Wound-healing ability is conserved during periods of chronic stress and costly life history events in a wild-caught bird.

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Abstract

Chronic stress, potentially through the actions of corticosterone, is thought to directly impair the function of immune cells. However, chronic stress may also have an indirect effect by influencing allocation of energy, ultimately shifting resources away from the immune system. If so, the effects of chronic stress on immune responses may be greater during energetically-costly life history events. To test whether the effects of chronic stress on immune responses differ during expensive life history events we measured wound healing rate in molting and non-molting European starlings (*Sturnus vulgaris*) exposed to control or chronic stress conditions. To determine whether corticosterone correlated with wound healing rates before starting chronic stress, we measured baseline and stress-induced corticosterone and two estimates of corticosterone release and regulation, negative feedback (using dexamethasone injection), and maximal capacity of the adrenals to secrete corticosterone (using adrenocorticotropin hormone [ACTH] injection). After eight days of exposure to chronic stress, we wounded both control and chronically stressed birds and monitored healing daily. We monitored nighttime heart rate, which strongly correlates with energy expenditure, and body mass throughout the study. Measures of corticosterone did not differ with molt status. Contrary to work on lizards and small mammals, all birds, regardless of stress or molt status, fully-healed wounds at similar rates. Although chronic stress did not influence healing rates, individuals with low baseline corticosterone or strong negative feedback had faster healing rates than individuals with high baseline corticosterone or weak negative feedback. In addition, wound healing does appear to be linked to energy expenditure and body mass. Non-molting, chronically stressed birds decreased nighttime heart rate during healing, but this pattern did not exist in molting birds. Additionally, birds of heavier body mass at the start of the experiment healed wounds more rapidly than lighter birds. Finally, chronically stressed birds lost body mass at the start of chronic stress, but after wounding all birds regardless of stress or molt status started gaining weight, which continued for the remainder of the study. Increased body mass could suggest compensatory feeding to offset energetic or resource demands (e.g., proteins) of wound healing. Although chronic stress did not inhibit healing, our data suggest that corticosterone may play an important role in mediating healing processes and that molt could influence energy saving tactics during periods of chronic stress. Although the experiment was designed to test allostasis, interpretation of data through reactive scope appears to be a better fit.

1. Introduction

Chronic stress can cause behavioral and physiological effects in organisms that have implications for survival and reproduction (McEwen and Wingfield, 2003; Romero et al., 2009; Sapolsky et al., 2000). Several frameworks have been proposed to understand when stress will have negative consequences for animals, and the literature provides examples that support and refute these frameworks (Cyr et al. 2008; DuRant et al. 2008; Romero et al. 2000; Romero and Wikelski 2001, 2010). One framework, the allostatic overload model, is rooted in energy expenditure and availability, and predicts that stress will cause pathology in an organism when the energetic cost of the stressor and the animal's basic maintenance costs (termed allostatic load) become too great an energetic burden (McEwen and Wingfield 2003). The allostasis model thus predicts that the ability of an individual to withstand stress should vary with their life history stage, such that life history stages associated with increased energy expenditure (e.g., reproduction, or molt in birds) should also correspond with a reduction in an animal's ability to cope with stress. An alternative framework, reactive scope, posits that an organism has a range in which various physiological mediators of stress can increase or decrease (e.g., heart rate, corticosterone, etc.) and only when an organism fluctuates outside this range, termed the reactive homeostasis range, does an animal experience pathology from stress (Romero et al. 2009). Thus reactive scope allows a suite of variables, including energy-associated variables, to cause or protect an individual from stress-induced pathology. For instance, marine iguanas with robust negative feedback of corticosterone (the primary glucocorticoid in reptiles and birds) release were more likely to survive famine than individuals with poor negative feedback ability (Romero and Wikelski, 2010). Shutting down corticosterone presumably prevented these iguanas from moving outside the reactive homeostatic range.

One of the pathological effects often associated with chronic stress, is reduced immune function. (e.g, Dhabar, 2006, 2014; French et al., 2006; Segerstrom and Miller, 2004; Walburn et al., 2009). In humans, periods of intense stress (e.g., nursing a parent or spouse through chronic illness) are often followed by the development of disease (Fonareva, and Oken, 2014; Tsukamoto & Machida, 2014). Although relationships between chronic stress and immune function are less well studied in wild populations of animals (Martin, 2009), existing studies suggest that chronic stress can negatively impact immune function of wild animals (Archie, 2013; Archie et al. 2012; French et al., 2006; Kinsey et al., 2003; Morales et al., 2004). Stress could directly impact the immune system through the actions of glucocorticoid hormones (Sternberg, 2006), which are known to alter immune function (Apanius, 1998;

Dhabhar, 2002; Sternberg, 2006). Tissues and cells associated with immune processes contain glucocorticoid receptors (Rabin, 1999; Planey and Litswack, 2000), and glucocorticoids have well-documented anti-inflammatory effects (Sternberg, 2006). Glucocorticoids can inhibit production of some T-helper cells which are important in adaptive immune responses, and suppress maturation and proliferation of most immune cells associated with innate immunity by inhibiting gene expression (Sternberg, 2006). Negative feedback of glucocorticoid release influences how long an individual is exposed to elevated glucocorticoids, and has been associated with higher rates of survival during chronic stress events (Romero and Wikelski, 2010). Thus, high negative feedback efficacy could reduce the negative effects of chronic stress on physiological processes like immune responses.

Chronic stress may also indirectly influence immune processes by altering energy expenditure and allocation, an idea that fits nicely within the allostatic overload model. Corticosterone is important in liberating energy stores (Sapolsky et al., 2000), and during times of stress, energy may be allocated to immediate survival needs and away from other energetically costly behavioral or physiological processes (Wingfield et al., 1998; McEwen and Wingfield, 2003). Thus, physiological processes like immune responses may be reduced in times of chronic stress as an energy-saving mechanism, or because of energetic constraints induced by chronic stress (Apanius, 1998). Research suggests that immune responses can upregulate energy expenditure, increase protein and lipid breakdown, and are greater in individuals of better condition (Hasselgren and Fischer, 1998; Lochmiller and Deerenberg, 2000; Martin et al., 2003, 2008; Navarro et al., 2003). If immune processes are down-regulated during periods of chronic stress to help conserve energy that can be allocated towards immediate survival needs (Segerstrom and Miller, 2004), then costly life-history events with high maintenance costs (i.e., high allostatic load) could exacerbate immune-stress interactions.

In birds, molt is a particularly costly life-history event and is one of the most physiologically-costly processes birds undergo (Klaassen, 1995; Lindstrom et al., 1993). Molt has been shown to alter physiological responses to chronic stress, and increase nighttime energy expenditure by 60% and protein synthesis by 3.5 fold (Murphy, 1996; Cyr et al., 2008; Kostelanetz et al. 2009). During molt there is increased deposition of keratins and increased protein synthesis in the liver, muscle, and whole body (Murphy and Taruscio, 1995). Thus, based on the allostatic overload type I model whereby the sum of energetic requirements exceeds available energy, a molting bird, which has increased basic maintenance costs, may attempt to cope with chronic stress by mounting a weaker immune response compared to a non-molting bird (McEwen and Wingfield, 2003).

To test whether chronic stress differentially affects immune processes in relation to life-history events, we measured wound healing, an integrative measure of immune function (French et al. 2006), in molting and non-molting European Starlings either exposed to chronic stress or control conditions (Table 1). We predicted that chronic stress and molt would have negative and additive effects on wound healing. We also examined the role energy expenditure plays in mediating immune-stress interactions by measuring nighttime heart rate, which strongly correlates with energy expenditure (Cyr et al., 2008), throughout the study. Finally, we measured various components of corticosterone release and regulation prior to wounding to determine whether they predicted wound healing ability. Specifically, we hypothesized that individuals with lower baseline corticosterone and better negative feedback ability would heal wounds more quickly than birds with higher corticosterone and less robust negative feedback ability (Romero and Wikelski 2001; 2010).

2. Materials and methods

2.1 Study species and animal husbandry and collection. We used adult European starlings (*Sturnus vulgaris*, Linneaus) in this study, because they were well-suited for studies of chronic stress. Physiological changes associated with stress in both wild-caught and free-living starlings are well understood, including heart rate and hormonal responses to acute and chronic stress. In addition, the chronic stress protocols described below successfully induce chronic stress in starlings (e.g., Cyr et al., 2007, Kostelenatz et al., 2009; Rich and Romero, 2005). Starlings have also been the focus of several laboratory studies regarding molt (Cyr et al., 2008; De Bruijn and Romero, 2013; Dawson, 2003; Kostelenatz et al., 2009).

We trapped wild starlings on the Tufts University, Medford campus and in surrounding neighborhoods. Birds used in this study were captured at different times throughout the year. Due to logistical constraints of the heart rate software we could only conduct the experiment on 8 birds at a time. The first round of birds were captured in January-February 2012, the second round of birds were captured in May-June 2012, the third round of birds were captured in August 2012, and the last round was captured in September-October 2012. All birds had at least 2 weeks to acclimate to captive conditions, and time to acclimate varied across birds. For each round of the experiment we allocated similar numbers of birds to the control and chronic stress treatment. For birds in the molt treatment, half of the molting birds had begun molting naturally, while we stimulated the remaining birds to molt by housing them on a long day light cycle for 6 weeks prior to the start of the experiment. Both

naturally-molting and induced-molting birds were equally represented in the chronic stress and control treatments.

In the lab, we housed starlings in two experimental rooms, one for chronic stress birds and one for control birds, held on a 14L:10D light cycle. We housed starlings in individual cages (45 cm x 37 cm x 33 cm) and provided food (Mazuri Insectivore diet 5MK8/5MM3) and water *ad lib*. All methods followed IACUC approved procedures.

2.2 Study design. To test for the interactive effects of chronic stress and molt on wound healing and heart rate telemetry we used a 2 X 2 factorial design (see Table 1; factor 1: control and chronic stress; factor 2: molt and non-molt). Sample sizes for each treatment are as follows: Total $N = 31$; non-molt control: $n = 9$; molt control: $n = 7$; non-molt chronic stress: $n = 7$; molt chronic stress: $n = 8$). Each treatment was comprised of mixed sexes (non-molt control: $n = 5M, 4F$; molt control: $n = 4M, 3F$; non-molt chronic stress: $n = 3M, 4F$; molt chronic stress: $n = 3M, 4F$). After birds were weighed (average mass = 81.7 g; range: 71-98g) and initial blood samples were taken for corticosterone analyses (see detailed description below), we began our 28 day chronic stress protocol. Eight days after starting the chronic stress protocol we wounded all birds, a time point chosen because chronic stress begins to impact corticosterone responses at approximately this time (Rich and Romero, 2005). All birds completely healed wounds before the end of the chronic stress protocol. We monitored body mass, molt progression, wound size, and nighttime heart rate repeatedly throughout the duration of the study.

2.3 Chronic stress protocol (CSP). We used a well-established method to create chronic stress in our chronic stress birds (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. After 28 days of exposure to the CSP all birds were euthanized.

2.4 Body mass and Molt scores. We measured body mass of birds every 2-3 days throughout the study. We followed the progression of molt in the molting birds following the methods of (Dawson, 2003). Briefly, every seven days we assigned a score of 0-5 to each of the nine primary feathers on a bird's left and right wing. A score of zero means the bird had not dropped its old feather, and five represented a

fully-grown new feather. We added the scores of the 18 feathers to generate a molt score between 0-90 for each bird.

2.5 Wound healing. After eight days of the chronic stress protocol, we administered a small superficial wound to the left thigh of control and chronic stress birds using a 4mm biopsy punch (Miltex 33-34, York PA) following the methods of (Carter et al., 2013; French et al., 2006). Prior to wounding we anesthetized birds using isoflurane. We photographed wounds daily every morning along-side a ruler and determined wound size (mm^2) using Image J software (NIH) by measuring the perimeter of the wound (measurer was blind to treatment). Birds were captured and handled for ~1 min for photographing. Wounds were considered fully-healed when a scab was no longer present and the underlying tissue had rejoined.

2.6 Heart rate telemetry. Prior to the start of the experiment, we implanted all birds with a small heart rate transmitter (Data Sciences International TA 10EA-F20, St. Paul, MN following the methods of (Cyr et al., 2008; Nephew and Romero, 2003). We collected nighttime heart rate data from birds for two nights prior to the start of the CSP, at nights 4 and 5 during the CSP, the first and second night post wounding, and every other two nights thereafter until 25 days post the start of the CSP. All birds completed healing within this time frame. For each night of data collection, we measured heart rate of birds for two hours, between 0000 hours and 0200 hours. We measured heart rate at night because there is less activity in the building that can intermittently and unpredictably alter heart rate. In addition, previous data suggest heart rate and energy expenditure of birds remain low and stable throughout night (Cyr et al., 2008; Hawley et al., 2012), whereas heart rate can be more variable during the day (Cyr et al. 2008) and could limit our ability to detect effects of our treatments on heartrate.

2.7 Blood sampling and hormone injection. Before starting the CSP we collected blood samples for plasma corticosterone analyses from all birds (total volume collected was ~ 300 μ l). 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. Immediately following collection of the stress-induced sample we injected birds with 1mg/kg of dexamethasone (Vedco dexamethasone solution 2mg/ml) and collected another blood sample 90 minutes post dexamethasone injection. We then injected birds with 100 IUs/kg adrenocorticotropin hormone (ACTH; Sigma A6303) and collected a final blood sample 15 minutes later. Injection with dexamethasone allowed us to assess negative feedback ability of the birds (expressed as a percent decline from the 30 min sample to control for individual

responses to stress, Lattin et al., 2012), and injection with ACTH allowed us to assess maximal capacity of the adrenals to secrete corticosterone (Dickens et al., 2009; Rich and Romero, 2005). We kept samples on ice (~1-2 hr) until we could centrifuge blood to separate plasma, and then stored the plasma at -20°C.

2.8 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 resuspended in 550 µL of phosphate buffered saline. We determined individual extraction efficiency using 100 µL of the sample (mean recoveries was 72%). For the two 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 11.1 and 5.4%, respectively, as determined by running standards in the two assays.

2.9 Statistical analyses. All statistical analyses were run in SAS 9.1 (SAS Institute Inc., Cary, NC, USA) or Microsoft Excel and statistical significance was recognized at $\alpha < 0.05$. Where appropriate, we tested for normal distribution of the data and homoscedasticity using Ryan-Joiners and Bartlett's tests, respectively. Unless otherwise noted, raw data were used in statistical models, and sexes were combined for analyses because sample sizes were not large enough to adequately test for sex effects. For the body mass, wound size and heart rate data sets we tested for effects of day of study (time), chronic stress treatment, molt status, and all interactions using a mixed model repeated measures ANOVA with experimental round (1-4) included as a random effect. The three-way interaction (day of study X chronic stress treatment X molt status) was dropped from the final model of body mass because it was highly insignificant ($p > 0.7$). The wound healing and heart rate data sets also contained initial body mass (body mass at the beginning of the experiment) as a covariate. We also tested for the effects of stress treatment, molt, and their interaction on the total number of days it took for birds to fully-heal a wound using a two-way mixed model ANCOVA with experimental round included as a random effect to control for seasonal and/or other differences among rounds. Initial body mass was included as a covariate. To test for differences in molt scores between control and chronic stress birds over time we used a repeated measures mixed model ANOVA model which included day of study, chronic stress treatment and their interaction as fixed effects, and experimental round as a random effect.

Corticosterone concentrations were analyzed using a mixed model repeated measures ANCOVA with body mass as the covariate and experimental round as the random effect. The model included molt status, but not stress as a main effect, since animals had yet to be exposed to chronic stress. Because we predicted that various components of corticosterone production and regulation may predict healing rates, we regressed days to full recovery against baseline, stress-induced, % decrease from stress-induced concentrations (i.e negative feedback induced by dexamethasone injection), and maximal corticosterone.

3.Results

3.1 Body mass. Body mass of starlings varied over the course of the study, and changes in mass differed in stressed and control birds (time: $F_{13,382} = 13.8$, $p < 0.001$; stress X day of study: $F_{13,382} = 2.49$, $p = 0.003$; Fig. 1). Body mass initially increased in control birds at the start of the study, but decreased in stress birds just before wounding; however all groups began to gain weight 2-8 days post wounding. Weight gain continued until the end of the study. Molt had no influence on body mass (molt: $p = 0.22$, molt X stress: $p = 0.46$, molt x time: $p = 0.10$).

3.2 Molt scores. Molt progressed similarly in chronic stress and control birds during the study (day of study: $F_{4,63} = 40.30$, $p < 0.001$; stress $F_{1,63} = 1.07$ $p = 0.31$; stress x day of study: $F_{4,63} = 0.03$, $p = 0.99$; data not presented). At the start of the experiment control and stress birds received molt scores of 30.8 ± 2.7 and 24.5 ± 6.6 , respectively, and molt scores of 51 ± 1.9 , and 43.1 ± 6.7 , respectively, by the end of the experiment four weeks later.

3.3 Wound healing. Molt birds initially (Days 1-7) tended to heal wounds more slowly compared with non-molting birds, while later healing progressed at similar rates (molt X day of study: $F_{16,454} = 1.86$, $p = 0.02$; Fig. 2A,B). Contrary to predictions, chronic stress did not influence healing patterns (stress: $p = 0.17$; stress X day of study: $p = 0.78$; stress X molt: $p = 0.42$; stress x molt x day of study: $p = 0.08$). Heavier birds experienced faster decreases in wound size than lighter birds ($F_{1,454} = 337$; $p < 0.001$)

All birds fully-healed wounds in 11 ± 0.5 days (range: 6-17 days) regardless of stress or molt status (stress: $F_{1,22} = 0.01$, $p = 0.97$; molt: $F_{1,22} = 0.19$, $p = 0.67$; stress X molt: $F_{1,22} = 0.02$, $p = 0.89$; Fig. 2C). Initial body mass significantly correlated with healing rates; heavier birds fully-healed wounds more quickly than lighter birds (mass: $F_{1,22} = 5.28$; $p = 0.03$; Fig. 3).

3.4 Nighttime heart rate. Heart rate varied differently over the course of the study depending on birds' molt status and exposure to chronic stress (Fig. 4A,B; stress x molt x day of study: $F_{12,338} = 1.75$, $p = 0.055$; stress x molt: $F_{1,338} = 8.30$, $p = 0.004$; molt X day of study: $F_{12,338} = 1.63$, $p = 0.08$; stress x day of study: $F_{12,338} = 1.42$, $p = 0.15$). This interaction is driven by a decrease in heart rate immediately following wounding that persisted for ~ 10 days in non-molting chronic stress birds relative to non-molting control birds, while there is no difference in heart rate between control and chronic stress molting birds. There was no significant relationship between initial body mass and heart rate ($p = 0.48$).

3.5 Corticosterone. Plasma corticosterone concentrations changed significantly over the corticosterone sampling period (Fig. 5; time: $F_{3,87} = 104.58$, $p < 0.001$), but were not influenced by molt status (molt: $F_{1,112} = 2.27$ $p = 0.0.13$; molt x day of study: $F_{3,112} = 0.59$ $p = 0.62$), or body mass ($F_{1,112} = 1.18$; $p = 0.28$). Corticosterone was lowest immediately following capture from the cage (baseline; molt: 1.9 ± 0.7 ng/ml; nonmolt: 2.49 ± 0.9 ng/ml) and after injection with dexamethasone (molt: 4.9 ± 2.0 ng/ml; nonmolt: 8.5 ± 3.2 ng/ml), whereas corticosterone was highest after 30 minutes of restraint (molt: 18.3 ± 2.3 ng/ml; nonmolt: 17.9 ± 2.2 ng/ml) and after injection with ACTH (molt: 37.7 ± 3.7 ng/ml; nonmolt: 42.7 ± 2.4 ng/ml). Baseline corticosterone predicted wound healing (Fig. 6A; $R^2 = 0.23$ $p = 0.007$); birds with lower baseline corticosterone concentrations healed more quickly than birds with higher corticosterone concentrations. In addition, the strength of negative feedback may play a role. Four birds had no response to DEX (corticosterone concentrations were similar to stress-induced concentrations), but it is unclear whether these birds failed to respond or there were technical difficulties in administering the DEX. If those four birds are included, there were no correlations with wound healing ($R^2 = 0.01$ $p = 0.82$). However, if those four birds are excluded, the birds with the strongest negative feedback response to DEX healed wounds faster (Fig. 6B; $R^2 = 0.20$ $p = 0.02$). Comparison of corticosterone to all other variables: $R^2 \leq 0.015$; $p \geq 0.51$.

4. Discussion

In this study, we induced chronic stress in birds during different life history stages that should be associated with differing energetic burdens to determine how these factors influenced wound healing and energy expenditure (Table 1). Although captivity itself can be stressful, the chronic stress protocols used in this study successfully induces chronic stress in wild caught European Starlings (e.g., Cyr et al., 2007; Kostelenatz et al., 2009; Rich and Romero, 2005). Thus, control birds not exposed to the CSP

should experience less stress than birds exposed to the stress protocol. Indeed, we successfully induced chronic stress in birds exposed to the chronic stress protocol, as noted by the decrease in body mass of chronically stressed birds that occurred after 6 days of chronic stress (2 days prior to wounding; Fig. 1). Loss of body mass is the most conserved effect of chronic stress across lab and field studies (Dickens and Romero, 2013). Furthermore, we found that chronic stress and molt had interactive effects on energy expenditure of birds after wounding (Fig. 4). Despite these effects and contrary to our predictions, we did not detect an effect of chronic stress or molt on the time it took to fully-heal wound healing (Fig. 2C), but molting birds did initially reduce wound size faster than non-molting birds (Fig. 2A,B). Initial increases in wound size are associated with inflammation at the wound site (Martin 1997), and sometimes, but not always, greater inflammation is associated with faster healing (Archie 2013), which was not the case in this study. It is surprising that we did not find an effect of chronic stress on wound healing, but other studies have also found that chronic stress or chronic corticosterone application in wild animals did not alter immune performances (McCormick and Langkilde 2014). It may be that healing rates would have been affected if wounding occurred after longer exposure to chronic stress, which is what would be predicted by reactive scope. Interestingly, individual condition or quality appear to be important to wound healing since heavier birds and birds with low baseline corticosterone healed faster than lighter birds and birds with high baseline corticosterone (Figs. 3, 6A). Low baseline corticosterone and heavy body mass are often associated with individuals in good condition (e.g., Kitasky et al., 1999; Moore et al., 2000; Romero and Wikelski, 2001). In addition, we found that individuals better capable of shutting down corticosterone release healed wounds faster than birds with less robust negative feedback (Fig. 6B). This finding is consistent with research on marine iguanas (*Amblyrhynchus cristatus*, Bell); individuals with strong negative feedback during low stress conditions were more likely to survive drought (Romero and Wikelski, 2010). Ability to shut down corticosterone release after exposure to a stressor or during chronic stress reduces the duration in which individuals are exposed to high levels of corticosterone, and appears to protect an individual from pathological effects of stress. We did not detect a significant relationship between healing rates and stress-induced or ACTH-induced corticosterone, possibly because these measures of corticosterone were less predictive of the duration of high corticosterone exposure in birds throughout the study.

Energetic burdens and constraints are an important component of the allostatic framework for understanding the negative consequences of stress. According to allostatic overload type I, pathology occurs when additive costs of stress and maintenance exceed available energy (McEwen and Wingfield, 2003). Interestingly, our results do not support the allostatic model of stress. The current study was

designed to create treatments that experienced progressively greater energetic burdens. Non-molting control birds should have experienced the lowest energetic demands, non-molting chronic stress and molting control birds should have experienced moderate energetic demands, and molting chronic stress birds should have experienced high energetic demands (Table 1). Based on the allostasis model, we expected decreasing healing ability with increasing energetic burden (McEwen and Wingfield, 2003) but, this was not the case. However, we cannot entirely rule out the allostatic model because our birds had *ad lib* access to food and may have been able to compensate for any energetic costs associated with stress and/or molt by increased feeding (supported by the increase in mass after wound healing, Fig. 1). Additionally, albeit unlikely, wound healing, the CSP, or molt may not have sufficiently increased allostatic load of our birds. In contrast to the allostatic model, the reactive scope model predicts that as long as an organism's physiological response to stress stays within the reactive homeostasis range, stress will not result in pathology (Romero et al., 2009). Perhaps birds experiencing stress and/or molt were still responding within this range, which is why neither chronic stress nor molt influenced wound healing.

Although neither molt nor stress reduced healing time, energy expenditure, stress, and immune function do appear to be linked. Non-molting chronic stress birds down-regulated nighttime energy expenditure immediately after wounding and this effect persisted until healing was complete (average healing time was ~11 d). This suggests that non-molting chronic stress birds were employing an energy saving strategy at night. In contrast, molting chronic stress birds did not use a nighttime energy saving tactic, potentially resulting from increased maintenance costs associated with feather growth (Cyr et al., 2008), or because heart rate in molting birds may be under greater parasympathetic nervous system regulation (Kostelenatz et al., 2009). Body mass data also suggest a link between stress, energy expenditure, and immune function. Several days after wounding all birds regardless of treatment began to gain weight, and this trend continued through the end of the study. Birds had *ad lib* access to food, thus weight gain could indicate compensatory feeding in the birds. It is possible that birds mitigated energetic challenges induced by chronic stress and wounding by consuming more food while simultaneously using energy saving tactics when possible, such as reduced daytime activity and energy expenditure, or nighttime energy expenditure. Birds also may have increased feeding after wounding in anticipation of additional immune or stress challenges. Future studies examining the relationships between molt, chronic stress and healing that restrict food availability, or manipulate food quality, and measure daytime activity and energy expenditure will be helpful in determining how birds navigate the energetic demands of stress and immune processes during different life-history events. Daytime energy

expenditure may be particularly relevant for starlings during molting because day length is greatest at this time of year.

The lack of an effect of molt on corticosterone concentrations and time needed to fully-heal wounds was unexpected. Baseline and stress-induced corticosterone in birds are sometimes depressed during molt (e.g., Romero and Wingfield 1998; Romero et al. 1998a,b,c; Rich and Romero 2001), but not always (Romero and Wingfield 2001; Perfito et al. 2002). Corticosterone concentrations of molting starlings in our study were similar to those of other starlings undergoing molt in captivity, starlings held on long and short days in captivity, and free-living starlings during late summer (Dawson and Howe 1983; Ramage-Healey and Romero 2001, 2002). When assessing effects of molt on healing patterns, it is worth noting that non-molting birds do appear to begin healing faster than molting birds, but these differences disappear by 7 days post wounding, and it is unclear whether this difference would be of ecological relevance to a wild animal. During molt, significant blood vessel development occurs at the feather along with substantial increases in daily protein synthesis and keratin deposition (Lillie, 1940; Murphy and Taruscio, 1995). Given the resource demands of molt, one would predict that molt would detract from other costly physiological processes such as immune function. Perhaps the increased risk of developing an open wound if feathers are injured during molt necessitates robust immune function during this time. A small song bird in heavy molt, for example, would have over 20 potential wound sites if growing feathers, which are not attached to the bone and have extensive vascularization, should become dislodged. Further, some of the requirements of molt, e.g. tissue renovation, keratinocytes, and protein synthesis, are also important for immune processes, thus the tools necessary for healing are already upregulated, which could allow for nearly normal wound healing (Gouin and Keicolt-Glaser, 2011; Martin, 1997; Murphy and Taruscio, 1995). The lack of effect of molt on wound healing also may relate to seasonality of immune processes (Bilbo et al., 2002; Martin et al., 2008; Nelson et al., 2002). In birds, cell-mediated immune responses increase during times of short day length (winter) for several species, including European Starlings (Bentley et al., 1998; Martin et al., 2008). During winter, many small endotherms increase cellular maintenance and other processes that promote survival (Nelson et al., 2002). Perhaps starlings in our study were beginning to upregulate immune responses around the same time molt occurs. Finally, the progression of molt in our study was slower than in other studies (Dawson 1991), thus normal healing rates during molt may have been at the expense of decreased rate of feather regrowth. It is not clear why molt did not correspond with increased nighttime energy expenditure in control birds in this study (Cyr et al., 2008; but see Kostelenatz et al., 2009), but this

result may partially explain why wound healing rates did not differ between molting and non-molting birds.

4.1 Conclusions. As evidenced in the current study, the costs of immune responses and the interplay between chronic stress, corticosterone, and wound healing in relation to costly life-history events are complex. Our results suggest that animals may be able to preserve immune processes, a vital component to survival, even during periods that should be associated with increased energetic demands on the individual. However, studies manipulating food quality or quantity may produce conditions that do cause trade-offs with the immune system, even during life-history events that would benefit from robust immune responses (e.g., molt). Lastly, our findings indicate that although stress did not influence healing rates, baseline corticosterone and body mass are both important to wound healing.

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Figure Legend

Figure 1: Body size of European starlings fluctuates during chronic stress. Body mass (means $\pm$ 1 SE) of molting and non-molting European starlings (*Sturnus vulgaris*) during exposure to a chronic stress protocol (chronic stress birds) or left undisturbed (control birds). All birds received a small superficial wound eight days after the start of the chronic stress protocol, which is indicated by the dotted line.

Figure 2: Changes in wound size differed over time between molting and non-molting starlings, but time to fully heal wounds is similar. Wound size (means $\pm$ 1 SE) of a small superficial wound administered to European starlings (*Sturnus vulgaris*) that were exposed to control or chronic stress conditions when not molting (A) or during molt (B). The total number of days (means $\pm$ 1 SE) needed for starlings to fully heal wounds (C)

Figure 3: Body mass positively correlates with ability to quickly heal wounds. The number of days needed for European starlings (*Sturnus vulgaris*) to fully heal wounds regressed against body mass ($R^2 = 0.27$; $y = -0.22x + 28.55$) of birds prior to the start of chronic stress.

Figure 4: Non-molting chronic stress birds reduce nighttime energy expenditure after wounding. Nighttime heart rate (means $\pm$ 1 SE) of non-molting (A) and molting (B) European starlings (*Sturnus vulgaris*) during exposure to a chronic stress protocol (chronic stress birds) or left undisturbed (control birds). All birds received a small superficial wound eight days after the start of the chronic stress protocol, which is indicated by the dotted line.

Figure 5: Corticosterone profiles are similar between molting and non-molting birds. Plasma corticosterone concentrations (means $\pm$ 1 SE) of molting and non-molting European starlings (*Sturnus vulgaris*) prior to the start of a chronic stress protocol. Birds were sampled serially within three minutes of capture (baseline), after thirty minutes of restraint (stress-induced), ninety minutes after injection with dexamethasone (Dex), and fifteen minutes after injection with adrenocorticotropin hormone (ACTH). Dexamethasone stimulates negative feedback of corticosterone release, whereas ACTH induces maximal corticosterone release from the adrenals.

Figure 6A,B: Low baseline corticosterone (A) and high negative feedback (B) are associated with the ability to quickly heal wounds. The number of days needed for European starlings (*Sturnus vulgaris*) to fully heal wounds regressed against baseline corticosterone ($R^2 = 0.23$; $y = 0.44x + 10.00$) or negative feedback ($R^2 = 0.20$; $y = -0.07x + 16.51$) of birds prior to the start of chronic stress. Negative feedback is expressed as a percent decline from the 30 min stress-induced sample to 90 minutes after dexamethasone injection (DEX) to control for individual responses to stress. Individuals with a score of 100% have completely shut-off corticosterone release.

		Chronic Stress	
		No	Yes
Molt	No	Low Allostatic Load = Fastest Wound Healing N=9	Medium Allostatic Load = Slower Wound Healing N=7
	Yes	Medium Allostatic Load = Slower Wound Healing N=7	High Allostatic Load = Slowest Wound Healing N=8

Table 1: Experimental design and predicted results based upon allostasis

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6A,B