1 **Title:** A test of reactive scope: reducing reactive scope causes delayed wound healing.

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Abstract:

Reactive scope predicts that all animals have an adaptive ability to respond to stressors in their environment, termed reactive homeostasis, and that only when an animal's response to stressful stimuli exceeds a certain threshold (homeostatic overload) will stress have pathological effects. While this framework has successfully helped interpret effects of stressors on wildlife, no study has designed an experiment to directly test this framework. This study was designed to expose house sparrows (*Passer domesticus*) to treatments that would result in varying ranges of reactive homeostasis during chronic stress, which based on the reactive scope model should cause birds with the lowest reactive homeostasis range to exhibit signs of pathology during a subsequent challenge. To modulate the reactive homeostasis range, we altered allostatic load of birds by exposing them to chronic stress while either elevating, blocking, or not manipulating corticosterone. After concluding chronic stress treatments, birds were exposed to the subsequent challenge of a superficial wound. Individuals treated with corticosterone during chronic stress (high allostatic load) experienced the most pathology, including both weight loss and slower wound healing. Unmanipulated birds (medium allostatic load) also experienced weight loss but had normal healing rates, while birds with blocked corticosterone (low allostatic load) had minimal weight loss and normal healing rates. Our results indicate that increased allostatic load reduces the reactive homeostasis range, thereby causing individuals to cross the homeostatic overload threshold sooner, and thus support the reactive scope framework.

1. Introduction

Reactive scope provides a framework for understanding how and when stress may have pathological effects on an individual (Romero et al., 2009). Animals all have the capacity to respond to stress, termed reactive homeostasis in the reactive scope model, an adaptive process whereby an organism modulates physiological variables to respond to unpredictable and/or threatening changes in their environment. The reactive scope model proposes that only when an organism's responses are pushed outside their reactive homeostasis range and into homeostatic overload does stress begin to have pathological effects (Romero et al., 2009). Thus, a reduction in an animal's reactive scope, or reactive homeostasis range, should increase the likelihood that a subsequent stressor will push the animal into homeostatic overload and induce pathology. To put this in terms of a physiological variable associated with responses to unpredictable stimuli, like glucocorticoids, an organism needs varying circulating concentrations of glucocorticoids throughout the day or season to maintain basic physiological processes, termed predictive homeostasis in the reactive scope model. Upon exposure to a stressful stimulus an organism

can increase glucocorticoid concentrations (i.e., reactive homeostasis). Although these transient increases in glucocorticoids are adaptive, repeated or chronic exposure to stress can cause pathology by reducing the reactive homeostasis range, causing normal glucocorticoid responses to stress to push an animal into homeostatic overload.

Many factors can constrain an individual's reactive scope. For instance, as physiological parameters of an animal vary over the season or throughout the day, referred to as predictive homeostasis, so does the animal's ability to cope with stress in their environment (Romero et al., 2009). An animal would have a reduced reactive scope when a physiological variable was high (e.g., increased metabolic rate; Cyr et al., 2008). Another possibility for altering an individual's reactive scope is a decrease in the threshold at which an animal is pushed into homeostatic overload. A reduction in the threshold for homeostatic overload can occur when a prior event, such as an early developmental experience, has long term consequences. For instance, reduced grooming early in life increased stress-induced corticosterone in rat pups resulting in individuals that are more fearful later in life (Meaney, 2001; Weaver, 2004). A reduction in the homeostatic overload threshold (i.e. decreased reactive scope) can also be temporary, decreasing as an organism is faced with a persistent stressor in their environment (e.g., low food availability; Romero, 2012) and then recovering once the stressor is removed. If an animal is exposed to a subsequent stressor while reactive scope is decreased, they are more likely to be pushed into overload, but less likely if they have sufficient time to recover from the initial stressor.

Although numerous studies have discussed their findings in the context of reactive scope (198 studies to date based on a web of science search), no study has empirically tested this framework. In this study, we designed an experiment to test the reactive scope model. We pre-treated three groups of house sparrows (*Passer domesticus*) with different protocols that the reactive scope model predicts would result in different magnitudes of a decreased threshold to enter homeostatic overload. In other words, at the end of the pre-treatment period, each group should have a different range of reactive scope. Then, each group was challenged with the identical stressor of a subcutaneous wound. If the reactive scope model is correct, then only the treatment with the smallest reactive scope (i.e. lowest threshold) will enter homeostatic overload during the period of wound healing (Fig. 1). To produce treatments with differing reactive scopes we manipulated allostatic load of birds by exposing them to chronic stress while also manipulating corticosterone concentrations. Allostatic load is the summation of an individual's cost to live, including physiological and behavioral factors (e.g., circulating hormones, metabolic rate, social interactions, foraging, etc.), and life history events, like reproduction or molt

(McEwen and Wingfield, 2003). During chronic stress we artificially elevated corticosterone in one treatment group (high allostatic load), blocked corticosterone production in another treatment group (low allostatic load), and did not manipulate corticosterone in the control treatment group (moderate allostatic load). After a two day recovery from our chronic stress protocol (described below), we exposed all treatments to a subsequent increase in allostatic load, specifically a small superficial wound. Stress is known to impact immune processes; acute stressors often have stimulatory effects, while chronic stressors or more severe stressors have inhibitory effects (Dhabar, 2003, 2009; Dhabar and McEwen, 1999; Carter et al., 2013). We predicted that individuals with the highest allostatic load would cross the homeostatic threshold and experience reduced healing and exhibit other indicators of chronic stress (e.g., weight loss), whereas individuals with the lowest allostatic load would not cross this threshold, exhibiting normal healing and no deleterious effects of chronic stress (Figure 1).

2. Materials and Methods

2.1 Study species, trapping, and animal husbandry. We used house sparrows in this study because they are invasive to North America and also have been a popular model organism for stress physiology studies (e.g., Hegner and Wingfield, 1987; Kuhlman and Martin, 2010; Martin et al., 2011; Lattin et al., 2012a, 2012b, 2014). Twenty-four wild house sparrows were captured with baited Potter traps in Medford, MA in May, 2013. Birds were taken to indoor facilities and housed in mixed sex or female only pairs based on treatment groups in 45 cm x 37 cm x 33 cm cages. All cages were housed in the same room. Sparrows were held on a 14L:12D light cycle at 22 °C with food and water available ad libitum. Birds were allowed at least 2 weeks to acclimate to captivity before starting the experiment. Birds were fed on a mixed seed diet (Supreme Wild Bird Mix, Lebanon Seaboard Corporation) and were also provided grit. All experiments were approved by the Institutional Animal Care and Use Committee at Tufts University and were carried out according to the guidelines of the Association for Assessment of Laboratory Animal Care

2.2 Experimental design and timeline. To understand how an individual's reactive scope affects their likelihood of entering homeostatic overload, we manipulated corticosterone concentrations of birds during exposure to a 10 day chronic stress protocol and monitored changes in body mass, corticosterone, and their ability to handle a subsequent increase in allostatic load—wound healing (Figure 2). Based on assumptions of the reactive scope model, we hypothesized that the 10 days of

chronic stress would gradually reduce the threshold at which animals enter homeostatic overload (Fig. 1). The degree of underlying allostatic load will then alter how likely an individual animal is to enter homeostatic overload. To augment allostatic load (thereby reducing reactive scope) we increased circulating corticosterone in the corticosterone-elevated treatment group (n=8; 5F and 3M) (described below). We ameliorated allostatic load (thereby increasing reactive scope) by blocking corticosterone release in the corticosterone-blocked treatment group (n=7; 4F and 3M). Finally, we did not manipulate allostatic load (resulting in moderate reactive scope from the chronic stress protocol) in control birds (n=8; 6F and 2M). Using previously-established methods, corticosterone-elevated birds received dermal application on the neck between the feather tracts of 20 µg corticosterone dissolved in 20 µL DMSO twice daily throughout the duration of chronic stress (Schallin-Busch et al., 2008). To block secretion of corticosterone we injected 180 mg/kg body weight mitotane (ortho, para-DDD); dissolved in peanut oil into the pectoralis muscle of birds daily for 3 days (Breuner et al., 2000 Lattin et al., 2012, 2015) prior to the start of chronic stress, and again on day five of the chronic stress protocol. Control and CORT treated birds received injections of peanut oil. To ensure our treatments were effective, we measured baseline and stress-induced corticosterone of birds at the start of the chronic stress protocol, on day five of the chronic stress protocol, two days after the 10 day chronic stress protocol ended (day 12 of the experiment), and again 12 days later (day 24 of the experiment; see Figure 2 for a timeline). Blood samples were collected before daily corticosterone administration in the corticosterone-elevated group. Two days after we ended the chronic stress protocol we wounded birds and monitored wounds until healing was completed. This two day interval would allow us to measure how our manipulations during chronic stress altered the birds' ability to respond to a new challenge, avoiding any direct effects of chronic stress on wound healing. Wound healing in adult passerines takes ~11-12 days, thus our final blood sample should have corresponded with the completion of healing in our control birds (House Sparrows: Love et al., In prep; Lendvai unpub data; European starlings: DuRant et al., 2016). One mitotane-treated individual died for unknown reasons just before birds were wounded.

2.3 Chronic Stress Protocol. We used a well-established method to create chronic stress (e.g., Cyr et al., 2007; Kostelenatz et al., 2009; Rich and Romero, 2005). Briefly, we exposed birds to four different 30 minute stressors four times a day, with stressors spaced 2 or more hours apart. In addition, every day we randomized the order and time at which birds were exposed to each stressor to prevent habituation. Our four stressors consisted of 1) tapping on cages, 2) crowding four birds into one cage, 3) playing loud music, and 4) exposure to human presence and voice. Birds were exposed to the protocol for 10 days,

and effects of chronic stress on hormonal profiles are detectable within 8-12 days of exposure (Rich and Romero, 2005).

2.4 Blood collection and stress series. Between 0905 and 0920 hours we collected baseline samples from birds in < 3 minutes (Romero and Reed, 2005), then placed birds in a cloth bag and 30 minutes later collected a stress-induced sample. We collected samples from birds just before starting the chronic stress protocol (day 1), five days after the start of the stress protocol (day 5 – just prior to injecting with mitotane), on the day birds were wounded (day 12—two days after the stress protocol was terminated), and again twelve days later (day 24). Blood samples were always collected prior to treatment administration (corticosterone, mitotane) for that day. We kept samples on ice (~1-2 hr) until we could centrifuge blood to separate plasma, and then stored the plasma at -20°C.

2.5 Body mass. We weighed birds to the nearest 0.5 g eight days prior to the start of the chronic stress protocol, on the day the protocol began, and every three to four days until 36 days after the start of the stress protocol.

2.6 Wound healing. Two days following the end of the chronic stress protocol, we administered a small superficial wound to the left thigh of all birds using a 4mm biopsy punch (Miltex 33-34, York PA) following the methods of (Carter et al., 2013; French et al., 2006). Birds were anesthetized using isoflurane prior to the biopsy. We photographed wounds daily along-side a ruler and determined wound size (mm²) by tracing the perimeter of the wound and calculating its area using Image J software (NIH).

2.7 Radioimmunoassay. We determined plasma concentrations of corticosterone in our samples using radioimmunoassay (RIA) techniques following extraction based on the methods of Wingfield et al. (1992). To determine sample extraction efficiency we equilibrated each sample overnight with 2000 cpm of tritiated steroid. Each sample was extracted with 4 mL of redistilled dichloromethane, dried using nitrogen gas and re-suspended in 550 µL of phosphate buffered saline. We determined individual extraction efficiency using 100 µL of the sample (mean recoveries were 70%). For the assay we allocated 200 µL of the sample to each of two duplicates. We then compared duplicates to a standard curve, which contained known amounts of corticosterone, run with each assay. Inter- and intra-assay variation were 20.6 and 5.1%, respectively, as determined by running standards in each assay.

2.8 Statistical Analyses. All statistical analyses were run in SAS 9.1 (SAS Institute Inc., Cary, NC, USA) and statistical significance was recognized at $\alpha < 0.05$. Where appropriate, we tested for normal distribution of the data and homoscedasticity using Anderson-Darling and Bartlett's tests, respectively. Unless

otherwise noted, raw data were used in statistical models. We used four separate repeated measures ANOVAs (Proc Mixed) to test for the effects of treatment, time, and their interaction on house sparrow body mass, log₁₀-transformed wound size, baseline corticosterone and stress-induced plasma corticosterone concentrations of birds. We tested for the effect of corticosterone manipulation on the time it took for birds to fully-heal wounds using an ANOVA (Proc GLM).

3. Results

3.1 Corticosterone. Baseline and stress-induced corticosterone differed among treatments over the course of the experiment (Figure 3A-C; Baseline: treatment X day: $F_{6,60} = 8.86$; $p < 0.001$; Stress-induced: treatment X day: $F_{6,60} = 3.83$; $p = 0.003$). Baseline and stress-induced plasma corticosterone were undetectable in mitotane birds at the start of the chronic stress protocol, whereas control and corticosterone-treated birds had baseline concentrations of 3.3 ± 0.99 and 1.07 ± 0.59 ng/ml, respectively, and stress-induced concentrations of 7.73 ± 3.2 and 2.63 ± 1.2 ng/ml, respectively. During the chronic stress protocol baseline concentrations remained low in mitotane-treated birds (2.3 ± 1.1 ng/ml) relative to controls (5.2 ± 2.6 ng/ml), but these birds were able to mount a stress-response (15.6 ± 8.8 ng/ml) similar to control birds (11.1 ± 3.14 ng/ml). When wounding occurred mitotane-treated birds had similar baseline corticosterone concentrations as control birds, and they continued to be able mount a stress response. Baseline and stress-induced corticosterone of corticosterone-treated birds increased relative to control birds once dermal application began and were returning to baseline concentrations elevated 24 days later.

3.2 Body mass. Body mass of birds varied over the course of the experiment and were affected by treatment (Figure 4; treatment: $F_{2,20} = 15.75$, $p < 0.001$; time: $F_{12,90} = 3.03$, $p = 0.001$; treatment X time: $F_{24,90} = 0.41$, $p = 0.99$). In general, birds with blocked corticosterone release were heavier than control and corticosterone-treated birds. All birds experienced some weight loss during chronic stress and just after wounding, but this was more pronounced in control and corticosterone-treated birds.

3.3 Wound healing. Initially, wound healing progressed similarly among all birds, but birds treated with corticosterone healed more slowly later in the healing process (Figure 5A; treatment X time: $F_{38,376} = 1.54$, $p = 0.024$). Although birds treated with exogenous corticosterone took ~ 2 days longer to completely heal wounds, (15.1 ± 1.0 days) than control and mitotane treated birds (control: 13.0 ± 0.3 days,

mitotane: 12.9 ± 0.6), there was not a strong statistical influence of treatment on days to fully heal (Figure 5B; treatment: $F_{2,20} = 2.71$, $p = 0.090$).

4. Discussion

Our data provide support for the reactive scope model. The purpose of this study was to test whether chronically-elevated allostatic load could change the threshold at which homeostatic overload (mass loss, decreased wound healing rate) occurred. Our findings support this idea, as we found that individuals with the greatest allostatic load, birds treated with corticosterone during chronic stress, experienced more severe weight loss and slower healing. However, individuals with the lowest allostatic load during chronic stress, birds with blocked corticosterone release, experienced transient and less severe weight loss and normal healing. Control birds, which should have experienced moderate allostatic load, exhibited similar weight loss as corticosterone-treated birds and normal healing. It is important to keep in mind that corticosterone manipulation and chronic stress ended 2 days prior to wounding, implying that results were a consequence of altered allostatic load and not direct effects of corticosterone.

The findings of our current study and the reactive scope framework help explain why exposure to chronic stress did not impair healing in birds in a previous study (DuRant et al., 2016). In that study, European starlings (*Sturnus vulgaris*) were exposed to chronic stress for 28 days and wounded on the eighth day of the protocol, but chronic stress did not alter healing rates but did cause weight loss. Allostatic load may not have been sufficiently increased in chronic stress birds compared with controls at the time of wounding, thus chronic stress birds never sufficiently surpassed the homeostatic threshold to cause both weight loss and reduced healing. Importantly, birds used in the starling study also had similar healing rates as control birds and corticosterone-blocked birds of the current study, approximately 11-12 days, further supporting that allostatic load was not sufficiently elevated in the starlings to impair healing. It is not surprising that the current study and the starling study found that chronic stress does cause weight loss, the most consistent response to chronic stress across organisms and contexts (Dickens and Romero, 2013). Currently, it is not clear why weight loss occurs before other pathological effects, like impaired healing, but may be the result of reduced feeding, or increased metabolic rates (Martí et al., 1994; Lankford et al., 2005; Cyr et al., 2008). Finally, our data also fit nicely with the previously documented relationship between high baseline corticosterone and slower healing, and poor negative feedback and slower healing (DuRant et al., 2016) found in the starling study.

Starlings with high baseline corticosterone and reduced negative feedback ability were likely exposed to more corticosterone during chronic stress, thus increasing their allostatic load and reducing reactive scope, which caused slower healing.

As indicated by previous studies, corticosterone may be important to the healing process in wild animals (French et al., 2006; DuRant et al., 2016), including birds, since individuals with low baseline corticosterone and/or robust negative feedback of corticosterone release before starting chronic stress healed wounds faster than birds with high baseline corticosterone and/or poor negative feedback ability. We did not have a large enough sample size in our control group to test for this effect in the current study. However, exogenous application of corticosterone to birds in this study did result in elevated corticosterone concentrations and decreased healing rates, even though blocking of corticosterone did not enhance healing. It is still unclear whether corticosterone is having a direct or indirect effect on healing, but it is important to note that the corticosterone manipulations ended well before wounding took place. Circulating corticosterone could directly alter healing through its interactions with immune related cells and effects on inflammation (Rabin, 1999; Planey and Litswack, 2000; Sternberg, 2006). Alternatively, direct effects of corticosterone on healing may operate on a more localized level, for instance, through localized changes in skin corticosterone production (Taves et al., 2011; Vukelic et al., 2011) or receptors in the skin that bind to corticosterone (Tiganescu et al., 2014; Lattin et al., 2015) and independent of plasma corticosterone concentrations. It is important to note that blocking of corticosterone was no longer apparent at the time of wounding; therefore, any direct positive effects of reduced corticosterone on healing may have no longer been present.

Existing evidence (French et al., 2006; DuRant et al., 2015; Love et al., In prep) and the results of this study suggest that elevated corticosterone release during stressful events may inhibit an individual's ability to cope with subsequent stressors, particularly immune-related stressors (but see McCormick and Langkilde, 2014). Thus, individuals or populations of individuals with higher circulating corticosterone due to low resource availability (Romero and Wikelski, 2001; MacLarnon et al., 2014), altered developmental conditions (Kitasky et al., 2006; Meaney, 2001), predation (Boonstra, 1998; 2014), social subordination (Virgin and Sapolsky, 1997), etc., are at greater risk of experiencing pathological effects of stress. Finally, our study provides the first empirical support for the reactive scope framework. The results of other studies have fit nicely within the framework (e.g., Romero, 2012; MacLarnon, 2015; DuRant et al., 2016), and indicate it may be useful in assessing physiological constraints or consequences preventing animals from accessing certain habitats (e.g., MacLarnon, 2015), or faring well in severe

277 climatic events (e.g., Romero, 2012) or during periods of high allostatic load (DuRant et al., 2016). Taken
278 together, those studies and the current experiment underscore the utility of the reactive scope
279 framework in understanding how adaptive responses to stress can ultimately result in pathological
280 effects in an individual.

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Figure 1: Conceptual portrayal of study within context of the reactive scope model. A conceptual figure depicting the primary components of the reactive scope model in relation to our experimental design. Predictive homeostasis represents the range (due to diel or seasonal rhythms) at which various mediators (e.g. hormones, metabolic rate, etc.) vary, the grey bar represents the theoretical allostatic load of birds in this study in the absence of stress. Reactive homeostasis is the range in which an organism can alter a physiological mediator to respond to a stressful stimulus without experiencing any deleterious effects of stress. Homeostatic overload represents the threshold at which a response to stress becomes deleterious. In this study, we manipulated reactive scope of our treatments by altering allostatic load of birds *via* exposure to chronic stress while simultaneously either increasing circulating corticosterone (high allostatic load), blocking corticosterone release (low allostatic load), or leaving corticosterone unmanipulated (moderate allostatic load; controls). The reactive scope model proposes that the threshold to entering homeostatic overload would steadily decrease over the 10 day course of chronic stress, rebound once the stressors are removed, then decrease again once the subsequent challenge, healing a wound, was applied. Groups with higher allostatic load should experience deleterious effects during the subsequent challenge, whereas those with lower allostatic load should not.

Figure 2: Experimental timeline. Corticosterone was blocked using mitotane injection in one group of birds, elevated in a second group of birds via exogenous application of corticosterone, and unmanipulated in a third group (controls) during a 10 day exposure to a chronic stress protocol (CSP). Two days after chronic stress, birds in all treatments were wounded. Blood samples to assess baseline and stress-induced corticosterone concentrations were taken just before and during the CSP, at the time of wounding and at the end of the healing process, which takes ~ 12 days in adult passerines. Body mass was monitored throughout the study until all birds had healed wounds (36 days after starting the CSP).

Figure 3A-C: Plasma corticosterone concentrations vary over time and across treatments. Baseline and stress-induced plasma corticosterone (means $\pm$ 1 SE) of house sparrows (*Passer domesticus*) exposed to a chronic stress protocol (CSP; represented by the gray bars) for 10 d, and then given a small superficial wound (n = 7-8 birds/treatment), which is indicated by the arrow. A) Corticosterone during the CSP was unmanipulated in control birds B) blocked using mitotane injections in cort-blocked birds, and C) artificially-elevated in cort-elevated birds via dermal corticosterone application. Mitotane treatments were administered for three days prior to the start of the CSP, and again on day 5 of the CSP. Corticosterone application occurred daily during the 10 day CSP. Note difference in scale of y-axis.

Figure 4: Body mass varies across time and treatments. Changes in body mass (means $\pm$ 1 SE) of house sparrows (*Passer domesticus*) exposed to a chronic stress protocol (CSP; indicated by the gray bar) for 10 days (n = 7-8 birds/treatment), and then given a small superficial wound (indicated by the arrow). During the CSP, corticosterone release was decreased using mitotane in one group (cort-blocked), artificially increased using exogenous corticosterone application in a second group (cort-elevated), or unmanipulated in the control group. Mitotane treatments were administered for three days prior to the start of the CSP, and again on day 5 of the CSP. Corticosterone application occurred daily during the 10 day CSP. The dotted line represents average body mass of birds at prior to beginning the experiment.

Figure 5: Wound size and healing rates of house sparrows. (A) Change in wound size (means $\pm$ 1 SE) and (B) the number of days (means $\pm$ 1 SE) needed for house sparrows (*Passer domesticus*) to heal a small superficial wound. Birds (n = 7-8 birds/treatment) were wounded immediately following 10 days of exposure to a chronic stress protocol. During the chronic stress protocol, corticosterone release was decreased using mitotane in one group (cort-blocked), artificially increased using exogenous corticosterone application in a second group (cort-elevated), or unmanipulated in the control group.

Figure 1

Figure 2

Figure 2.

Figure 3

Figure 4

Figure 5