Stress-responsiveness influences baseline glucocorticoid levels: revisiting the under 3 min sampling rule

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1 Plasma glucocorticoid (CORT) levels collected within 3 min of capture are commonly believed
2 to reflect pre-stressor, baseline CORT levels. Differences in these “baseline” values are often
3 interpreted as reflecting differences in health, or the amount of social and environmental stress
4 recently experienced by an individual. When interpreting “baseline” values it is generally
5 assumed that any effect of capture-and-handling during the initial sampling period is small
6 enough and consistent enough among individuals to not obscure pre-capture differences in
7 CORT levels. However, plasma CORT increases in less than 3 min post-capture in many free-
8 living, endothermic species in which timing has been assessed. In addition, the rate of CORT
9 secretion and the maximum level attained (i.e., the degree of stress-responsiveness) during a
10 severe stressor often differs among individuals of the same species. In Florida scrub-jays
11 (*Aphelocoma coerulescens*), an individual’s stress-responsiveness during a 30 min post-capture
12 stressor is correlated with CORT levels in samples collected within 1.5 min of capture,
13 suggesting there is an intrinsic connection between stress-responsiveness and pre-capture CORT
14 levels. Although differences in stress-responsiveness accounted for just 11% of the variance in
15 these samples, on average, higher stress-responsive jays (top third of individuals) had baseline
16 values twice that of lower stress-responsive jays (bottom third). Further, plasma CORT levels
17 begin to increase around 2 min post-capture in this species, but the rate of increase between 2
18 and 3 min differs markedly with CORT increasing more rapidly in jays with higher stress-
19 responsiveness. Together, these data indicate that baseline CORT values can be influenced by an
20 individual’s stress response phenotype and the differences due to stress-responsiveness can be
21 exaggerated during sample collection. In some cases, the effects of differences in stress-
22 responsiveness and the increase in CORT during sample collection could obscure, or supersede,
23 differences in pre-capture plasma CORT levels that are caused by extrinsic factors.

Keywords: Baseline; Stress response; Glucocorticoid; Individual variation;
Hypothalamic-pituitary-adrenal axis; Handling stress

1. Introduction

Glucocorticoids (corticosterone and cortisol; CORT) are a hormonal end product of the hypothalamic-pituitary-adrenal (HPA) axis and, while their primary function is metabolic, they have wide-ranging effects on an organism (Sapolsky et al., 2000). During short-term, extreme environmental challenges, high levels of CORT (stress-induced CORT [SI-CORT]) help facilitate the response to, and recovery from, immediate life-threatening circumstances. At the relatively low “baseline” levels commonly found in unperturbed animals, CORT is thought to support the predictable daily and seasonal patterns of changing energetic needs (see Landys et al., 2006; Romero, 2004; Sapolsky et al., 2000).

An important consideration for the measurement of baseline CORT in plasma is the time between initiation of the stressor (e.g., removal from an enclosure, being caught, etc.) and sample collection. Within the first few seconds of encountering a stressor, corticotropin-releasing hormone (CRH) is secreted from the hypothalamus. CRH travels to the anterior pituitary where it stimulates adrenocorticotrophic hormone (ACTH) secretion into general circulation, resulting in increased CORT synthesis and subsequent release from the adrenal cortex “over the course of minutes” (Sapolsky et al., 2000). Much of what is known about the timing and rate of the post-stressor increase in HPA axis activity, including CORT secretion, is derived from studies on captive bred rodents (Dallman and Bhatnagar, 2001). However, the HPA axes of captive-bred animals often respond more slowly and reach lower peak activity in comparison to their free-living counterparts (Awata et al., 2011; Douxfils et al., 2011; Ericsson et

al., 2014; Homberger et al., 2015; Künzl and Sachser, 1999; Plyusnina et al., 2011; Suzuki et al., 2012; Takahara et al., 2014; Trut et al., 2009; Verbeek et al., 2008), suggesting that captive studies will overestimate the time between the onset of a stressor and the subsequent increase in plasma CORT levels in a free-living animal.

While some sources indicate that CORT levels start to increase between 3 to 5 min post-capture in free-living animals (Boonstra, 2005; Sheriff et al., 2011), Romero and Reed (2005) conducted the most thorough investigation on this topic and their study has been widely interpreted to support an “under 3 minute rule” as the standard time frame for sampling baseline CORT in birds and some mammals (Johnstone et al., 2012; Owen, 2011). In their study, change-point analyses were used to determine the primary time at which CORT levels began to increase post-capture for five species of songbird and one species of iguana. Each bird species was sampled during at least two, but up to four, different life-history stages (e.g., breeding, molt, wintering, etc.). The iguana species showed no significant change-point within 3 min of capture, but in the 13 songbird data sets, seven had a change-point of 2 min or less, four had change-points at 3 min, and two had no significant change-point, indicating that CORT levels began to rise in less than 3 min in over half of the data sets. However, even when CORT began to increase within 3 min of capture, levels were still significantly lower than CORT levels after 30 min of capture stress. Based on this, the authors concluded, “Even when samples collected within 3 min do not reflect unstressed concentrations, they will likely be near enough to baseline to be distinguishable from stress-induced concentrations.”

Although “near baseline” CORT samples are well below levels attained in response to a 30 min severe stressor, they may still be high enough to obscure important information found in “true” baseline samples. Studies that use baseline CORT levels as a predictor of fitness, or to

investigate whether CORT levels are repeatable, may require greater precision (e.g., Bonier et al., 2009; Ouyang et al., 2011; Small and Schoech, 2015). Further, the changes that occur within 3 min of capture may vary among individuals.

Once CORT levels begin to increase in response to a severe stressor, its rate of increase, maximum blood concentration, and the duration of elevation, can differ greatly among individuals in many animal species (Cockrem, 2013a). These inter-individual differences can be transient or they can be repeatable over time (Cockrem, 2013a; Cook et al., 2011; Hruschka et al., 2005; Narayan et al., 2013; Romero, 2002; Small and Schoech, 2015; Wada et al., 2008). These differences also have been linked to among individual differences in behavior, often characterized on a “bold-shy” or “proactive-reactive” scale (Cockrem, 2013b; Koolhaas et al., 1999). For example, great tits (*Parus major*) that explored a novel environment more slowly had higher “baseline” CORT (collected < 3 min) and higher SI-CORT (collected 90 min post-capture) when compared to faster explorers (Baugh et al., 2013). However, CORT levels increased during the 3 min post-capture sampling period and slower explorers had generally higher “baseline” CORT levels in samples collected between 2-3 min post-capture. Baugh et al. (2013) argued that the under 3 min baseline values were “stress-contaminated” and that the higher baseline CORT levels of slower explorers did not represent “differences in true ‘baseline’ concentrations”. This conclusion highlights the potential problems that can arise when differences in stress-responsiveness are not accounted for when measuring “baseline” CORT.

These among individual differences in SI-CORT may be caused by life-long differences in HPA axis activity (i.e., stress response phenotypes; Baugh, 2012; Carpenter et al., 2007; Claessens, 2011; Pottinger and Carrick, 1999). Repeatable among individual differences in HPA axis activity still allow for internal and external cues to modulate an individual’s HPA axis

activity, but the degree of modulation may be constrained by the broader phenotype (i.e., endocrine flexibility/reaction norms; Hau and Goyman, 2015; Taff and Vitousek, 2016). Whether individual differences in HPA axis activity during a severe stressor reflect similar differences in normal CORT regulation at baseline levels is still unclear. It is possible that the observed differences in SI-CORT regulation are entirely independent of baseline CORT regulation given that the biological activity and negative feedback regulation of CORT at baseline and stress-induced levels are primarily regulated through different receptor types (mineralocorticoid and glucocorticoid receptors, respectively; Atkinson et al., 2008; Funder, 1997; Landys et al., 2006; Spencer et al., 1998).

In Florida scrub-jays (*Aphelocoma coerulescens*), individual differences in SI-CORT levels are detectable within weeks of fledging and these differences are repeatable within adult males and females sampled over an eight year period (repeatability score > 0.5 for both sexes), suggesting that the general magnitude of the CORT stress response (the stress response phenotype) is a largely “fixed” physiologic trait that persists throughout an individual’s adult life (Rensel and Schoech, 2011; Small and Schoech, 2015). Similarly, baseline CORT levels in adult males are moderately repeatable, and baseline CORT values are often correlated with SI-CORT values in both males and females of this species.

To investigate the relationship between the life-long differences in the magnitude of an individual’s stress response and baseline CORT, we first determined which characteristics of the CORT stress response (e.g. rate of CORT increase, timing of negative feedback, rate of decline, etc), were most responsible for individual differences in SI-CORT (area under the curve for samples collected from capture to 30 min post-capture; Cockrem and Silverin, 2002). We then compared CORT levels collected within 3 min of capture and SI-CORT levels to test two non-

mutually exclusive hypotheses. First, differences in the stress response phenotype observed during an acute, severe stressor are indicative of differences in baseline CORT regulation, such that highly stress-responsive individuals (high SI-CORT) also maintain higher baseline CORT levels. Second, differences in stress-responsiveness influence when CORT levels begin to increase (i.e., the change-point) or the rate that CORT increases within 3 min of capture.

Because individual differences in SI-CORT are known to be repeatable through the life of adult Florida scrub-jays (Small and Schoech, 2015), SI-CORT will be used as the measure of an individual's "stress-responsive phenotype" and compared to CORT levels collected within 3 min of capture. Further, to control for individual variation in CORT values due to experience prior to capture, a repeated measures design was used in which a subset of jays was sampled two or three times within 3 min of capture to investigate within individual changes in CORT values during the "baseline" sampling period.

To reduce confusion, throughout the remainder of the manuscript we will use the term "baseline CORT" to refer to the concentration of plasma CORT *prior* to capture. The term "initial CORT" will be used for samples collected within 3 min *post*-capture, which are often believed to reflect baseline CORT values. Samples collected after the 3 min initial CORT sampling period will be referred to as "SI-CORT" samples and these SI-CORT values will be used as a measure of an individual's "stress-responsive phenotype".

2. Materials and Methods

2.1. Study species and site

Florida scrub-jays are non-migratory corvids that maintain territories year-round in coastal and inland Florida scrub oak ecosystems. Data presented herein were collected from adult (1 year of age or older) males and females from January to March of 2011-2014 as part of our ongoing long-term research on this cooperatively breeding species at Archbold Biological Station (ABS) in south-central Florida (27°10'N, 81°21'W, 38-68 m ASL). The study population occupies 9 km² of scrub habitat in the south 'half' of ABS and has been studied by SJS and colleagues for over two decades (Mumme, 1992; Schoech et al., 1991, 2012; Small and Schoech, 2015). All jays in the population are given a unique combination of two or three colored leg-bands and a USGS aluminum band for field identification so they can be monitored from hatching until death (up to 16 yr). Individuals are sexed using both genetic and behavioral markers. From 2011-2014, 233 Florida scrub-jays (118 female and 115 male; after hatching year or older) were sampled. Of these birds, 131 were sampled in only one year, 62 were sampled in two different years (once per year), 28 were sampled in three years, and 12 were sampled in all four years resulting in 387 captures.

2.2. Blood sample collection

Florida scrub-jays were caught in peanut-baited, walk-in Potter traps during the pre- to early-breeding season. Jays were trained to associate the traps with food for three or more days prior to trapping. On the trapping day, traps were constantly monitored and the exact capture time was recorded. When the trap door closed, a stopwatch was started to measure the elapsed time from capture to sample collection. After capture, a researcher removed the bird from the

trap, transferred it to a processing station (typically within 20 meters), and initiated blood sampling as rapidly as possible. Blood samples were collected in heparinized microhematocrit tubes following venipuncture of the brachial vein with a 25G sterile needle. The first blood sample was collected within 3 min of capture, and in all years, samples were also collected at 5, 15, and 30 min post-capture to measure SI-CORT (Romero and Reed, 2005; Schoech et al., 2007; Small and Schoech, 2015; Wingfield et al., 1995). Between collection of the 5, 15, and 30 min samples, individual jays were held in a loosely-woven cloth bag in the shade. In 2013, to allow for repeated measures analysis, if a sample was collected in less than 2.5 min post-capture, an attempt was made to collect a second and, in some cases, a third sample before 3 min had elapsed. For all samples, blood was kept on ice in the field for 1-4 hours until return to the lab where samples were separated into cellular and plasma fractions with centrifugation, after which the plasma portion was drawn off and stored at -20 °C until assayed for CORT (see below). All samples were collected between 7-11AM to help control for diel patterns in hormone secretion. In all cases, the total blood volume taken from an individual was below the 1% body weight safety limit recommended for birds (averaged 0.5%; max 0.7%; Owen, 2011). In 2013 and 2014, the time the trap was opened at each site was recorded to measure how long it took to catch each jay, thus allowing us to examine whether there is an anticipatory CORT response caused by an individual's (or other group members') interaction with the open trap. All procedures in this study were approved by the University of Memphis Institutional Animal Care and Use Committee.

2.3 Corticosterone assays

All plasma samples were assayed for CORT during the calendar year in which they were

collected. However, due to changes in corticosterone antibody availability over the four years of the study, samples were assayed using two different protocols. Samples collected in 2011 were assayed with radioimmunoassay (RIA) using the Fitzgerald 20-CR45 antibody (Fitzgerald Industries International, Acton, MA), and samples collected in 2012-2014 were assayed with Cayman 500655 Corticosterone Enzyme-Linked Immunosorbent Assay (ELISA; Cayman Chemicals, Ann Arbor, MI). The values from these assays are highly comparable (see Small and Schoech [2015] for validation of inter-assay comparisons). All samples collected from the same bird in the same year (initial [< 3 min], 5, 15, and 30 min) were run in the same assay.

2.3.1 Radioimmunoassay

CORT was extracted from plasma samples (5 to 40 μ l) using diethyl ether. The aqueous fraction of the extraction solution was snap-frozen, the ether fraction containing steroids was poured off, and the ether was evaporated under nitrogen gas. The dried sample was reconstituted in 550 μ l of phosphate-buffered saline with gelatin. Subsequently, 200 μ l of the sample in PBSG were aliquoted into duplicate assay tubes to which we also added a known amount of antibody and tritiated corticosterone (Perkin Elmer, Inc.). From the remaining 150 μ l, 100 μ l were dispensed into a vial to calculate recovery (see below). A standard curve was prepared by serially diluting CORT standard (2,000-7.8 pg; Sigma Aldrich) and adding the same amounts of labeled CORT and antibody that were combined with the unknown samples. CORT levels were determined by correcting for sample volume and percent recovery (mean recovery was $76.5 \pm 7.3\%$ [standard deviation; SD]). Percent recoveries were calculated from a small volume of radiolabeled CORT that had been added to samples prior to extraction. Samples from a total of 4 assays were included in this study. Two internal controls (IC; i.e., CORT samples of known concentrations) were included in duplicate at the beginning and end of each assay and used to

assess the inter- and intra-assay coefficients of variation (CV). The intra-assay CVs were 5.3%, 6.5%, 7.2% and 10.9%, whereas the inter-assay CV was 6.7%.

2.3.2 Enzyme-linked immunosorbent assays

Samples collected in 2012-2014 were assayed with the protocol and reagents provided with the Cayman Chemical ELISA corticosterone kit. Similar to extracted samples in the RIA, the Cayman assay measures total CORT in Florida scrub-jay samples (Schoech et al., 2013; Small and Schoech, 2015). Fifteen plates were used to assay samples in duplicate, and each plate also included IC CORT samples for assessment of intra- and inter-assay variation. The intra-assay CVs range from 1.1% to 17.8% (mean 8.3%) and the inter-assay CVs for 2012-2014 were 15.4%, 6.1%, and 10.2%, respectively. The inter-assay CV for all years combined was 13.9%.

The same Sigma Aldrich CORT stock solution was used to make the ICs for both the ELISAs and the RIAs. However, due to the difference in sensitivity between the two types of assays, the ELISA ICs were prepared at a lower concentration than those of the RIA to ensure that the ICs were distributed on the linear part of the standard curve. When the ELISA ICs were corrected for the dilution difference, the inter-assay CV for all RIAs and ELISAs combined was 12.9%.

2.4 Statistical analysis

To characterize the SI-CORT response for each individual, we calculated the area under the curve for the capture and restraint stress response (initial, 5, 15, and 30 min samples; integrated CORT). We also calculated the area under the curve after subtracting the initial CORT value from each value in the stress series (corrected integrated CORT; i.e., setting baseline to zero) to evaluate the change in CORT over initial CORT (Cockrem and Silverin, 2002). It is

unclear whether integrated or corrected integrated CORT values are the more biologically relevant measure of SI-CORT, so all analyses were done with integrated CORT and again with corrected integrated CORT. Results using these two measures of SI-CORT did not qualitatively differ. Only results using corrected integrated CORT are reported because it is the more conservative measure of SI-CORT for this study. By subtracting the initial CORT value from the SI-CORT calculation, the only potential bias introduced is a negative correlation between SI-CORT and initial CORT (the opposite of the predicted direction in this study) because the larger the value of initial CORT, the smaller the corrected integrated CORT value will be in comparison to the integrated CORT value.

In Florida scrub-jays, initial CORT and SI-CORT levels decline from 7 to 11AM each day due to a normal diel rhythm in CORT secretion (Rensel and Schoech, 2011; Small and Schoech, 2015; Wilcoxon et al., 2011). To correct for this effect, the residuals from a regression of initial CORT vs. capture time of day (hr since sunrise; $N = 387$, $\beta = -0.23 \pm 0.11$, $P < 0.001$) and the residuals from a regression of SI-CORT vs. capture time of day ($N = 387$, $\beta = -0.32 \pm 0.11$, $P < 0.001$) were used in each analysis.

2.4.1 Change-point

Similar to the methods of Romero and Reed (2005), non-parametric change-point analysis (Andersen et al., 2009; Pettitt, 1980) was used to estimate the timing of the post-capture increase in CORT. To avoid inclusion of non-independent data, for each jay captured in more than one year, a single sample was randomly selected for inclusion. Also, for jays sampled more than once in less than 3 min, only the 1st samples were included (see section 2.4.4 below). First, we calculated the overall change-point for samples collected under 3 min post-capture. Second, to investigate whether SI-CORT phenotype influenced when the post-capture increase in CORT

began, jays were near evenly divided into three SI-CORT categories (see Fig. 1A), and the CORT change-point was calculated separately for each: Low responders (mean: 441 [ng/ml] $\times$ min, range: 147-560, $n = 78$), Medium responders (Med; 658, 565-759, $n = 77$), or High responders (970, 775-1679, $n = 78$).

Prior to analysis, mean initial CORT values were centered for each year to control for inter-annual variation in initial CORT (Schoech et al., 2007; Small and Schoech, 2015). In the change-point analysis, each time point (sampling time post-capture) can only have one value associated with it. Because blood samples took multiple seconds to collect from the brachial vein, and sample times were recorded at the completion of blood collection, if more than one CORT value was associated with the same time point (e.g., three different jays had CORT samples at 2 min post-capture), extra values were moved to unoccupied time points within 3 seconds of the original time point. When no earlier time points were available, all CORT values associated with the same time point were averaged. The CORT values were then ranked (ties received a mid-rank) prior to each of the analyses and the change-point was calculated using the mean square error estimator technique in Change-Point Analyzer 2.3 (Taylor Enterprises, Inc., Libertyville, IL USA; Taylor, 2000). Change-point confidence levels and 95% confidence intervals (CI) were determined by performing 10,000 bootstrap simulations for each analysis (Hinkley and Schechtman, 1987). The data in each of the four analyses met the assumption of independent error structure (Taylor, 2000).

2.4.2 Rate of CORT increase during 30 min capture and handling stress

To determine when the rate of CORT increase was greatest during the 30 min standardized stressor, and when the greatest differences exist in the rate of increase among individuals with Low, Med, and High SI-CORT responses, we calculated the rate at which

CORT increased for the three time periods between sample collections (i.e., initial to 5 min, 5 to 15 min, and 15 to 30 min). Rate of change was used instead of comparing absolute concentration of CORT at each time point because the rate of change provides a standardized comparison between samples that were not equally distributed across time. In each case, the absolute change in CORT between sample points was divided by the elapsed time (e.g., [15 min CORT – 5 min CORT]/10 min) to produce a rate of change ([ng/ml]/min). For these calculations, initial CORT samples used 2 min as the initial CORT sample time instead of their true sample time because change-point analysis identified 2 min as the approximate time point when CORT began to increase (see results and Table 1). For samples collected prior to 2 minutes, using the true time would have underestimated the rate of change because it is unlikely that CORT levels appreciably increased prior to 2 min. Similarly, for those samples collected more than 2 min post-capture, the period of time between 2 min and the true sample time is likely to have the slowest rate of increase in plasma CORT concentration during the first 5 min, thus, including the additional time in the calculation would have less of a biasing effect compared to the overestimation that would result from using the true time.

To allow for post hoc comparisons that would more closely test the above stated hypotheses, we subdivided jays into Low, Med, and High SI-CORT categories (see 2.4.1 above). The rate of CORT change (response variable) was compared to SI-CORT using a linear mixed model (LMM) fitted by restricted maximum likelihood with SI-CORT, time period, and subject sex as fixed factors. Subject identity (ID) was included as a random variable nested within SI-CORT (repeated measures approach; Wallace and Green, 2002). Sex was included as a fixed factor because it was previously shown that sex differences exist in the repeatability of initial and SI-CORT values of Florida scrub-jays (Small and Schoech, 2015) indicating that sex differences

in CORT regulation may exist in this species. Tukey-Kramer HSD was used for post hoc comparisons of means to further investigate significant effects in the LMM.

2.4.3 Initial CORT and SI-CORT phenotype under 3 min

The effects of SI-CORT phenotype on initial CORT values were investigated using a LMM. CORT values from jays sampled once within 3 min of capture (2011-2014; $n = 350$) and the first sample collected from jays sampled more than once within 3 min (2013; $n = 37$) were used. Time since capture, SI-CORT phenotype, subject sex, and their interactions were included as fixed factors. The model was first run with SI-CORT and time since capture as continuous data. Similarly, a subsequent model was run with SI-CORT phenotype and time since capture treated as categorical data to facilitate post hoc analyses. SI-CORT phenotype categories were divided into Low, Med, and High (as 2.4.1 above), and time since capture was divided into samples collected between capture and 1.5 min post-capture (< 1.5 min), between 1.51 and 2 min (2 min), between 2.01-2.5 min (2.5 min), or between 2.51 and 3 min (3 min). The time categories were selected to coincide with the CORT change-point (~ 2 min) and to generally encompass the change-point 95% CIs for all three categories of SI-CORT phenotype (Table 1). For both models, year (categorical) was included as a random variable to control for inter-annual variation in initial CORT values and subject ID was included as a random variable to control for birds sampled in more than one year. Tukey-Kramer HSD was used for post hoc comparisons.

We estimated the relative effect size of the fixed factors on initial CORT values using two methods. First, we calculated the standardized regression coefficients (β) which expresses the slope of a line as the number of standard deviations the dependent variable changes per standard deviation of the independent variable. As such, for β values we report the slopes and 95% CIs of each explanatory variable and their interactions in the LMM containing SI-CORT

and time since capture as continuous data, but prior to analysis, all continuous independent and dependent variables were converted to their z-scores and the categorical variable, sex, was converted to a binary variable (Schielzeth, 2010). Second, we used a mixed model analogue of the linear model coefficient of determination (R^2) called R^2_{GLMM} (R^2_{G} ; Nakagawa and Schielzeth, 2013) to report the proportion of the variance in the initial CORT values that is explained by the independent variables. Similar to R^2 , R^2_{G} ranges from 0-1 and is a general expression of the “variance explained” by a model. There are two forms of R^2_{G} : conditional R^2_{G} (estimates the variance explained by both fixed and random factors), and marginal R^2_{G} (estimates the variance explained by the fixed factors alone). Using the `sem.model.fits` function in the `piecewiseSEM` package (Lefcheck, 2015) for R statistics (R Core Team, 2015), both conditional and marginal R^2_{G} were calculated for the LMM model as described above (Time since capture, SI-CORT phenotype, subject sex, and their interactions as fixed factors). To estimate the influence of each fixed factor on its own, both forms of R^2_{G} were also calculated for three additional models, each containing only one of the three fixed factors. The random variables were the same as above in all four models used to calculate R^2_{G} .

2.4.4 Repeated measures of initial CORT

To further investigate the influence of SI-CORT phenotype on initial CORT values, 37 jays were sampled either twice ($n = 28$) or three times ($n = 9$) within 3 min of capture during the 2013 pre-breeding season. Samples were analyzed using a mixed-model repeated measures approach (Wallace and Green, 2002). As above (2.4.3), the repeated measures model was first run with SI-CORT and time since capture as continuous data and a subsequent model was run with SI-CORT phenotype and time since capture included as categorical fixed factors, allowing post-hoc comparisons. Subject ID nested in SI-CORT phenotype was included as a random

variable. Subject sex was not included as a fixed factor due to the smaller sample size and because the prior analysis indicated sex had no effect on initial CORT values. Tukey-Kramer HSD was used for post hoc comparisons.

To test if the repeated measures design had carryover effects (i.e., collecting the first sample influenced the CORT values of subsequent samples), samples collected in 2013 from individuals that were sampled only once within 3 min were compared to the second or third samples from individuals that were repeatedly sampled under 3 min. In individuals sampled three times, only the third samples were used so that individuals were included only once in the analysis and because this last sample would likely best detect any carryover effect. Time since capture was limited to the period in which second and third samples were collected (1.8 to 3 min). The initial CORT values (response variable) were compared to SI-CORT (continuous), time since capture (continuous), sampling type (single vs repeated samples) and their interactions (fixed factors) in a general linear model (GLM) fitted by least squares.

2.4.5 Initial CORT and SI-CORT under 1.5 min

To more closely investigate whether baseline CORT levels covary with, or are a function of, SI-CORT phenotype, the following analysis was restricted to samples collected within 1.5 min of capture. The 1.5 min threshold was used because it fell below the lower 95% CI for the CORT change-point of each for the three SI-CORT categories (Table 1) and thus was unlikely to be influenced by capture stress and likely represented pre-capture baseline values. SI-CORT and time since capture were included as continuous fixed factors in the LMM to increase the power of the analysis and because non-linear relationships were not of interest. Sample year was included as a random variable.

Similar to 2.4.3, we estimated β using the slope and 95% CIs and conditional and marginal R^2_G for the under 1.5 min LMM model (Time since capture, SI-CORT phenotype, and their interactions as fixed factors) and then for two additional models, containing either time since capture, or SI-CORT phenotype as the fixed factors. The random variables were the same as above in all three models.

2.4.6 Capture effort

Jays must enter a trap to be caught and the experience of approaching and interacting with the trap could be a stressor that influences CORT levels prior to capture. The time it takes to catch a jay can vary greatly (mean 34 ± 37 [SD] min; median 18; range 1.2-169 min; elapsed time since a trap is set and baited at a site until capture; henceforth, “capture effort”). To investigate whether the pre-capture experience influenced the initial CORT values of Florida scrub-jays, in 2013-2014 we recorded the time a group of jays were first exposed to a baited trap and the exact capture time of each jay thereafter. In the first analysis, capture effort, SI-CORT level, time since capture (all continuous fixed factors) and their interactions were compared to initial CORT values collected within 3 min of capture. Second, the same analysis was repeated but only for samples collected within 1.5 min. Lastly, a subset of data from all birds caught within 10 min of encountering the trap were analyzed to assess whether exposure to the trap had a rapid effect on baseline CORT values that quickly stabilized. Samples collected within 2 min post-capture were used instead of 1.5 min to avoid the post 2 min increase in CORT while still providing a large enough sample size for analysis.

Because multiple jays were often caught on the same day at a site, witnessing other jays being captured prior to a jay’s own capture could have influenced baseline CORT values independently of capture effort. The order in which jays were captured at a site and capture effort

were not included as fixed factors in the same analysis because, as expected, they were strongly correlated ($F_{1,175} = 98.8$, $P < 0.0001$, $R^2=0.61$). When capture effort was replaced with capture order in the first and second analyses, results did not meaningfully differ, so the capture order results were not included.

Unless otherwise stated, statistical analyses were performed in JMP 10 (SAS Institute Inc., Arlington, VA.). CORT data were square-root transformed to meet assumptions of normality and homogeneity of variance. For ease of interpretation and comparison, non-transformed values are used in the text, tables, and figures.

3. Results

Male and female jays did not differ in any aspect of sample collection (DOY, $F_{1,381.5} = 0.384$, $P = 0.536$; time since sunrise, $F_{1,382.4} = 0.86$, $P = 0.355$; time post-capture, $F_{1,181.1} = 0.087$, $P = 0.768$) or in initial CORT levels ($F_{1,381.1} = 0.17$, $P = 0.680$; Female mean 3.15 ± 2.16 ng/ml [SD]; Male mean 3.22 ± 1.99 ng/ml). For jays sampled once under 3 min (all years), sample time post-capture ranged from 45 to 180 sec (114 ± 29). For jays sampled multiple times under 3 min, time of first sample ranged from 53 to 145 sec (105 ± 26) and last sample time ranged from 115 to 180 (166 ± 18). The three SI-CORT categories also did not differ in sample collection time ($F_{2,381.8} = 0.26$, $P = 0.771$; Low 50 to 180 sec (118 ± 29), Med 45 to 178 sec (111 ± 27), High 47 to 180 sec (114 ± 30).

3.1 Change-point

Change-point analysis identified 1.97 min post-capture as the primary change-point when all samples collected under 3 min were analyzed (Table 1). When the data were subdivided by

SI-CORT phenotype, change-points ranged from 1.80 min to 2.12 min and did not differ from each other or from the change-point derived for the full dataset (overlapping 95% confidence intervals; Table 1).

3.2 Rate of CORT increase

The rate that CORT increased differed among the three different time periods (initial to 5 min, 5 to 15 min, and 15 to 30 min; $F_{2,454} = 153$, $P < 0.0001$). CORT also increased at different rates in jays with different SI-CORT phenotypes ($F_{2,602} = 44.2$, $P < 0.0001$) and there was an interaction between SI-CORT phenotype and time period ($F_{4,454} = 10.5$, $P < 0.0001$). High SI-CORT phenotypes increased more rapidly than Med and Low SI-CORT phenotypes in initial to 5 min samples ($P < 0.001$ for both), and again from 5 to 15 min samples (Low, $P < 0.001$; Med, $P = 0.004$). Med SI-CORT phenotypes increased more rapidly than Low SI-CORT phenotypes from initial to 5 min ($P = 0.045$; Fig. 1A, B). There was no evidence that Sex affected the rate of CORT change ($F_{1,602} = 0.22$, $P = 0.6386$) and the interaction terms between sex, SI-CORT phenotype, and time period were all statistically non-significant (All $P > 0.50$).

3.3 Initial CORT under 3 min and SI-CORT phenotype

Initial CORT levels from birds sampled once within 3 min of capture were positively related to SI-CORT phenotype ($F_{2,339.5} = 10.49$; $P < 0.0001$, all factors as categorical data in the analysis; Fig. 3A; for SI-CORT and Time as continuous data in the analysis see Table 2A). CORT increased with time post-capture ($F_{3,360.5} = 7.53$; $P < 0.0001$) and post hoc analyses indicated that birds sampled prior to 2 min (< 1.5 min and 2 min time points) had lower CORT than those in the 3 min time point ($P = 0.0006$, $P < 0.0229$, respectively; Fig. 3A). There was no effect of sex nor were there interactions between sex, sampling time, and SI-CORT phenotype ($P > 0.30$).

For data collected in less than 3 min of capture, conditional and marginal R^2_G values for the whole model (all fixed factors) and the three models with single fixed factors are listed in Table 3. Most notable is that marginal and conditional R^2_G values for SI-CORT were similar to those of the whole model, strongly suggesting that SI-CORT was the dominant factor in the whole model and the single best explanatory variable for initial CORT levels. The results from the LMMs for the models with single fixed factors (SI-CORT, $F_{2,289.5} = 22.09$, $P < 0.0001$, $\beta = 0.22 \pm 0.09$; Time, $F_{1,384.2} = 12.27$, $P = 0.0005$, $\beta = 0.18 \pm 0.10$; Sex, $F_{1,161.6} = 0.16$, $P = 0.689$, $\beta = -0.02 \pm 0.10$) were similar to those of the whole model (Table 2A).

3.4 Repeated measures of initial CORT

In birds repeatedly sampled under 3 min, samples were collected on average 0.8 ± 0.3 min (mean $\pm$ SD of 1st to 2nd and 2nd to 3rd) apart. Sampling time post-capture (all factors as categorical data; $F_{2,61.8} = 6.37$; $P = 0.0031$), SI-CORT phenotype ($F_{3,40.3} = 30.11$; $P < 0.0001$), and their interaction ($F_{6,40.1} = 3.20$; $P = 0.0116$) influenced initial CORT values (Fig. 3B; for SI-CORT and Time as continuous data in the analysis, see Table 2B). Post hoc analyses did not detect a significant change in Low SI-CORT phenotype individuals within 3 min of capture ($P > 0.05$). Within Med SI-CORT birds CORT increased from < 1.5 to 3 min ($P = 0.0033$) and from 2 to 3 min ($P = 0.042$), but did not differ between the other time points. High SI-CORT birds increased CORT between times 1.5 and 3 min ($P < 0.0001$), 2 and 2.5 min ($P = 0.0120$), 2 and 3 min ($P < 0.0001$), 2.5 and 3 min ($P = 0.0013$). High SI-CORT birds differed from Low SI-CORT birds at the < 1.5 ($P = 0.0359$), 2.5 ($P = 0.0038$), and 3 min time points ($P = 0.0002$). Med SI-CORT birds differed from High SI-CORT birds at 3 min ($P = 0.0013$; see Fig. 3B).

Repeated sampling of initial CORT did not influence CORT values. Second and third sample values ($n = 37$) collected under 3 min did not differ from the values of jays sampled once

during the same time period ($n = 46$; $F_{1,75} = 0.299$, $P = 0.5861$). As previously seen, initial CORT levels were related to time since capture ($F_{1,75} = 15.0$, $P = 0.0002$) and SI-CORT phenotype ($F_{1,75} = 39.4$, $P < 0.0001$), but there were no significant interactions among time since capture, SI-CORT, and sampling method (single vs repeated samples; all $P \geq 0.8775$).

3.5 Initial CORT (under 1.5 min) and SI-CORT

There was a positive relationship between SI-CORT levels and initial CORT levels in samples collected under 1.5 min (2011-2014; $F_{1,107.1} = 14.56$, $P = 0.0002$, $\beta = 0.33 \pm 0.17$ [95% CI], $n = 113$), but there was no effect of sampling time on samples collected between 45 and 90 sec post-capture ($F_{1,109} = 1.48$, $P = 0.2262$; $\beta = -0.24 \pm 0.39$) nor was there an interaction between time and SI-CORT ($F_{1,106.3} = 2.70$, $P = 0.1032$; $\beta = 0.26 \pm 0.32$). Conditional and marginal R^2_G values for the whole model (all fixed factors) and the two models with single fixed factors on CORT collected under 1.5 min of capture are listed in Table 3. The results from the LMMs for the models with single fixed factors (SI-CORT; $F_{1,109.1} = 13.57$, $P = 0.0004$; $\beta = 0.32 \pm 0.17$, Fig. 3; Time, $F_{1,111} = 1.42$, $P = 0.2362$; $\beta = -0.25 \pm 0.41$) produced similar results to those of the model containing both fixed factors.

3.6 Capture effort

Capture effort had no effect on initial CORT values in any of the three analyses, but similar to previous analyses, initial CORT levels in each of the three analyses were influenced by SI-CORT levels (Table 4). Time since capture had an effect on samples collected within 3 min of capture but not samples collected within 1.5 min. Time since capture also did not affect birds captured within 10 min of trapping effort and (sampled within 2 min of capture).

4. Discussion

The primary findings of this study are: 1) on average, jays with higher SI-CORT had higher CORT in samples collected within 1.5 min of capture (i.e., prior to the change-point 95% CIs), suggesting that factors associated with differences in SI-CORT are also associated with baseline CORT levels; 2) plasma CORT began to increase at approximately 2 min post-capture but, based on change-point analysis, the SI-CORT phenotype did not influence the general timing of the increase; 3) starting approximately 2 min post-capture, plasma CORT increased more rapidly in jays with higher SI-CORT phenotypes; 4) the differences in the rate of CORT increase were still detected between 5 and 15 min post-capture but were no longer detectable between 15 and 30 min post-capture, and 5) there was no evidence that pre-capture interactions with the trap, or observing other jays being trapped, influenced CORT values.

4.1 Baseline CORT

The correlation between SI-CORT and initial CORT levels collected before the CORT change-point supports the hypothesis that baseline (i.e., pre-capture) CORT regulation is influenced by the general stress response phenotype in Florida scrub-jays. Based on the linear relationship between initial CORT values collected < 1.5 min of capture and SI-CORT levels, the most stress-responsive jays have baseline CORT values that are, on average, approximately two or more times higher than the least stress-responsive jays (Fig. 4). However, the marginal R^2_G for the < 1.5 min SI-CORT model (Table 4) indicates that SI-CORT only accounts for approximately 11% of the variation in the CORT values. Visual inspection of Figure 4 also highlights the considerable variability in initial CORT concentrations among individuals with similar SI-CORT phenotypes. Taken together, these data suggest that the factors responsible for inter-individual differences in SI-CORT levels also influence baseline CORT regulation in

Florida scrub-jays. However, this effect at baseline levels, prior to the influence of capture and handling stress, does not appear to overshadow the influence of other factors.

It is presently unknown why inter-individual differences in baseline and SI-CORT concentrations are correlated in Florida scrub-jays or whether this is a common phenomenon in other species. It is possible that this phenomenon is exclusively found in species with long-term, repeatable phenotypic differences in stress-responsiveness. Inter-individual differences in stress-responsiveness have been shown to covary with a variety of persistent behavioral and physiological traits that may have different energetic requirements (see Cockrem, 2013b; Schoech et al., 2011 for reviews). These suites of covarying traits are often called behavioral types, personalities, or behavioral syndromes, and individuals are commonly ranked on a “bold-shy,” “reactive-proactive,” or “fast-slow” continuum (for reviews see Carter et al., 2013; Dall et al., 2012; Dall and Griffith, 2014; Sih et al., 2012; Sih and Del Giudice, 2012). Individuals on the bold, proactive, and fast end of the spectrum are hypothesized to have a faster “pace-of-life” that requires higher CORT levels to support their more energetically demanding life-style (Careau and Garland, 2012; Carere et al., 2010). There is evidence that a similar relationship between SI-CORT phenotype and behavior exists in Florida scrub-jays, with lower SI-CORT jays being “bolder” and more “proactive” than higher SI-CORT jays (Bebus et al., 2013; Jones et al., 2013). However, the relationship between baseline CORT levels and bold/proactive behavior in Florida scrub-jays is in the opposite direction of what is commonly predicted by the “pace-of-life” hypothesis. The “bolder”, lower SI-CORT jays have lower baseline CORT, not the higher levels that would support their presumed faster lifestyle. This potential contradiction does not exclude the possibility that differences in behaviors or energetic needs are driving differences in baseline CORT, as similar relationships between lower baseline CORT and “faster” life histories have

526 been observed (Palacios et al., 2012; Versteegh et al., 2012). If higher SI-CORT jays do have
527 higher energetic requirements that cause, or are caused by elevated baseline CORT levels, those
528 behaviors and metabolic processes remain unclear.

529 Understanding how inter-individual differences in stress-responsiveness are regulated
530 could provide important insight into why baseline and SI-CORT levels covary. The differences
531 could be regulated at the level of the adrenal gland, further up the HPA axis, in the cognitive
532 processing of environmental stimuli, or a combination of these factors (Herman et al., 2003;
533 Øverli et al., 2007). In a wide variety of vertebrates, differences in stress-responsiveness are
534 associated with differences in adrenal sensitivity to ACTH (Carsia and John-Alder, 2003; Ingram
535 et al., 1999; King et al., 1993; Michopoulos et al., 2012; Singh et al., 1999; Slawik et al., 2004;
536 Veenema et al., 2004), and adrenal production of CORT (Heidinger et al., 2008; Romero et al.,
537 1998; Romero and Wingfield, 1998). Differences in the general functioning of the HPA axis
538 during an acute, extreme stressor could also influence CORT regulation at or near baseline levels
539 (Veenema et al., 2004). In such a case, the observed correlation between baseline and SI-CORT
540 levels in Florida scrub-jays could simply be due to a functional difference in the HPA axis of an
541 individual that consistently influences CORT production throughout the entirety of CORT's
542 concentration range.

543 Despite the covariance of baseline and SI-CORT concentrations, these inter-individual
544 differences may not result in physiologically meaningful effects at both low and high CORT
545 concentrations. The effects of CORT at baseline levels are primarily regulated through high-
546 affinity mineralocorticoid receptors, while the effects of SI-CORT are largely mediated through
547 low-affinity glucocorticoid receptors (Funder, 1997; Landys et al., 2006). Individual differences
548 in stress-responsiveness may be accompanied by differential regulation of these receptor types,

or their downstream signaling pathways (Lattin et al., 2015; Lattin and Romero, 2013). If this is the case in Florida scrub-jays, higher concentrations of mineralocorticoid receptors in lower SI-CORT jays could compensate for their, on average, lower baseline CORT levels and render the phenotypic differences in baseline CORT physiologically meaningless (i.e., high SI-CORT jays require higher baseline CORT levels to elicit the same physiological response as lower baseline levels elicit in low SI-CORT jays). At the same time, an absence of differences in glucocorticoid receptor concentrations across different stress-responsive phenotypes would allow for the differences in SI-CORT concentrations to have different physiological and behavioral effects in the jays. Similarly, higher corticosteroid binding globulin (CBGs; Breuner and Orchinik, 2002) in higher stress-responsiveness individuals could result in similar “free-CORT” concentrations across stress-responsive phenotypes despite there being differences in total baseline CORT levels; however, we are unaware of any studies to date that have identified differences in CBGs associated with differences in behavioral or stress-responsive phenotypes (Malisch et al., 2010, 2008). More work is needed to understand if these differences in plasma CORT regulation are accompanied by differences in tissue sensitivity to CORT.

4.2 SI-CORT phenotype and rate of CORT increase during initial sampling

Our results also support the hypothesis that differences in stress-responsiveness are reflected in the rate at which CORT increases during the 3 min post-capture sampling period. The changes in CORT concentration between 2 and 3 min post-capture mirrored the differences in the rate CORT increased between initial sample collection and 5 min post-capture, and again between 5 and 15 min (Fig. 1B, 3). Between 2 and 3 min post-capture, CORT increased 2-3 times more in high SI-CORT phenotype jays (approx. 2.6 ng/ml averaged between single- and repeated-measure studies) compared to low SI-CORT birds (0.9 ng/ml increase; not a

statistically significant increase). Combined with the differences in baseline CORT that existed before CORT began to rise, high SI-CORT jays had CORT concentrations between 2.5 and 3 min that were nearly twice that of the low SI-CORT jays (see Fig. 3). It could be argued that the general increase in CORT during the initial sampling period does more to exaggerate the pre-capture differences in baseline CORT rather than obscure those differences. However, this argument assumes that all post-capture samples are collected in a very narrow window of time. Instead, once CORT begins to rise, as little as a 30 sec difference in sampling time could produce large differences in CORT values between individuals that previously had little or no differences in CORT (Fig. 2B). Correcting for such a time effect would be difficult and, at the very least, would require knowledge of individual differences in SI-CORT and an estimate of the CORT change point.

Although it remains to be determined whether a similar phenomenon occurs in other species, there are reasons to think it is common. Differences in stress-responsiveness are well documented throughout vertebrates (Breuner et al., 2008; Cockrem, 2013a; Hing et al., 2014; Øverli et al., 2007) and, in comparison to other species studied, Florida scrub-jays have moderate to low inter-individual variation in SI-CORT levels. Cockrem (2013a) calculated the coefficient of variation (CV) for SI-CORT within 66 species of fish, amphibians, reptiles, birds, and mammals and found the average within species SI-CORT CV (initial sample to 30 min) to be 53.1% (48.6% in birds), compared to 35.8% for the Florida scrub-jays in this study (250/698 ng/ml [SD/mean]). These data suggest that once CORT begins to increase in a species with individual differences in stress-responsiveness, SI-CORT values will diverge at a rate that is similar or faster than what occurs in Florida scrub-jays.

CORT is likely to increase within 3 min of an acute stressor in many animals. Based on

studies by Romero and Reed (2005) and others (Beuving and Vonder, 1978; Dawson, 1983; Harvey et al., 1980; Heidinger et al., 2006; Maho et al., 1992; Schwabl et al., 1991), most species of birds that have been closely studied have a CORT change-point of 2 min or less during much of their annual cycle. Although far less is known about the exact time CORT begins to increase in other types of free-living animals, many mammals, and in some circumstances reptiles and fish, share a similar rapid increase in CORT that reaches peak, or near peak, levels within 10-15 min of the initiation of a stressor (Baker et al., 2013; Cartledge and Jones, 2007; Cockrem, 2013a; Reeder and Kramer, 2005; Romero and Wikelski, 2001). In these cases, the initiation of a rise in CORT levels (change-point) may occur at a similar time point to that of many birds.

Given the potential prevalence of this phenomenon, it is surprising that correlations between SI-CORT and CORT sampled within 3 min of capture are not reported more often (but see Baugh et al., 2014; Deviche et al., 2014; Malisch et al., 2013; Vitousek et al., 2014). However, baseline CORT levels can rapidly change in response to recent experiences, such as the amount of physical activity (Girard and Garland, 2002; Mastorakos et al., 2005; Rees et al., 1985; Rees and Harvey, 1987), encounters with con- or hetero-specific individuals (Jones et al., 2016; Ros et al., 2014; Van Duyse et al., 2004), and the fed state of the animal (Fokidis et al., 2012; Herring and Gawlik, 2013; Kitaysky et al., 1999). Because of this, individual variation in experience in the minutes to hours prior to sample collection could obscure longer-term patterns (e.g., days to weeks) in baseline CORT levels (Landys et al., 2006). We investigated whether our use of walk-in baited traps to catch jays provided a “standardized” experience prior to capture that made the effect of stress-responsiveness on baseline values more easily detectable. However, there was no effect of the amount of time a jay spent interacting with the trap prior to capture (capture effort) and initial CORT values. Additionally, we found a strong correlation

between initial CORT (sampled within 2 min of capture) and SI-CORT in jays caught within 10 min of setting out the trap. Together these data suggest that the trapping protocol either had no effect on baseline CORT levels or it influenced baseline CORT within a few minutes of exposure, and the effect did not increase over time.

Another explanation for why a correlation between initial and SI-CORT values exists in Florida scrub-jays, but is less commonly observed in other species, is that the jays were always sampled during the pre- to early-breeding season (sampling occurred on average 48 ± 17 days [SD] prior to clutch initiation by the breeder female of a given group), a period when baseline CORT levels may be less responsive to environmental factors. Some animals dampen HPA axis responsiveness throughout the breeding period to prevent elevated CORT levels from adversely affecting reproductive activities, such as egg production and parental care (Angelier and Chastel, 2009; Ouyang et al., 2012; Pitk et al., 2012; Saino et al., 2005; Schoech et al., 2012). Under these conditions, broader phenotypic differences might be easier to detect. Consistent with this idea, Florida scrub-jay SI-CORT levels measured during this same time period are highly repeatable among years and initial CORT values are repeatable among years in males (Small and Schoech, 2015).

4.3 Collecting CORT using the “3 min” rule

Our findings raise concerns about the measurement and interpretation of baseline CORT values when stress-responsiveness varies among individuals. When CORT begins to rise during the initial sampling period, it cannot be assumed that the changes are small and similar among individuals (Fig. 2B). The overall increase in CORT levels between 2-3 min post-capture accounts for less than 10% of the variance in the data (comparing marginal R^2_G for time < 1.5 and < 3 min; Table 3). This relatively small impact is consistent with the previous observations

by Romero and Reed (2005) that samples collected within 3 min of capture are “near baseline”. However, the concern does not specifically arise from the amount of additional variance added to the entire data set; instead, the primary concern is that the impact of time since capture on CORT values is both unevenly and non-randomly distributed (Figs. 2-4). Most of the variance due to time since capture is from the 27% of samples collected between 2 and 3 min post-capture (see sample sizes Fig. 3A), and within that 27% subset, individuals with higher SI-CORT phenotypes are responsible for a larger proportion of the increase in CORT (Fig. 2B).

If differences in stress-responsiveness are unrelated to the independent variable under investigation, this uneven, non-random increase in CORT may only decrease the likelihood of detecting differences in baseline values. However, stress-responsiveness is known to vary with numerous intrinsic and extrinsic factors (Baker et al., 2013; Crespi et al., 2013), such as life-history stage (Romero, 2002; Romero et al., 2008; Wingfield et al., 1992), fluctuating weather conditions (Romero et al., 2000; Rubenstein, 2007), social status (Cavigelli and Chaudhry, 2012; Creel et al., 2013; Summers et al., 2005), age (Elliott et al., 2014; Heidinger et al., 2010), resource availability (Schoech et al., 2007), habitat characteristics (Bauer et al., 2013; Fokidis et al., 2009; Li et al., 2011; Lucas and French, 2012), and reproductive effort and success (Lendvai and Chastel, 2010; Miller et al., 2009; Schmid et al., 2013; Vitousek et al., 2014). Many of these same factors are also associated with differences in baseline CORT (Baker et al., 2013; Bonier et al., 2009). When differences in stress-responsiveness are unevenly distributed among the groups being compared, as would be expected in the above cited examples, the differences in capture-and-handling time along with the phenotypic differences in rates of CORT increase could obscure or supersede moderate pre-capture differences in baseline CORT levels. The work of Baugh et al. (2013) on great tits clearly demonstrates that just such an error could occur if stress-

responsiveness and the timing the rise of CORT are not considered. If such errors are common, they could explain some of the contradictory results found in the baseline CORT literature (Bonier et al., 2009; Crespi et al., 2013; Dickens and Romero, 2013).

4.4 Conclusion

In Florida scrub-jays, an individual's stress response phenotype appears to influence measures of baseline CORT in two important ways. First, what we consider to be "true" baseline CORT concentrations (collected within 1.5 min of capture) mirror an individual's stress response phenotype, with higher SI-CORT jays having, on average, higher baseline CORT concentrations. The reason this connection exists is presently unknown but the among individual differences related to SI-CORT are small enough as to not obscure moderate to large differences in CORT caused by other factors. However, knowledge of an individual's stress response phenotype may be important for understanding what differences in baseline values mean for these animals. Second, differences in stress response phenotypes are primarily driven by differences in the rate at which CORT increases in the first few minutes of an extreme stressor, such as capture and handling. Once CORT begins to increase at about 2 min post-capture, the phenotypic differences in rates of increase complicate any attempt to determine pre-capture CORT levels (i.e., "true" baseline), not to mention its links with any number of potential variables of interest (e.g., fitness, health, reproduction, etc.).

These findings affirm many of the conclusions of Romero Reed (2005). In Florida scrub-jays, plasma CORT "samples collected in less than 2 min can be assumed to reflect unstressed concentrations with a high degree of confidence", and samples collected within 3 min are "near enough to baseline to be distinguishable from stress-induced concentrations". However, in

Florida scrub-jays, it cannot be assumed that samples collected between 2 and 3 min accurately represent pre-capture differences in CORT levels. This phenomenon does not appear to be unique to Florida scrub-jays (Baugh et al., 2013), but it is presently unknown how common it is.

We do not believe that these results indicate that an under 2 min post-capture sampling limit should be broadly adopted, as it may be unnecessarily restrictive. We also do not believe that sampling after the post-capture rise in CORT begins will always produce inaccurate estimates of among individual differences in baseline CORT. Instead, we recommend that at the very least, studies report the range and mean $\pm$ standard deviation of sampling times for each group being compared (e.g., each life history stage, social status level, habitat type, etc.), instead of only reporting the maximum sampling time for all samples collected (e.g., < 3 min). With this information, sample collection time can be taken into consideration when comparing results across studies.

We also recommend validating if CORT increases during the baseline sampling period, the magnitude of any changes that do exist, and if there are inter-individual differences in the rate of change. Ideally, this should be done for each group being compared within a study to determine if differences exist among groups that could obscure or bias the results. When more than one blood sample can be safely collected from a subject, we encourage validating the timing of CORT sample collection using a repeated measures design. In this study, the repeated measures analysis of samples collected under 3 min contained less than one tenth as many subjects as the independent measures analysis, yet it still produced highly robust results (Table 2). In many studies, a small subset of subjects could be repeatedly sampled during the initial sampling period with few or no modifications to the experimental protocol, and for little additional cost. Even with small sample sizes, and no measure of stress-induced CORT, visual

inspection of repeated measures baseline CORT data (Fig. 2B) could provide important insight into the dynamics of, and inter-individual variation in, the rise of CORT during the initial sampling period. Armed with this information, researchers should be better able to determine the most appropriate baseline CORT sampling protocol for their study system.

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Table 1

Nonparametric change-point detection results for the first change-point detected in samples collected up to 3 min post-capture based on all jays (All) or jays subdivided by SI-CORT phenotype (High, Med, Low).

<i>SI-CORT</i>	<i>n</i>	<i>Time range (min)</i>	<i>Change-Point (min)</i>	<i>95% CI (min)</i>	<i>Conf. Level</i>	<i>CORT Change</i>
All	387	0.75-3.0	1.97	1.90,2.13	99%	3.02/5.44
High	119	0.75-3.0	2.12	1.67,2.35	97%	3.56/4.90
Med	140	0.77-3.0	1.82	1.52,2.37	100%	2.86/4.36
Low	128	0.83-3.0	1.80	1.67,2.27	99%	2.39/2.86

95% confidence intervals (CI) and confidence levels (Conf. Level) are based on 10,000 bootstrap simulations.

CORT Change shows the average CORT value (ng/ml) before and after the change-point (before/after).

Table 2

A) Effect of SI-CORT phenotype, time since capture (Time), and sex on single CORT (ng/ml) samples collected within 3 min of capture (n = 387 samples; 2011-2014). B) Effect of SI-CORT phenotype and Time on individuals sampled 2 or 3 times within 3 min of capture (n = 37 birds; 83 samples; 2013). SI-CORT and Time were analyzed at continuous data.

<i>Fixed</i>	<i>F(df)</i>	<i>p</i>	<i>β</i>	<i>β 95% CI</i>
<i>A) Single sample/1st sample</i>				
SI-CORT (C)	23.1 (1,283.7)	<0.0001	0.23	0.13, 0.32
Time (T)	13.6 (1,378.6)	0.0003	0.19	0.09, 0.28
Sex (S)	1.08 (1,164.2)	0.3012	-0.05	-0.14, 0.04
C×T	0.25 (1,373.9)	0.6200	0.02	-0.06, 0.11
C×S	0.51 (1,285.9)	0.4745	0.03	-0.06, 0.13
T×S	0.25 (1,374.9)	0.6149	-0.02	-0.12, 0.07
C×T×S	1.26 (1,374.0)	0.2623	0.05	-0.04, 0.13
<i>B) Repeated sampling</i>				
SI-CORT (C)	29.8 (1,35.3)	<0.0001	0.57	0.36, 0.78
Time (T)	96.5 (1,47.3)	<0.0001	0.39	0.31, 0.48
C×T	30.8 (1,47.9)	<0.0001	0.25	0.16, 0.34

Table 3

Conditional (Con; fixed and random factors) and marginal (Mar; only fixed factors) R^2_{GLMM} (R^2_G) values for linear-mixed models (Nakagawa and Schielzeth, 2013) with either stress-induced CORT (SI-CORT), time since capture (Time), or a model containing SI-CORT, Time, and their interaction (Whole) for CORT samples collected within 1.5 min of capture (< 1.5 min) or within 3 min of capture (3 min). All models contained sampling year (2011-2014) as a random variable.

<i>Model</i>	<i><1.5min</i>		<i><3 min</i>	
	<i>Mar</i>	<i>Con</i>	<i>Mar</i>	<i>Con</i>
	R^2_G	R^2_G	R^2_G	R^2_G
SI-CORT	0.11	0.18	0.12	0.32
Time	0.00	0.09	0.08	0.29
Sex	na	na	0.00	0.20
Whole	0.13	0.21	0.18	0.34

Table 4

Effect of trapping effort (Effort; the elapsed time between trap deployment and capture) on initial CORT values in birds caught in 2013- 2014 (without repeated measures). SI-CORT phenotype and time since capture (Time) were included as continuous variables to investigate interaction effects on plasma CORT samples from jays that had experienced between 1.2-169 min of exposure to the trap prior to capture (All pre), and then were sampled within A) 1.5 min of capture (<1.5 min post; n = 43) or B) 3 min of capture (< 3 min post; n = 178). C) Jays caught within 10 min of pre-capture trap exposure (< 10 min pre) and then sampled within 2 min post-capture (< 2 min post; n = 28).

<i>Fixed</i>	<i>A) All pre, <1.5 min post</i>		<i>B) All pre, <3 min post</i>		<i>C) <10 min pre, <2 min post</i>	
	<i>F(df)</i>	<i>p</i>	<i>F(df)</i>	<i>p</i>	<i>F(df)</i>	<i>p</i>
SI-CORT (C)	11.5 (1,32)	0.0018	19.8 (1,162.6)	<0.0001	8.88 (1,20)	0.0074
Time (T)	0.71 (1,32)	0.4070	11.9 (1,161.3)	0.0007	2.71 (1,20)	0.1151
Effort (E)	1.88 (1,32)	0.1797	0.34 (1,163)	0.5580	0.00 (1,20)	0.9453
C×T	0.00 (1,32)	0.9502	0.33 (1,166)	0.5675	0.00 (1,20)	0.9495
C×E	0.04 (1,32)	0.8486	0.03 (1,144.2)	0.8534	0.06 (1,20)	0.8079
T×E	1.84 (1,32)	0.1850	1.70 (1,165.9)	0.1939	1.14 (1,20)	0.2983
C×T×E	1.63 (1,32)	0.2104	0.02 (1,138.5)	0.8875	2.09 (1,20)	0.1636

Figure Legends

Fig. 1. General characteristics of the stress-induced corticosterone (SI-CORT) response of Florida scrub-jays. A) Variation in plasma CORT concentration in response to being captured and held for 30 min (SI-CORT). Stress responses have been divided into Low (N=78), medium (Med; N=77), and High (N=78) ranks. B) Rate of increase in corticosterone (CORT) levels in the blood between sampling time points. Bars not sharing a letter are different ($P < 0.05$; Tukey-Kramer HSD test; also see Table 2) and shading of bars matches the legend in panel A. Data points (A) and bars (B) represent means $\pm$ standard errors (error bars).

Fig. 2. Raw CORT values collected within 3 min of capture. A) Values from birds sampled once within 3 min of capture in 2011-2014. White points and solid lines are birds with High stress-induced CORT (SI-CORT), gray points and dashed lines are medium (Med) SI-CORT, and black points and dotted lines are Low SI-CORT. B) A representative subset of values from nine birds repeatedly sampled (2 or 3 times) within 3 min of capture in 2013. Each line connects the CORT values for one individual. For the purpose of comparison, the subset was limited to birds with a CORT concentration between 1-2 ng/ml for their first sample collected within 2 min of capture, and a last sample collected between 2.5-3 min.

Fig. 3. Changes in initial CORT concentrations as a function of time since capture and the individual's stress-induced CORT phenotype (SI-CORT). A) Values from birds sampled once within 3 min of capture in 2011-2014. B) Values from birds repeatedly sampled (2 or 3 times) within 3 min of capture in 2013. SI-CORT phenotypes are divided into Low, Medium (Med), or High groups. Time since capture is divided into samples collected between 0.75-1.5 min (< 1.5 min), 1.5-2.0 min, 2.0-2.5 min, and 2.5-3.0 min post-capture. Sample sizes are shown at the base of each bar. Averages across SI-CORT phenotypes are shown as points connected with a dashed line. Bars and points are means ($\pm$ standard error). Points that do not share a letter are significantly different ($P < 0.05$). Differences between bars are described in the Results section.

Fig. 4. The relationship of initial CORT and stress-induced CORT (SI-CORT) in 113 Florida scrub-jays sampled within 1.5 min of capture (2011-2014). Dashed lines indicate the divisions between high (> 760 [ng/ml] $\times$ min), medium, and low (< 565) SI-CORT categories used in other analyses. Gray area is the 95% confidence interval around the line. Conditional (Con; fixed and random factors) and marginal (Mar; only fixed factors) R^2_{GLMM} (R^2_G) values for linear-mixed models are based on Nakagawa and Schielzeth (2013).

Individual samples <3 min

Subset of repeat samples

Individual samples <3 min (2011-2014)

Repeated samples <3 min (2013)

Collected under 1.5 minutes