Individual variation in natural or manipulated corticosterone does not covary with circulating glucose in a wild bird.

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Other people who helped:

* General field work for multiple years and multiple sites worth of field crews. Not sure any single person from those years really needs to be added on this particular paper, but could be.
* Currently I have the population comparison in here. I think it could be fine without it, but it’s also kind of nice since the main patterns seem robust across all the populations. If we do leave the populations in that might mean adding authors from those populations? I’m fine with that but just let me know what makes sense.

Open questions:

* I originally had dex in here as well, but I realized in preparing this that it doesn’t actually fit all that well because even though dex should decrease cort, dex itself should be triggering a similar glucose increase through its actions at GC receptors. That might be interesting in a similar way as Cortrosyn, but since we can’t measure actual concentrations of cort + dex, there isn’t any real way to do the between individual covariation that the rest of the paper focuses on. The results aren’t really changed by adding dex, but I think it’s probably simpler to just keep it out.

**ABSTRACT**

Animals respond to sudden challenges with a coordinated set of physiological and behavioral responses that enhance the ability to cope with and recover from stressors. While general characteristics of the vertebrate stress response are well described, it is not as clear how individual components of the response covary between- or within-individuals. A rapid increase in glucocorticoids coordinates the stress response system and one of the primary downstream actions of glucocorticoids is an increase in glucose availability. Here, we asked whether between- and within-individual variation in either the absolute levels or degree of upregulation in corticosterone directly predicted variation in glucose regulation. We collected 2,673 paired glucose and corticosterone measures from 776 individual tree swallows (*Tachycineta bicolor*) from four populations spanning the species range. In adults, glucose and corticosterone both increased during a standardized handling restraint protocol and this result was robust across four populations. Moreover, in one population experimentally increasing corticosterone resulted in a further increase in both measures. In contrast, nestlings did not show a robust glucose response to handling or corticosterone manipulation. Despite this group level variation, there was very little evidence in any population that between-individual variation in corticosterone predicted any aspect of between-individual variation in glucose regulation. Using a subset of individuals sampled four or more times, we also found that within-individual variation in corticosterone did not predict within-individual variation in glucose. Our results highlight the fact that a strong response in one aspect of the coordinated acute stress response (corticosterone) does not necessarily indicate that an individual is a strong responder overall. These results have important implications for understanding both how individuals differ in their response to challenges and for studying how the full stress-response system is likely to respond to selection.

***Keywords:*** *stress response, glucose regulation, evolutionary endocrinology*

**INTRODUCTION**

Wild animals live in capricious environments where sudden challenges are encountered regularly. Successfully navigating these challenges requires an integrated physiological and behavioral response (cite). In vertebrates, acute challenges trigger both the catecholamine regulated ‘fight-or-flight’ response and the glucocorticoid mediated acute stress response (cite). When rapidly elevated, glucocorticoids organize a variety of physiological and behavioral changes that facilitate the successful response to an acute challenge along with recovery and preparation for subsequent challenges (cite). In the past fifteen years, attention has shifted from describing the general response to investigating how individuals differ and what consequences this variation may have for fitness and the evolution of physiological systems (Bonier et al., 2009; Breuner et al., 2008). While some patterns have emerged (Schoenle et al., 2021), the lack of consistent links between fitness and corticosterone, coupled with substantial within-individual variation in components of the stress response across time, contexts, or even different tissues in one animal has led to some debate about how to interpret studies that seek to link fitness with corticosterone without measuring other aspects of the multifaceted stress response (Gormally et al., 2020; Lattin et al., 2015; Romero and Gormally, 2019). Addressing these critiques will require more studies of how different aspects of the stress response system covary within and between individuals.

One of the primary actions of glucocorticoids during an acute stress response is to alter glucose homeostasis and increase the availability of glucose (cite). Regulation is accomplished through multiple routes; first, glucocorticoids act antagonistically to insulin in peripheral tissue and decrease glucose uptake across cell membranes, thereby increasing circulating glucose availability (Horner et al., 1987; Remage-Healey and Romero, 2001). At the same time, glucocorticoids promote gluconeogenesis in the liver and alter glucose homeostasis through a variety of other indirect pathways (Kuo et al., 2015). In the context of the acute vertebrate stress response, glucocorticoids are thought to increase the availability of glucose for use by the brain and to allow animals to cope with and recover from an acute challenge (Kuo et al., 2015; Remage-Healey and Romero, 2001).

Although the general pattern of acute challenges increasing glucose availability has been demonstrated many times (cite), there is a great deal of variation and context dependence in this response both within and between species (cite). For example, in captive European starlings (*Sturnus vulgaris*), handling stress increases both corticosterone and glucose, but only in samples taken during or shortly after nighttime (Remage-Healey and Romero, 2000). In a wild population of Rufous-winged sparrows (*Peucaea capalis*), glucose either increases, decreases, or stays the same in response to handling stress, depending on the life history stage (Deviche et al., 2016a). Thus, the magnitude of the glucose response to a stressor—and whether a response occurs at all—may differ with season, time of day, and nutrition. A small number of studies published to date demonstrate that baseline glucose can be moderately repeatable and associated with individual performance (Montoya et al., 2018; Montoya et al., 2020). Across many more species, baseline and stress-induced glucocorticoid levels are also moderately repeatable within individuals (Taff et al., 2018), but repeatability of each trait does not necessarily imply covariation within or between individuals.

Despite the mechanistic link between glucocorticoids and glucose, relatively few studies have investigated changes in both measures simultaneously in wild birds (but see Deviche et al., 2016a; Deviche et al., 2016b). Even fewer studies have investigated whether between-individual variation in the absolute value or magnitude of change in glucocorticoids during an acute stress response predicts the degree of change in glucose availability. In a recent study of Wandering gartersnakes (*Thamnophis elegans vagrans*), Neuman-Lee et al. (2020) used both observational and experimental data to show that while glucose did increase with an acute stress response, individual differences in the amount of glucocorticoid change did not predict the magnitude of change in circulating glucose (see also Gangloff et al., 2017). The generality of this lack of relationship has important implications for understanding how selection operates on variation in the physiological stress response. There is an assumption—often implicit—in evolutionary physiology that a stronger glucocorticoid response to a stressor will result in a similarly stronger change in downstream regulation, including a larger increase in glucose (Romero and Gormally, 2019). However, this assumption is rarely directly tested and if substantial regulation occurs in other components of the system (e.g., in tissue specific receptor density; Lattin et al., 2015) then a similar glucose response might require different levels of corticosterone in two different animals. Moreover, individuals may differ in their degree of within-individual variation (how tightly linked corticosterone and glucose are within the same individual when measured multiple times). Even if corticosterone and glucose are tightly coupled within-individuals, there is no guarantee that within-individual trait correlations will scale to between-individual covariation (Agrawal, 2020).

Here, we studied between- and within-individual covariation in glucose and corticosterone regulation during an acute stress response in the breeding season of wild tree swallows (*Tachycineta bicolor*). We measured glucose and corticosterone repeatedly at baseline and stress-induced levels for adults from four populations with different climate variability along with nestlings from one population. In the main population, we also coupled observational data with experimental manipulations that directly increased circulating corticosterone in adults and nestlings to determine whether there was a causal effect of additional corticosterone on subsequent glucose levels.

We first predicted that glucose and corticosterone would be relatively low in baseline samples, elevated in stress-induced samples, and highest in samples where corticosterone was experimentally elevated. Next, we predicted that if variation in corticosterone levels is the direct cause of glucose elevation, then individual variation in circulating corticosterone and in the magnitude of the natural or experimental increase in corticosterone would be correlated with glucose levels or the increase in glucose levels over the same time period. Alternatively, if glucose and corticosterone regulation do not covary, it would suggest that these two aspects of the acute stress response can be regulated relatively independently or that their link depends critically on additional unmeasured traits (e.g., nutritional state). Finally, using a subset of individuals that were measured multiple times, we assessed the degree to which corticosterone and glucose covaried within-individuals.

**METHODS**

*General Field Methods*

We studied tree swallows breeding near Ithaca, New York, USA (42.4°N, 76.5°W) from 2016-2019. In each year, we monitored nest boxes using established protocols for this population (Winkler et al., 2020). Adult females were captured at the nest box up to three times each breeding season (1-2 times during incubation and 1-2 times after nestlings had hatched). Adult males were typically captured once per year 3-8 days after nestlings had hatched. Nestlings were sampled on days 12 and 15 after hatching. All adults were captured between 6 a.m. and 10 a.m. and nestlings were sampled between 12 p.m. and 3 p.m. to limit variation in physiological measurements associated with circadian patterns.

At each capture, we took a measurement of individual mass to the nearest 0.25 grams and added a unique USGS aluminum band to any individual that was not previously banded. For most captures, we took a series of blood samples by brachial venipuncture to measure corticosterone and glucose. First, we collected a baseline sample within 2 minutes of disturbance (< 70 μl). After 30 minutes, we collected a stress-induced blood sample (< 30 μl). For a subset of adults, immediately after the stress-induced sample, we manipulated the course of the stress-response by injecting Cortrosyn and collected a final blood sample 30 minutes later (< 30 μl; see details below). We performed the same manipulation on nestlings, except in the case of nestlings the Cortrosyn sample was collected on a separate day from the baseline and stress-induced sample (see below).

We measured glucose from baseline, stress-induced, and post-Cortrosyn blood samples at the time of collection using a handheld glucose meter and test strips (FreeStyle, Abbott Diabetes Care, Alameda, CA, USA). Similar devices have been used in previous studies of wild birds (Clinchy et al., 2004; Malisch et al., 2018), and this device was previously validated to provide repeatable measures of glucose in this population of tree swallows (Taff et al., 2019c). The remaining blood sample was stored on ice in the field for <3 hours. Plasma was then separated by centrifugation and stored frozen until corticosterone was measured with an ELISA kit that was previously validated in this population (Arbor Assays K014-H5; Taff et al., 2019a).

During these years, adult females in the population were subjected to a variety of manipulative experiments (Taff et al., 2018; Taff et al., 2019b; Zimmer et al., 2019). In this study, we excluded samples from individuals that had been subject to direct manipulations of corticosterone (Vitousek et al., 2018a), but we included samples from treatments that manipulated flight costs, perceived predator presence, or plumage coloration. Males did not receive any direct manipulation and we include all male samples. In the one year that nestling glucose was measured (2019), adult females had received plumage dulling or simulated predation treatments prior to nestling sampling. However, these treatments only targeted adult females and we included all nestling samples. As part of the adult experiments during this year, eggs at most nests were cross fostered prior to the onset of incubation so that each nest typically included eggs from multiple females; we included nest identity as a random effect in all of our models but did not specifically investigate any effects of cross-fostering.

*Comparative Population Study*

In parallel with the study described above in New York, we collected similar data from adult tree swallows breeding in McCarthy, Alaska (2016-2017; 61.4°N, 143.3°W), Burgess Junction, Wyoming (2018; 44.5°N, 107.3°W), and Chattanooga, Tennessee (2018; 35.1°N, 85.2°W). Sampling schedules and details of sampling in each location were identical to those described above for New York. Full descriptions of these study locations can be found in Zimmer et al. (2020). Inclusion criteria were the same as described above from the New York population. Corticosterone measurements from the other populations were not available for most adult males, so analyses comparing the four populations are restricted only to adult females. We did not collect glucose measurements from nestlings in these populations and we do not report data from any post-injection measures in these additional populations. Baseline and stress-induced samples were collected exactly as described above.

*Manipulating the Corticosterone Response*

In the New York population only, we used manipulations to artificially increase the magnitude of the corticosterone response to handling stress in 2019 to determine if there was a direct causal effect of continued corticosterone release on circulating glucose levels. Most adults and nestlings in 2019 received an injection of Cortrosyn (a synthetic version of adrenocorticotropic hormone). Because we had not used this injection previously, we also conducted a validation experiment with a separate set of birds, which confirmed that Cortrosyn injection produced a reliable increase in circulating corticosterone compared to saline injection in both adults and nestlings (supplemental methods; Figure S1-S2; Table S1).

For nestlings, the time course of sampling differed slightly from adults. For all nestlings, we collected a series of samples on day 12 that included a baseline and stress-induced sample as described above for adults. On day 15, we returned to each nestling and collected a post-Cortrosyn sample at a single time point. For this last sample, nestlings were injected immediately after removal from the nest and then a single blood sample was collected 30 minutes later.

*Data Analysis*

Using a subset of adult samples where multiple measures were available, we first evaluated overall unadjusted repeatability in baseline, stress-induced, and induced minus baseline glucose and corticosterone in a linear mixed model fit with the ‘rptR’ package in R (Stoffel et al., 2017). We next sought to determine whether glucose and corticosterone differed at a group level for the three different sample types (baseline, induced, and post-Cortrosyn). We fit a single linear mixed model separately for adults and nestlings to address this question with glucose or corticosterone as the response variable and sample type as a categorical predictor. The adult model included an additional fixed effect for sex and a random effect of bird identity to account for repeated sampling from the same individual. The nestling model included random effects for individual identity and for nest to account for the fact that nestlings sampled from the same nest are not independent.

We next asked whether between individual variation in circulating corticosterone predicted variation in glucose levels. For these analyses, we fit a set of three models separately for adults and nestlings with baseline glucose or change in glucose (base to stress-induced or induced to post-Cortrosyn) as the response variable. Predictor variables included baseline corticosterone or the change in corticosterone over the same sampling interval. The adult model included a random effect for individual identity to account for repeated sampling and the nestling model included a random effect for nest identity to account for non-independence. The adult post-Cortrosyn model did not include any repeat sampling, so it was fit as a simple linear model with no random effects. In these models, we also included mass and an interaction between mass and the corticosterone predictor to test whether corticosterone and glucose were more tightly linked under conditions of food limitation. If there was no support for the mass by corticosterone interaction, we removed this effect from the final model for simplicity.

To determine whether there were population differences in glucose regulation, we fit three linear mixed models with baseline, stress-induced, or induced minus baseline glucose as the response variable and population as a categorical predictor. These analyses included only baseline and stress-induced sample types in adult females. Next, we fit models similar to those describe above with either baseline or induced minus baseline glucose as the response and with corticosterone over the same interval, mass, and a corticosterone by mass interaction as predictors. These models were fit separately for each population and included female identity as a random effect.

Finally, we assessed whether there was any evidence for within-individual covariation between glucose and corticosterone using a subset of individuals that had four or more sampling events. Using this subset, we centered and standardized both glucose and corticosterone within each individual and then fit models with baseline, induced, or the change in glucose as the response variable and with corticosterone over the same time period as the response. By centering within individuals, we used these models to ask whether an increase in corticosterone—relative to an individual’s own mean corticosterone level over all observations—was associated with an increase in glucose relative to their own mean glucose level across observations (see discussion of within-individual mean centering in Westneat et al., 2020).

All linear mixed models except for repeatability estimates were fit using the ‘lme4’ package version 1.1-26 (Bates et al., 2015, 4). For all linear mixed models with categorical comparisons, we extracted means and 95% confidence intervals for each group using the ‘emmeans’ package in R with the Satterthwaite approximation (Lenth, 2020). We interpret groups whose confidence intervals do not overlap to be significantly different. In cases where an interaction was supported, we illustrated the interaction by calculating confidence intervals across a range of predictor values by drawing 1,000,000 samples from the multivariate normal distribution of the fit model using the ‘mvrnorm’ function in R package MASS version 7.5-53 (Ripley et al., 2013) and then calculating the highest posterior density interval with default settings (‘HPDI’ function) using package ‘rethinking’ version 2.01 in R (McElreath, 2020) All figures and analyses were produced in R version 4.0.2 (R Core Development Team, 2020). Complete raw data and the full set of code required to reproduce all of the analyses and figures presented here is publicly available on GitHub and will be permanently archived upon acceptance (<https://github.com/cct663/glucose_cort>).

**RESULTS**

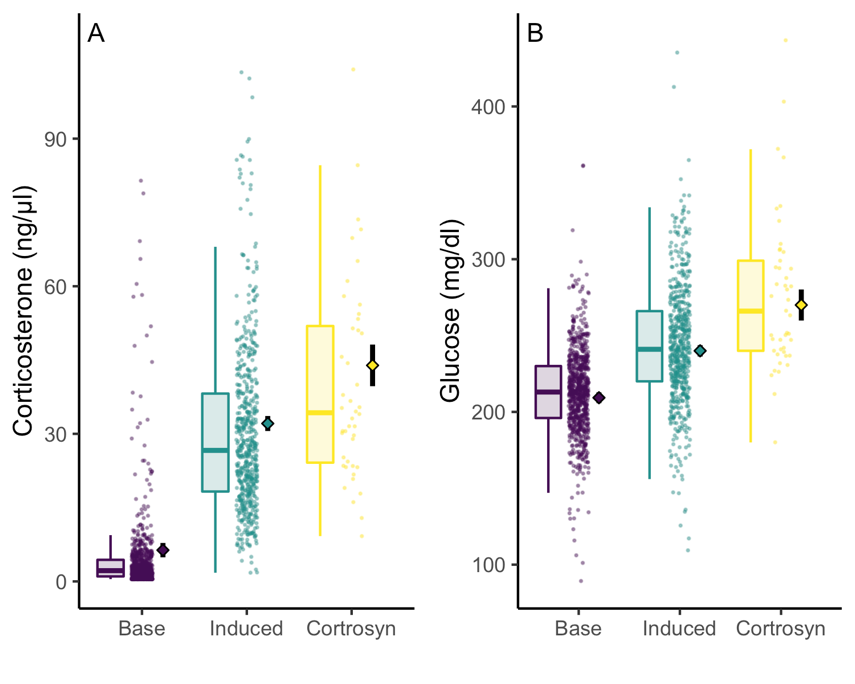
In total, our New York analyses included corticosterone and glucose samples from 331 adults with 776 baseline, 586 stress-induced, and 45 post-Cortrosyn samples. For nestlings, we included samples from 187 nestlings in 43 nests. The population comparison also included baseline and stress-induced corticosterone and glucose measurements from 71, 75, and 112 adult females from Alaska, Tennessee, and Wyoming, respectively. A full table of sample sizes by age, location, year, and sample type is included in the supplementary material (Table S2).

Baseline, stress-induced, and the change in glucose levels had low, but significant repeatability (baseline r = 0.11, CI = 0.02 to 0.20, likelihood ratio test P = 0.02; stress-induced r = 0.21, CI = 0.12 to 0.30, P < 0.001; change in glucose r = 0.11, CI = 0.02 to 0.20, P = 0.01). Baseline corticosterone was not repeatable in this sample (baseline corticosterone r = 0.01, CI = 0 to 0.10, P = 0.42). Stress-induced corticosterone and the change in corticosterone had the highest repeatability (stress-induced corticosterone r = 0.32, CI = 0.23 to 0.41, P < 0.001; corticosterone response r = 0.25, CI = 0.16 to 0.35, P < 0.001). Note that repeatability estimates for corticosterone, but not glucose, in a subset of these birds were previously reported with similar effect sizes (see Table 5 in Vitousek et al., 2018b).

*Overall Changes in Glucose and Corticosterone*

In adults, mean corticosterone levels differed significantly for all three sample types, although there was substantial overlap in the distribution of individual corticosterone measures (Figure 1A; Table S3). Corticosterone was lowest in the baseline sample (‘emmeans’ estimated mean from mixed model = 6.4 ng/μl; 95% CI = 4.9 to 7.8) and increased substantially by the stress-induced sample (32.1 ng/μl; CI = 30.6 to 33.6). Injection with Cortrosyn resulted in a further increase of corticosterone (43.9 ng/μl; CI = 39.7 to 48.2).

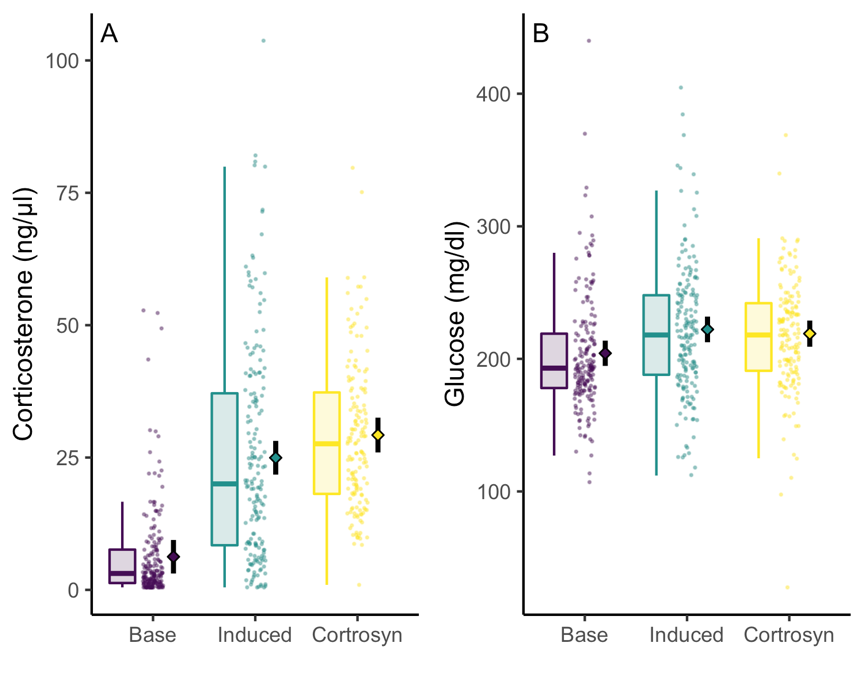
Overall, glucose concentrations showed a similar pattern of variation between sample types to corticosterone (Figure 1B; Table S3). Glucose was lowest in the initial sample (mean = 209.3 mg/dl; CI = 205.7 to 212.8) and increased by the stress-induced sample (240.0 mg/dl; CI = 236.3 to 243.7). Cortrosyn injection resulted in a further increase in circulating glucose levels (270.0 mg/dl; CI = 259.8 to 280.2).



**Figure 1.** Corticosterone (A) and glucose (B) levels for adult tree swallows in the New York population by sample type. Boxes and whiskers show the interquartile range and largest value within 1.5 times the IQR, respectively. Small points show raw data. Solid diamonds and solid black lines show the point estimate and 95% confidence interval for each group mean as calculated by ‘emmeans’ and the linear mixed model described in text.

In nestlings, corticosterone increased from the initial to stress induced sample (Figure 2A; initial mean = 6.3 ng/μl; 95% CI = 3.1 to 9.4; stress-induced = 25.0; CI = 21.8 to 28.1). Cortrosyn injection resulted in the highest corticosterone levels, although the confidence interval for post-cortrosyn samples overlapped that for stress-induced samples (29.2; CI = 26.0 to 32.5).

In contrast to adults and despite clear variation in corticosterone levels, the confidence intervals for glucose concentrations overlapped across all three sample types (Figure 2B). Although they did not differ significantly, glucose levels were lowest in the initial sample (mean = 204.2 mg/dl; CI = 194.6 to 213.8) and increased at the stress-induced sample (222.2; CI = 212.5 to 231.9). Glucose did not increase further after Cortrosyn injection (219.0; CI = 209.1 to 228.9).

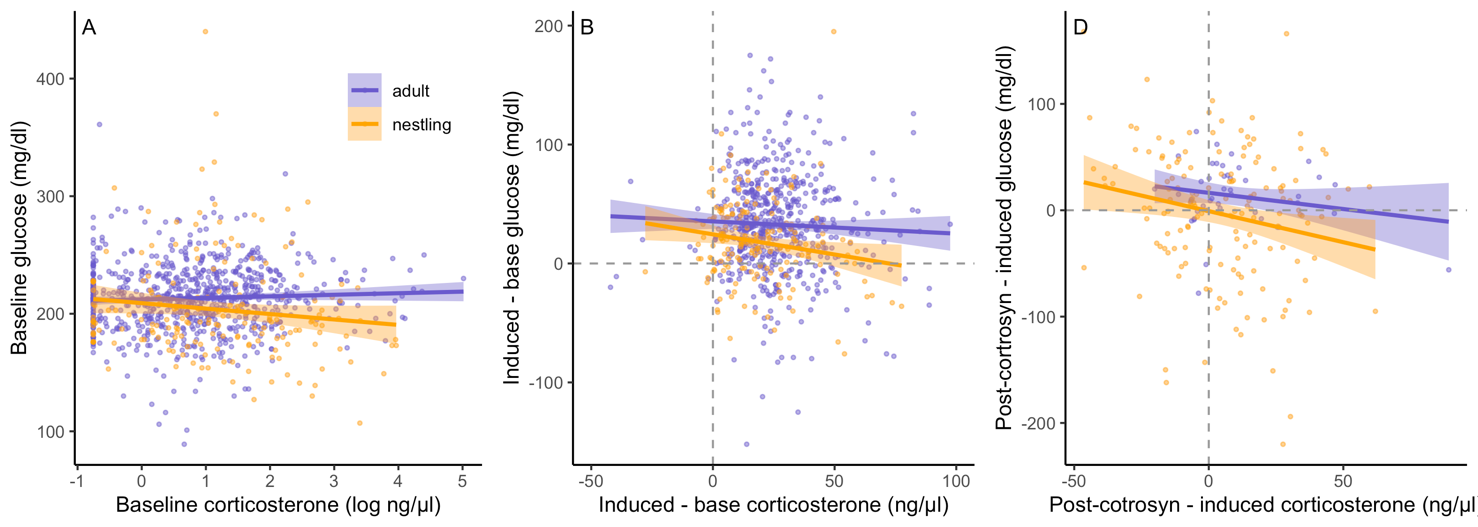


**Figure 2.** Corticosterone (A) and glucose (B) levels for nestling tree swallows in the New York population by sample type. Boxes and whiskers show the interquartile range and largest value within 1.5 times the IQR, respectively. Small points show raw data. Solid diamonds and solid black lines show the point estimate and 95% confidence interval for each group mean as calculated by ‘emmeans’ and the linear mixed model described in text.

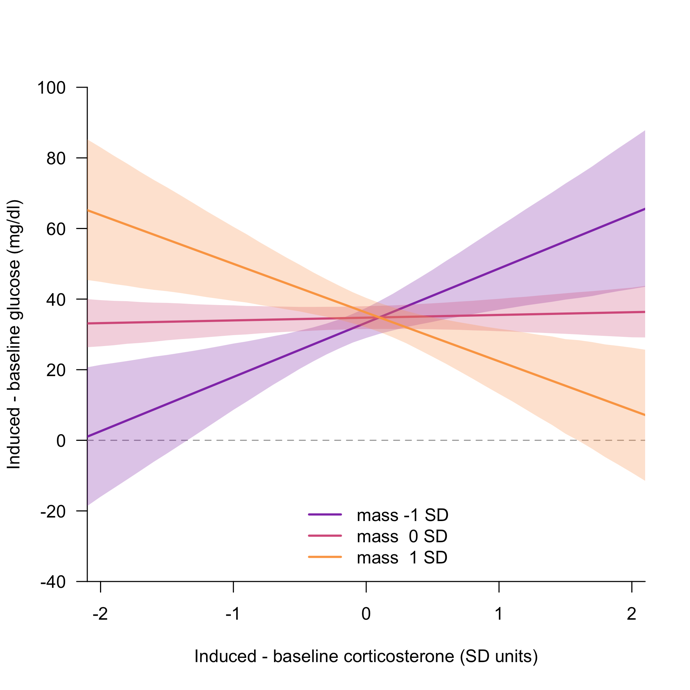
*Between-Individual Covariation in Glucose and Corticosterone*

For adults and nestlings in New York, there was no relationship between baseline corticosterone and baseline glucose, although nestlings with higher baseline corticosterone tended to have lower glucose levels (Figure 3A; Table S4 & S5; adult β = 1.7; CI = -0.4 to 3.7; nestling β = -3.6; CI = -10.7 to 3.5). Among nestlings, a greater increase in corticosterone from baseline to stress-induced samples was associated with a smaller increase in glucose during the same period (Figure 3B; β = -5.5; CI = -11.0 to -0.1).

In contrast, among adults there was an interaction between mass and the change in corticosterone from baseline to stress-induced samples (Figure 3B; corticosterone by mass interaction β = 4.3; CI = 0.7 to 8.0). Adults that were below average mass had a positive relationship between corticosterone increase and glucose increase during this period, while adults that were above average mass had a negative relationship (Figure 4). However, this model only explained a small amount of variation in the glucose response (full model marginal R2 = 0.03; Table S4). Neither mass nor the change in corticosterone from stress-induced to post-Cortrosyn measurements were related to the change in glucose in adults or nestlings (Figure 3C-D; Table S4 & S5).

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**Figure 3.** Covariation in between-individual variation in corticosterone and glucose for adults (blue) and nestlings (orange) in the New York population. Panel A shows baseline corticosterone measures versus baseline glucose (note baseline corticosterone is log transformed for easier visualization). Panel B shows the change in corticosterone from baseline to induced samples versus the change in glucose over the same period. Panel C shows the change in both measures from stress-induced to post-cortrosyn samples. In panels B & C, dashed lines indicate positive changes in corticosterone (right of the vertical line) and glucose (above the horizontal line).

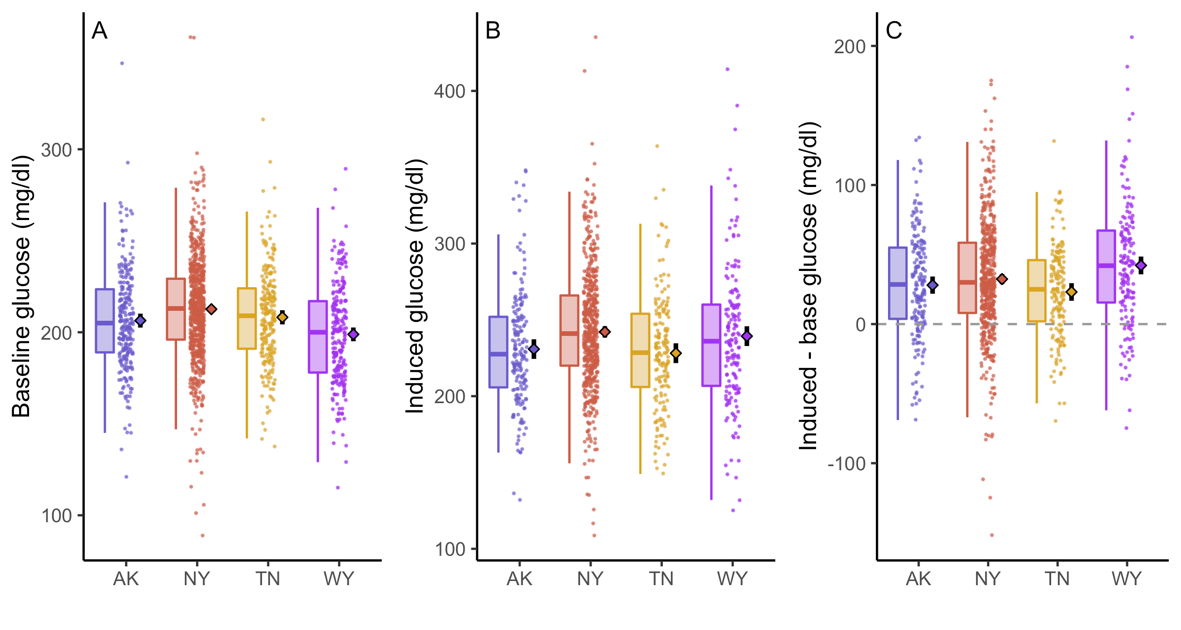


**Figure 4.** Illustration of the interaction between adult mass and the change in corticosterone from baseline to stress-induced samples as a predictor of the change in glucose concentration over the same time period. Solid lines and shaded regions show the maximum likelihood estimate and 95% confidence interval for the relationship among adults 1 SD below the mean (purple), at the mean mass (red), or 1 SD above the mean (orange). Confidence intervals were computed based on sampling from the fit model (see text).

*Population Comparison*

When comparing population baseline glucose levels, the NY population had a higher circulating level than the AK and WY populations, but the confidence intervals for all other two-way comparisons overlapped (Figure 5A; ‘emmeans’ estimate for AK = 206.4, CI = 202.6 to 210.2; NY = 212.6, CI = 210.4 to 214.8; TN = 208.2, CI = 204.3 to 212.1; WY = 198.9, CI = 195.1 to 202.6). For stress-induced glucose, the NY population had higher levels than the AK and TN populations, but the other two-way comparisons had overlapping confidence intervals (Figure 5B; AK = 230.8, CI = 224.4 to 237.3; NY = 242.1, CI = 238.4 to 245.8; TN = 228.1, CI = 221.6 to 234.6; WY = 239.3, CI = 232.8 to 245.8).

All four populations increased in mean glucose levels between baseline and stress-induced samples (Figure 5C). However, the WY population showed a greater increase over this period than AK or TN, while the other two-way comparisons did not differ (mean change in glucose AK = 28.0, CI = 21.8 to 34.2; NY = 32.4, CI = 28.7 to 36.0; TN = 23.1, CI = 16.8 to 29.5, WY = 42.2, CI = 35.8 to 48.6).

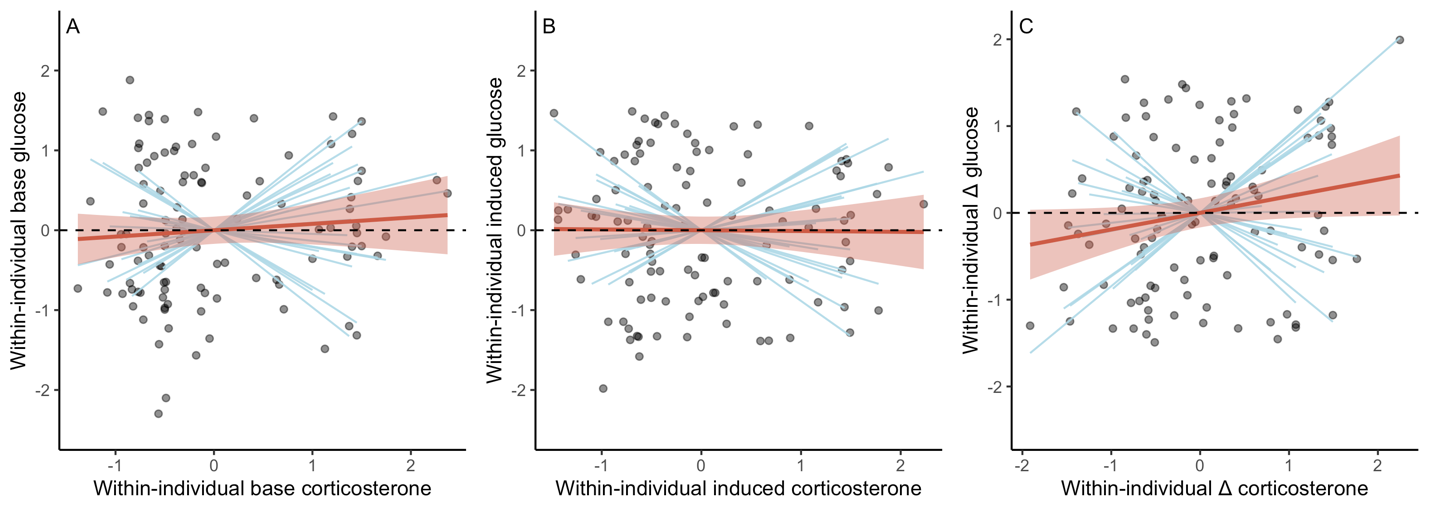
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**Figure 5.** Population comparison among adult females of baseline (A), stress-induced (B), and stress-induced - baseline glucose (C). Boxplots show the interquartile interval and points < 1.5 times IQR. Points include all raw data. Filled diamond and black lines show the estimated mean and 95% CI of the mean as estimated by the ‘emmeans’ package in R.

For baseline glucose, there was no evidence that between-individual variation in adult female corticosterone was related to variation in circulating glucose in any of the four populations (Table S6; coefficient estimate for baseline corticosterone AK β = 1.5, CI = -2.4 to 5.5; NY β = 1.5, CI = -0.7 to 3.7; TN β = -2.2, CI = -6.6 to 2.3; WY β = -2.2, CI = -7.3 to 2.9). Mass was negatively associated with baseline glucose levels in all four populations, although the confidence interval crossed zero in the TN population (Table S6; estimate for mass AK β = -4.1, CI = -8.0 to -0.1; NY β = -3.0, CI = -5.2 to -0.8, TN β = -3.2, CI = -7.2 to 0.8; WY β = -4.8, CI = -9.1 to -0.4). There was no support for an interaction between mass and baseline corticosterone on baseline glucose in any population (Table S6). For the change in glucose from baseline to induced measures, there was no support for either a direct effect of the change in corticosterone, for mass alone, or for an interaction between corticosterone and mass in any population other than NY (Table S7). The NY population had a similar effect size for the corticosterone by mass interaction to that described above, although the confidence interval was wider in this subset of data with males excluded (see above).

*Within-Individual Glucose and Corticosterone Covariation*

For both baseline and stress induced glucose, there was no evidence that deviations from the observed within-individual average corticosterone level were associated with similar deviations in the within-individual average glucose level (Figure 6A-B; overall baseline β = 0.1, CI = -0.1 to 0.3; overall stress-induced β = -0.01, CI = -0.2 to 0.2). For the induced minus baseline measures, there was a tendency for higher within-individual corticosterone stress responses to be associated with stronger within-individual glucose responses, though the confidence interval for this effect included zero (Figure 6C; overall response β = 0.2, CI = 0.0 to 0.4).



**Figure 6.** Covariation between baseline (A), stress-induced (B), and induced – base (C) corticosterone and glucose with both measures standardized within-individual. Points show raw data, blue lines are regressions for each individual sampled at least 4 separate times, red line and confidence interval show the overall relationship across all birds. The black dashed line is included as a reference of no effect. By definition, each individual’s regression passes through the origin where both corticosterone and glucose are at the mean value observed for that bird.

**DISCUSSION**

At the group level, our results fit well with the canonical stress response. In adults, corticosterone and glucose increased from baseline to stress-induced samples and this result was robust across four widely spread populations. Furthermore, injection with Cortrosyn in the New York population resulted in an additional increase in both measures in adults, though not in nestlings. However, these group level patterns largely failed to scale down to the between- and within-individual level. Between-individual variation in corticosterone was unrelated to variation in glucose at any time scale that we measured, except in one case when considered as an interaction with mass. Even in that instance, the corticosterone response explained a very small amount of variation in the glucose response, suggesting that it is not safe to assume that strong responders in one aspect of the acute stress response will also have strong changes in other facets of the response. Furthermore, even at the within-individual level, there was no clear evidence that modulation of the corticosterone response was associated with similar modulation of the glucose response.

This lack of between- and within-individual covariation between corticosterone and glucose has important implications for evolutionary physiologists interested in understanding variation in the acute stress response. Many studies make an implicit assumption that strong corticosterone responders will be strong responders in other downstream components of the stress response (see critique in Romero and Gormally, 2019). However, much of the basis for this assumption comes from group level comparisons (e.g., overall comparisons of baseline vs. stress-induced samples) and recent papers have challenged the degree to which different components of the stress response system are correlated within- or between-individuals (Lattin et al., 2015; Neuman-Lee et al., 2020). Making inferences about individuals based on group level differences is a classic logical error known as the ecological fallacy (Piantadosi et al., 1988). To varying degrees, evolutionary physiologists fall into this trap when trying to understand the fitness consequences of variation in the acute stress response from studies that measure only a single component (i.e., corticosterone). Understanding how and when different components of the stress response system covary—and when those correlations do or do not transfer across the within-individual, between-individual, and between population or species level—will require both better empirical data and a more robust scale-dependent framework for understanding trait correlations (Agrawal, 2020)

To a large extent, the limitation inherent in studies of the stress response that only measure corticosterone has long been recognized and the major barrier to progress is more logistical than conceptual. Studies in wild animals are still limited to disruptive sampling at a limited number of single time points. Recent conceptual papers have sought to explain the variation across these different time points by incorporating the time course of a stress response with systems biology (Del Giudice, 2012; Del Giudice et al., 2011) and within-individual reaction norms (Lema, 2014; Michaela Hau et al., 2016; Taff and Vitousek, 2016; Wada and Sewall, 2014). These approaches are powerful because they allow for a full description of the hierarchical nature of trait variation from within-individuals up to population and species levels in a single coherent framework. However, fully embracing these approaches in empirical studies—particularly for multiple simultaneous physiological measures—requires the ability to repeatedly sample individual animals many times across different contexts. Despite a large number of samples over many years in our study, we only had 24 individuals that were sampled four or more times. Using those samples, we found no evidence for within-individual covariation in glucose and corticosterone, but this result would be much more robust if we were able to sample the same individual continuously (see discussion of power for within-individual reaction norms in van de Pol, 2012). Bio-logging devices capable of recording key physiological parameters continuously may revolutionize this field, though they are not yet capable of measuring many of the parameters that are the most relevant for stress physiology (see discussion in Romero et al., 2015). In the meantime, an increased focus on theory and on empirical work that targets key assumptions (e.g., within- or between-individual covariation), rather than just documenting between individual variation, will be useful.

Despite the fact that there was no between- or within-individual covariation in glucose and corticosterone, we found that both of these parameters were somewhat consistent within individuals. For corticosterone, we previously reported negligible repeatability of baseline values and moderate repeatability of stress-induced and induced minus baseline values (Vitousek et al., 2018b) and we report similar patterns here. For glucose, all three measures were repeatable, but identity explained only a fairly small amount of variation in glucose levels (11-21%). These repeatability estimates are somewhat lower than those found in recent studies of zebra finches (*Taeniopygia guttata*), where both baseline and induced glucose were found to be individually repeatable and associated with early life conditions and lifespan (multiple repeatability estimates reported ranging from 30-50% Montoya et al., 2018; Montoya et al., 2020). There are few published estimates of glucose repeatability and isn’t clear what resulted in our lower estimates, but one obvious difference is that our measurements were taken under more variable natural conditions rather than in captivity.

It is also unclear what drove the difference that we observed between adults and nestlings. In general, nestling glucose was less responsive to both corticosterone elevation after handling and to experimental increases in corticosterone. In chickens, several components of the glucose regulation system in chicks differ from those of adults (reviewed in Braun and Sweazea, 2008). It is possible that the 12- to 15-day old nestlings that we sampled had not yet fully developed the physiological systems that would result in a robust glucose response. However, nestlings at this age do show a fully developed corticosterone response to experimental challenges. Alternatively, it is possible that the difference between adults and nestlings is an artefact of our sampling design. Nestlings were only sampled in a single year and the amount of variation in ecological conditions was narrower than for adults that were sampled over several years and populations. Moreover, we sampled nestlings only in the afternoon, while adults were only sampled in the morning. Previous work in captive adult European starlings found that handling resulted in a glucose response during the night and early morning, but that this response disappeared during the daytime hours (Remage-Healey and Romero, 2000; Remage-Healey and Romero, 2001). Because our samples were restricted to narrow times of day for each age class, we could not evaluate whether the glucose response differed by time of day in this study.

When comparing different populations, we found that two general patterns—a negative association between mass and baseline glucose and a stress-induced increase in glucose—were robust across all populations. Despite large differences in ecological conditions and environmental predictability across these locations (Zimmer et al., 2020), there was little evidence for between-individual covariation between corticosterone and glucose in any population. It is interesting to note, however, that both the absolute levels of glucose and the magnitude of glucose increase differed in some cases. With only four populations to compare it is unclear whether these differences are related to the different ecological challenges experienced by each population or whether they result from phenotypic plasticity versus local adaptation. While several comparative studies have investigated differences in baseline glucose in relation to mass and life history characteristics (Braun and Sweazea, 2008; Tomasek et al., 2018), we are not aware of any comparative data on differences in the magnitude of the change in glucose during an acute stress response between species. Large scale comparative studies of baseline and stress-induced corticosterone have recently begun to illuminate how hormonal regulation covaries with life history differences (Vitousek et al., 2019). Using this approach to understand variation in the patterns of covariation between different physiological components of the acute stress response is a promising direction as multifaceted physiological responses are measured in more species. It is increasingly possible to measure a panel of physiological parameters in the field for many species with point-of-care devices (Morales et al., 2020). Moreover, this comparative approach is less constrained by the logistical challenges described above at the within- and between-individual level.

Taken together, our results demonstrate that physiological or behavioral changes that co-occur as part of the canonical stress response at a population or species level, do not necessarily covary at the between- or within-individual level. While this presents major challenges for linking fitness to variation in the stress response at the individual level (Romero and Gormally, 2019), it also adds to a growing number of studies calling for a diversification of measures used to assess variation in the vertebrate stress response (Gormally et al., 2020). In particular, we advocate for integrating multiple measures with flexible analytical frameworks that are able to formally consider the hierarchical nature of variation between different components of the stress response system, as has been promoted recently for understanding behavior variation (Allegue et al., 2016; Araya-Ajoy et al., 2015; Westneat et al., 2014). Moving forward, careful and clear thinking will be needed to ensure that studies intending to answer a question at one level (e.g., between-individuals) actually have a study design and sampling regime that will work at that level and to be ensure that implicit assumptions are tested empirically. A greater embrace of simulation and theory coupled with advances in bio-logging and a rethinking of the ‘standard’ stress restraint protocol may be necessary to satisfactorily address questions at the logistically challenging within- and between-individual level.

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**COMPETING INTERESTS**

The authors declare no competing or financial interests.

**ETHICAL NOTE**

All work described here was approved by the Cornell University Institutional Animal Care & Use Board (IACUC protocol numbers xxx & xxx). Capture and sampling of wild birds was approved by appropriate federal and state agencies (federal permit # xxx, AK permit # xx, NY permit # xx, TN permit # xx, WY permit #). Maren, can you also fill this in. Not sure if we also want to list who each permit was to. I checked in the Sci Reports paper but all the permits aren’t listed.

**AUTHOR CONTRIBUTIONS**

All authors contributed to field data collection and study conceptualization. CCT and CZ performed the lab work for corticosterone measurement. CCT analyzed the data and wrote the first draft of the manuscript. All authors contributed to revisions of the final manuscript.

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SUPPLEMENTARY MATERIALS FOR:

Individual variation in natural or manipulated corticosterone is unrelated to variation in circulating glucose in a wild bird.

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**SUPPLEMENTARY METHODS**

To ensure that Cortrosyn® had the desired effect in elevating the corticosterone response, we conducted two validation studies on a separate set of nestlings and adults that were not part of the main study presented here. Once reconstituted, Cortrosyn is not stable at room temperature. Therefore, rather than delivering exact doses based on individual mass, we reconstituted vials of lyophilized Cortrosyn (Amphastar Pharmaceutical Incorporated, Item #054881) and prepared aliquots pre-measured into syringes based on the average mass in our population (on day 15 nestlings weigh approximately the same amount as full grown adults and the same dose was used for both adults and nestlings). Aliquoted Cortrosyn doses were stored frozen for < 2 weeks and thawed immediately before injection.

In 2018, we carried out a validation experiment on 15-day old nestlings from 9 nests. At each nest, individual nestlings were alternately assigned to a Cortrosyn injection group (n = 23 nestlings) or a control group that received a saline injection (n = 20 nestlings). For all nestlings, a baseline blood sample (< 30 μl) was collected within 3 minutes of disturbance and then nestlings were immediately injected with either 50 μl of saline or 50 μl of 0.1 microgram per μl freshly thawed Cortrosyn. Following injection, two additional blood samples (< 30 μl) were collected 15 and 30 minutes after injection.

In 2020, we carried out a separate validation experiment on adult females captured during incubation that were not part of the main study presented here. For each female, we collected a baseline blood sample (< 70 μl) within 3 minutes of disturbance. All females received a saline injection 50 μl immediately after this baseline sample was collected and then had a second blood sample (< 30 μl) taken 30 minutes later. Immediately after this second sample, females were injected with either an additional saline dose of the same volume (n = 9) or a dose of Cortrosyn (n = 9; 50 μl at 0.1 microgram per μl). Thirty minutes after this second injection, a final blood sample (< 30 μl) was collected. For both adults and nestlings in these validation experiments, blood samples were processed and corticosterone measured exactly as described in the main text.

To compare corticosterone between treatment groups, we fit a single model for each dataset (adults and nestlings). For nestlings, we fit a linear mixed model with corticosterone measurement as the response, an interaction between treatment and sampling time point (fit as a factor) as predictors, and individual identity as a random effect. Because multiple nestlings were sampled from the same nest, this model included a random effect for nest of origin. For adults, we fit a similar model, except that there was no need for a random effect for nest since only one female was sampled at each nest. We used the full models to compare circulating corticosterone in saline versus Cortrosyn injected birds at each of the three timepoints. Significance in these mixed models was assessed with p-values based on the Satterthwaite's Method implemented by the `lmerTest` package in R (Kuznetsova et al., 2017).

**SUPPLEMENTARY RESULTS**

Injection with Cortrosyn led to a clear increase in circulating corticosterone in both nestlings (Figure S1) and adults (Figure S2). For nestlings, corticosterone increased in both groups from baseline to 15 minutes but increased significantly more in the Cortrosyn injected group when compared to saline injection. From 15 to 30 minutes, saline injected nestlings declined in circulating corticosterone, but Cotrosyn injected nestlings continued to rise, resulting in an even larger difference in circulating corticosterone between the two groups at 30 minutes (Table S2, P < 0.003).

Adult females similarly increased circulating corticosterone from baseline to 30 minutes. The two treatment groups did not differ at the first or second timepoint (before Cortrosyn was injected). However, by the final timepoint (30 minutes after injection), the Cortrosyn injected group had significantly higher circulating corticosterone. This difference was driven by a continued increase in corticosterone from 30 to 60 minutes in the Cortrosyn group coupled with a stable circulating level from 30 to 60 minutes in the saline injected group (Table S2, P < 0.001).

**SUPPLEMENTARY TABLES & FIGURES**

*Chart

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**Figure S1.** Circulating corticosterone before and after Cortrosyn or saline injection in adult females.

*Chart

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**Figure S2.** Circulating corticosterone before and after Cortrosyn or saline injection in 15 day old nestling tree swallows.

**Table S1.** Model details for the effect of Cortrosyn injection on nestling and adult circulating corticosterone level. Timepoints 2 and 3 are at 15- and 30-minutes post injection for nestlings and at 30 and 60 minutes for adults.

**Table

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**Table S2.** Total sample sizes for glucose measurements by year, location, and sample type.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | Adults | | | Nestlings | | |
| State | Year | Base | Induced | Post-Cortrosyn | Base | Induced | Post-Cortrosyn |
| NY | 2016 | 244 | 178 | - | - | - | - |
| NY | 2017 | 140 | 138 | - | - | - | - |
| NY | 2018 | 188 | 123 | - | - | - | - |
| NY | 2019 | 204 | 147 | 45 | 185 | 177 | 159 |
| AK | 2016 | 117 | 84 | - | - | - | - |
| AK | 2017 | 134 | 100 | - | - | - | - |
| TN | 2018 | 228 | 167 | - | - | - | - |
| WY | 2018 | 236 | 160 | - | - | - | - |

**Table S3.** Results of linear mixed models with glucose or corticosterone as the response and sample type as a categorical predictor.

**Table

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**Table S4.** Results of linear mixed models on New York adults with glucose as the response variable and corticosterone, mass, a corticosterone by mass interaction, and sex as predictors. In each case, the corticosterone measure included spans the same interval as the glucose response variable. Unsupported interactions were dropped. Corticosterone and mass are scaled to a mean of zero and standard deviation of one to make effect sizes easier to interpret.

Table

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**Table S5.** Results of linear mixed models on New York nestlings with glucose as the response variable and corticosterone, mass, a corticosterone by mass interaction, and sex as predictors. In each case, the corticosterone measure included spans the same interval as the glucose response variable. Unsupported interactions were dropped. Corticosterone and mass are scaled to a mean of zero and standard deviation of one to make effect sizes easier to interpret.

**Table

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**Table S6.** Results of linear mixed models with baseline glucose as the response and corticosterone, mass, and their interaction as predictors in each of the four studied populations. Only adult females are included. Predictors are scaled to a mean of zero and standard deviation of one.

**Table

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**Table S7.** Results of linear mixed models with induced - baseline glucose as the response and induced – baseline corticosterone, mass, and their interaction as predictors in each of the four studied populations. Only adult females are included. Predictors are scaled to a mean of zero and standard deviation of one.

Table

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**SUPPLEMENTARY REFERENCES**

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