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Recovery from repeated stressors: Physiology and behavior are affected on different timescales in house sparrows

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ABSTRACT

For decades, researchers across disciplines have been captivated by classifying, diagnosing, and avoiding the consequences of chronic stress. Despite the vast body of literature this has generated, we still lack the ability to predict which individuals or populations may be susceptible to stress-related pathologies. One critical unanswered question is whether the impacts of repeated stressors are reversible, or if instead they permanently alter an individual. In this study, we exposed house sparrows (*Passer domesticus*) to 6 days of random, repeated stressors, permitted them 0, 1, 3, or 6 days to recover, and then assessed changes in their body mass, hypothalamic–pituitaryadrenal (HPA) axis (baseline, stress-induced corticosterone, negative feedback strength), immune function, uric acid concentrations, DNA damage levels, and perch hopping activity. Body mass did not vary between groups after recovery. We found that the HPA axis and perch hopping were not significantly impacted by the 6 days of stressors, but that uric acid and DNA damage increased. Short recovery periods tended to negatively affect the HPA axis and reduced uric acid levels, but these were reversed with longer recovery periods. Following the recovery periods, the birds experienced an additional 6 days of random stressors and their responses were assessed again. All recovery times reduced perch hopping and immune function, but paradoxically, DNA damage was highest in the birds that had the longest amount of time to recover. These results show that recovery time affects responses to subsequent chronic stress in complex ways, and highlight the importance of multimodal, interdisciplinary approaches to studying stress physiology.

1. Introduction

The stress response is driven by a combination of hormones (e.g. glucocorticoids) and neurotransmitters (e.g. catecholamines) that elicit downstream physiological and behavioral adaptations to cope with unpredictable events (Romero and Wingfield, 2016; Sapolsky et al., 2000). The acute stress response, which is activated within seconds, is necessary for vertebrates to react to stimuli, however overactivation of these mechanisms can lead to what is referred to as chronic stress. Though many aspects of 'stress' are still debated, it is generally agreed that chronic exposure to repeated stressors results in negative physiological effects, including altered cardiovascular regulation (Cohen et al., 2007; Cyr et al., 2009; Fischer et al., 2018; Rupp, 1999), reduced efficacy of glucocorticoids (Cort; Cyr et al., 2007; Dickens et al., 2009a,b; Rich and Romero, 2005), immunosuppression (Dhabhar and McEwen, 1997; Gormally and Romero, 2018; Martin, 2009), or DNA damage (Gormally et al., 2019a).

Much of the confusion surrounding the impacts of chronic stress is a result of an incomplete understanding of the timing of wear-and-tear on an organism. In other words, we do not yet fully understand the intricate temporal dynamics of how repeated stressors impact an animal. Similarly, it is unknown whether chronic stress can permanently impact an organism or if the effects can be reversed. For example, it is usually presumed that if wild animals survive a chronic stressor their stress physiology reverts to the functioning prior to exposure (e.g. Romero and Wikelski, 2010, but see Clinchy et al., 2013); but chronic stress, especially during development, can permanently alter stress physiology (e.g. Landys et al., 2011). These opposite responses are crucial aspects in need of exploration in order to clarify how chronic stress impacts vertebrates.

Recovery from chronic stress has previously been examined in biomedical contexts in rodent model systems. Perhaps most well-known are the effects on hippocampal structure. Chronic restraint stress has resulted in reduced hippocampal volume and dendritic atrophy of neurons in the CA3 region (Conrad et al., 1999; Sousa et al., 2000). Surprisingly, during rest periods following chronic stress, these brain regions have shown plasticity and have regained much of their structure (Conrad et al., 1999; reviewed by Ortiz and Conrad, 2018); one

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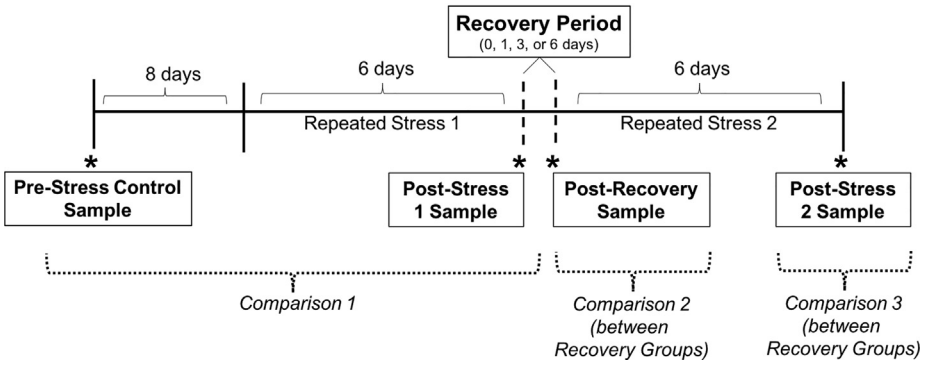

Fig. 1. Experimental design. House sparrows were captured from the wild and after at least 3 weeks of acclimation in captivity a Pre-Stress Control Sample was taken. After an additional 8 days, the chronic stress protocol commenced for 6 consecutive days. This consisted of 4 30-minute acute stressors experienced at random, unpredictable times of the day. A Post-Stress 1 sample was then taken before birds were permitted a recovery period, which was randomly assigned. After 1, 3, or 6 days a Post-Recovery Sample was taken. Finally, the birds were exposed to 6 additional days of a chronic stress protocol and a final Post-Stress 2 sample was taken. At each of the 4 sample points, blood and video samples were taken. Three major statistical comparisons were made: between the Pre-Stress Control and Post-Stress 1 samples; between the recovery groups at the Post-Recovery sample; and between recovery groups at the Post-Stress 2 sample.

study even showed that cognitive deficits resulting from chronic stress could be improved (Sousa et al., 2000). Other laboratory-focused studies have found that recovery from chronic variable stress initially results in hypoactivity of the hypothalamic pituitary adrenal (HPA) axis, but normal function eventually returns after 30 days (Ostrander et al., 2006). Furthermore, some, but not all aspects of the heart rate response—a common proxy for the catecholamine-mediated fight-or-flight response—have been shown to recover following random, repeated stressors (Park et al., 2017).

It quickly becomes obvious that the impacts of recovery can be as complex as those of chronic stress. Perhaps most significantly, studies have yet to assess recovery using an interdisciplinary approach. The stress response is traditionally studied by focusing on the catecholamine and glucocorticoid mediators (Romero and Wingfield, 2016); however, in reality these are merely the signals that elicit a suite of downstream physiological and behavioral effects that help an individual cope with an unpredictable event in its environment (Sapolsky et al., 2000). Nearly all studies that have examined recovery periods and chronic stress have either done so using single metrics of the stress response and/or have used domesticated laboratory rodents. It is particularly important to examine these questions using an interdisciplinary approach and undomesticated animals since an increasing number of studies seek to link stress susceptibility to fitness in wild animals

We sought to test how rest periods impact the responses to short (6 days) bouts of repeated stressors in house sparrows (*Passer domesticus*). Specifically, we assessed body mass, HPA axis function and regulation (baseline and stress-induced Cort and negative feedback strength), immune function (bacterial killing capacity), metabolism (uric acid), DNA damage (comet assay), and behavior (perch hopping). Each of these metrics have previously been shown to significantly change in response to repeated stressors (Cyr et al., 2007; Cyr and Romero, 2007; Gormally et al., 2019a, 2018; Gormally and Romero, 2018; Lattin et al., 2012; Lattin and Romero, 2014; Rich and Romero, 2005). It was therefore hypothesized that longer recovery periods would enable these parameters to 'reset' and therefore animals would be better able to respond to future repeated stressors by exposing them to a second 6 days of repeated stimuli. Importantly, this experimental design also tested whether recovery improved the animals' ability to cope with additional stressors. To our knowledge, this is one of the few studies that aims to test this. Specifically, in response to the bouts of repeated stressors we expected body mass to decrease (DuRant et al., 2016; Gormally et al., 2019b), baseline and stress-induced Cort to decrease (Cyr et al., 2007; Gormally et al., 2018; Rich and Romero, 2005), for negative feedback strength to weaken (Gormally and Romero, 2018; Lattin et al., 2012), for bacterial killing capacity to decline (Dhabhar and McEwen, 1997; Gormally et al., 2018), uric acid to decrease (Gormally et al., 2018), and DNA damage to increase (Flint et al., 2007; Gormally et al., 2019a; Hara et al., 2011). Note that while it has been shown that administration of exogenous Cort increases uric acid in birds (Lin et al., 2004a), in this system Cort is not expected to increase (Lattin and Romero, 2014). Prior studies have shown that repeated stressors decrease uric acid in house sparrows (Gormally et al., 2019a, 2018), perhaps indicating an attempt at regaining redox balance.

2. Materials & methods

2.1. Experimental design

Between October 2017 and April 2018, 34 house sparrows (15 females and 19 males) were captured in Medford, MA, USA. The birds were brought to Tufts University where they were housed in male-female pairs (n = 26) or in direct view of other birds (n = 8); there was no effect of being housed in pairs v. alone ($p > 0.10$ in all cases), except in the behavioral data (see Results). Previous experiments indicate that this sample size is appropriate for the variance associated with these physiological parameters based on a power analysis assuming a significance level of 0.05. All birds were maintained in cages (45 cm × 37 cm × 33 cm) and on a 12L:12D light cycle with seed, grit, and water provided *ad libitum*. All birds were permitted a minimum of 3 weeks to acclimate to captivity prior to the experiment as this period of time has been shown to be sufficient to stabilize Cort and heart rate (Fischer et al., 2018).

After this minimum acclimation period, blood and video samples (Pre-Stress Sample in Fig. 1) were taken to quantify baseline physiology and behavior. Eight days later, birds were subjected to a chronic stress protocol that consisted of 30-minute stressors presented at 4 random times throughout the day for 6 consecutive days. The chosen stressors were cage tapping (tapping on the cages at intermittent times), cage rolling (randomly moving the wheeled cage racks), food removal (food was removed for the stressor period), human voice (a researcher read out loud in the animal facility), and radio. Each of these have been used in the past and have been shown to elicit acute hormonal responses (Cyr et al., 2009; Gormally et al., 2018; Lattin and Romero, 2014; Rich and Romero, 2005). A random number generator was used to establish which of these five stressors would be presented for each 30-minute session. This initial 6-day period was meant to push animals towards, but not yet into, homeostatic overload (Romero et al., 2009). We hypothesized that during this period, wear-and-tear or allostatic load would increase, thus increasing susceptibility of the animals (McEwen and Wingfield, 2003; Romero et al., 2009). A second sample was taken after the 6-day period (Post-Stress 1 Sample in Fig. 1). Next, the animals were randomly divided into different treatment groups; birds either immediately proceeded to the second stress period (0 recovery days; n = 4 females, 4 males) or where permitted recovery time (1 days,

n = 3 females, 5 males; 3 days, n = 3 females, 5 males; or 6 days, n = 5 females, 5 males). Despite these sex differences in sample sizes, no parameter measured here was affected by sex ($p > 0.14$ in all cases). During these recovery periods, birds were minimally disturbed only for husbandry purposes. A third sample was taken after recovery (Post-Recovery Sample in Fig. 1) except for the animals that received 0 recovery. An additional 6 days of stressors was then applied, after which a fourth and final sample was taken (Post-Stress 2 Sample in Fig. 1). At each of these four sample points, we took blood samples for physiological measurements and video samples to assess changes in behavior. Finally, birds were weighed before the experiment, after the first 6 days of repeated stressors, after recovery, and after the final 6 days of repeated stressors.

All experiments were conducted with approval from the Tufts Institutional Animal Care and Use Committee and in accordance with the Guidelines to the Use of Wild Birds in Research (Fair et al., 2010).

2.2. Blood sampling

Three different types of blood samples were taken at each point. Firstly, a baseline sample was taken within 3 min of entering the room (Romero and Reed, 2005). Secondly, birds were placed in a breathable, opaque cloth bag for 30 min after which a stress-induced sample was taken; this reflects the acute stress response to a unique stimulus that was not part of the chronic stress protocol. Finally, the synthetic glucocorticoid dexamethasone was used to stimulate negative feedback (Carroll, 1982; Lattin et al., 2012). Dexamethasone (1 mg/kg; Phoenix Pharmaceuticals, St. Joseph, MO, USA) was injected intramuscularly into the pectoralis muscle of house sparrows immediately following the collection of the stress-induced sample; the third blood sample was taken after allowing the drug to circulate for 45 min.

All samples were stored on ice until processing. Whole blood was separated from plasma using centrifugation, separate aliquots of plasma were made for Cort, uric acid, and bacterial killing assays, and samples were stored at −20 °C until assayed.

2.3. Behavioral sampling

Security camera systems were used to remotely record the behavior of the house sparrows. On the mornings of each sample (Fig. 1) the cameras remotely turned on and recorded behavior for 20 min beginning at lights-on. Perch hopping was counted for the second half of this 20 min. A perch hop was considered as any movement throughout the cage, typically from the perch to the cage sides, bottom, or food/water dishes. All videos were coded by the same individual (HY) who was blinded to treatment.

2.4. Corticosterone assays

Corticosterone was quantified using a well-established radioimmunoassay (Wingfield et al., 1992). Briefly, diluted plasma was spiked with tritiated Cort and then extracted using dichloromethane. Nitrogen gas was used to dry down the extracted steroids and phosphate buffered saline with gelatin was used for rehydration. A portion of the sample was used to calculate recovery efficiency while the remaining was run in duplicate in the radioimmunoassay. B3-163 antibody (Esoterix, Calabasas Hills, CA, USA) and tritiated Cort were added to the sample tubes. A standard curve was used to extrapolate Cort concentration, with final values adjusted by the recovery efficiency. A total of seven assays were necessary to run all the samples; samples from one assay were lost because of a failed standard curve. Inter and intra assay coefficients of variation were 15% and 4.9% respectively. A total of 34 samples (out of 334) fell below the level of detection and therefore were assigned the floor value of 1.07 ng/mL.

2.5. Uric acid fluorometric assays

Uric acid was quantified in the baseline blood samples using the Amplex® Red Uric Acid/Uricase Assay Kit (Molecular Probes, Eugene, OR, USA). Protocol was followed according to assay instructions (Gormally et al., 2019a, 2018). Briefly, 2–3 μL of plasma was diluted with 200 μL of reaction buffer. Samples were run in duplicate and the reaction was started by added horseradish peroxidase; uric acid converts to hydrogen peroxide, which reacts with the Amplex Red Reagent in the presence of horseradish peroxidase to form a red fluorescent product. The reaction proceeded for 30 min in a 37 °C incubator and the 96-well plates were read in a spectrophotometer at 560 nm. Inter and intra assay coefficients of variation were 2.3% and 2.5% respectively.

2.6. Comet assays

Comet assays were performed on the day of sampling and according to previously published studies, with some minor modifications (Gormally et al., 2019a). Prior to centrifugation of the blood samples, 2 μL of whole blood was removed and diluted in 800 μL of phosphate buffered saline (Mg^{2+}, Ca^{2+} free). Samples were diluted 1:4 twice more with fresh PBS. A portion of this final dilution was combined with low melting agarose at a 1:10 dilution and then immediately added to a precoated microscope slide (R & D Systems, Minneapolis, MN, USA). The entire slide was submerged in lysis buffer for 1 h and then electrophoresis was performed (buffer: 300 mM sodium acetate, 100 mM Tris base, pH 10; electrophoresis conditions: 30 min, 21 V, 90 mA). Slides were then soaked in chilled deionized water, then ethanol and allowed to dry in 37 °C.

Slides were stored in the dark, with desiccant, until staining and microscopy. Slides were stained with SYBR Gold (Molecular Probes, Eugene, OR) for 30 min and imaged with the 10X objective on a fluorescent microscope using the green fluorescent protein filter.

Standardized damaged cells (Catalog #4257-010-NC, R & D Systems, Minneapolis, MN, USA) were run alongside samples during each assay. These were used to 1) calculate variation between assays, and 2) generate a standard curve to normalize damage results in three aberrant assays (see Section 2.8 for details). The inter assay coefficient of variation was 24%.

2.7. Bacterial killing assays

Bacterial killing assays were run on plasma collected at the Pre-Stress Control and Post-Stress 2 Samples; due to the fact that this assay requires a large amount of blood, assays were restricted to these samples to remain below the ethical limit of blood removal. Assays were begun within 5 h of sampling and conducted using a modified protocol with microplates (French and Neuman-Lee, 2012; Liebl and Martin, 2009; Millet et al., 2007). Preliminary experiments were conducted to determine appropriate plasma dilutions for this species (data not shown). All materials were sterilized by autoclave prior to conducting the assays. Briefly, one lyophilized pellet of *Escherichia coli* (ATCC® 8739) was added to 10 mL of pre-warmed phosphate buffered saline and incubated for 30 min. This suspension was then further diluted to 10^5 bacteria/mL, which was the working concentration. 4.5 μL of plasma was added to the microplate in triplicate. Next, 13.5 μL of CO_2-independent media (Catalog #18045088, ThermoFisher, Waltham, MA, USA) plus 4 mM L-glutamine was added to dilute each plasma replicate. 6 μL of diluted *E. coli* and 125 μL of tryptic soy broth were also added to each well. Positive and negative controls were included on each plate.

Covered plates incubated for 12 h at 37 °C before being read at 300 nm with a spectrophotometer. Bacterial killing capacity was calculated as 1 – (absorbance of sample/absorbance of positive control). Inter and intra assay coefficients of variation were 17% and 1.5% respectively.

2.8. Statistical analyses

All statistical analyses were performed in RStudio (RStudio Team, 2015). All datasets contained either unequal variances or were not normally distributed, therefore we opted to perform nonparametric tests. Three statistical comparisons were made (Fig. 1): between the Pres-Stress Control and Post-Stress 1 Samples; between recovery groups after the recovery times; and between recovery groups at the Post-Stress 2 Sample. For each of these comparisons, the nonparametric Kruskal-Willis test was used, followed by a Wilcox test for pairwise comparisons in the event of a significant p-value. Effect sizes were calculated using the ε^2 method (rcompanion package, Mangiafico, 2019; Tomczak and Tomczak, 2014).

The three Cort samples (baseline, stress-induced, and negative feedback strength) were analyzed separately. Baseline and stress-induced levels of Cort interact with different receptor subtypes and reflect unique physiological outcomes (Sapolsky et al., 2000). Any values higher than 150 ng/mL (greater than 4 standard deviations from the stress-induced mean) were removed from analyses as these outliers likely resulted from experimental error (4/334 samples). Changes in negative feedback strength were assessed by calculating the percent decrease in Cort concentration from the stress-induced level. Values of birds for which this calculation resulted in a negative number were standardized to 0. Therefore a value of 100% would indicate a bird that completely shut down the release of Cort due to the dexamethasone injection while a value of 0% would correspond to an animal that failed to show any negative feedback.

Body mass, uric acid, bacterial killing capacity, and behavior were all assessed using the nonparametric tests as indicated previously. Changes in body mass were compared within recovery group and were calculated as percent initial weight. Two uric acid samples (out of 136) fell in the negative range due to too little plasma and were therefore assigned a value of 0. When the bacterial killing assays resulted in values that were above 100 or below 0, the samples were standardized to the ceiling (6/67 samples) and floor respectively (2/67 samples).

Comet assay images were analyzed using the OpenComet plugin (Gyori et al., 2014) for Fiji (Schindelin et al., 2012). Comets from the standard control cells of 3 of 9 assays significantly deviated from the overall mean and therefore these results were normalized.

The OpenComet program automatically detects abnormal comets—mainly those that were overlapping—but also allows for user review. Aberrant comets were manually reviewed if the program improperly detected the heads or tails of comets. We compared changes in average percentage of tail DNA ("TailDNA%"; Gormally et al., 2019a,b) over the course of the experiment using the nonparametric tests indicate above. We also compared the distributions of damage at each sample point for each group (4 samples and 4 groups, 16 total distributions). Prior work has noted unexpected patterns in the distributions of these data (Gormally et al., 2019a). The modality of each distribution was assessed using Hartigan's dip test (diptest package; Maechler, 2016) where a p-value that is less than 0.05 suggests a non-unimodal distribution (Hartigan and Hartigan, 1985). For this distribution analysis, normalized data points that were less than 0 were removed (~3% of points).

3. Results

3.1. Comparisons 1 and 2—Impacts of recovery following repeated stressors

Body mass varied little across the experiment, however the 3-day group did tend to increase after the initial 6 days (0 days $\chi^2 = 2.96$, df = 3, p = 0.40; 1 day $\chi^2 = 4.53$, df = 3, p = 0.21; 3 days, $\chi^2 = 9.07$, df = 3, p = 0.03; 6 days, $\chi^2 = 1.56$, df = 3, p = 0.67; Fig. 2). Neither baseline nor stress-induced Cort changed over the course of the first 6 days of repeated stressors (baseline, $\chi^2 = 0.006$, df = 1, p = 0.94; stress-induced, $\chi^2 = 0.005$, df = 1, p = 0.94; Fig. 3A) or due to recovery (baseline, $\chi^2 = 3.45$, df = 3, p = 0.33; stress-induced, $\chi^2 = 4.48$, df = 3, p = 0.21; Fig. 3B). Similarly, negative feedback strength did not significantly change (Comparison 1, $\chi^2 = 0.1$, df = 1, p = 0.75; Comparison 2, $\chi^2 = 7.12$, df = 3, p = 0.07; Fig. 3C). Birds that recovered for 24 h had 0% negative feedback strength; in other words, all birds in this group either had the same amount or more Cort circulating post-dexamethasone injection as they did after a 30-minute acute stressor.

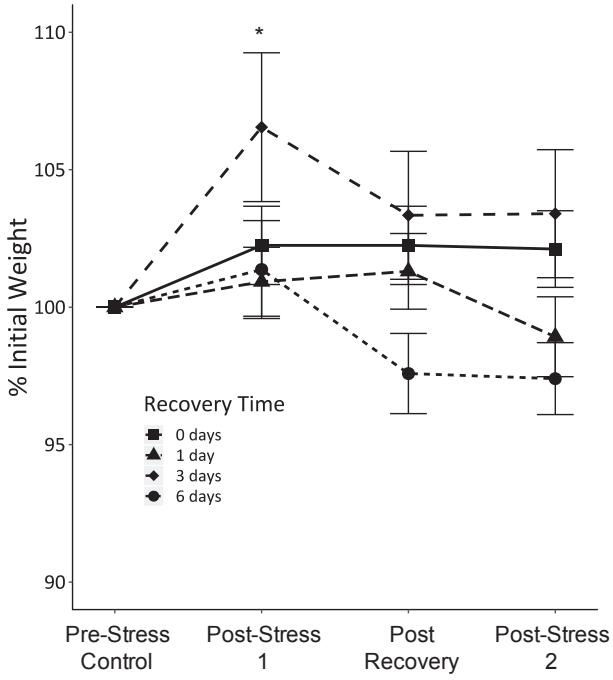

Fig. 2. Impact of repeated stressors and recovery on body mass. Comparisons were made within recovery group, across the 4 different samples. * denotes significance at p = 0.05. Error bars represent ± SEM.

Uric acid significantly increased following the 6-day chronic stress period ($\chi^2 = 11.89$, df = 1, p = 0.0006, $\varepsilon^2 = 0.87$; Fig. 3D). Recovery time reduced this level, with birds that experienced 3 and 6 days of recovery having significantly lower levels than the 0-day group ($\chi^2 = 13.03$, df = 3, p = 0.005, $\varepsilon^2 = 0.93$; 3 v. 0 days, p = 0.03; 6 v. 0 days, p = 0.01; 1 v. 0 days, p = 0.11; Fig. 3D).

DNA damage also significantly increased due to chronic stress ($\chi^2 = 12.6$, df = 1, p = 0.0004, $\varepsilon^2 = 1$; Fig. 3E). Recovery time had a complex effect on damage with birds in the 3-day group having significantly reduced damage, while other groups remained elevated ($\chi^2 = 20.65$, df = 3, p = 0.0001, $\varepsilon^2 = 1$; 3 v. 0 days, p = 0.0009; 3 v. 1 day, p = 0.0009; 3 v. 6 days, p = 0.002). The damage in individual cells was unimodally distributed in all groups of birds prior to the experiment (p = 0.99), and remained that way except in the 3-day group ($p < 0.001$; Fig. 4). After the recovery time, only birds in the 6-day group exhibited a non-unimodal distribution (p = 0.02; Fig. 4).

Finally, perch hopping activity did not significantly change after 6 days of chronic stress ($\chi^2 = 0.30$, df = 1, p = 0.59; Fig. 3F) nor did recovery time significantly alter behavior ($\chi^2 = 1.88$, df = 3, p = 0.60; Fig. 3F). Cage status affected perch hopping after the repeated stressors; individually housed birds tended to be less active than those housed in pairs ($\chi^2 = 7.23$, df = 1, p = 0.007, $\varepsilon^2 = 0.96$). However, this effect was not detected following recovery ($\chi^2 = 1.29$, df = 1, p = 0.26).

3.2. Comparison 3—Impacts of repeated stressors after recovery

No significant differences in baseline or stress-induced Cort were detected between recovery groups following a second period of repeated stressors (baseline, $\chi^2 = 1.89$, df = 3, p = 0.60; stress-induced,

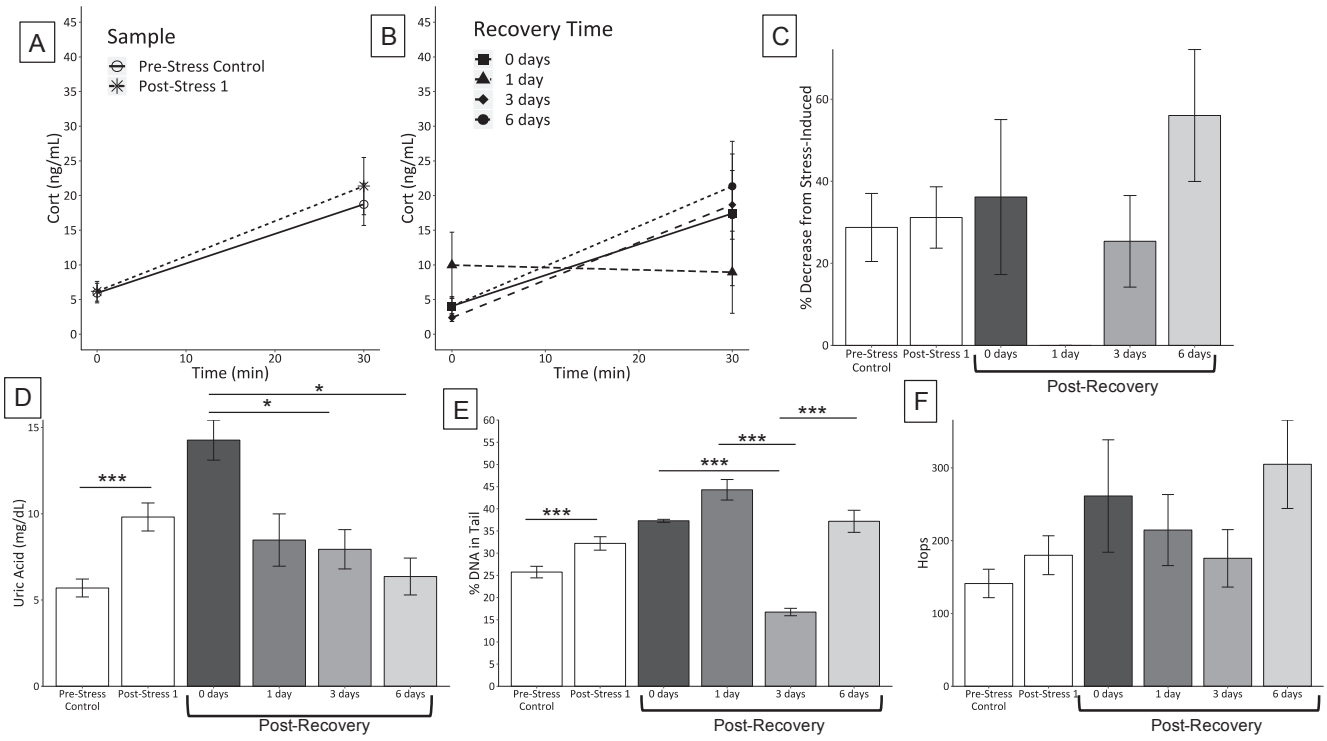

Fig. 3. Impacts of recovery on the physiological and behavioral effects of chronic stress. (A) and (B) Acute corticosterone response. (C) Negative feedback strength assessed by dexamethasone injection. (D) Uric acid. (E) DNA damage assessed using the comet assay. A greater % DNA in Tail value corresponds to more damage. (F) Perch hopping activity. Statistical comparisons were made between the Pre-Stress Control and Post-Stress 1 samples as well as between recovery groups at the Post-Recovery group. * denotes significance at p = 0.05, ** denotes significance at p = 0.01, *** denotes significance at p = 0.001. Error bars represent mean ± SEM.

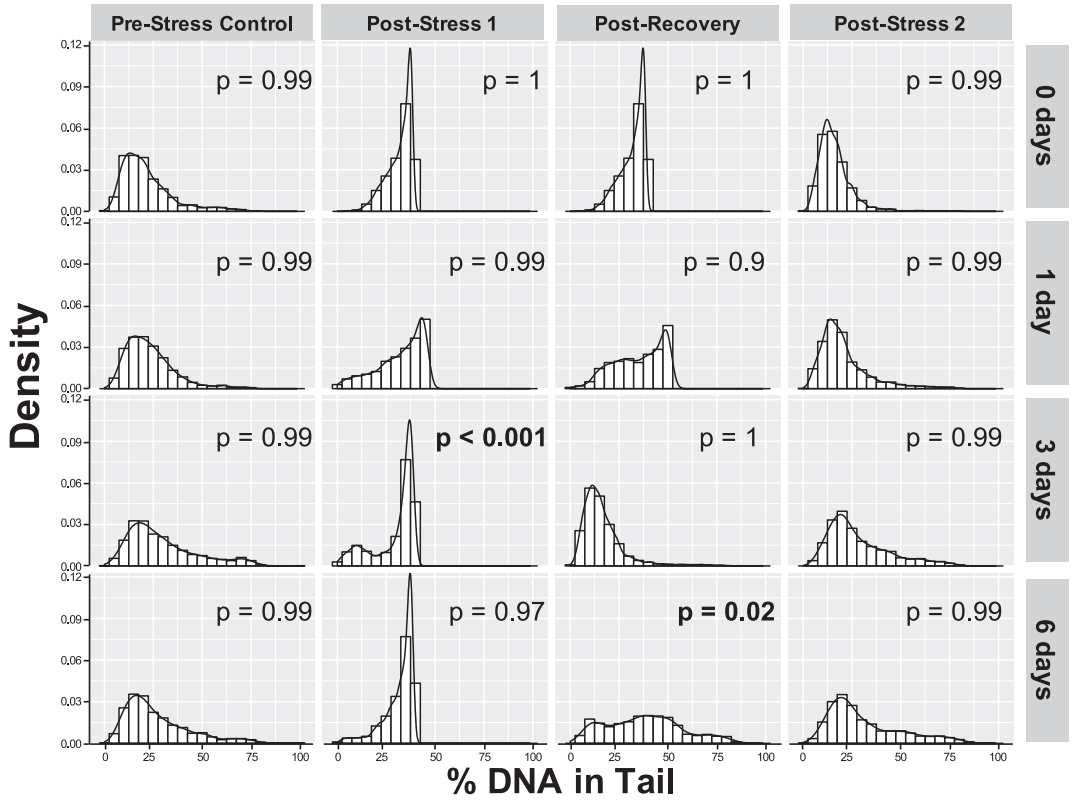

Fig. 4. Distribution of DNA damage in individual red blood cells. Each plot is a histogram of the results from all the cells at each sample overlaid with a kernel density curve. To simplify visualization, the plot for the Post-Stress 1 sample of the 0-day group is repeated for the Post-Recovery samples as only one sample was taken. Insets include the p-values that are the result of Hartigans' Dip Test for Unimodality. Bolded numbers indicate values that fall below a significance value of 0.05.

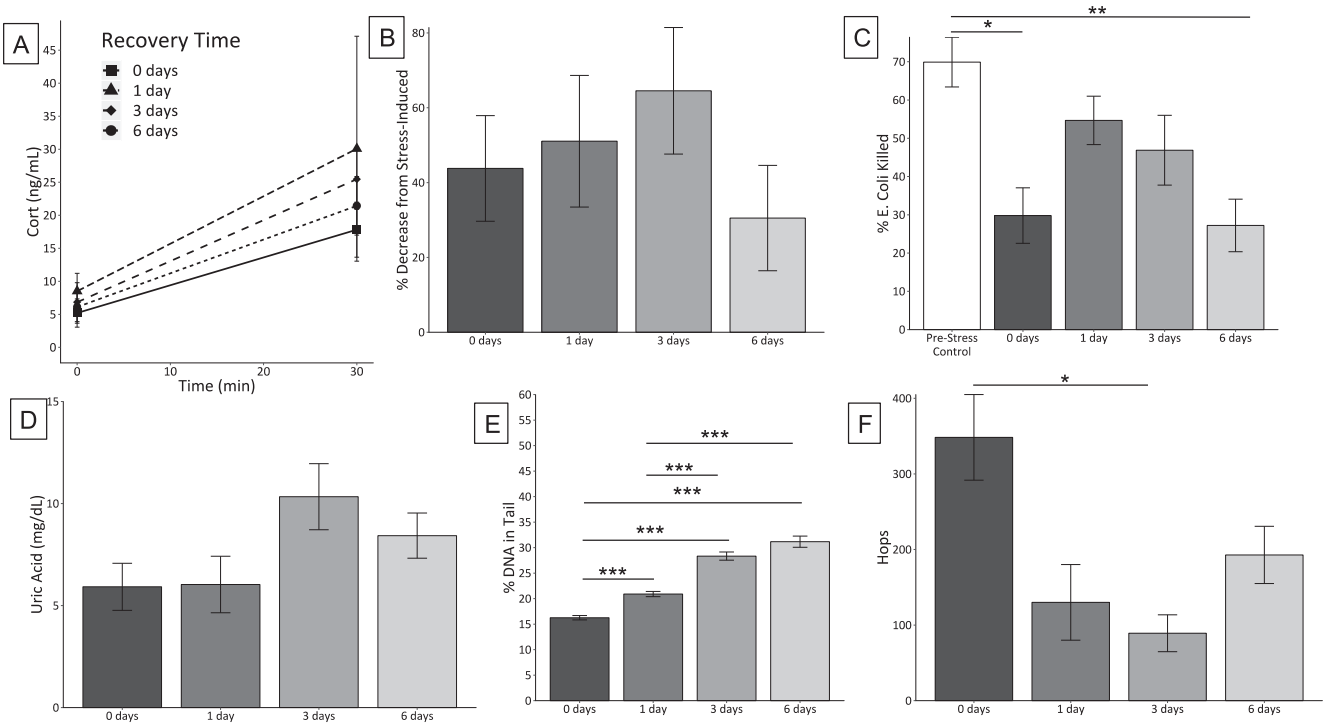

Fig. 5. Impacts of recovery time following a second period of chronic stress. Note that birds in the 0 days group experienced 12 consecutive days of the chronic stress protocol. (A) Acute corticosterone response. (B) Negative feedback strength. (C) Immune function was assessed using the bacterial killing capacity. A lower % *E. coli* killed indicates a more immunocompromised individual. (D) Uric acid. (E) DNA damage measured using the comet assay. (F) Perch hopping activity. * denotes significance at p = 0.05, ** denotes significance at p = 0.01, *** denotes significance at p = 0.001. Error bars represent mean ± SEM.

$\chi^2 = 2.49$, df = 3, p = 0.48; Fig. 5A); however, the recovery groups do visually appear to non-significantly separate after this final 6 days (Fig. 5A). Similarly, there were no differences in negative feedback strength between the recovery groups ($\chi^2 = 2.21$, df = 3, p = 0.53; Fig. 5B).

Immune function was only assessed at the start and end of the experiment due to restrictions on blood sampling. Birds that had either 0 or 6 days to recover between the sets of repeated stressors had significantly reduced bacterial killing capacity relative to before the experiment ($\chi^2 = 16.56$, df = 4, p = 0.002, $\varepsilon^2 = 0.99$; Fig. 5C). Uric acid approximately returned to pre-experiment levels in all groups and there were no significant differences between groups ($\chi^2 = 5.45$, df = 4, p = 0.14; Fig. 5D).

Average DNA damage also differed significantly between recovery groups with the 6-day birds having the highest level ($\chi^2 = 28.11$, df = 3, p < 0.001, $\varepsilon^2 = 1$; 0 v. 1 day, 0 v. 3 days, and 1 v. 3 days p = 0.0006, 0 v. 6 days p = 0.0003, 1 v. 6 days p = 0.0002; Fig. 5E). All groups exhibited unimodal distributions after the second set of repeated stressors (p = 0.99; Fig. 4).

Finally, behavior in response to repeated stress was significantly affected by recovery time ($\chi^2 = 10.96$, df = 3, p = 0.01, $\varepsilon^2 = 0.99$; Fig. 5F). Birds that experienced 12 consecutive days of the chronic stress protocol were more active relative to those that had recovery time; this was a significant difference only with the 3-day group (p = 0.04; Fig. 5F). Again, following a second period of repeated stressors, birds in pairs tended to be more active than those housed individually ($\chi^2 = 5.08$, df = 1, p = 0.02, $\varepsilon^2 = 0.92$).

4. Discussion

In this study we tested whether recovery following repeated stressors could change house sparrow responses to an additional bout of repeated stressors. Importantly, the first 6 days of repeated stressors was not meant to drive animals immediately into homeostatic overload (Romero et al., 2009). Instead, the purpose of the design was to only have the birds in the 0-day group experience enough consecutive days to enter overload. Prior studies that examined heart rate regulation and HPA axis function suggest that this experimental protocol elicits significant differences by 10–12 days (Cyr et al., 2007; Rich and Romero, 2005) in another passerine the European starling (*Sturnus vulgaris*). Another prior study showed that 10 days of this same chronic stress protocol significantly reduced wound healing rates in house sparrows (DuRant et al., 2016). Thus, we assumed that 6 days would simply build wear-and-tear on the birds, whereas the complete 12 days would initiate chronic stress (e.g. significant changes in HPA axis function and regulation). In other words, we assumed that increasing consecutive days—in this case 12—of repeated stressors would build wear-and-tear on the animals. By enabling the other groups time to recover, we hoped to assess how those rest periods changed responses to repeated stimuli. In sum, we found that recovery was reflected in some, but not all, metrics that were tracked.

4.1. Short recoveries negatively impact Cort regulation

We found that 6 consecutive days of random, repeated stressors was substantial enough to alter some aspects of physiology measured here; however, not all metrics were affected suggesting that some systems may be more susceptible to repeated stressors. Surprisingly, body mass did not decline as was expected and as has been previously shown in this species (DuRant et al., 2016; Gormally et al., 2019b). This highlights the variation in individual responses to chronic, or repeated stressors (Dickens and Romero, 2013). Neither baseline nor stress-induced Cort were impacted by the first part of the experiment (Fig. 3A). This meant there were no alterations for the recovery groups to recover from (Fig. 3B). This result was not surprising, however, since prior studies have shown that European starlings take 10–12 days to exhibit significant changes in Cort regulation (Cyr et al., 2007; Rich and Romero, 2005). What was surprising was the distinct effects of 1 day of

recovery on baseline and stress-induced Cort. Though this group was not statistically different from the other groups, it appears quite visually distinct (triangles, Fig. 3B). These birds tended to have higher baseline Cort (0 min) and a completely absent acute response (30 min). This is further reflected in the negative feedback strength of these birds; following the dexamethasone injection, birds failed to shut down the release of Cort (Fig. 3C). Both an inability to mount an acute stress response (Fig. 3B) and failure to shut down the release of Cort (Fig. 3C) are indicative that this aspect of the stress response is malfunctioning (Romero, 2004).

Though initially unexpected, this pattern could indicate that after 6 consecutive days, the birds are essentially anticipating an additional day of repeated stressors. When that does not become a reality, however, the anticipation of a stressor can itself be a stressor, and this anticipation manifests itself through elevated baseline Cort and a reduced Cort response to restraint. The physiological consequences of frustration and anticipation have been noted many times in historic studies (reviewed by Levine et al., 1989, 1972). It is worth noting, however, that these previous studies were not focusing on chronic stress (e.g. random, repeated stressors) and often classified 'stress' as an enhanced Cort response. Though a stronger Cort response is not the pattern we see in this study, we think it is likely that the result presented here indicates that 1 day of recovery was initially more disruptive to house sparrows. A similar result has been seen in a closely related study in which 1 day of recovery following 4 days of a stimulus resulted in a decreased Cort response (Gormally et al., 2019b). The combination of these studies could suggest that there is an initial minimum threshold of time in order for recovery to be beneficial.

4.2. Recovery improves responses of other systems

While 6 days was not enough to significantly affect Cort function and regulation, other systems were impacted by the initial experimental protocol. Firstly, uric acid became significantly elevated following 6 days of repeated stressors (white bars, Fig. 3D). Particularly important is that this increase subsided in all the recovery groups. Uric acid is both the primary product of nitrogen breakdown (Wright, 1995) and also a key antioxidant that quenches free radicals (Ames et al., 1981). Uric acid has been shown to be elevated in response to the administration of Cort both on a short (hours) and long (days) timescale (Lin et al., 2004a,b). The elevation in uric acid in this study could indicate that birds were either upregulating the breakdown of proteins or attempting to regain redox balance through antioxidants. The former, however, seems improbable since most proteins are only broken down as an energy source in dire situations and only once carbohydrates and fats have been depleted (Romero and Wingfield, 2016); it seems unlikely that 6 days of mild, repeated stressors would have elicited this response. Curiously, in other studies of house sparrows, uric acid has been shown to decrease both in response to captivity (Gormally et al., 2019a) and 4 days of a single repeated stressor (Gormally et al., 2018), suggesting that 'chronic stress' may not always result in elevations of the molecule. These prior results, in conjunction with the present study, suggest that uric acid changes in unique ways depending on the specific stimuli being tested. Note also that, as with Cort, it currently is not known whether increases in uric acid are reflective of the body attempting to cope with deviations from homeostasis, or if instead the elevations themselves are indicative of pathologies (Costantini, 2008). Whatever the underlying cause of the increase in uric acid seen here, recovery periods reverted the levels to pre-experiment conditions.

Recovery time also affected DNA damage, which was measured in the nucleated red blood cells. As expected, the repeated stressors increased damage (white bars, Fig. 3E). DNA damage has rarely been studied in direct connection to repeated stressors, however one prior study found that introduction to captivity significantly elevated damage in house sparrows within 3 days (Gormally et al., 2019a). This damage could be a result of a number of different mechanisms, including altered DNA protection and repair (Feng et al., 2012; Hara et al., 2011) or exposure to free radicals (Bayir, 2005). Recovery time had unexpected effects on DNA damage, with birds that had 1 day of recovery exhibiting significantly elevated damage relative to those in the 3-day group, but not the 6-day group. The 6-day group also had a significantly non-unimodal distribution (Fig. 4), a pattern that has been seen in other chronic stress situations (Gormally et al., 2019a). This would suggest that, as with the Cort data, there is a minimum threshold below which recovery could actually be disruptive. The mechanisms that are causing this relationship between stressors, recovery, and DNA damage have not yet been elucidated, but likely involve the temporal intricacies of red blood cell removal and replacement.

Finally, perch hopping behavior was not significantly affected by 6 days of repeated stressors, nor were there any differences between recovery groups (Fig. 3F). In prior studies of house sparrows, shorter periods of repeated stressors elicited decreases in both perch hopping activity and neophobic behavior (Gormally et al., 2018; Gormally and Romero, 2018). Other studies have shown that acute Cort administration directly upregulates hopping in other passerines (Breuner et al., 1998). The differences in this study could again reflect the variety of responses that particular stimuli elicit.

4.3. Prior recovery causes system-specific responses to additional repeated stressors

As with the first part of the experiment, recovery time differentially impacted responses to an additional 6 days of random stressors depending on each system. Baseline and stress-induced Cort still were not significantly affected by this set of stressors, nor was negative feedback strength (Fig. 4A,B). Perhaps if the experiment had persisted for longer, statistical significance would have emerged. It seems likely that for this species, 12 days was at the cusp of significance. Finally, the disruptive impacts that were seen in the 1-day group (Fig. 2B,C) seem to disappear after the birds experience an additional round of stressors.

Immune function tended to decrease in all groups relative to the Pre-Stress Control sample, with the birds in the 0 and 6-day groups showing significant changes (Fig. 5C). Changes in immune function are commonly associated with stress (reviewed by Martin, 2009). On an acute time scale (hours to days), stress (and specifically glucocorticoids) have been shown to enhance immunity by redistributing immune cells (Dhabhar, 2002; Kuhlman and Martin, 2010) and stimulating the production of cytokines including interferon gamma and interleukins 1 and 6 (Bulloch, 2001; Dhabhar et al., 2000; Spencer et al., 2001). On a chronic time scale (weeks to months), however, stress is often associated with immunosuppression (Cyr et al., 2007; Dhabhar and McEwen, 1999, 1997; Gao and Deviche, 2019; Gormally et al., 2018; McCormick et al., 2015). Despite this seemingly clear biphasic response, many studies involving wild animals often find different results depending on what sort of chronic stressors are being tested (e.g. captivity, repeated stressors) and which test of immune function is used (e.g. bacterial killing assay, hemagglutination assay, inflammatory response), suggesting more complex underlying mechanisms are involved. In the present study, the results suggest that while repeated stressors resulted in a reduction of killing capacity, longer recovery times decrease while shorter periods enhance immune function. Additional work needs to be done to assess whether this is a biologically relevant difference.

Though uric acid was significantly elevated after the initial 6 days of repeated stressors (Fig. 3D), there were no differences between the recovery groups at the final sample (Fig. 5D). Even in birds that experienced 12 consecutive days of stressors, uric acid remained at pre-experiment levels, approximately 5 mg/dL. Avian species tend to have substantially higher levels of uric acid relative to mammals (Costantini, 2008), and house sparrows have some of the highest levels among that taxon (Gavett and Wakeley, 1986; Harr, 2002). The levels reported here and in other studies of house sparrows in captivity are, on average,

lower than those of wild birds (Gormally et al., 2019a, 2018). Without measuring other aspects of the antioxidant system (e.g. total antioxidant capacity, enzymatic antioxidants), we cannot be certain whether the changes—or lack thereof—in uric acid are reflective of changes in nutrient source or of redox balance. Our data suggest that the transient increase in uric acid (Fig. 3D) supports the latter hypothesis because it implies that the birds were attempting to cope with free radical production; however this enhancement could not be sustained, which is why at the final sample there were no longer differences between the recovery groups (Fig. 5D). If this interpretation were correct, we would expect that continued repeated stressors would again elicit a transient increase in uric acid in the birds regardless of exposure to recovery periods.

This hypothesis is partially supported by the DNA damage data, which suggests that there could have been a larger prevalence of free radicals after 6 days of repeated stressors (white bars, Fig. 3E). After recovery and an additional 6 days of stressors, the recovery groups do differ, but in an unexpected way. The birds stratify from least to most damaged, from shortest to longest recovery (Fig. 5E). The opposite trend was expected as recovery time was thought to provide time for the damaged red blood cells to be removed from circulation through erythrophagocytosis and be replaced with new, young, undamaged cells (Arias and Arias, 2017). We do not yet have enough of an understanding of whether or how red blood cell DNA is repaired, on what timescale damaged red blood cells are removed, and how both these factors interact with external stressors.

Finally, though behavior was not initially affected by the first part of the experiment, after birds experienced the entire course of stressors they were significantly more active than those that had any recovery at all (Fig. 5F). Therefore, 6 days was not long enough to induce a change in behavior, but 12 days was; and having recovery between rounds of stressors resulted in avoidance of this effect. Few studies have examined perch hopping as it relates to artificially induced stress (e.g. not direct Cort administration), but it's possible that this increase could correlate with the birds becoming more anxious. Finally, housing condition influenced perch hopping activity with birds housed in pairs tending to be more active than those housed alone. This effect was only detected following the repeated stress periods, but not following recovery, suggesting activity might be more sensitive to housing conditions during challenging periods.

5. Conclusions

In this study we showed that permitting house sparrows recovery periods in between bouts of repeated stressors can alter the impacts of those stimuli. Of particular interest were the stark differences between the various physiological and behavioral responses measured here. The HPA axis regulation, immune function, antioxidant levels, DNA damage, and activity each changed on distinct timescales in response to both the repeated stressors and recoveries. This is not surprising, however, since the stress systems interact with and influence many aspects of physiology; even the metrics that were chosen here do not represent the full spectrum of the effects of the stress response generally and glucocorticoids more specifically. The concept of interdisciplinary approaches to questions about stress are not new, however these data further emphasize that very different conclusions can be made depending on which parameters are measured in an experiment. Finally, we feel it is important to acknowledge that there are many different options when choosing physiological metrics. Though the ones examined here represent a broad spectrum, even more measurements are likely be necessary to get a fully complete characterization of a chronically stressed animal.

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