The seasonal glucocorticoid response of male Rufous-winged Sparrows to acute stress correlates with changes in plasma uric acid, but neither glucose nor testosterone

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Abstract

We sought to clarify functional relationships between baseline and acute stress-induced changes in plasma levels of the stress hormone corticosterone (CORT) and the reproductive hormone testosterone (T), and those of two main metabolites, uric acid (UA) and glucose (GLU). Acute stress in vertebrates generally stimulates the secretion of glucocorticoids, which in birds is primarily CORT. This stimulation is thought to promote behavioral and metabolic changes, including increased glycemia. However, limited information in free-ranging birds supports the view that acutely elevated plasma CORT stimulates glycemia. Acute stress also often decreases the secretion of reproductive hormones (e.g., T in males), but the role of CORT in this decrease and the contribution of T to the regulation of plasma GLU remain poorly understood. We measured initial (pre-stress) and acute stress-induced plasma CORT and T as well as GLU in adult male Rufous-winged Sparrows, *Peucaea carpalis*, sampled during the pre-breeding, breeding, post-breeding molt, and non-breeding stages. Stress increased plasma CORT and the magnitude of this increase did not differ across life history stages. The stress-induced elevation of plasma CORT was consistently associated with decreased plasma UA, suggesting a role for CORT in the regulation of plasma UA during stress. During stress plasma GLU either increased (pre-breeding), did not change (breeding), or decreased (molt and non-breeding), and plasma T either decreased (pre-breeding and breeding) or did not change (molt and non-breeding). These data provide only partial support to the hypothesis that CORT secretion during acute stress exerts a hyperglycemic action or is responsible for the observed decrease in plasma T taking place at certain life history stages. They also do not support the hypothesis that rapid changes in plasma T influence glycemia.

Keywords: Annual cycle; antioxidant; glycemia; passerine; seasonality; sparrow.

Abbreviations:

ANOVA: analysis of variance

CORT: corticosterone

CP: cloacal protuberance

GLU: glucose

HPA: hypothalamic-pituitary-adrenal axis

IQ: interquartile interval

KW test: Kruskal-Wallis test

MST: mountain standard time

MW test: Mann-Whitney U-test

P: probability

- 51 rmANOVA: repeated measure analysis of variance
- 52 SNK test: Student-Newman-Keuls test
- 53 T: testosterone
- 54 UA: uric acid

1. Introduction

Animals are regularly exposed to events (e.g., predation and inclement weather) that incite a stress response. Hormonal and physiological changes that occur during this response include the release of catecholamines and the activation of the hypothalamic-pituitary-adrenal (HPA) axis, which controls the production and secretion of glucocorticoids. A component of the stress response consists of a rapid (within minutes) increase in plasma glucocorticoids above resting (initial) levels (Mora et al. 2012). As understood from mammalian studies, glucocorticoids act synergistically with catecholamines and glucagon to stimulate glycogenolysis and gluconeogenesis, ultimately increasing plasma glucose (GLU) and its availability to the brain, muscles, and other tissues (Eigler et al., 1979; McMahon et al., 1988; Sapolsky et al., 2000). This, in turn, is thought to improve the chances of survival in a threatening situation (Astheimer et al., 1992; Sandi et al., 1996; Sapolsky et al., 2000; Miles et al., 2007).

The hyperglycemic effect of glucocorticoids is observed in domestic poultry (Kobayashi et al., 1989; Yuan et al., 2008). However, the few studies investigating effects of acute stress on glycemia in wild birds have yielded variable results. For example, acute stress increased plasma GLU in free-ranging Abert's Towhees, *Melospiza aberti* (Davies et al., 2013) and captive European Starlings, *Sturnus vulgaris*, but in the latter case only at certain times during the daily and annual cycle (Remage-Healey and Romero, 2001, 2002). By contrast, acute stress did not influence glycemia in captive Curved-billed Thrashers, *Toxostoma curvirostre* (Fokidis et al., 2011) and decreased it in free-ranging Rufous-winged Sparrows *Peucaea carpalis* (RWSP; Deviche et al., 2014). In all these species, acute stress increased plasma glucocorticoids (primarily corticosterone (CORT) in birds), indicating that this increase is not necessarily accompanied with elevated glycemia.

Birds differ from other vertebrates in that they normally maintain high glycemia and yet are resistant to the effects of oxidative stress that are generally associated with elevated plasma GLU (Braun and Sweazea, 2008). Surprisingly, the regulation of GLU metabolism has received little investigation in wild birds, with studies primarily focusing on migrating birds (Jenni and Jenni-Eiermann, 1998; Maillet and Weber, 2006; reviewed in Scanes and Braun, 2013). Migration includes extended periods of physical exertion and is characterized by numerous metabolic and endocrine adjustments that are specific to this life history stage (Speakman and Selman, 2003; Landys et al., 2004). Whether information on the control of glycemia in migratory birds applies to sedentary species is, therefore, uncertain.

Resistance to the effects of oxidative stress that are associated with high glycemia in avian species is thought to involve circulating antioxidants such as uric acid (UA), a product of amino acid catabolism (Cohen et al., 2007; Braun and Sweazea, 2008). In many avian species, plasma UA decreases during an acute stress response, but this effect is not universal (Cohen et al., 2007; Davies et al., 2013, 2014). The decrease may result from UA being used in antioxidant actions associated with the metabolic

effects of elevated CORT. However, the relationship between UA and both CORT and GLU remains unclear.

The CORT stress response of temperate region birds often changes seasonally and concurrent with transitions between life history stages (e.g., reproduction, molt, and in some species migration (Ramage-Healey and Romero, 2000; Romero, 2002; Cyr et al., 2008). If a primary role of CORT is to regulate GLU metabolism, then it would be expected that seasonal and stress-induced changes in plasma CORT are consistently associated with corresponding changes in plasma GLU. Naturally occurring seasonal changes in the CORT stress response can, therefore, be used to mechanistically investigate the relationship between this hormone and plasma GLU and UA.

In addition to its above roles, the CORT stress response is believed to limit the amount of energy that is directed to non-essential functions such as digestion, immune defense, and reproduction (Angelier et al., 2007), and to redirect energy to activities and metabolic processes that are critical for survival (Wingfield and Sapolsky, 2003). In some circumstances, acute stress decreases the activity of the hypothalamic-pituitary-gonadal (HPG) axis and the secretion of reproductive hormones, including testosterone (T) in male birds (Cyr and Romero, 2007; Schoech et al., 2009; Goutte et al., 2010; Deviche et al., 2012, 2014). As the acute stress response is associated with increased plasma CORT (Lynn et al., 2010; Deviche et al., 2012, 2014), these observations are often taken to suggest a causal relationship between an increase in plasma CORT and a concurrent decrease in plasma T. However, recent evidence, including from the species investigated here (Deviche et al., 2012), indicates that changes in plasma CORT such as during stress or social interactions, are not necessarily associated with changes in the opposite direction in plasma T (Goutte et al., 2010). These observations suggest a lack of or more complex proximate relationships between these hormones.

In many free-ranging male birds, plasma T varies seasonally, decreasing from high levels during breeding to low levels during molt and outside the breeding period (Dawson, 1983; Wingfield, 1984; Goymann et al., 2007). A primary function of T is to promote reproductive behaviors, but mammalian studies demonstrate a regulatory role of T also in carbohydrate, fat, and protein metabolism (reviewed in Kelly and Jones, 2013). These studies overwhelmingly point to an anabolic function of T, acting to increase glycogen synthesis and decrease plasma GLU (Leonard, 1952; Bergamini et al., 1968; Marin et al., 1992; Haffner et al., 1994; Ramamani et al., 1999; Kelly and Jones, 2013). The relationship between T and GLU in birds remains, however, poorly defined: few studies on this subject have been performed in free-ranging subjects and in chickens, research involving experimental manipulation of plasma T has yielded inconsistent results (Lepkovsky et al., 1967; Chen et al., 2005).

Due to the sparse number of studies investigating changes in metabolites during acute stress in free-living birds, the primary objective of the present study was to use such birds to clarify the

relationships between CORT, T, GLU, and UA. To this aim, we measured pre-stress (initial) and acute stress-related plasma CORT and T across seasons in relation to plasma GLU and UA in free-living male RWSPs. This Sonoran Desert passerine breeds in association with the summer monsoon, and adults are sedentary and maintain the same territory year-round (Lowther et al., 1999). Thus results are not influenced by the potentially confounding effects of factors related to seasonal migration or dispersion.

In a previous but independent study on adult male RWSP, we sampled birds shortly before (June) and during (July and August) the breeding season, as well as during post-breeding molt (September; Deviche et al., 2014). In response to stress, plasma CORT and T of these birds consistently increased and decreased, respectively, with the CORT response to stress showing attenuation during molt. Plasma metabolites (UA and GLU) were measured only in samples collected from breeding and molting sparrows, so that no data on this subject are available for birds sampled at other life history stages. In order to expand our understanding of how acute stress influences plasma CORT, T, UA, and GLU, to better understand relationships between these hormones and metabolites, and to test the robustness of these relationships, we measured all four plasma chemicals in RWSP sampled not only before and during breeding as well as molt, but also during winter, when birds are not molting and their reproductive system is quiescent.

Given the demonstrated role of CORT in regulating GLU metabolism in mammals, we hypothesized that CORT exhibits a similar function in wild birds. Based on previous work showing a decrease in plasma GLU and UA during acute stress (Deviche et al., 2014), we predicted a negative correlation during stress between changes in plasma CORT and those in both plasma GLU and UA. We then utilized the seasonally variable CORT stress response to investigate the relationship between plasma CORT and T during the stress response. If the hypothesis that acute stress-associated elevated plasma CORT is responsible for the concurrent decrease in plasma T is supported, we predicted a consistent inverse relationship between these hormones across life history stages. We also investigated the potential of T to influence plasma GLU and, for this, utilized stress-induced and seasonal changes in plasma T to investigate this relationship. We predicted that if T modulates plasma GLU, changes in plasma T, be they related to the life history stage or resulting from stress, would be consistently associated with changes in plasma GLU.

In a recent study on Blue Tits, *Cyanistes caeruleus*, changes in plasma GLU during stress were associated with changes in ambient temperature: the higher the minimum ambient temperature the night preceding capture, the more plasma GLU increased during stress (Kalinski et al., 2014). To our knowledge this type of relationship has not been researched in other free-ranging species and the mechanism involved is unknown. Depending on the season, sparrows in the present study were naturally exposed to minimum ambient temperature ranging over one order of magnitude ($< 2^{\circ}\text{C}$ to $> 22^{\circ}\text{C}$; see

Results section) at the time of sampling. The body mass of these small songbirds (15-16 g; see Results) is similar to that of Blue Tits (10-11 g; Charmantier et al., 2004) and the present investigation, therefore, offered an excellent opportunity to test the generality of findings in this species.

2. Materials and methods

2.1. Study site, species, and capture

We sampled adult male Rufous-winged Sparrows in the Santa Rita Experimental Range, Pima County, Arizona, USA (altitude: 979 m.a.s.l.; latitude: 31° 49' N; longitude: 110° 55' W) in 2013 (5th August: n = 7; 16th December: n = 10) and 2014 (16th June: n = 10; also 5th August: n = 10; 29th September: n = 10). Rufous-winged Sparrows breed primarily during the summer monsoon (July-August; Lowther et al., 1999), when gonads in males are fully developed and plasma T is seasonally highest (Deviche and Small, 2006; Deviche et al., 2006; Small et al., 2008). Sampling dates were, therefore, selected to represent the pre-breeding (June), breeding (August), molt (September) and non-breeding (December) life history stages of the species. On each date, we caught sparrows using a mist net and conspecific song playback. Birds were caught between 05:40 AM and 13:20 PM (MST). The average duration of song playback to which males were exposed before capture equaled $4:27 \pm 0:47$ (SEM) minutes. Previous studies found no change in plasma T or GLU after song playback exposure for up to 30 min (Deviche et al., 2006, 2014). The latter study reported a moderate increase in plasma CORT and decrease in plasma UA in response to song playback exposure for 30 min, but no information is available on whether exposure to song playback for a brief period (less than five minutes as was the case here) affects plasma CORT or UA. Times of capture to the nearest 10 min were noted and converted to time elapsed after local civil twilight on the date of capture. Ambient temperatures (minimum temperature the night before sampling in Green Valley, which is located less than 10 km from sampling sites) were obtained from USClimatedata.com.

2.2. Blood collection and acute stress protocol

We collected all blood samples (75 µl) from the right jugular vein using a heparinized syringe. Within two minutes of capture, we collected a first blood sample (initial) to quantify pre-stress levels of plasma hormones and metabolites (see below). To elicit an acute stress response, we then confined birds to individual breathable cloth bags, which we kept in the shade. Ten and 30 min after capture, we collected a second and a third blood sample, respectively, to characterize the plasma hormone and metabolite responses to acute stress. All blood samples were immediately placed on ice until they were

centrifuged and the plasma separated later the same day. Plasma was frozen at -80° C until assayed for
CORT, T, UA, and GLU (see below).

Following the stress protocol and blood collections, we measured body mass (± 0.1 g), wing
chord (± 1 mm), and cloacal protuberance (CP) width (± 0.1 mm), which is a T-dependent secondary
sexual trait. We also visually scored the amount of fat in the furcular region (0-5; Rogers, 1991) and the
degree of contour feather molt (0-3). Birds then received an injection of saline solution (i.m., pectoral
muscle: 200 μ l of 0.9% NaCl in water) to compensate for the volume of blood collected, were marked
with a uniquely numbered US Geological Survey aluminum tarsal band, and were released on site.

2.3. Plasma hormone and metabolite assays

Each plasma sample was assayed for CORT, T, GLU, and UA in duplicate following validated
methods described by Deviche et al. (2014). Briefly, we quantified plasma T and CORT using
commercial enzyme-linked immunoassay kits (Enzo Life Sciences, Farmingdale, NY; Deviche et al.,
2006, 2010, 2014). Plasma was diluted 20x in assay buffer containing displacement reagent (see
manufacturer's specifications) before assay. For CORT, the average inter- and intra-assay coefficients of
variation were 10.4% and 7.5%, respectively, and the assay sensitivity was 0.048 ng/ml. For T, the
average inter- and intra-assay coefficients of variation were 5.1% and 7.9%, respectively, and the average
assay sensitivity was 0.035 ng/ml. We quantified plasma GLU and UA using colorimetric assay kits
(GLU: Cayman Chemical Co., Ann Arbor, MI; UA: BioAssay Systems, Hayward, CA; Deviche et al.,
2014). Plasma used for UA assay was diluted 4x in assay buffer before the assay. The average inter- and
intra-assay coefficients of variation for the GLU assay were 1.2% and 1.1%, respectively, and the assay
sensitivity was 20 mg/dl. For UA, the average inter- and intra-assay coefficients of variation were 5.6%
and 4.7%, respectively, and the assay sensitivity was 1 mg/dl. For all assays, we randomly assigned
samples to assay plates but such that the three samples collected from a given bird were assayed on the
same plate.

Seven (out of a total of 141, i.e., 5%) and one plasma samples were not assayed for CORT and
UA, respectively, for technical reasons. We estimated missing values using multiple imputation and the
NORM program (<http://sites.stat.psu.edu/~jls/misoftwa.html>; Schafer, 1999). As advised by others (Little,
1988), multiple imputation is more appropriate than other approaches (e.g., case deletion or replacement
of missing values by group means) to deal with missing values because it relies on more plausible
assumptions, properly accounts for uncertainty about missing values (leading to appropriate standard
errors), and retains original sample sizes.

2.4. Statistical analyses

Comparisons of data obtained for males sampled on the same calendar date (5th August) in 2013 and 2014 were performed using Student's t-tests (wing chord, body mass, CP widths, and capture times) or a Mann-Whitney U-test (MW; fat scores). Plasma CORT, T, UA, and GLU data obtained for these birds were analyzed using two way repeated measures ANOVA (rmANOVA) with date and stress duration (initial, 10 min, or 30 min) as independent variables. Data sets that were not normally distributed were transformed to square root (plasma CORT) or Log₁₀ (T and UA) before analysis to reach normal distribution. When appropriate, rmANOVAs were followed with pairwise multiple comparisons using Student-Newman-Keuls (SNK) tests.

Plasma CORT, T, UA, and GLU data collected at different life history stages were likewise analyzed using rmANOVA followed, when appropriate, with pairwise comparisons using SNK tests. Corticosterone, T, and UA data sets were not normally distributed and were converted to ranks before analysis (Conover and Iman, 1981). Changes in body mass and CP width across life history stages were analyzed using one-way ANOVA followed with SNK tests, with body mass data ranked before this analysis to reach normality (Conover and Iman, 1981). Seasonal changes in capture times, fat reserves, and body molt scores were analyzed using Kruskal-Wallis ANOVA on ranks (KW) followed with Dunn's pairwise comparison tests.

To determine associations between changes in plasma levels of hormones and metabolites over time, we calculated, for each parameter, Ln (10 min stress/corresponding initial level) and Ln (30 min stress/corresponding initial level) and then determined associations between parameters using linear regressions.

We used effect sizes to measure the strength of statistically significant group differences or correlations between variables. Following MW tests we calculated effect sizes using Cureton's (1956) rank-biserial correlation (r). After Student's t-tests we measured effect size using Cohen's d. Following KW tests we calculated effect sizes using eta-squared (η^2_H ; Tomczak and Tomczak 2014). After ANOVA and rmANOVA, effect sizes were estimated using eta-squared (η^2) and partial eta-squared, respectively (Levine and Hullett, 2002). The coefficient of determination (r^2) served as estimate of effect size after linear regression. Consistent with convention, effect sizes were deemed to be small if equal to or smaller than 0.2, medium if equal to approximately 0.5, and large if equal to or larger than 0.8.

We performed statistical analyses using SigmaStat 12 (Systat Software, Inc., San Jose, CA) and Statistica 10 (StatSofts, Inc., Tulsa, OK) with an alpha of 0.05. We present data sets that we analyzed using parametric statistical methods as means $\pm$ standard errors (s.e.m.) and data sets that we analyzed using non-parametric tests as medians $\pm$ 0.5 interquartile intervals (IQ).

3. Results

3.1. Comparison of males sampled in August 2013 and 2014

Males sampled on the same date (5th August) in 2013 and 2014 did not differ with respect to wing chord, body mass, and cloacal protuberance (CP) width ($P_s > 0.5$). Males had higher furcular fat scores in 2013 (1.5 ± 0.5) than 2014 (1.0 ± 0.3 ; MW: $U = 10$; $P = 0.013$, $r = 0.86$). This difference may relate to the fact that birds were caught later in the day in 2013 ($11:46 \text{ AM} \pm 0:22 \text{ hr}$) than in 2014 ($8:04 \text{ AM} \pm 0:28 \text{ hr}$; $t_{15} = 5.83$; $P < 0.001$; Cohen's $d = 2.9$).

Plasma CORT, T, UA, and GLU changed as a function of the stress duration but overall, did not differ in 2013 and 2014 (Table 1). There was a significant stress duration $\times$ year interaction for T and UA, but effect sizes associated with these interactions were modest ($\eta^2 \leq 0.21$; Table 1) and pair-wise group comparisons did not reveal differences at 0, 10, or 30 min for either parameter (SNK: $P_s > 0.05$). Given the overall similarities between males sampled in August 2013 and 2014, data for the two groups ($n = 17$) were combined for subsequent analyses.

3.2. Capture times

Capture times differed between seasons (KW: $H_3 = 14.68$, $P = 0.002$), with birds being caught, on average, earlier in the day in June ($7:10 \text{ AM} \pm 0.55 \text{ hr}$) than in August ($10:00 \text{ AM} \pm 2:00 \text{ hr}$) or December ($10:35 \text{ AM} \pm 1:25 \text{ hr}$; $P_s < 0.015$). However, differences in capture time were relatively small ($\eta^2_H = 0.27$) and in addition, the amount of time elapsed between civil twilight on the sampling dates and capture times ($2:20 \pm 0.55 \text{ hrs}$ (June) to $4:45 \pm 2:00 \text{ hrs}$ (August)) did not differ seasonally (KW: $P > 0.08$).

3.3. Seasonal morphological changes

Body mass. The body mass of the study birds underwent statistically significant but small seasonal changes (ANOVA: $F_{3,46} = 3.19$; $P = 0.033$; Cohen's $d = 0.2$; Table 2). Birds were, on average, lightest during the breeding season and heaviest during molt, with pre-breeding and non-breeding birds having intermediate masses.

Cloacal protuberance width. Cloacal protuberance width underwent robust seasonal changes (ANOVA: $F_{3,46} = 65.28$; $P < 0.001$; Cohen's $d = 0.8$; Table 2), with average width during each life history stage differing from each other. Cloacal protuberance was largest during the breeding season and smallest during the non-breeding season. Birds sampled during the pre-breeding season had smaller CP than during the breeding season. The CP size of sparrows sampled during molt was intermediate in size between those of breeding and non-breeding sparrows.

Fat score. Fat score changed seasonally (KW: $H_3 = 20.97$, $P \leq 0.001$; $\eta^2_H = 0.42$; Table 2). Birds during the breeding season had higher fat score than during molt or the non-breeding season. Pre-breeding birds had intermediate fat score that did not differ significantly from that of birds at other life history stages.

Contour feather molt. Molt score underwent substantial seasonal changes (KW: $H_3 = 33.67$, $P \leq 0.001$; $\eta^2_H = 0.71$; Table 2). Sparrows sampled in September were undergoing heavy contour feather molt whereas birds sampled at other times were not molting.

3.4. Seasonal changes in plasma testosterone and corticosterone

Stress affected plasma CORT and T and, overall, plasma T but not CORT changed as a function of the life history stage (Table 3). Furthermore, in both cases there was a statistically significant, but relatively small ($\eta^2 < 0.20$), life history stage x stress interaction.

Plasma CORT during stress increased in all groups within 10 min of capture and restraint (SNK: $P_s < 0.001$), and further increased in all groups (SNK: $P_s < 0.001$) except in non-breeding birds during the following 20 min (Fig. 1). Pair-wise comparisons revealed no group differences either in initial plasma CORT or in plasma CORT measured after capture and restraint for 10 min or 30 min. These results provide no evidence that the plasma CORT response to acute stress was seasonally modulated.

Pre-breeding and breeding sparrows had similar initial plasma T, which likewise did not differ between molting and non-breeding birds (Fig. 2). Furthermore, initial plasma T in pre-breeding and breeding birds was higher than in molting and non-breeding birds (SNK: $P_s < 0.03$). Plasma T decreased in response to 30 min of capture and restraint in pre-breeding birds (SNK: $P < 0.001$) and only 10 min in breeding birds (SNK: $P = 0.019$). By contrast, we found no evidence that plasma T changed during stress in molting or non-breeding birds.

Initial plasma T did not correlate with initial plasma CORT (Spearman rank order correlation: $P = 0.48$). There also was no correlation between plasma levels of the two hormones during stress (10 min: $P = 0.90$; 30 min: $P = 0.18$).

3.5. Seasonal changes in plasma glucose and uric acid

Plasma GLU changed as a function of the life history stage and considering all life stages, was significantly but weakly influenced by stress ($\eta^2 = 0.1$; Table 3 and Fig. 3). There was, however, a moderate life history stage x stress duration interaction ($\eta^2 = 0.35$). Plasma GLU at the time of capture did not differ between life history stages. In response to acute stress, it increased within 10 min in pre-breeding birds (SNK: $P = 0.007$), did not change in breeding birds, and decreased in molting and non-breeding birds (SNK: $P_s \leq 0.013$). In molting but not non-breeding birds, a further decrease was observed

between 10 min and 30 min of stress (SNK: $P = 0.005$). Stress-associated plasma GLU 30 min after capture was, on average, 17.6% higher (pre-breeding), and 3.4% (breeding), 26.2% (molt), and 12.9% (non-breeding) lower relative to corresponding plasma GLU at capture (see Section 3.7).

Plasma UA varied as a function of the life history stage and was markedly influenced by stress ($\eta^2 = 0.71$; Table 3 and Fig. 4). There was no life history stage $\times$ stress duration interaction, indicating that the effect of stress on plasma UA was similar across life history stages. Pre-breeding birds had higher plasma UA than breeding birds (SNK: $P = 0.006$; Fig. 4), but plasma UA in neither group differed from that in molting or non-breeding birds. Across groups, plasma UA decreased within 10 min of capture and restraint (SNK: $P < 0.001$) and again between 10 min and 30 min of stress (SNK: $P < 0.001$).

3.6. Correlations between changes in plasma hormones and metabolites

We determined whether changes in plasma CORT during exposure to stress for 10 min or 30 min during each life history stage correlated with corresponding changes in plasma GLU. Changes in plasma CORT and GLU were positively correlated in breeding sparrows exposed to stress for 10 min ($P = 0.04$, $r^2 = 0.28$) and negatively correlated in non-breeding sparrows exposed to stress for 30 min ($P = 0.05$, $r^2 = 0.41$). The significance of these observations is uncertain because correlations were statistically insignificant (i.e., $P > 0.006$) after adjustment for the number of regressions performed (2 times $\times$ 4 life history stages). Considering all birds, the increase in plasma CORT during stress for 30 min correlated negatively with the corresponding decrease in plasma UA ($P = 0.014$, $r^2 = 0.13$; Fig. 5): males whose plasma CORT increased more during stress also experienced a larger decrease in plasma UA.

There was no relationship between changes in plasma T during exposure to stress for 10 min or 30 min and corresponding changes in plasma GLU when each life history stage was considered separately. Combining data for all birds, the decrease in plasma T during exposure to stress for 30 min correlated negatively with corresponding changes in plasma GLU ($P = 0.04$; Fig. 5). However, this correlation was weak ($r^2 = 0.09$) and, after exclusion of a statistical outlier (see Fig. 5; Grubb's test, $P < 0.05$), was no longer statistically significant. Changes in plasma T during stress were statistically unrelated to corresponding changes in plasma UA.

3.7. Relationships between environmental factors and glycemia

In an attempt to identify factors that could account for the changes in plasma GLU observed during acute stress, we analyzed these changes (% change at 30 min relative to initial plasma GLU) as a function of the capture times and dates, and of the minimum ambient temperatures experienced during the night preceding capture.

Stress-associated changes in plasma GLU were independent of the time of day that birds were caught (linear regression: $P > 0.06$) and the amount of time elapsed between twilight and capture ($P > 0.3$). Confirming rmANOVA results (see above and Fig. 3), the magnitude of these changes varied seasonally (ANOVA: $F_{3,43} = 9.88$, $P < 0.001$, $\eta^2 = 0.41$) and such that it differed between sparrows sampled in September (decrease), December (decrease), August (no change), and June (increase; SNK: $P_s \leq 0.007$; Fig. 6). Changes in glycemia during stress correlated positively but weakly with minimum ambient temperature during the night preceding capture (linear regression: $n = 47$; $P = 0.03$; $r^2 = 0.1$) and negatively with capture date ($P < 0.001$; $r^2 = 0.22$; Fig. 6). However, these two factors were not independent because minimum nightly temperatures decreased considerably from June to December (linear regression: $n = 4$ dates; $P = 0.05$; $r^2 = 0.90$). Therefore, variations in plasma GLU sensitivity to stress as a function of the life history stage may have resulted from effects of ambient temperature, but alternatively from effects of unidentified temperature-unrelated and seasonally changing factors.

4. Discussion

We used a free-ranging sedentary and seasonally breeding male passerine to analyze life history stage- and acute stress-associated changes in plasma levels of two hormones, CORT and T, and of two metabolites, GLU and UA. Functional relationships between plasma CORT, T, GLU, and UA in free-ranging birds are rarely investigated and remain poorly understood. Therefore, a main objective of our investigation was to infer potential relationships through analysis of correlations between temporal changes in these parameters. In an independent study on RWSP, Deviche et al. (2014) determined effects of acute stress only for 30 min and only in breeding and molting birds. Two main objectives of the present work were to (1) confirm and extend previous findings by measuring effects of stress also in pre-breeding and non-breeding birds and (2) better characterize the latency of endocrine and metabolic effects of acute stress by determining these effects in birds exposed to acute stress not only for 30 min, but also 10 min. Based on previous work, we predicted a negative correlation during stress between changes in plasma CORT and those in both plasma GLU and UA, and that a decrease in plasma T, be it related to the life history stage or resulting from stress, would be consistently associated with changes in plasma GLU.

With regard to plasma UA and consistent with our prediction, plasma CORT and UA increased and decreased, respectively, at all life history stages during stress. Further, at the individual level, changes in plasma CORT and UA during stress were negatively correlated. With previous studies in RWSP and other species, these observations suggest that CORT contributes to mediating the inhibitory effect of acute stress on plasma UA, although as discussed below, more research is warranted to evaluate this hypothesis. With regard to plasma GLU, we found that depending on the life history stage, stress

exposure either increased (pre-breeding stage), did not change (breeding stage), or decreased (molting and non-breeding stages) glycemia. Thus, parallel changes (i.e., increases) in plasma CORT and GLU were observed only in pre-breeding sparrows. Additionally, at the individual level we found no consistent relationship between changes in plasma CORT and GLU during stress. Taken together, the results offer little support for the hypothesis that CORT exerts a rapid hyperglycemic action. Finally, during stress, plasma T decreased in pre-breeding and breeding birds whereas at the same time, glycemia increased (pre-breeding stage) or did not change (breeding stage). Furthermore, in molting and non-breeding sparrows stress decreased plasma GLU without also influencing plasma T. The results do, therefore, not show consistent relationships between rapid changes in plasma T and glycemia.

4.1. Stress-induced plasma glucose regulation

Elevated plasma CORT during stress is often associated with hyperglycemia (Sapolsky et al., 2000; Rand et al., 2002) and acute stress in birds can elevate plasma GLU (Rock Pigeon, *Columba livia*: Scope et al., 2002). However, to our knowledge, a single study on free-ranging birds has previously investigated relationships between plasma CORT and GLU during stress (Deviche et al., 2014). In the present study and consistent with a hyperglycemic effect of CORT, we found in pre-breeding sparrows that acute stress rapidly (within 10 min and for the next 20 min) increased plasma CORT and GLU. However, during other life history stages, plasma CORT also increased during stress while plasma GLU did not change (breeding birds) or decreased (molting and non-breeding birds). Consistent with the latter observation, Deviche et al. (2014) reported in molting RWSP that plasma CORT increases whereas plasma GLU concurrently decreases during acute stress. Furthermore, in the present study, at the individual level and considering each life history stage separately, we found no consistent associations between stress-induced changes in plasma CORT and GLU. Overall, these data provide only partial support for the hypothesis that plasma CORT mediates rapid changes in plasma GLU. Studies on other avian species also provide weak support for the hypothesis that CORT plays a major role in the control of glycemia. For example, chronic CORT administration to captive male Abert's Towhees, *Melospiza aberti*, did not elevate their plasma GLU (Davies et al., 2013). In the European Starling, *Sturnus vulgaris*, CORT treatment likewise did not increase plasma GLU when given to short- or long-day exposed captive birds (Remage-Healey and Romero, 2001). Corticosterone administration to molting starlings did elevate glycemia (Remage-Healey and Romero, 2002) but the treatment produced supraphysiological plasma CORT levels and so the relevance of the findings to the situation in intact birds is questionable.

Testosterone administration to chickens often increases their glycemia (Chen et al., 2005; Mohammadi and Ansari-Pirsaraei, 2014; but see Lepkovsky et al., 1967), but whether endogenous T also exerts a hyperglycemic action in birds is not well understood. Plasma T in male RWSP changes across

life history stages and we used this variation to research relationships between this hormone and plasma GLU. However, we found only a weak association between plasma T and GLU, an observation that provides no evidence that the androgen normally stimulates plasma GLU in the RWSP.

Due to their small size (body mass approx. 15 g), RWSP have a high metabolic rate relative to larger birds and presumably also a high rate of GLU absorption into tissues. Furthermore, RWSP store little body fat (Table 2: fat scores ≤ 1 on a 0-5 scale) and birds generally store little glycogen in their liver and muscles (Scanlon and Braun, 2013). In these birds, therefore, maintaining or elevating plasma GLU presumably depends heavily on gluconeogenesis. This dependence may be exacerbated during mild restraint, as used here, because birds in this situation have no access to food and so cannot replenish their energy reserves. We previously proposed that the rapid decrease in plasma GLU that, in some cases, occurs during acute stress (molting and non-breeding birds in the present study) may result from tissues absorbing plasma GLU at a faster rate than it can be provided into circulation following gluconeogenesis, thereby leading to net plasma GLU depletion (Deviche et al., 2014). This remains a possibility, but it should be noted that birds primarily use glycerol and amino acids as substrates for gluconeogenesis (Sweazea and Braun, 2008). If the above proposition is correct, we would expect the ability to elevate or maintain plasma GLU during stress to be positively associated with factors such as body fat scores or body masses, but this was not the case. For example, sparrows were heaviest during molt, when plasma GLU decreased the most during stress. Furthermore, fat scores in the study birds were generally small and were largest during the breeding period, when plasma GLU did not change in response to stress. Thus, the mechanism that accounts for rapid changes in glycemia during acute stress remains speculative and its identification requires additional research.

Changes in plasma GLU during stress were associated with ambient temperature: the higher the minimum ambient temperature the night before capture, the more plasma GLU increased during stress. A similar relationship was recently described for the Blue Tit (Kalinski et al., 2014), but whether this association reflects a causal relationship is unknown. When exposed to relatively cold nocturnal temperature, birds may use a larger proportion of their energy reserves than when exposed to warmer temperature and so subsequently become less capable of increasing plasma GLU in response to acute stress. In mammals, acute stress in some conditions is thought to stimulate the transcription of the preproglucagon gene (Zhang et al., 2009). In fasting birds, as may have been the case during restraint in the present study, stress increases plasma glucagon (review: Dupont et al., 2015). Glucagon in birds plays a major stimulatory role in glycemia (Brown and Sweazea, 2008) and in the Red-winged Blackbird, *Agelaius phoeniceus*, adipose tissue contains more glucagon receptors in birds that are chronically exposed to high than to low ambient temperatures (Hintz, 2000). The same may apply to RWSP and in particular to glucagon-responsive tissues, such as the liver, that play a major role in gluconeogenesis. If so,

GLU secretion by these tissues may increase with rising ambient temperatures and this could account for the present observations. Further research on this subject is warranted for two reasons. First, the present relationship between ambient temperature and glycemia, while statistically significant, was relatively weak as shown by low associated effect size. Second, changes in minimum ambient temperature were negatively correlated with calendar dates of capture, i.e., this temperature gradually decreased during the study period (June to December). It can, therefore, not be excluded that environmental factors(s) other than, but correlated with, ambient temperature influenced the observed seasonal variation in the plasma GLU response to stress.

4.2. Stress-induced plasma uric acid regulation

Consistent with a previous but independent study on the RWSP (Deviche et al., 2014) and studies on other species (Cohen et al., 2007; Davies et al., 2013), stress caused a rapid (within 10 min) decline in plasma UA. This decrease occurred at all life history stages and consistent with Deviche et al. (2014), at the individual level, males whose plasma CORT increased more during stress also experienced a larger decrease in plasma UA. Deviche et al. (2014) also found that the negative relationship between plasma CORT and UA held not only during capture and restraint-associated stress but also during a social challenge, when plasma CORT increased and plasma UA concurrently decreased. Furthermore, in September – when the plasma CORT response to acute stress was dampened – there was no detectable change in plasma UA. Collectively, these data indicate that in birds exposed either to acute stress or to social challenge, the plasma UA response to acute stress negatively mirrors the plasma CORT response. It must be noted, however, that while acute stress consistently decreases plasma UA in the RWSP, this is not necessarily the case in other species (Cohen et al., 2007; Fokidis et al., 2011). Manipulative experiments using CORT administration also suggest that the relationship between plasma CORT and UA is species-specific: increasing plasma UA in chickens (Lin et al., 2004) but having no effect in the Abert's Towhee (Davies et al., 2013). Therefore, overall the present data are consistent with the hypothesis that changes in plasma CORT are causally related to changes in the opposite direction in plasma UA, but further research is needed in order to confirm this hypothesis.

Uric acid is a potent antioxidant that may mitigate oxidative stress associated with GLU utilization. Accordingly, the stress-associated decrease in plasma UA may result from UA being absorbed into tissues and then quenching free radicals produced by increased tissue GLU oxidation (Deviche et al., 2014). Whether this mechanism is correct, however, requires further research because in some birds (e.g., Mourning Dove, *Zenaidura macroura*), high glycemia does not appear to be associated with oxidative damage (Smith et al., 2011). Further, when compared to other vertebrates, birds have multiple biochemical processes which can protect against oxidative damage, and White-throated Sparrows,

Zonotrichia albicollis, can mount an effective antioxidant response independent of UA (Sepp et al., 2012; Alan and McWilliams, 2013). Finally, we found no consistent relationship between stress-induced changes in plasma GLU, which varies seasonally, and plasma UA, which always decreased during stress.

4.3. Initial and stress-induced plasma testosterone regulation

Consistent with our previous studies on the same population of RWSP (Deviche et al., 2006), initial plasma T changed seasonally, with pre-breeding and breeding birds having higher plasma T than molting and non-breeding birds, which did not differ. In addition, and congruent with previous work (Deviche et al., 2010, 2012), plasma T was affected by acute stress, decreasing within 10 min of capture and restraint in breeding sparrows, and within 30 min of this manipulation in pre-breeding birds. By contrast, plasma T did not decrease during stress in molting or non-breeding males. An independent study measuring plasma T in molting male RWSP likewise found no inhibitory effect of acute stress on plasma T (Deviche et al., 2014). The current findings extend previous observations showing that plasma T is acutely sensitive to stress and this sensitivity is a function of initial plasma T. Effectively, birds decrease plasma T more during stress when initial plasma T is higher. It should be noted that as initial plasma T varied as a function of the life history stage, it cannot be determined whether changes in plasma T sensitivity to acute stress are related to initial plasma T *per se* as compared to other factors that co-vary with the life history stage.

An objective of the present study was to investigate relationships between stress-related changes in plasma CORT and T. Consistent temporal relationships between these changes across life history stages would suggest that they are causally related. As expected, plasma CORT increased rapidly (within 10 min) after capture and restraint and further increased during the next 20 min of restraint. Furthermore, we found no evidence that the increase in plasma CORT during stress differs across life history stages, i.e., that is seasonally modulated. Contrasting with this conclusion, Deviche et al. (2014) observed that acute stress increased plasma CORT more in breeding than in molting birds. The reason for this difference between studies is unknown. Nevertheless, the present data do not support the hypothesis that initial plasma T or the T response to acute stress is mechanistically related to plasma CORT. In the Zebra Finch, *Taeniopygia guttata*, *in vitro* CORT treatment stimulates the gonadal expression of gonadotropin inhibitory hormone (GnIH) mRNA (McGuire et al., 2013) and birds with higher plasma CORT during stress also express more testicular GnIH (Lynn et al., 2015). Furthermore, there is evidence in the Zebra Finch that GnIH acts locally to inhibit T secretion (McGuire et al., 2010). In the Zebra Finch, therefore, testicular GnIH may contribute to mediating inhibitory effects of CORT on T secretion. Even if future studies on RWSP revealed a causal relationship during stress between changes in plasma CORT and T, it is unlikely that testicular GnIH in this species plays the same role as postulated in the Zebra Finch. Indeed,

the short latency (within 10 min) for acute stress to decrease plasma T in RWSP by itself points to a non-genomic rather than genomic (e.g., increase in GnIH expression) mechanism orchestrating this decrease.

4.4. Conclusions

In many vertebrates, including birds, acute stress stimulates the secretion of glucocorticoids, and many studies have described how factors such as the life history stage, habitat, and sex modulate this stimulation. Elevated plasma glucocorticoids during acute stress are generally considered to help organisms deal with the potentially detrimental effects of stressors, but the underlying endocrine and metabolic mechanisms remain poorly understood, and particularly so in free-ranging vertebrates. This lack of understanding stems partly from the fact that many studies on this subject have measured levels of plasma glucocorticoids without also measuring plasma reproductive hormones and metabolic substrates and products. To address this issue, we determined the effects of acute stress on plasma CORT as well as T, GLU, and UA in a free-ranging avian species as a function of the life history stage. As predicted based on previous work, plasma CORT increased during stress. Furthermore, this increase was consistently associated with a decrease in plasma UA, but not with changes in plasma GLU or T. These findings question the frequent assumption that elevated plasma CORT during acute stress increases glycemia and decreases the secretion of reproductive hormones, and new mechanistic studies are warranted to determine whether and in what conditions these assumptions are correct. In order to fully appreciate the diversity and complexity of the endocrine and metabolic roles of glucocorticoids during acute stress in free-ranging vertebrates, studies on this subject should not only measure circulating levels of plasma glucocorticoids, but also of multiple endocrine and metabolic markers.

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Figure legends

Fig. 1. The effect of acute stress on plasma corticosterone in free-ranging adult male Rufous-winged Sparrows, *Peucaea carpalis*, is similar across life history stages. Plasma corticosterone (mean $\pm$ s.e.m) was measured within 2 min of capture (0 min: initial) and again after 10 and 30 min of restraint in sparrows sampled before (June: Pre-breeding; n = 10) and during (August: Breeding; n = 17) the breeding period, as well as during molt (September; n = 10) and the non-breeding period (December; n = 10). * indicates a significant difference ($P \leq 0.05$) relative to initial plasma corticosterone. + indicates a significant difference between two consecutive time points within the same group of birds.

Fig. 2. Plasma testosterone (mean $\pm$ s.e.m) in adult male Rufous-winged Sparrows, *Peucaea carpalis*, rapidly decreases in response to capture and restraint in pre-breeding and breeding sparrows, but not in molting or non-breeding sparrows with lower initial plasma T. See Fig. 1 legend and Results for additional information.

Fig. 3. The magnitude and direction of changes in plasma glucose (mean $\pm$ s.e.m.) of adult male Rufous-winged Sparrows, *Peucaea carpalis*, vary as a function of the life history stage. See Fig. 1 legend and Results for additional information.

Fig. 4. Plasma uric acid (mean $\pm$ s.e.m) in adult male Rufous-winged Sparrows, *Peucaea carpalis*, decreases rapidly (within 10 min) in response to capture and restraint. The magnitude of this decrease is similar across life history stages. See Fig. 1 and Results for additional information.

Fig. 5. *Upper panel:* Changes in plasma uric acid during acute stress (30 min) in free-ranging adult male Rufous-winged Sparrows, *Peucaea carpalis*, are negatively related to corresponding changes in plasma corticosterone (CORT; $r^2 = 0.13$, $P = 0.014$). *Lower panel:* In the same birds as shown in the upper panel, changes in plasma testosterone (T) during acute stress (30 min) are negatively related to corresponding changes in plasma glucose ($r^2 = 0.09$, $P = 0.040$). but I thought after excluding outlier this is no longer significant.

Fig. 6. Changes in plasma glucose (% change relative to initial plasma glucose; mean $\pm$ s.e.m.) in free-ranging adult male Rufous-winged Sparrows, *Peucaea carpalis*, after capture and restraint for 30 min vary as a function of the minimum ambient temperature during the night preceding capture and the life

752 history stage. Means with different superscript letters differ significantly ($P \leq 0.05$; Student-Newman-
753 Keuls test). See Fig. 1 legend and Results for additional information.

Table 1: Results of repeated measure analyses of variance on changes in four plasma parameters (corticosterone, testosterone, glucose, and uric acid) in adult male Rufous-winged Sparrows, *Peucaea carpalis*, as a function of the year of sampling (2013 vs. 2014) and the time (0, 10, or 30 min) after capture and beginning of restraint.

	Corticosterone	Testosterone	Glucose	Uric acid
Year	$F_{1,15} = 0.47$ $P = 0.50$ $\eta^2 = 0.03$	$F_{1,15} = 0.00$ $P = 1.00$ $\eta^2 = 0.00$	$F_{1,15} = 1.19$ $P = 0.29$ $\eta^2 = 0.07$	$F_{1,15} = 0.03$ $P = 0.87$ $\eta^2 = 0.00$
Time	$F_{2,30} = 54.42$ $P < 0.0001$ $\eta^2 = 0.78$	$F_{2,30} = 45.47$ $P < 0.0001$ $\eta^2 = 0.75$	$F_{2,30} = 4.20$ $P = 0.02$ $\eta^2 = 0.22$	$F_{2,30} = 28.43$ $P < 0.0001$ $\eta^2 = 0.65$
Year x Time	$F_{2,30} = 0.81$ $P = 0.45$ $\eta^2 = 0.05$	$F_{2,30} = 3.84$ $P = 0.03$ $\eta^2 = 0.20$	$F_{2,30} = 0.52$ $P = 0.06$ $\eta^2 = 0.03$	$F_{2,30} = 4.05$ $P = 0.03$ $\eta^2 = 0.21$

P = probability associated with corresponding F value. η^2 = partial eta-squared (a measure of effect size). Statistically significant effects ($P < 0.05$) and medium or large effect sizes ($\eta^2 > 0.5$) are shown in bold.

755 **Table 2.** Morphological characteristics of free-ranging adult male Rufous-winged Sparrows, *Peucaea*
756 *carpalis*, from the same Arizona population and sampled before (June: Pre-breeding; n = 10) and during
757 (August: Breeding; n = 17) the breeding period, as well as during molt (September; n = 10) and the non-
758 breeding period (December; n = 10).

	Pre-breed.	Breeding	Molt	Non-breed.
Body mass (g) ¹	15.7 ± 0.1 ^{ab}	15.3 ± 0.2 ^a	16.1 ± 0.2 ^b	15.9 ± 0.1 ^{ab}
Cloacal protuberance width (mm) ¹	4.2 ± 0.1 ^a	5.0 ± 0.1 ^b	3.8 ± 0.1 ^c	3.3 ± 0.1 ^d
Fat score ²	0.5 ± 0.3 ^{ac}	1.0 ± 0.4 ^a	0.3 ± 0.3 ^{bc}	0.5 ± 0.5 ^{bc}
Body molt score ²	0 ± 0 ^a	0 ± 0 ^a	3 ± 1 ^b	0 ± 0 ^a

¹: Means ± s.e.m.; ²: Medians ± 0.5 interquartile intervals. For a given parameter, means/medians with the same superscript letter do not differ significantly (P > 0.05).

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Table 3: Results of repeated measures analyses of variance on changes in four plasma parameters (corticosterone, testosterone, glucose, and uric acid) in adult male Rufous-winged Sparrows, *Peucaea carpalis*, as a function of the life history stage (pre-breeding, breeding, molting, or non-breeding) and the time (0, 10, or 30 min) after capture and beginning of restraint.

	Corticosterone	Testosterone	Glucose	Uric acid
Life history stage	$F_{3,43} = 1.20$ $P = 0.32$ $\eta^2 = 0.08$	$F_{3,43} = 8.78$ $P = 0.0001$ $\eta^2 = 0.38$	$F_{3,43} = 9.39$ $P < 0.0001$ $\eta^2 = 0.4$	$F_{3,43} = 4.38$ $P = 0.009$ $\eta^2 = 0.23$
Time	$F_{2,86} = 223.74$ $P < 0.0001$ $\eta^2 = 0.84$	$F_{2,86} = 32.85$ $P < 0.0001$ $\eta^2 = 0.43$	$F_{2,86} = 4.82$ $P = 0.010$ $\eta^2 = 0.1$	$F_{2,86} = 103.29$ $P < 0.0001$ $\eta^2 = 0.71$
Life stage x Time	$F_{6,86} = 2.67$ $P = 0.020$ $\eta^2 = 0.16$	$F_{6,86} = 2.98$ $P = 0.011$ $\eta^2 = 0.17$	$F_{6,86} = 7.88$ $P < 0.0001$ $\eta^2 = 0.35$	$F_{6,86} = 2.19$ $P = 0.52$ $\eta^2 = 0.13$

P = probability associated with corresponding F value. η^2 = partial eta-squared (a measure of effect size). Statistically significant effects ($P < 0.05$) and medium or large size effects ($\eta^2 > 0.5$) are shown in bold.

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775 Fig. 6. Deviche et al.
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