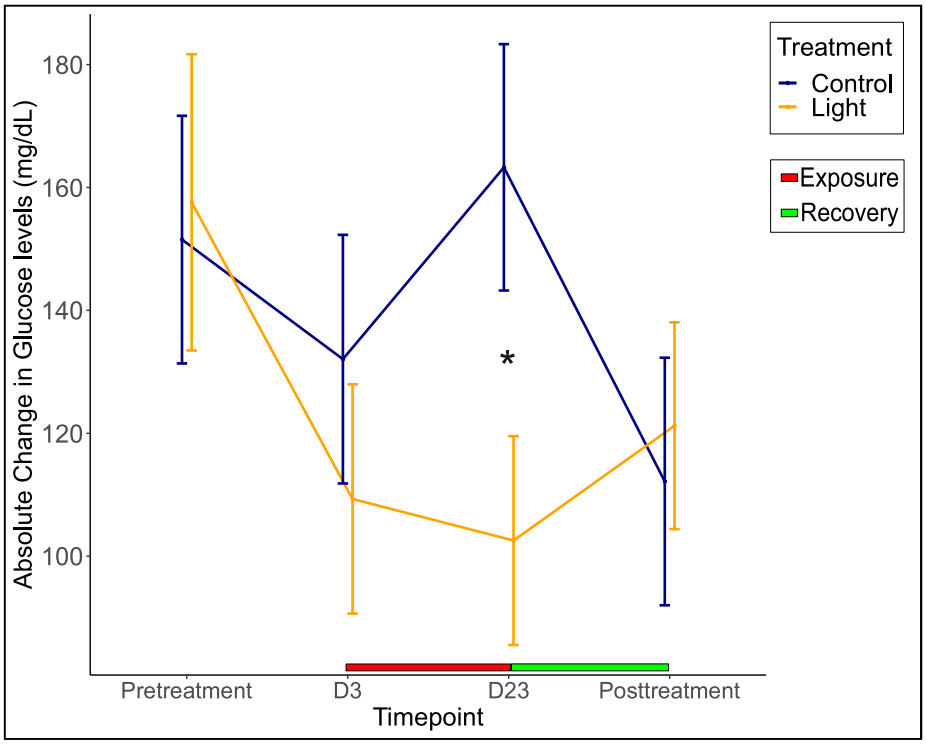

Fig. 6. Glucose stress response. Treatment birds (n = 16) are indicated by the orange line while control birds (n = 16) are indicated by the navy line. The solid red bar represents the exposure period of the light treatment (23 days total), while the green bar represents the recovery period (12 days total). Average absolute change in glucose levels (mg/dL) of both groups is plotted across timepoints using raw data with error bars indicating ± the standard error of the mean (SEM). Statistical significance within the light group (light-exposed birds) is shown with a solid black line with asterisks denoting associated p values (post hoc analysis; *≤ 0.05; **≤ 0.01, ***≤ 0.001). Statistical differences between groups at a particular timepoint are shown with just asterisks. See supplementary materials for entire statistical output. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

response, but only within the treatment group. Our findings indicate that diurnal zebra finches were able to maintain adrenocortical responses under constant light. Furthermore, we show that physiological and morphological traits differed in their ability to recover from chronic stress. However, we were unable to determine whether this stress resilience was driven by a trade-off in recovery between traits. There was a significant positive relationship between GR and the glucose stress response in the light group, suggesting that GR may be a mechanism critical in restablishing sufficient glucose responses after recovery from stress. Future studies should characterize whether individuals incur a trade-off when given the opportunity to recover from stress. Furthermore, exploration of the mechanisms that confer stress resilience should be considered, especially in the face of rapid environmental change.

Our finding of an increase in body mass under constant light is consistent with another study in zebra finches (Malik et al., 2020), in which two weeks or longer of light at night is necessary to induce changes. It is possible that this increase in body mass is due to higher food consumption in response to a constant light environment. However, increased feeding rates in great tit (*Parus major*) nestlings exposed to ecologically relevant levels of light did not lead to an increase in body mass, suggesting patterns in body mass differs between species, light regimes, and life history (Titulaer et al., 2012; Zuo et al., 2023). Alternatively, the rise in body mass may stem from a shift in the timing of food intake, rather than from increased food consumption (Fonken et al., 2010). Indeed, zebra finches under dim light at night showed no differences in food consumption, but did show stark differences in their feeding pattern during the inactive phase, resulting in excessive weight gain and lipid accumulation in the liver (Batra et al., 2019). However, we found that the effect of constant light on body mass was ephemeral, as the addition of a 12-day recovery period reversed the rise in body mass and returned finches to pretreatment levels, indicating stress resilience in the trait. Other studies have reported varying durations of recovery periods to reverse the effects of chronic stress on morphological and physiological traits (Gormally et al., 2019; Beattie et al., 2022). For example, in house sparrows (*Passer domesticus*) subjected to repeated cycles of stressors (cage rolling, cage tapping, etc.), individuals declined in body mass, and did not return to pretreatment levels even 2 weeks after cessation of treatment, indicating a persistent effect of a chronic stressor (Beattie et al., 2022). These studies show that the recovery of some traits may be prioritized over others (*i.e.,* trade-offs) or that the length of recovery required to elicit beneficial effects differs across traits. Our results, together with published data, suggests variation in the capacity of morphological or physiological traits in avian species to recover from different types of chronic stress.

To understand how physiological systems respond to and recover from constant light, we measured the adrenocortical response and blood glucose levels. Interestingly, we found no differences in either baseline Cort or the reactivity of the HPA axis between treatment groups at any point during the experiment. This could be due to several reasons. First, it is possible that our light treatment was not strong enough to disrupt the birds' circadian rhythm. This is unlikely however, as the light level in our study was well above 150 lx and Prabhat et al. 2020 showed that constant light at 150 lx can disrupt central clock gene expression (*Bmal1, Per2, reverb β)* in the hypothalamus of zebra finches that were reared under constant light as hatchlings (Prabhat et al., 2020). Although we did not measure Cort or clock gene expression over 24-hours, such data would provide more information on whether circadian rhythmicity was maintained or disrupted in light-exposed birds. Second, it is possible that constant light phase delayed, or phase advanced, the circadian peak in Cort in the constant light group; thus, circulating levels of Cort at the time of sampling (0800-hours and 1000-hours) may not have been representative between groups. However, Jha et al. (2021) found that F1 zebra finches under constant light showed no differences in baseline Cort 2 h before and after lights were turned on, and 4 h before and after

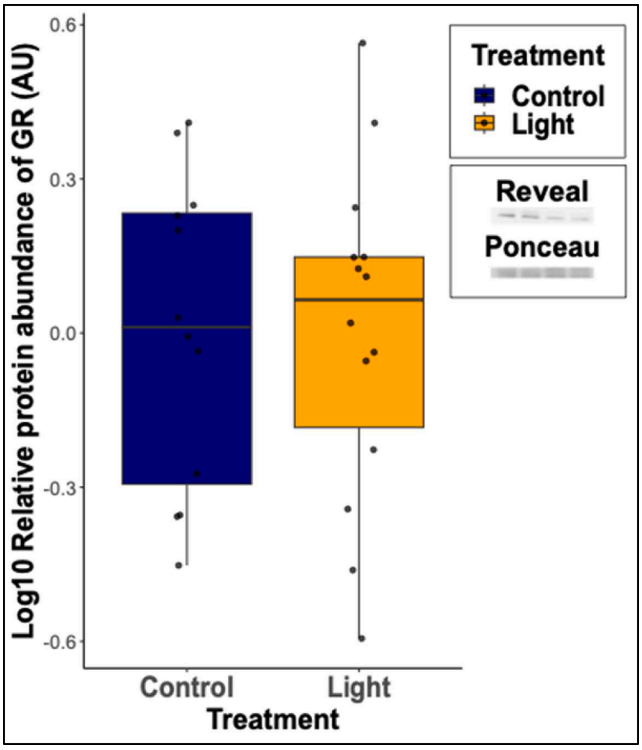

Fig. 7. Relative glucocorticoid receptor (GR) protein levels (measured post-euthanasia). Treatment birds (n = 14) are indicated by the orange, while control birds (n = 12) are indicated by the navy bar. Relative abundance of liver GR (arbitrary units; AU) is plotted between groups using a boxplot with the solid black line indicating the median value on log transformed data. Western blot reveal image is of the raw data (two control samples on the left and two treatment samples on the right). Normalization ponceau stain is also shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

lights turned off, suggesting that constant light may not affect the peak or nadir of Cort in zebra finches (Jha et al., 2021; but see Mishra et al., 2019). Our birds were measured 1 to 2 h after the lights had turned on; thus, it is reasonable to assume that circulating levels of baseline Cort were representative between groups at the time of sampling. Alternatively, it is possible that female zebra finches may be more resilient to changes in HPA axis function under stress as sex-specific differences in managing stress responses have been reported in a variety of avian species (Edwards et al., 2013; Khan and Robert, 2013; Ninnes et al., 2010; Tetel et al., 2022). Indeed, Jha et al. (2021) further found that there were no changes in baseline Cort in female adult zebra finches under constant light but found that males tended to have lower levels of baseline Cort (Jha et al., 2021). These data suggest that there may be sex-specific differences in how male and female zebra finches maintain HPA axis function under circadian disruption. In our study of female zebra finches, both acute and chronic exposure (at least 23 days) to constant light did not affect baseline levels of Cort, nor HPA axis reactivity. Based on this finding, female zebra finches were able to maintain neuroendocrine responses to acute challenges (*i.e.,* capture and restraint) under a perturbed light environment.

Circulating glucose levels have been widely used as a physiological indicator of wildlife health as they can signify overall condition, but can vary both seasonally and based on environmental quality (McGraw et al., 2020; Remage-Healey and Romero, 2000; Scanes and Braun, 2013). Furthermore, variation in both the baseline and glucose stress response can reflect differences in how glucose is mobilized in response to acute stressors or changes in the environment (Ryan et al., 2023; Schradin et al., 2015). Contrary to our predictions, we found that baseline levels of glucose decreased in light-exposed birds compared to control birds over the course of the experiment. A similar finding was found in western mosquitofish (*Gambusia affinis)* in which constant light decreased glucose levels in the brain (Miner et al., 2021). Differences in behavior or activity patterns may explain why glucose levels decreased. Although those traits were not measured in our study, light at night has been shown to induce sleep loss and result in higher activity and foraging in many avian species (Lebbin et al., 2007; Raap et al., 2015). This increased energetic demand may result in higher glucose utilization over time and could explain why we detected low circulating levels of baseline glucose in our light-exposed birds. However, this effect was transient, as once the stressor ended, baseline glucose levels in light-exposed birds increased and returned to pretreatment levels, indicating stress resilience. In contrast, Beattie et al. (2022) found that baseline glucose levels in house sparrows (under a fed state) did not return to pretreatment levels and remained elevated 2 weeks after the stress regime had ended (Beattie et al., 2022). These data indicate species-specific differences in how glucose levels may respond to chronic stress and recovery, but also how various stressor types may illicit different glucose patterns. Long periods of hyperglycemia or hypoglycemia could result in cellular damage through glycated hemoglobin or reductions in cellular function because of the lack of sufficient energy provided to cells; thus, further research is warranted on how glucose homeostasis is affected in response to different stressors, and the potential physiological consequences. We also found that the glucose stress response was suppressed after 23 days of high intensity constant

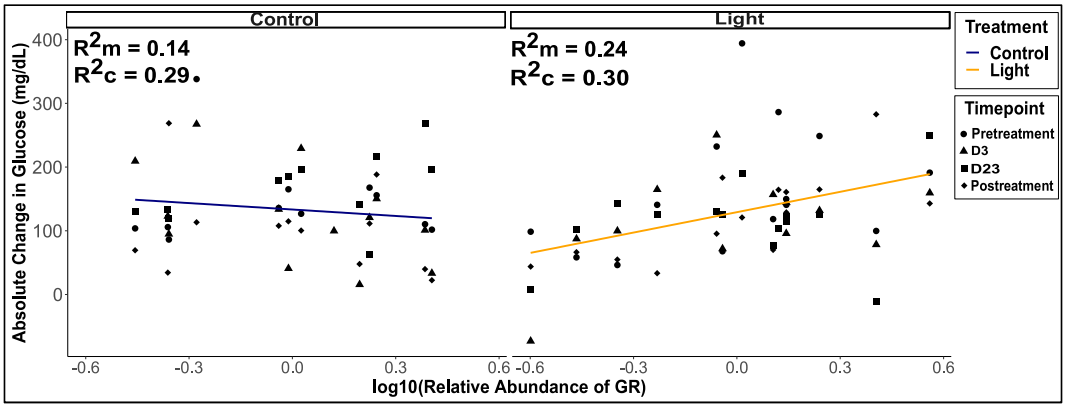

Fig. 8. Relationship between GR and the glucose stress response. Absolute change in glucose at each timepoint is represented by circles (posttreatment), triangles (D3), squares (D23) and diamond (posttreatment). Treatment birds (n = 14) are indicated by the orange line while control birds are indicated by the navy line (n = 12). The marginal and conditional R^2 are listed from two separate linear regression model outputs, one with just Control and one with just Treatment birds. Best fit lines are depicted.

light and remained blunted after 12 days of recovery, indicating a persistent effect. It is possible that our zebra finches had a depletion of energy stores due to altered activity levels stemming from constant light (Zuo et al., 2023) and could not mount a strong glucose response to an acute challenge; however, all birds had access to *ad libitum* food. Nevertheless, a blunted glucose stress response could have implications on survival as a weak response would indicate a lower ability to mobilize glucose to supply energy in face of immediate threats in the environment (Marik and Bellomo, 2013).

In the liver, GR activate genes associated with gluconeogenic pathways through Cort signaling, resulting in stress-induced hyperglycemia that promotes greater cellular glucose uptake and anti-apoptotic pathways to maximize function and aid in survival during and after environmental challenges (Kuo et al., 2018; Marik and Bellomo, 2013). GR are also responsible for regulating the adrenocortical response, particularly by facilitating negative feedback in the brain to terminate elevated levels of Cort (Sapolsky et al., 1984). Thus, the presence of increased GR can augment the organismal and cellular stress response, but may be tissue specific in their effect (Bekhbat et al., 2017; Jimeno and Zimmer, 2022; Lattin and Romero, 2014). In our study, we did not detect differences between experimental groups in relative protein expression of GR in the liver. It is possible we were unable to capture differences due to the timepoint of collection, as we measured GR 13 days after the stressor had ended. While GR are essential in activating molecular pathways, it is possible that chronic stress leads to glucocorticoid receptor resistance, meaning that intracellular receptors have reduced efficiency in activating or repressing genes associated with multiple cellular pathways (immune or metabolic) in response to hormone signaling across tissue (Cohen et al., 2012; Marques et al., 2009). In this context, higher presence of GR would not augment physiological responses. This could explain why the glucose stress response remained blunted in response to capture and restraint after recovery from constant light. Yet, we found an interesting, positive relationship between GR and the glucose stress response in the constant light group. Even though treatment birds had a blunted glucose stress response, they were able to mount adrenocortical responses comparable to controls. This suggests that GR in the liver is coupled with Cort signaling, such that downstream responses (*i.e.,* the glucose stress response) remains sufficient under stressors such as constant light. This is likely as liver GR proteins are essential transcription factors that regulate glucose homeostasis (Kuo et al., 2018). However, we are unable to determine whether GR proteins detected in this study are functional. To our knowledge, this is the first study to measure protein levels of GR in the liver of an avian model under constant light. Future studies should evaluate GR as a mechanism modulating HPA axis function and downstream physiology across multiple tissues. Glucocorticoid receptors may augment or suppress cellular and molecular responses to stressors, particularly circadian disruption induced by light at night, to maintain metabolic, immune, and organismal function, and are a potential mechanism conferring stress resilience (Jimeno & Rubalcaba, 2024).

5. Conclusions

Here, we show varying degrees of stress resilience under high intensity constant light in the zebra finch (*Taeniopygia castanotis*) depending on the morphological or physiological trait of interest. While some metrics returned to pretreatment levels 12 days after cessation of constant light, such as body mass and circulating levels of baseline glucose, the glucose stress response remained blunted after the same period. These results suggest that 1) traits responded differently when given the opportunity to recover from chronic high intensity light 2) the effects of chronic stressors can persist after stress exposure ends. We found no differences in baseline Cort, HPA axis reactivity, or GR abundance, indicating that zebra finches can maintain HPA axis function under constant light. We found a positive relationship between liver GR and the glucose stress response in the treatment birds only, indicating that GR is essential is modulating glucose levels under constant light. Future work should characterize how individuals display stress resilience to various environmental perturbations, including light-induced circadian disruption, particularly by probing various aspects of the HPA axis and downstream physiological, behavioral, and morphological traits.

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7. IACUC Statement

All procedures done in this experiment were approved by the Institution of Animal Care and use Committee at Auburn University (IACUC) (protocol #2020–3805).

CRediT authorship contribution statement

Kevin Pham: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Madeline Lazenby:** Writing – review & editing, Validation, Methodology, Investigation, Data curation, Conceptualization. **KayLene Yamada:** Writing – review & editing, Methodology, Formal analysis. **Christine R. Lattin:** Formal analysis, Methodology, Validation. **Haruka Wada:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2024.114644.

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