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Recovery periods during repeated stress impact corticosterone and behavioral responses differently in house sparrows

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ABSTRACT

A number of studies have shown that chronic stress can negatively impact both physiology and behavior in a variety of organisms. What has yet to be extensively explored is whether these changes permanently alter an animal's functioning, or if they can be reversed. In this study, we used wild-caught house sparrows (*Passer domesticus*) to assess how recovery periods influence the physiological and behavioral impacts of an initial four days and subsequent four days of repeated stressors. Birds were randomly assigned to a recovery group and either experienced 0, 24, or 72 h of recovery between the two sets of stressors (cage rolling and cage tapping). We measured the regulation of the hypothalamic pituitary adrenal (HPA) axis by quantifying baseline and stress-induced corticosterone as well as negative feedback strength. We also assessed behavior using neophobia trials to measure how birds altered their approach towards novel objects and their overall activity. Both behavior and corticosterone responses were assessed before the experiment, after the recovery time, and following the final 4 days of stressors. We found that birds that experienced 24 h of recovery had reduced stress-induced corticosterone, but enhanced negative feedback relative to the pre-experiment sample. Additionally, 4 days of stressors was enough to significantly reduce approach latency towards novel objects; however, pre-experiment levels returned with longer periods of recovery. Finally, recovery time did not significantly influence responses to the second 4 days of stressors. Our results indicate that brief recovery periods partially ameliorate the hormonal and behavioral effects of repeated stress.

1. Introduction

Researchers from a variety of fields including psychology (e.g. Lepore et al., 1991; Mayer et al., 2017), medicine (e.g. McEwen and Gianaros, 2010; Moloney et al., 2014), and wildlife conservation (e.g. Dickens et al., 2009a, 2009b; Seltmann et al., 2017) have become interested in determining the diagnostic features of chronic stress and how to counteract its harmful impacts. Though physicians focus on combatting stress at an individual level, ecologists hope to use it as a means to assess the physiology of at-risk populations. While some progress has been made in field-based studies, many questions remain and stress continues to be difficult to define, model, and predict (Romero et al., 2015). Especially in wild birds, the features of chronic stress are often dependent on context (e.g. Cyr and Romero, 2007; Dickens and Bentley, 2014; Fischer et al., 2018; Lattin and Romero, 2014; Love et al., 2017), species (Romero, 2004), and life-history stage (Landys et al., 2006; Lattin et al., 2012).

Many studies on chronic stress focus on how the function and regulation of the hypothalamic pituitary adrenal (HPA) axis are impacted. The HPA axis is responsible for eliciting a cascade of hormones that culminates in the release of glucocorticoids (cortisol or corticosterone, Cort) (Romero and Wingfield, 2016). Cort has become extraordinarily popular to measure, with particular attention paid to understanding how baseline and stress-induced Cort changes under circumstances of chronic stress. Cort play crucial roles at baseline levels across different life-history stages (Landys et al., 2006), but have more traditionally been associated with their roles in the acute stress-response, during which they become elevated. One common definition of chronic stress, or homeostatic overload (Romero et al., 2009), is an overactivation of this acute response, either due to stronger stressor intensity or longer stressor duration (Harbuz and Lightman, 1992; McCormick et al., 2015). Despite this seemingly straightforward definition, chronic stress has a complex relationship with the endocrine system. Different studies have shown that chronic stress can both elevate and reduce baseline (e.g. Dickens et al., 2009a; Rödl et al., 2007) and stress-induced Cort (e.g. Cabezas et al., 2007; Romero and Wikelski, 2002), weaken and strengthen HPA axis negative feedback strength (e.g. Dickens et al., 2009b; Lattin et al., 2012), and alter the capacity of the adrenals to

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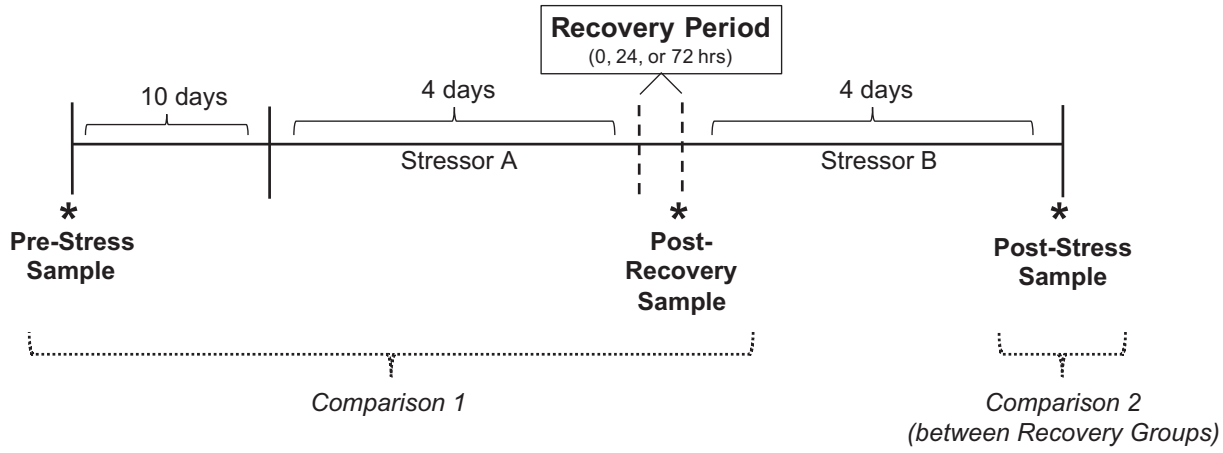

Fig. 1. Experimental design to test the effects of recovery time during chronic stress. Male and female house sparrows were captured and acclimated for at least 3 weeks. They were randomly assigned to a recovery period group (0, 24, or 72 h), which would correspond to how much recovery they were permitted between the bouts of stressors. A Pre-Stress Sample was taken and 10 days later the birds were exposed to a stressor randomly (cage rolling or cage tapping) and repeatedly for 4 consecutive days. Then, the birds were allowed to recover for their allotted time with minimal disturbances. A post-recovery sample was taken following this period, after which the birds experienced a second, new stressor. The dotted brackets indicate the comparisons that were focused on. Firstly, the post-recovery samples were compared to pre-stress sample (Comparison 1). Secondly, the recovery groups were compared to each other after the second round of stressors at the post-stress sample (Comparison 2).

release Cort (e.g. Lattin et al., 2012). In fact, a recent review has shown that a single, consistent endocrine phenotype of chronic stress does not exist in wild animals (Dickens and Romero, 2013). It is currently hypothesized that these variations partially result from differences in experimental design, specifically in focal species and their life-history stage.

Despite these differences in results, it is generally agreed that chronic stress—no matter the origin—results in measurable physiological changes. A less explored avenue of research has been examining whether these changes can be reversed or if they permanently modify an organism. Some studies have examined this in rodent models. One study found that increasing intensity, but not duration, of immobilization compromised recovery of the HPA axis (García et al., 2000). In contrast, a separate study in rats found that longer durations of stressors led to more severe immune system damage and slower recovery (Sarjan and Yajurvedi, 2018). Additionally, some, but not all, aspects of the sympathetic nervous system—the mechanism responsible for the fight-or-flight response—recover from chronic, random stressors (Park et al., 2017). Finally, a number of studies have found that chronic stress-induced hippocampal neuronal atrophy can be reduced following periods of rest (Conrad et al., 1999; Ortiz and Conrad, 2018; Sousa et al., 2000).

To our knowledge, the studies that have previously examined the effects of recovery periods after chronic stress have primarily done so in domesticated, laboratory rodents. In addition, it still remains unknown how these recovery periods influence animals' responses to future stressors. To address this gap, in the present study we tested how recovery periods affect both physiology and behavior in response to four contiguous days of one stressor experienced twice per day at random times, followed by three different periods of recovery (0, 24, and 72 h), and ending with a second set of four contiguous days of a second stressor. The initial period of repeated stressors was not meant to cause animals to enter homeostatic overload (Romero et al., 2009). Instead, we intended this 4-day period to build wear-and-tear (Romero et al., 2009) and allostatic load (McEwen, 1998) on the animals, making them more susceptible to experience pathologies during the second period of repeated stress. The 4-day period was chosen because 8–10 days has been shown to be long enough period to influence the HPA axis in both house sparrows and European starlings (*Sturnus vulgaris*) (Cyr et al., 2007; Gormally et al., 2018; Gormally and Romero, 2018; Rich and Romero, 2005). Thus, we predicted that two periods of 4 days of repeated stressors would elicit significant effects. We examined both HPA axis function and regulation (baseline and stress-induced Cort, negative feedback strength and adrenal capacity) as well as behavior at three different points: before the experimental protocol; following the recovery periods; and after the second set of stressors. Behavior has been examined far more infrequently, but it is known that both acute and chronic stress can influence neophobic responses and overall activity (e.g. Astheimer et al., 1992; Breuner et al., 1998; Gormally et al., 2018; Marin et al., 2007). If recovery could alleviate some of the effects of repeated stressors, we predicted that longer periods of rest would reset the physiological and behavioral changes to pre-experiment levels. Alternatively, if there was no effect of recovery periods, we predicted changes insensitive to recovery time.

2. Materials and methods

2.1. Experimental design

Between May 8 and May 12, 2017, 26 house sparrows (15 females, 11 males) were captured via mist-nets and potter traps in Medford, MA, USA. The birds were brought to Tufts University, where they were randomly assigned to one of three groups that differed as to how much recovery time they would be permitted during the experiment (0 h, 24 h, 72 h). They were housed in male-female or female-female pairs in cages (45 cm × 37 cm × 33 cm), maintained on a long-day light cycle (15 L:9D), and provided mixed seed, grit, and water ad libitum. Birds were permitted at least 3 weeks to acclimate to the conditions of captivity prior to beginning the experiment.

After this acclimation period, a set of pre-experiment samples were taken from each bird (Pre-Stress Sample in Fig. 1). This included both blood samples as well as video samples. Ten days after this initial sample, the chronic stress protocol commenced. Two thirds of the birds experienced intermittent cage tapping for 30 min twice per day for 4 consecutive days while the remaining birds experienced cage rolling for 30 min twice per day for the same period of time. Brief bouts of cage tapping were performed by an experimenter running a pen along the rungs of the cages. This was strong enough to elicit behavioral agitation in the birds. Cage rolling was conducted by moving the cage racks around on wheels; this also caused birds to become more active in their cages. Both of these stimuli have been shown to elicit acute Cort responses in European starlings (*Sturnus vulgaris*, Rich and Romero,

2005). After these 4 days, birds were permitted varying periods of recovery. Those in the 0-hour group were sampled on day 5 of the experiment and proceeded directly to the second 4 days of stressors. The 24 and 72-hour groups received their respective amounts of recovery time during which they were minimally disturbed. During the recovery periods, birds were removed from the experimental "stressor" room to allow for the other birds to proceed with the stressors. During the recovery periods, birds were separated from the disturbances by 2 doors. After this recovery time, they were sampled a second time and then proceeded with the final 4 days of stressors. Birds that initially experienced cage rolling had cage tapping as their second stressor and vice versa. After this second 4 days of stressors, a final set of blood and video samples was taken. Therefore, all birds experienced both stressor types. Finally, birds were weighed on the first day of the experiment, following their recovery time, and on the last day of the experiment.

This study was approved by the Tufts University Institutional Animal Care and Use Committee, performed in compliance with the Guidelines for Use of Wild Birds in Research (Fair et al., 2010), and all animals were collected with a Massachusetts collection permit.

2.2. Plasma sampling

At each of the sample points (Fig. 1), 4 different types of blood samples were taken. Firstly, a baseline blood sample was taken within 3 min of disturbing the birds (Romero and Reed, 2005). Birds were then placed in opaque, cloth bags. After 30 min, a stress-induced sample was taken, followed by injection of the synthetic glucocorticoid dexamethasone (1 mg/kg; Phoenix Pharmaceuticals, Inc., St. Joseph, MO, USA) into the pectoralis muscle. This drug stimulates negative feedback of the HPA axis and thus is a way to assess how effectively this system is working (Carroll, 1982). Birds were returned to the cloth bags for an additional 90 min, after which a third sample was taken. Next, an ACTH-challenge was conducted to assess the maximal capacity of the HPA axis. To do so, a second intramuscular injection of adrenocorticotropic hormone (100 IU/kg; Sigma Aldrich, St. Louis, MO, USA; Catalog No. A6303) was administered. After allowing the drug to circulate for 15 min, a fourth and final blood sample was taken. All samples were kept on ice until processing by centrifugation after which they were frozen at −20 °C until conducting radioimmunoassays.

2.3. Corticosterone assays

Cort concentrations were quantified using a radioimmunoassay (Wingfield et al., 1992). Briefly, each ~20 μL plasma sample was diluted to 200 μL with distilled water. 20 μL of tritiated Cort was then added to each of these samples to assess extraction efficacy. Cort was extracted using dichloromethane. After aspirating the steroids, extracts were dried under N_2 gas and rehydrated with phosphate buffered saline with gelatin. The radioimmunoassay was performed using the B3–163 antibody (Esoterix, Calabasas Hills, CA, USA). Assay sensitivity was 1.07 ng/mL and samples that fell below this limit were assigned this value. Inter and intra assay CVs were 9.8% and 2.0% respectively.

2.4. Behavioral recordings

In addition to the blood samples, three sets of behavioral samples were taken. Behavior was assessed during neophobic trials during which the birds' latency to approach a novel object was measured. Neophobia has previously been used to assess how stress impacts house sparrows (Fischer et al., 2016; Gormally et al., 2018; Gormally and Romero, 2018; Lendvai et al., 2011). Overall activity (perch hopping) was also measured during these same trials. Five days prior to the first neophobia trial, food dishes and excess food from the bottoms of the cages were removed within 1 h before lights off. The following morning, food was returned within 1 h after lights on. This process was repeated each night prior to the first trial to acclimate the birds. On the evenings before neophobia trials, opaque cardboard blinders were placed between each cage to avoid the birds being able to observe other birds' behavior. Neophobia trials were conducted by replacing the food dishes with a novel object, either a colored plastic egg, red ribbon, or red food dish. These objects have been used before in both house sparrows and European starlings and elicit delayed approach latencies (Fischer et al., 2016; Gormally et al., 2018; Gormally and Romero, 2018). Every bird saw each object only once over the course of the three neophobia trials, but presentation order was randomized across birds. Behavior was remotely recorded for 20 min using a security camera system. Approach latency was defined as the time it took birds to approach and land on the food dish after the experimenter left the room. After the 20-min trials, novel objects and blinders were removed. Perch hopping was counted during these same trials. Perch hopping has been associated with exogenous Cort administration (Astheimer et al., 1992; Breuner et al., 1998) and has also been shown to change during stress challenges (de Bruijn and Romero, 2013; Gormally et al., 2018; Gormally and Romero, 2018). It has also been suggested that perch hopping could be a displacement behavior that occurs in captivity and is representative of other 'natural' behaviors that are only expressed in the wild (Tinbergen and Van Iersel, 1948). Changes in perch hopping could reflect changes in underlying behavior or overall activity; combined with neophobia perch hopping can be used to assess overall changes in feeding activity. Counting began 5 min after food dish was replaced and continued for 15 min. A hop was considered to be any movement throughout the cage, typically between the sides of the cages, perches, and food/water dishes. All videos were coded for perch hopping by the same individual (HY), who was blinded to the treatment.

Additionally, no-object control trials were conducted 1–2 days before novel object trials. These were performed identically to the neophobia trials except food dishes were returned without an object. Perch hops were counted during these trials as well.

2.5. Statistical analyses

All statistical analyses were conducted in RStudio (RStudio Team, 2015). We constructed a number of linear mixed effects models with individual bird identity as a random effect to assess the impacts of recovery periods on Cort concentrations, HPA axis function and regulation, and behavior. Before conducting further analyses, we tested whether the variances of each group were equal using Bartlett's test. In the event of a violation, we transformed the data and re-tested; only negative feedback strength (cubic) and perch hopping (log) needed to be transformed. We also visually inspected residual plots of each linear mixed effects model. Finally, analyses were conducted without considering sex or stressor order as these factors have previously been shown to not impact results (Gormally et al., 2018; Gormally and Romero, 2018; Romero and Remage-Healey, 2000) and preliminary analyses showed no main effects in this study except for in one dataset. There was a main effect of sex on stress-induced Cort, which is reported below; however, because this did not impact the overall conclusion, we have only graphed the grouped data.

During data analyses, we had two primary goals—to assess the impacts of recovery periods on the physiological and behavioral responses 1) to an initial 4 days of stressors (Pre-Stress Sample v. Post-Recovery Sample; Comparison 1 in Fig. 1), and 2) to a subsequent 4 days of stressors (0-hour v. 24-hour v. 72-hour groups at the Post-Stress Sample; Comparison 2 in Fig. 1).

2.5.1. Mass data

Mass was analyzed by calculating percent change from the initial, pre-experiment levels. All birds were considered to be at 100% to begin and either lost or gained weight from that point. We analyzed changes in mass over the course of the experiment separately for each recovery group. We used a linear mixed effects model ('lmer' function, lme4

package; Bates et al., 2015) and generated significance values using the 'Anova' function (car package; Fox and Weisberg, 2011).

2.5.2. Corticosterone data

Each bleed type (baseline, stress-induced, negative feedback, and ACTH-challenge) was statistically analyzed separately. This is because different concentrations of Cort interact with different receptor subtypes and thus elicit distinct physiological and behavioral responses (Romero, 2004; Sapolsky et al., 2000). Negative feedback strength was calculated as the percent decrease from stress-induced to post-dexamethasone Cort-levels (Lattin et al., 2012). For each bleed type, we first assessed whether any pre-experiment differences existed between the three recovery groups. As expected, in each case, all recovery groups had the same pre-experiment Cort levels (baseline, $F_{2,21} = 0.62$, $p = 0.55$; stress-induced, $F_{2,22} = 0.83$, $p = 0.45$; negative feedback, $F_{2,22} = 0.71$, $p = 0.50$; ACTH challenge, $F_{2,23} = 0.61$, p = 0.55). For this reason, we combined the groups at this initial sample.

Because our first goal was to assess how recovery periods influence the effects of an initial 4 days of chronic stress (Comparison 1 in Fig. 1), we used a linear mixed effects model with individual bird identity to compare the pre-stress and post-recovery samples. To address our second goal of testing whether recovery groups differed in their responses to a second round of stressors, we constructed a model to test for differences between the three recovery groups at the end of the experiment (Comparison 2 in Fig. 1). In all cases, the 'Anova' function was used to test for significance. When significant ($p < 0.05$) results were found, the 'glht' function (multcomp package; Hothorn et al., 2008) was used to test for pairwise differences and effect sizes were also generated; partial η^2 ('eta_sq' function; sjstats package; Lüdecke, 2019) were calculated for ANOVAs while Cohen's d $\left(\frac{mean_{var1} - mean_{var2}}{SD_{avg}}\right)$ was used for pairwise comparisons.

2.5.3. Behavioral data

Birds that failed to approach the food dish in the allotted time were assigned the ceiling value of 20 min. Object type did not influence approach latency at the post-recovery sample (main effect, $F_{2,49} = 2.78$, $p = 0.08$; interaction, $F_{6,45} = 0.42$, $p = 0.86$) as well as at the post-stress sample (main effect, $F_{2,23} = 2.28$, $p = 0.13$; interaction, $F_{4,21} = 2.17$, $p = 0.16$). Therefore, data from all objects were pooled. We did find a weakly significant difference in approach latencies between the recovery groups during the pre-stress control trial ($F_{2,23} = 3.58$, $p = 0.04$); for simplicity, we chose to represent all behavioral data separated by group. The same statistical analyses were used as in the Cort dataset.

3. Results

3.1. Mass

Birds tended to lose weight over the course of the experiment (Fig. 2), however we did not detect statistically significant changes in any of the recovery groups (0 h, $F_{2,26} = 2.56$, $p = 0.11$; 24 h, $F_{2,26} = 2.62$, $p = 0.10$; 72 h, $F_{2,21} = 2.51$, $p = 0.12$). Interestingly, birds that were permitted 24 h of recovery tended to lose more weight following this recovery period than birds that had no recovery or had 72 h (Fig. 2). All birds exhibited similar changes in weight at the final sample.

3.2. Recovery following an initial period of chronic stress

Recovery periods did not influence baseline Cort in response to 4 days of stressors (post-recovery, $F_{3,46} = 0.66$, $p = 0.58$; Fig. 3A). Birds that experienced 24 h of recovery had significantly lower stress-induced Cort levels relative to the pre-stress control sample (post-recovery, $F_{3,46} = 3.55$, $p = 0.02$, $\eta^2 = 0.25$, d = 1.45; Fig. 3B). Birds in the 0 h recovery group had significantly weakened negative feedback strength (post-recovery, $F_{3,47} = 4.11$, $p = 0.01$, $\eta^2 = 0.29$; pre-stress control v. 0 h, $p = 0.03$, d = 0.69; Fig. 3C). Finally, recovery time did not influence the birds' maximum Cort capacity (post-recovery, $F_{3,47} = 0.69$, $p = 0.56$; Fig. 3D).

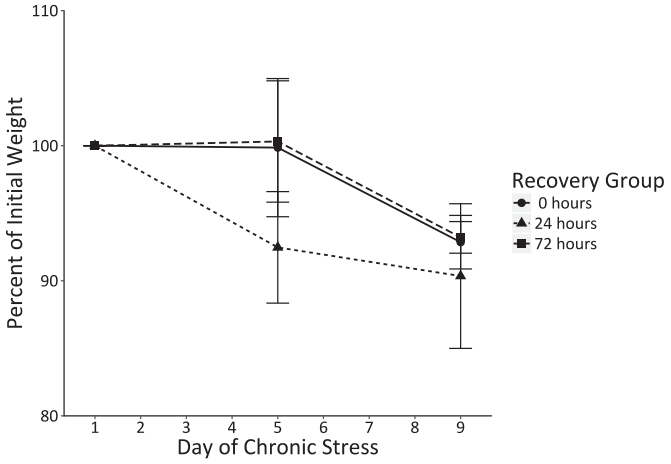

Fig. 2. Changes in mass during the experiment. Measurements on 'Day 5' were taken following the respective recovery time, then the birds proceeded with the final 4 days of stressors. Error bars represent ± SEM.

Behavior in the presence of novel objects was also influenced by recovery time. Birds that didn't receive any recovery time took significantly shorter time to approach novel objects relative to the pre-stress control sample (post-recovery, $F_{3,48} = 5.65$, $p = 0.003$, $\eta^2 = 0.31$, d = 1.54; Fig. 3E, Novel Object Trials). Perch hopping in the presence of novel objects did not significantly change at the post-recovery sample during (post-recovery, $F_{3,42} = 0.87$, $p = 0.47$; Fig. 3F, Novel Object Trials).

When novel objects were absent, birds significantly reduced their approach latencies after the recovery periods (post-recovery 0 h group, $F_{1,16} = 7.39$, p = 0.03, d = 1.35; post-recovery 24 h group, $F_{1,16} = 31.84$, $p = 0.0005$, d = 2.33; post-recovery 72 h group, $F_{1,14} = 20.19$, p = 0.003, d = 1.11; Fig. 3E, No-Object Control Trials). However, the pattern between groups prior to the experiment (with the group destined to get 72 h of recovery having longer latencies than the other two groups) was repeated after the recovery, even though all groups declined. During these no-object trials, perch hopping did not differ between recovery groups (post-recovery, $F_{3,41} = 1.86$, $p = 0.16$; Fig. 3F, No-Object Control Trials).

3.3. An additional period of chronic stress following recovery

We found no statistically significant differences in corticosterone between the recovery groups at the post-stress sample (baseline, $F_{2,23} = 0.52$, $p = 0.60$, Fig. 4A; stress-induced, $F_{2,22} = 3.40$, $p = 0.051$, Fig. 4B; negative feedback, $F_{2,20} = 0.81$, $p = 0.46$, Fig. 4C; maximum capacity, $F_{2,20} = 0.30$, $p = 0.74$, Fig. 4D). Additionally, recovery time did not influence behavioral responses after the final 4 days of stressors (post-stress neophobia, $F_{2,23} = 1.56$, $p = 0.23$, Fig. 4E, Novel Object Trials; post-stress perch hopping, $F_{2,21} = 0.42$, $p = 0.67$, Fig. 4F, Novel Object Trials).

In the absence of novel objects, the effect of recovery time on approach latency did not persist to the post-stress sample (post-stress, $F_{2,22} = 1.86$, $p = 0.18$; Fig. 4E, No-Object Control Trials). There were no inter-group differences in the perch hopping data (post-stress, $F_{2,21} = 1.89$, p = 0.18; Fig. 4F, No-Object Control Trials).

4. Discussion

The overall goal of this study was to test how the effects of chronic

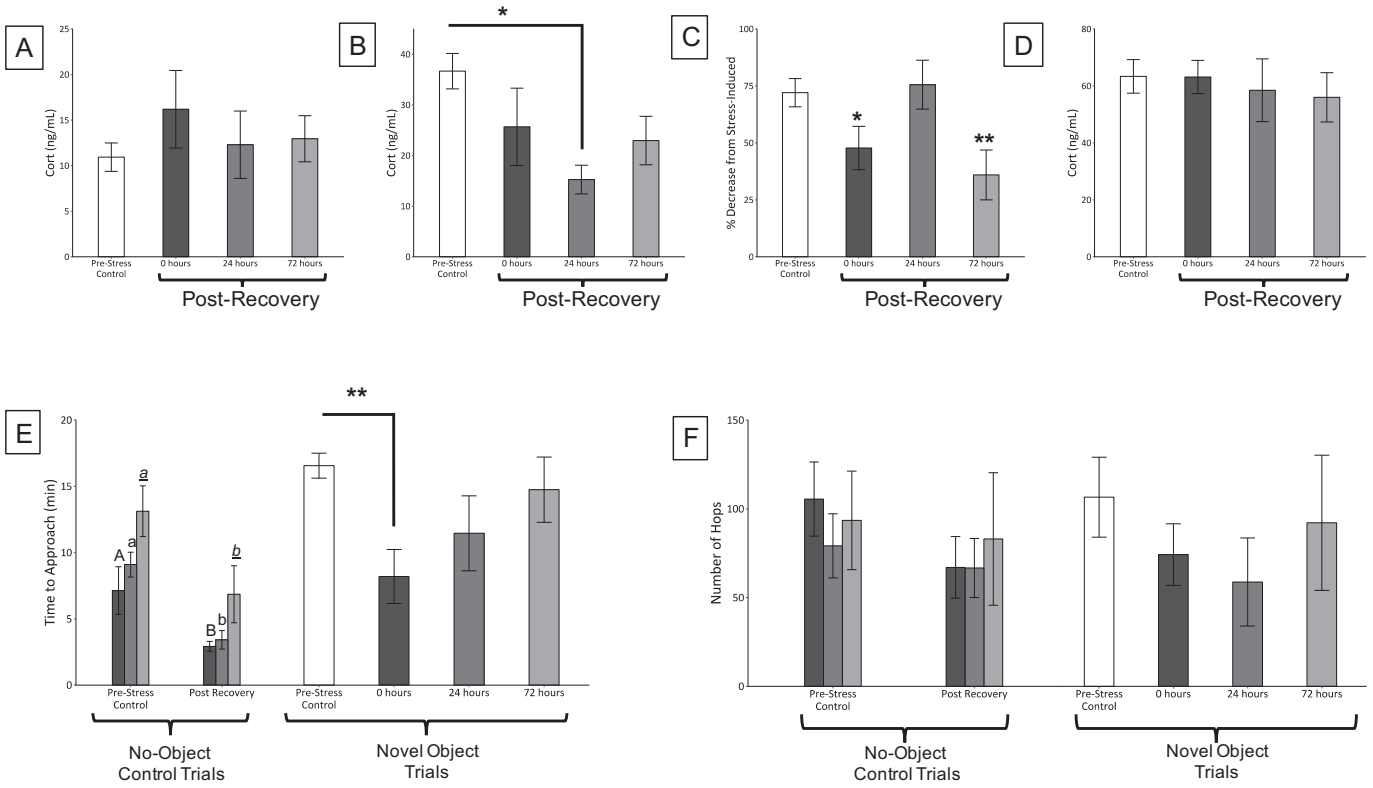

Fig. 3. Impacts of recovery on chronic stress. Cort changes after recovery periods compared to pre-stress samples at (A) baseline, (B) stress-induced, (C) negative feedback, and D) after ACTH-challenge. E and F show behavioral effects of recovery periods. No-object control trials are on the left while novel object trials are on the right. (E) Latency to approach the food dish. (F) Perch hopping. Data represented by different shades of grey correspond to the 3 recovery groups. Single asterisk indicates significance at $p < 0.05$, double asterisks indicates significance at $p < 0.01$. In (E), pairwise comparisons were made within recovery group; significant differences are indicated by different letters (A v. B, a v. b, a v. b). Error bars represent ± SEM.

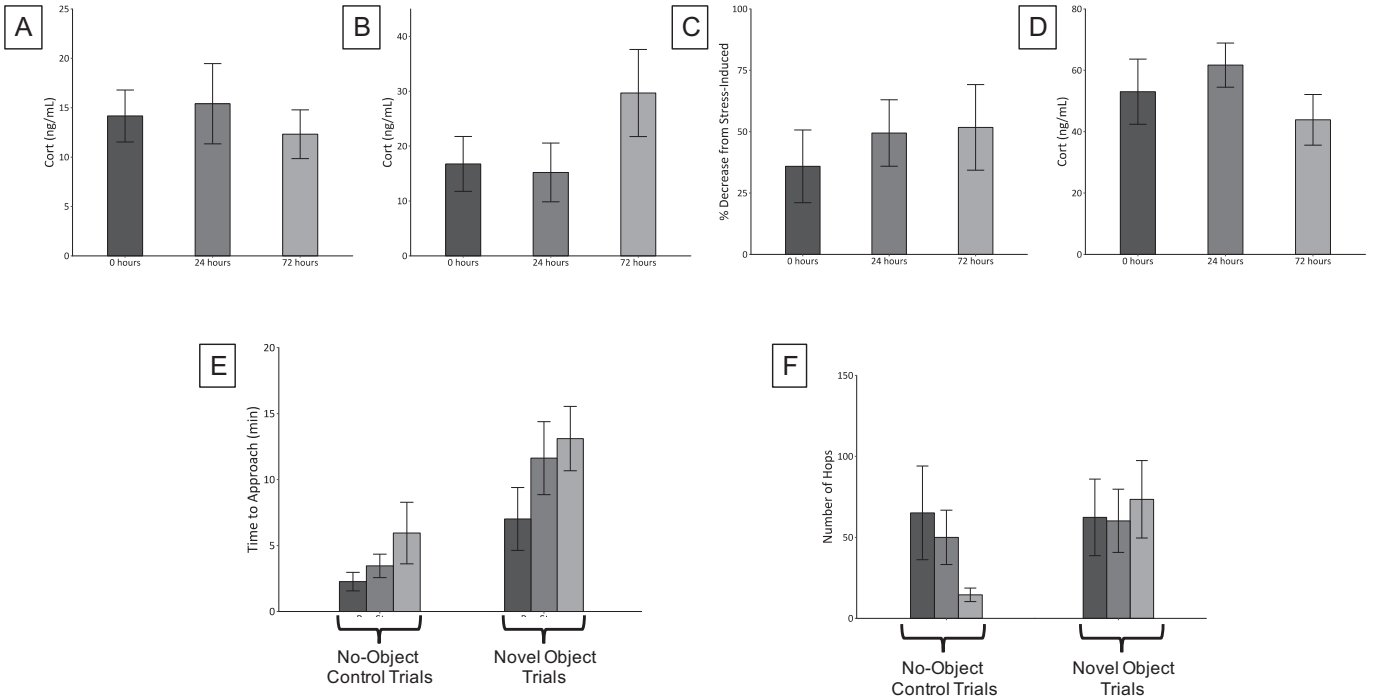

Fig. 4. Impacts of recovery on a second period of chronic stress. (A) baseline Cort, (B) stress-induced Cort, (C) negative feedback strength, (D) maximal Cort capacity, (E) approach latency towards novel objects, and (F) perch hopping. Data represented by different shades of grey correspond to the 3 recovery groups. Error bars represent ± SEM.

stress and recovery periods interact. We specifically were interested in exploring how recovery following a short period of chronic stress impacted animals' response to a novel chronic stress. To our knowledge, this is the first study to address these kinds of questions in a wild-caught species and to do so using a multimodal approach, assessing both endocrine and behavior responses. We found that brief (days) recovery periods can influence both HPA axis function and behavior, but that they do so in distinct ways. Interestingly, recovery time did not change the impacts of a second round of stressors.

4.1. Recovery following an initial period of chronic stress

Baseline Cort did not significantly differ between the three recovery groups following the initial 4 days of chronic stress (Fig. 2A). This was partially expected as previous studies involving house sparrows have found that baseline Cort does not become altered on this time scale (Gormally et al., 2018; Gormally and Romero, 2018; Lattin and Romero, 2014). Similar to closely related European starlings (*Sturnus vulgaris*), baseline Cort typically decreases within 8–12 days of chronic stress exposure (Cyr et al., 2007; Cyr and Romero, 2007; Dickens et al., 2009b; Rich and Romero, 2005).

While 4 days was not long enough to affect baseline Cort, this period did influence other aspects of HPA axis function and regulation. Specifically, this length of time significantly reduced negative feedback strength (Fig. 4A), indicating that birds were less efficient at terminating the stress response. This same effect was found in a previous study involving 4-days of a stressor presented to house sparrows (Gormally and Romero, 2018). Shutting off the release of Cort through negative feedback is important for avoiding the detrimental aspects of chronic stress (reviewed in Romero, 2004). Negative feedback strength was statistically restored after birds were permitted 72 h of recovery (Fig. 4C). In a number of recent studies, negative feedback strength has been implicated as a major effect of chronic stress (Gormally and Romero, 2018; Taff et al., 2018). Interestingly, the response to dexamethasone injection was strongest in birds that were permitted 24 h to recover (Fig. 3C). In other words, our results would suggest that 1 day of recovery restored HPA axis negative feedback, although longer periods elicited a weaker response.

To make sense of this result, it's necessary to consider the stress-induced Cort results. In this case, birds in the 24 h group experienced substantially reduced Cort responses relative to the samples from the pre-stress control, 0 h, and 72 h groups (Fig. 3A). Chronic stress protocols have been shown to reduce stress-induced Cort, likely through a controlled downregulation (Cyr et al., 2007; Cyr and Romero, 2007; Lattin and Romero, 2014; Rich and Romero, 2005). The finding in the present study would suggest that having 24 h of recovery following 4 days of stressors results in more significant effects on stress physiology than having no recovery at all. As a byproduct of this lower stress-induced Cort, the negative feedback—calculated as percent decrease from this level—of these birds was stronger.

The finding that a day of recovery appeared to enhance the effects of chronic stress suggests that this period is too short to be helpful to the birds. Our current hypothesis is that a one day recovery period was more disruptive to the HPA axis response than 4 consecutive days of stressors because the birds were both anticipating the stressors, which were less predictable. Anticipation and unpredictability can be a potent stressors themselves (Levine et al., 1989, 1972; Neubauer et al., 2018). In partial support of this interpretation, this group of birds tended to lose more weight than the birds in the other two groups following their recovery (Fig. 2). Though we did not detect a statistically significant difference, it is interesting that those 24 h appear to elicit weight loss, whereas when birds have no time or 3 days to recover, they are able to maintain their weight. These results also suggest that there may be a minimum threshold of recovery time to elicit beneficial effects. Interestingly, the 24 h recovery group was no more compromised than the 0 h recovery group following a second period of stressors (Fig. 4B).

Finally, maximum Cort capacity (as measured by the ACTH challenge) was unaffected by recovery time (Fig. 3D). This seems to be a less sensitive aspect of the HPA axis, as indicated from prior experiments (Gormally et al., 2018; Gormally and Romero, 2018). Interestingly, other kinds of chronic stress—like introduction to captivity—have been shown to influence HPA axis capacity (Lattin et al., 2012). There is increasing evidence that different chronic stress protocols change HPA axis function in distinct ways; for example, the protocol for inducing chronic stress used in this study usually decreases baseline Cort (Cyr et al., 2007; Cyr and Romero, 2007; Lattin and Romero, 2014; Rich and Romero, 2005), whereas chronic stress induced by introduction to captivity usually elevates baseline Cort (Fischer et al., 2018; Lattin et al., 2017; Love et al., 2017).

As expected, house sparrows approached their food dishes more slowly when novel objects where present (comparing No Object to Novel Object trials (Fig. 3E) and neophobia tended to decrease throughout the chronic stress periods (Fig. 3E). Because different objects were experienced during each trial, it's unlikely that these faster responses are indicative of habituation. Traditionally, increased neophobia is often associated with heightened states of anxiety or chronic stress (Korte, 2001; Skórzewska et al., 2006); however, these studies assumed a relationship between 'chronic stress status' and Cort levels. In other words, elevated Cort correlated to heightened expression of anxiety and fear behaviors. In the present study, we did not observe changes in baseline Cort (Fig. 3A) over the course of the experiment. This implies that changes in the neophobic response occur independently of circulating Cort. Additionally, a prior study of house sparrows also reported significant decreases in approach latency within 4 days of stressors (Gormally et al., 2018). Reductions in approach latency could suggest birds are prioritizing feeding behavior, or that the repeated stressors cause them to be unable to maintain their neophobic response. We are currently unable to tease apart these mechanisms. As recovery time lengthened, approach latency returned to pre-stress control levels (Fig. 3E). Though not statistically significant, it appears that the perch hopping data reflect similar effects, with birds becoming less active following the chronic stress period and more active following longer recovery periods. As with the endocrine data, recovery time did not influence behavioral responses following an additional stressor (Fig. 3F).

4.2. An additional period of chronic stress following recovery

Recovery periods did not appear to significantly influence responses to an additional stressor (Fig. 4). Firstly, no metric of HPA axis function/regulation statistically differed between the recovery groups following the second 4 days of stressors (Figs. 4A-D). This second period consisted of a unique, novel stressor that the birds had yet to experience. Stress-induced Cort was highest at the final sample point in the 72-hour group, however this difference was only weakly significant ($p = 0.051$). Similarly, these birds tended to have the strongest negative feedback strength. We think that this could indicate that this longer recovery period enabled birds to mount a "normal" acute stress response, while those with less recovery had a weakened response. If the experiment had been extended to include longer periods of repeated stressors, it is possible that statistical significance could have been found.

Secondly, there were no detectable statistical differences in the behavioral data (Figs. 4E and F). In contrast to the endocrine data, there does appear to be some visual stratification among the recovery groups, particularly in the approach latency data (Fig. 4E). It could suggest that birds in the longest recovery group retained the greatest neophobic response after the final 4 days of stressors. The differences in the two datasets of this study suggest the importance of taking a multimodal approach in questions regarding stress physiology. As has been shown previously (Gormally et al. 2019, 2018), chronic stress can impact physiological and behavioral systems on different time scales. The fact

that we see hints of these differences here further illuminates the complexities of these effects.

Our data do not show any differences between the recovery groups, indicating that perhaps the 0-hour group showed a maximal response that was not reversed with up to 72 h of recovery. Furthermore, the results could suggest that the system becomes permanently altered. This idea is also reflected in the mass data in which all three recovery groups wind up at roughly the same percentage of their mass at the end of the experiment (Fig. 2).

4.3. Do recovery periods "work"?

These data suggest that it is crucial to consider the length of both stress exposure and recovery time. While 72 h of recovery appeared long enough to elicit improvements in physiology and behavior after 4 days of chronic stress, surprisingly 24 h actually appeared more harmful than having no recovery at all. While unexpected, this result may reflect a minimum threshold of recovery. In other words, it is possible that shorter time periods may do more harm than good. Furthermore, different types of chronic stressors likely require different recovery periods. Because laboratory-induced chronic stress protocols result in unique physiological changes compared to introduction to captivity, it is likely that they also require different recovery periods. Furthermore, the data indicate that physiology and behavior can require different recovery timelines. These distinctions make it difficult to determine a priori how impacts of chronic stress can be alleviated.

This study also highlights the challenge of eliciting chronic stress in laboratory settings. Our main goal with this experimental design was to use single repeated stressors to increase wear-and-tear (Romero et al. 2009), or allostatic load (McEwen 1998), however this certainly is not the only way of inducing chronic stress. Some studies have used multiple, repeated stressors (Cyr et al. 2007; Cyr and Romero 2007; Lattin and Romero 2014; Rich and Romero 2005) while others have used the introduction to captivity (Fischer et al. 2018; Gormally et al. 2019; Lattin et al. 2012). A number of studies also exposed animals to exogenous Cort as a method of 'stressing' the animals (e.g. Herborn et al. 2014; Jorgensen et al. 2017; McCormick et al. 2015). Unsurprisingly, there is often great variation in results stemming from these studies, suggesting that there is not a 'one size fits all' approach when it comes to chronic stress; depending on what species is being studied, what experimental design is used, and what endpoints are measured, different conclusions can often be made. In fact, a recent review failed to find a consistent endocrine profile of 'chronically stressed' wild animals (Dickens and Romero 2013). This leads to the question of whether there is value in a common definition of chronic stress. Past results as well as the data from this study reinforce the idea that studies examining chronic stress should be clear about definitions and experimental designs and be careful when making conclusions.

In conclusion, these data indicate that brief recovery periods do influence how the physiology and behavior of house sparrows change due to chronic stress. However, we did not detect any differences in how our recovery groups responded to an additional 4 days of a novel stressor. This finding, along with the data that show mass declined similarly across groups, would suggest that the chronic stressors used in this experiment elicit more permanent effects and that recovery times used in this study did not 'reset' the systems after the initial 4 days of chronic stress.

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