

RESEARCH ARTICLE

Investigating the relationship between corticosterone and glucose in a reptile

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ABSTRACT

The glucocorticoid hormone corticosterone (CORT) has classically been used in ecophysiological studies as a proxy for stress and energy mobilization, but rarely are CORT and the energy metabolites themselves concurrently measured. To examine CORT's role in mobilizing glucose in a wild reptile, we conducted two studies. The first study measured natural baseline and stress-induced blood-borne CORT and glucose levels in snakes during spring emergence and again when snakes return to the denning sites in autumn. The second study manipulated the hypothalamic–pituitary–adrenal (HPA) axis in male snakes in the autumn by taking a baseline blood sample, then subjecting individuals to one of five treatments (no injection, saline, CORT, adrenocorticotropin hormone and metyrapone). Subsequent samples were taken at 30 and 60 min. In both studies, we found that glucose levels do increase with acute stress, but that the relationship was not directly related to CORT elevation. In the second study, we found that none of the HPA axis manipulations directly affected blood glucose levels, further indicating that CORT may play a complex but not direct role in glucose mobilization in snakes. This study highlights the need for testing mechanisms in wild organisms by combining *in situ* observations with manipulative studies.

KEY WORDS: Stress, Gluconeogenesis, Snake, Corticosterone challenge, Adrenocorticotropin, HPA axis

INTRODUCTION

The recognition that physiological systems and ecological processes are intricately linked and can inform each other has led to a dramatic increase in ecophysiological studies as a way to understand individual and population responses to the environment. A frequently used metric in this field is the measurement of glucocorticoids (GCs) in wild individuals. In many studies, GCs are used to quantify ‘stress’ in an organism, even though GCs are involved in much more than just facilitating changes in responses to stress (Bonier et al., 2009; Breuner et al., 2013; Busch and Hayward, 2009; Sapolsky, 2000). While GCs are typically released during a stressful event, their role is nuanced and they are involved in various activities such as mobilizing energy stores, immunomodulation and mediating other hormones (Dhabhar, 2009; Jimeno et al., 2018; Sapolsky, 2000; Sorrells and Sapolsky, 2007).

It is well understood that GCs are context dependent and can have variable effects under different conditions and at different time points during a stress response. Previous work has demonstrated that

one role during an acute stressor is hepatic gluconeogenesis, which makes glucose available in the blood stream for continued activity (Kuo et al., 2015). This GC release and subsequent upregulation of gluconeogenesis is coupled with the inhibition of transport and/or utilization of glucose in the peripheral tissues, which allows for more energy to be readily available for responding to the stressor (Dimitriadis et al., 1997; Herman et al., 2016; Sapolsky, 2000).

Many studies have set out to examine how stress influences the physiological responses of an organism by measuring GCs and glucose at baseline and post-stress time points. The positive relationship between stress and glucose has been demonstrated in several organisms (Clare and Thurby-Hay, 2009; Jessop et al., 2003). This relationship was not seen in all studies and may be dependent upon the nutritional state of the individual (Remage-Healey and Romero, 2001). Further, the vast majority of these studies have been conducted in endothermic species (birds and mammals) that have high metabolic rates and would be particularly sensitive to changes in glucose availability (Jimeno et al., 2018). When examining responses to acute stress in reptiles, studies have generally demonstrated a positive (Aguirre et al., 1995; Franklin et al., 2003; Gangloff et al., 2017; Hunt et al., 2016) or lack of (Flower et al., 2015) correlation between stress and glucose levels. The nascent research examining other factors involved in glucose and stress in reptiles has already uncovered that stress is not the only driver of glucose concentration. In response to stress, glucose concentration can differ between species (Telemeco et al., 2017), populations or ecotypes (Gangloff et al., 2016, 2017), with temperature (Telemeco et al., 2017), size (Gangloff et al., 2017) and even maternal variation (Gangloff et al., 2018).

These studies saw similar increases in both GCs and glucose after an acute stressor. Many of these studies have not explicitly tested the correlational relationship between these two physiological metrics (but see Gangloff et al., 2017). Glucose concentration increases are thought to be driven primarily by glucocorticoids, but glucose is regulated not only by glucocorticoids but also by catecholamines (Rizza et al., 1980; Smeets and González, 2000) and insulin (Strack et al., 1995).

Work conducted in reptiles has provided strong evidence that the role of glucose mirrors its role in mammals and birds, including its context dependency. In sexually dimorphic endotherms, energetic expenditure differs between males and females (Key and Ross, 1999). Because many reptiles are also sexually dimorphic in terms of size and have different life histories, the few studies that have examined differences in metabolic needs have revealed divergence between males and females that are invariably linked to seasonality and reproductive periods (Crews et al., 1987). It also is apparent that some reptiles may be able to evolve different set points for baseline glucose concentrations to better survive in resource-poorer areas and match morphological differences such as body and head size (Sparkman et al., 2018). Further, the relatively lower metabolic requirements in most reptiles adds complexity to the role of GCs which we are still attempting to elucidate.

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To examine whether corticosterone (CORT; primary GC in reptiles) directly affects glucose concentration in reptiles, we conducted two experiments. The first experiment examined the baseline concentrations of circulating CORT and glucose, as well as acute stress-activated levels of both metrics. Within this experiment, males and females from three population replicates were sampled in both the spring and autumn, and across multiple years to determine natural relationships among GCs and glucose. We hypothesized that we would see positive relationships between CORT and glucose, as in other studies. We further hypothesized that CORT and glucose concentrations would be directly correlated when examining the difference between baseline and post-stress samples. In our second study, to more directly test the role of CORT, we manipulated a subset of male snakes in the field during a single spring season. We took a baseline blood sample, then gave males a dose of one of five treatments [CORT, adrenocorticotropic hormone (ACTH), CORT-blocker metyrapone (META), vehicle injection and no injection] to test the effect of CORT on glucose. We hypothesized that CORT and the upstream hormone ACTH would show commensurate increases in CORT and glucose relative to animals either not injected or injected with a vehicle, while treatment with META would suppress both CORT and glucose production.

MATERIALS AND METHODS

In situ field experiment

Wandering gartersnakes, *Thamnophis elegans vagrans* (Baird and Girard 1853), were hand-captured between 29 March–13 April and 12 September–12 October 2015 and 12 March–2 April 2016 near three different hibernaculums in Cache County, UT, USA. These three sites (A, B, C) were selected because they had been monitored for several years and have different microenvironments. This would allow us to assess whether any relationships were universal or dependent upon the environmental context. Site A is located at a drainage pipe under a busy state highway. Emergence and mating occur in the ditches next to frequent traffic traveling at high speeds. Site B is a city park with high levels of pedestrian and bike traffic. Site C is an isolated location that requires walking approximately 2.6 km to access. While snakes here use an anthropomorphic structure and cattle rotate through the area in the autumn, there is little human presence.

Snakes were captured and a blood sample was taken within 3 min (typically less than 1 min) of capture (baseline). A single drop of whole blood was immediately measured on an AccuChek Aviva Plus (Roche Diagnostics, Indianapolis, IN, USA), while the rest of the blood was stored on ice until further processing (see below). The use of a whole-blood glucose monitor has been validated in several vertebrates (Stoot et al., 2014). Snakes were kept individually in a breathable cloth bag for 30 min, and then we took another blood sample (stress induced). We again measured their glucose levels using whole blood and retained the rest of the blood for further processing. Snakes were then sexed (male, female, juvenile), weighed, and snout–vent length (SVL) was determined. Snakes were considered juveniles if <20 g and <400 mm SVL. If snakes were unmarked, we cauterized their ventral and lateral scutes in an individual pattern using a medical cauterizer (Winne et al., 2006). Snakes were released at the site of capture.

Both the baseline and post-stress blood samples were transported back to Utah State University, where they were centrifuged to separate the red blood cells from the plasma. Plasma and the buffy layer were removed and frozen at -80°C until further processing (see below).

Manipulation experiment

Fifty-one male gartersnakes were captured between 28 September and 8 October 2016 from one site (site B) in Cache County, UT, USA. Only males were selected because it is logistically easier to capture a high sample size within a short period of time. A blood sample was taken from each snake (<3 min) and blood was processed as described above. Immediately after their blood draw (within 2 min), each male was randomly assigned to one of five treatment groups: No injection (NI), vehicle injection (VI) with 10% ethanol in Ringer's solution (Carolina Biological Supply Company, Burlington, VA, USA), $1.35\ \mu\text{g g}^{-1}$ CORT (Sigma–Aldrich, Inc., St Louis, MO, USA), $100\ \text{IU kg}^{-1}$ ACTH (porcine, Sigma–Aldrich, Inc.) or $25\ \mu\text{g g}^{-1}$ META (Sigma–Aldrich, Inc.). Previous work with reptiles and amphibians has demonstrated that META has a suppressive effect on acute CORT concentrations after a stressor when injected between 20 and 90 min prior to taking a post-stress sample (Neuman-Lee et al., 2015; Thaker et al., 2010; Yang and Wilczynski, 2003). Each snake received the same volume of liquid by mass (except the NI snakes). Snakes were stored in breathable bags as before and blood samples were taken at 30 and 60 min post-injection.

Radioimmunoassay

We measured the levels of CORT using a radioimmunoassay following Neuman-Lee and French (2017). Briefly, we assayed plasma samples in duplicate for CORT (corticosterone antibody, MP Biomedicals no. 07120016, Santa Ana, CA, USA). Each sample was extracted with 30% ethyl acetate:isooctane. Individual recoveries were measured and final concentrations were corrected. We assayed these samples in two different assays. Samples from snakes captured in 2015 for the *in situ* experiment were run in one assay and the coefficient of variation was 14.7% and the accuracy was 103.0%. Samples from snakes captured in 2016 for the *in situ* experiment and those for the manipulation experiment were run on a second assay with a coefficient of variation of 12.3% and accuracy of 91.6%. The coefficient of variation between the two assays was 11.3% and the accuracy was 97.7%. The coefficient of variation was calculated based on known standards distributed throughout each assay. The minimum level of detection for CORT was $0.3\ \text{ng ml}^{-1}$.

Statistical treatment of data

For all statistical models, we established an alpha level of 0.05 for statistical significance. If individuals were missing values for either CORT or glucose, we omitted the entire sample from the dataset to appropriately conduct analyses. We tested for normality in the residual distributions of all statistical models and compared across groups for equality of variance when appropriate. To help meet the assumptions of model normality, we \log_e -transformed values for glucose and CORT. When graphically presented, we calculated values for CORT and glucose based on the untransformed data. We performed all statistical analyses in R (version 3.5.1; <http://www.R-project.org/>) using the following packages: 'car' (version 2.1-6; <https://CRAN.R-project.org/package=car>), 'nlme' (version 3.1-137; <https://CRAN.R-project.org/package=nlme>) and 'lsmeans' (version 2.27-62; Lenth, 2018). For visual representation of data, we used the following R packages: 'ggplot2' (version 3.1.0; Wickham, 2016), 'gggnif' (version 0.6.0; <https://CRAN.R-project.org/package=gggnif>) and 'cowplot' (version 0.9.3; <https://CRAN.R-project.org/package=cowplot>).

In situ field experiment

We ran separate regression models for the continuous response variable (glucose) as a function of the explanatory variable (CORT)

to determine the extent that CORT and glucose were linearly related. Each model considered CORT and glucose at either baseline or stress-induced levels.

We also constructed separate multivariate mixed models for continuous response variables (CORT, glucose) by categorical fixed-effect parameters (season, sex, time) and random-effect parameters (individual identity, population, capture year). Beginning with full models, we used a process of backward selection in which we sequentially removed each variable (fixed or random) until reaching each null model. We used likelihood ratio tests to compare and select models based on their respective Akaike's information criterion (AIC) score (Burnham and Anderson, 2002). For CORT, we found an interaction model including season-by-time and sex-by-time as fixed-effect parameters and a random intercept accounting for individual identity to be optimal for our collected data. For glucose, we instead found an additive model including season, sex and time as fixed-effect parameters and a random intercept accounting for individual identity to best fit the data. We performed Type III sums of squares tests to determine the significance of fixed-effect parameters for each model. Summary outputs provided us with estimates of beta coefficients with 95% confidence intervals (CI) and *P*-values for each fixed-effect parameter as well as the variance (σ^2) with 95% CI for the random-effect parameter. To assess level differences of each significant fixed-effect parameter, we calculated least square means for multiple comparisons with Tukey adjustments.

Manipulation experiment

We ran separate analysis of covariance models between a continuous response variable (glucose) and the interaction of continuous covariate (CORT) and categorical predictor variable (treatment) at each time point. Testing the homogeneity of regression slopes allowed us to evaluate the relationship between baseline CORT and glucose, and determine whether and how each treatment affected the relationship at stress-induced levels. If this assumption was violated by a significant interaction, we disregarded any main effects and focused on interpreting the relationship between CORT and treatment on glucose. If CORT yielded a significant correlation with glucose, we ran regression models to determine the strength and directionality of the relationship.

Additionally, we constructed separate multivariate mixed models for each continuous response variable (CORT, glucose) by categorical fixed-effect parameters (treatment, time) and a random-effect parameter (individual identity). We evaluated

model fit by backward selection followed by likelihood ratio tests and AIC comparison (Burnham and Anderson, 2002). For CORT, we found an interaction model including treatment-by-time as fixed-effect parameters and a random intercept of individual identity to be optimal. For glucose, we instead found an additive model including treatment and time as fixed-effect parameters and a random intercept of individual identity to be the best fit. To assess overall significance and level differences of each fixed-effect parameter, we performed Type III sums of squares tests. If a fixed-effect parameter was significant, we conducted Tukey-adjusted multiple comparisons of least square means.

RESULTS

In situ field experiment

Overall relationship between CORT and glucose

We found baseline levels of CORT and glucose to be significantly correlated (Table 1; $n=72$, $r^2=0.115$, $P=0.002$), yielding a moderate, positive relationship (Fig. 1A). For stress-induced CORT and glucose, we instead found no significant relationship ($n=73$, $r^2=0.003$, $P=0.625$; Fig. 1B).

Seasonal effects on CORT and glucose between time points

For CORT, we found the interactive effects of season and time point to not be significant ($\chi^2=1.340$, $P=0.247$; Table 2). However, we found seasonal effects to be significant for CORT ($\chi^2=7.492$, $P<0.01$), wherein baseline CORT did not vary between seasons ($P=0.550$; Fig. 2A), but stress-induced CORT was 91.3% greater during spring ($P<0.05$). We also found time effects to be significant for CORT ($\chi^2=16.880$, $P<0.00001$), such that stress-induced CORT increased 65.3% by 30 min during spring ($P<0.01$), but significant changes did not occur during autumn ($P=0.114$). For glucose, we did not find seasonal effects ($\chi^2=0.079$, $P=0.780$), but time effects were significant ($\chi^2=206.64$, $P<0.000001$) in that stress-induced glucose increased by 46.3% during spring and 54.8% during autumn ($P<0.05$; Fig. 2B). Although seasonal effects were also not significant ($\chi^2=0.509$, $P=0.476$), we found time effects to be significant ($\chi^2=184.04$, $P<0.000001$), to the extent that stress-induced CORT increased by 46.3% during spring and 54.8% during autumn ($P<0.05$; Fig. 2B).

Sex effects on CORT and glucose between time points

For CORT, we found the interactive effects of sex and time point to be significant ($\chi^2=4.466$, $P<0.05$; Table 2). We determined the

Table 1. Longitudinal corticosterone and glucose concentrations from wandering gartersnakes (*Thamnophis elegans*) in *in situ* and manipulation experiments

| Fixed-effect parameter | CORT (ng ml ⁻¹) | | | | Glucose (mg dl ⁻¹) | | | |
|---------------------------------|-----------------------------|-------------|--------------|--------------|--------------------------------|------------|------------|------------|
| | <i>n</i> | 0 min | 30 min | 60 min | <i>n</i> | 0 min | 30 min | 60 min |
| <i>In situ</i> field experiment | | | | | | | | |
| Spring | 70 | 49.91±5.78 | 82.49±8.53 | – | 94 | 38.46±1.64 | 56.28±2.37 | – |
| Autumn | 60 | 38.12±7.23 | 43.12±5.92 | – | 68 | 35.15±1.62 | 54.41±1.72 | – |
| Male | 94 | 49.87±4.84 | 73.88±7.24 | – | 114 | 38.53±1.49 | 56.73±2.10 | – |
| Female | 36 | 29.74±10.44 | 42.37±7.77 | – | 48 | 33.58±1.67 | 54.00±1.75 | – |
| Manipulation experiment | | | | | | | | |
| ACTH | 30 | 18.89±3.99 | 95.00±35.81 | 108.67±6.41 | 24 | 27.40±2.86 | 48.56±4.34 | 53.56±5.55 |
| CORT | 30 | 23.08±5.89 | 348.57±46.70 | 339.40±23.99 | 24 | 31.22±2.73 | 51.10±3.05 | 62.78±4.12 |
| META | 30 | 31.81±7.55 | 52.26±8.08 | 61.71±4.86 | 18 | 33.50±7.23 | 42.30±6.85 | 55.80±7.09 |
| No injection | 36 | 16.98±3.71 | 49.66±11.26 | 75.34±10.10 | 30 | 29.25±3.18 | 49.27±5.36 | 67.70±6.80 |
| Vehicle | 30 | 21.80±7.94 | 141.54±27.52 | 147.24±21.86 | 27 | 31.20±2.35 | 50.90±3.44 | 59.11±4.44 |

CORT, corticosterone; ACTH, adrenocorticotropic hormone; META, metyrapone. Values are mean (±s.e.m.) concentrations derived from the untransformed data taken across time points (0, 30 and 60 min). Missing values at 60 min for the *in situ* experiment are due to study design. These data were gathered by using individuals as replicates in a field setting.

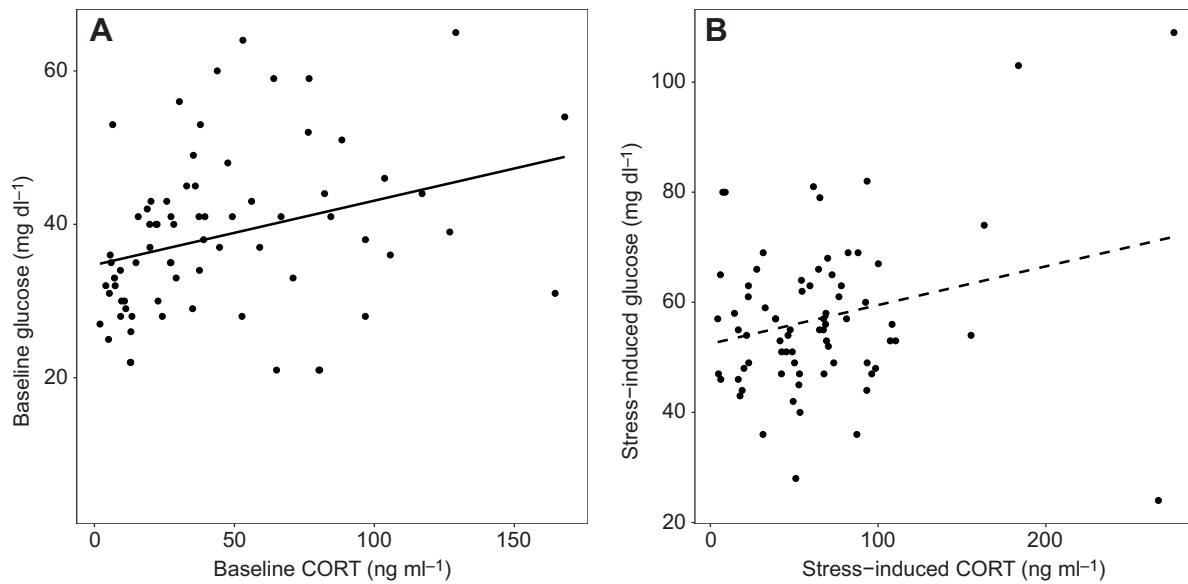


Fig. 1. Regressions of corticosterone (CORT) and glucose following stress in wandering gartersnakes (*Thamnophis elegans*). (A) Baseline levels. (B) Stress-induced levels. Values represent untransformed data. The solid line indicates a significant relationship whereas the dashed line indicates no significant relationship. These data were gathered by using individuals as replicates in a field setting.

time-dependent sex effects to be prompted by males exhibiting 67.7% greater baseline CORT than females ($P < 0.05$), as no significant differences were present following stress ($P = 0.910$; Fig. 3A). We also found that stress-induced differences in CORT were evident by 30 min with a 42.5% increase among females ($P < 0.05$), but no significant change occurred among males ($P = 0.346$). For glucose, we found no significance for the effects of sex ($\chi^2 = 0.515$, $P = 0.473$; Table 2), but we did find time effects to be significant ($\chi^2 = 206.664$, $P < 0.00001$) in that stress-induced glucose increased by 47.2% in males and 60.8% in females ($P < 0.05$; Fig. 3B).

Manipulation experiment

Treatment effects on CORT and glucose relationships

We found no significant interactive effects at baseline ($F_{4,37} = 1.61$, $P = 0.192$; Fig. 4A), 30 min ($F_{4,40} = 1.768$; Fig. 4B) and 60 min ($F_{4,37} = 1.460$, $P = 0.234$; Fig. 4C), indicating homogeneity among each of the treatment regression slopes between CORT and glucose. After removing the interaction from the model, we found no significant effect of treatment at baseline ($F_{4,41} = 0.366$, $P = 0.832$; Fig. 4A), 30 min ($F_{4,44} = 0.754$, $P = 0.561$; Fig. 4B) and 60 min

($F_{4,41} = 1.700$, $P = 0.169$; Fig. 4C). For baseline glucose, we found a significant relationship with CORT ($F_{1,41} = 8.839$, $P < 0.001$; Fig. 4A), such that 15.4% of the variance in glucose was explained by CORT ($r^2 = 0.154$). However, we found relationships between CORT and glucose to not be significant at 30 min ($F_{1,44} = 2.727$, $P = 0.106$; Fig. 4B) and 60 min ($F_{1,41} = 0.034$, $P = 0.854$; Fig. 4C).

Treatment effects on CORT and glucose between time points

We found significant interactive effects between treatment and time point for CORT ($\chi^2 = 65.871$, $P < 0.00001$; Tables 2 and 3). Treatment differences were evident at 30 min, as we found stress-induced CORT in the CORT group to be 146.3% greater than that in the vehicle group and 601.9% greater than that in the no-injection group ($P < 0.05$; Fig. 5A). We also found that the vehicle group had 185% greater stress-induced CORT than the no-injection group ($P < 0.05$). By 30 min, we found stress-induced increases in CORT occurred among all groups ($P < 0.05$), except for the META group ($P = 0.504$). Specifically, CORT increased by 192.5% for the no-injection group, 549.3% for the vehicle group, 403% for the ACTH group, 1410.3% for the CORT group and 64.3% for the META group. We found treatment differences at 60 min to be prompted by the CORT group, with stress-induced CORT levels that were 350.5% greater than in the no-injection group, 212.3% greater than in the ACTH group and 450% greater than in the META group ($P < 0.05$; Fig. 5A). By 60 min, stress-induced CORT was greater than baseline for all groups ($P < 0.05$), yet we found these changes to be only marginally different from those at 30 min ($P > 0.05$). Specifically, CORT increased by 51.7% for the no-injection group, 4% for the vehicle group, 14.4% for the ACTH group and 18.1% for the META group, but also decreased by 2.6% for the CORT group.

For glucose, we did not find treatment effects to be significant ($\chi^2 = 1.476$, $P = 0.831$), but we did find significant time effects ($\chi^2 = 503.855$, $P < 0.00001$; Table 2), such that all treatment groups demonstrated greater stress-induced glucose relative to baseline glucose at 30 and 60 min ($P < 0.005$; Fig. 5B). At 30 min, stress-induced glucose increased by 77.2% in the ACTH group, 63.7% in the CORT group, 26.3% in the META group, 68.4% in the

Table 2. Seasonal effects on CORT and glucose levels in wandering gartersnakes

| | log _e CORT | | log _e Glucose | |
|---------------------------------|-----------------------|--------------------|--------------------------|--------------------|
| | χ^2 | <i>P</i> | χ^2 | <i>P</i> |
| <i>In situ</i> field experiment | | | | |
| Season | 7.492 | <0.01 | 0.079 | 0.780 |
| Sex | 7.193 | <0.005 | 0.515 | 0.473 |
| Time | 16.880 | <0.00001 | 206.664 | <0.00001 |
| Season×time | 1.340 | 0.247 | – | – |
| Sex×time | 4.466 | <0.05 | – | – |
| Manipulation experiment | | | | |
| Treatment | 53.623 | <0.00001 | 1.476 | 0.831 |
| Time | 383.145 | <0.00001 | 503.855 | <0.00001 |
| Treatment×time | 65.871 | <0.00001 | – | – |

Type III sums of squares for multivariate mixed models of log_e-transformed CORT and glucose in response to fixed-effect parameters from *in situ* and manipulation experiments. Significant *P*-values are highlighted in bold.

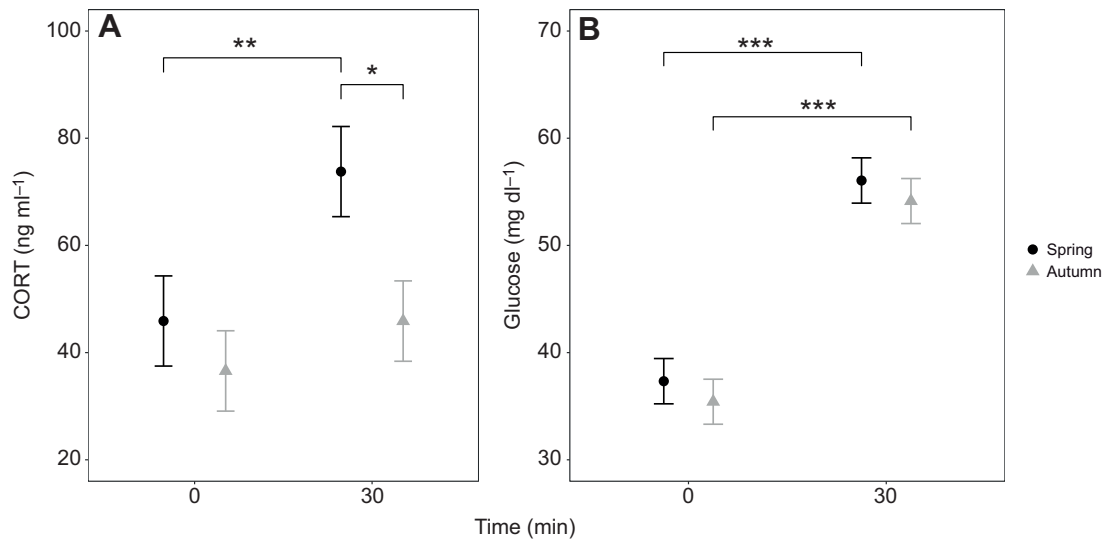


Fig. 2. Multiple comparisons of seasonal effects on CORT and glucose levels in wandering gartersnakes. (A) Baseline and stress-induced CORT levels. (B) Baseline and stress-induced glucose levels. Symbols represent untransformed least squares mean (\pm s.e.m.) concentrations at 0 min (baseline) and 30 min (stress induced). Asterisks represent the degree of significance between comparisons (* P <0.05, ** P <0.005, *** P <0.0005), whereas a lack thereof indicates no significant relationship. These data were gathered by using individuals as replicates in a field setting.

no-injection group and 63.1% in the vehicle group compared with baseline. We found greater stress-induced glucose at 60 min compared with 30 min only for the no-injection group (37.4% increase; P <0.005), as the other treatment groups only demonstrated insignificant differences (P >0.05; Fig. 5B).

DISCUSSION

The results of this study demonstrate that glucose and CORT are interrelated at basal levels in snakes, but that there are likely multiple other factors that contribute to their variation including sex (CORT), season (CORT) and stress state (both). In accordance with our

Table 3. Treatment effects on CORT and glucose levels at different time points in wandering gartersnakes

| | CORT | | Glucose | |
|--|---|--------------------|---|--------------------|
| | β estimate or σ^2 (95% CI) | P | β estimate or σ^2 (95% CI) | P |
| <i>In situ</i> field experiment | | | | |
| Fixed effect | | | | |
| Season (spring) | 1.349 (0.859–2.119) | 0.932 | 1.056 (0.908–1.228) | 0.780 |
| Sex (male) | 2.358 (1.427–3.899) | <0.005 | 1.532 (1.444–1.625) | 0.475 |
| Time (30 min) | 2.220 (1.31–3.763) | <0.005 | 1.02 (0.887–1.173) | <0.0001 |
| Season (spring) \times time (30 min) | 1.405 (0.859–2.119) | 0.251 | – | – |
| Sex (male) \times time (30 min) | 0.501 (0.261–0.963) | <0.05 | – | – |
| Random effect | | | | |
| Individual identity | 1.927 (1.336–4.397) | | 1.667 (1.517–1.872) | |
| Manipulation experiment | | | | |
| Fixed effect | | | | |
| Treatment (vehicle injection) | 1.094 (0.662–1.808) | 0.734 | 1.034 (0.801–1.334) | 0.793 |
| Treatment (CORT) | 1.307 (0.791–2.161) | 0.313 | 1.06 (0.815–1.378) | 0.656 |
| Treatment (ACTH) | 1.096 (0.663–1.812) | 0.728 | 0.909 (0.699–1.182) | 0.466 |
| Treatment (META) | 1.824 (1.104–3.015) | <0.05 | 0.998 (0.75–1.329) | 0.991 |
| Time (30 min) | 2.851 (1.904–4.269) | <0.00001 | 1.671 (1.563–1.787) | <0.00001 |
| Time (60 min) | 4.912 (3.28–7.356) | <0.00001 | 2.084 (1.949–2.228) | <0.00001 |
| Treatment (vehicle injection) \times time (30 min) | 2.39 (1.313–4.35) | <0.01 | – | – |
| Treatment (CORT) \times time (30 min) | 5.93 (3.258–10.794) | <0.00001 | – | – |
| Treatment (ACTH) \times time (30 min) | 1.558 (0.856–2.835) | 0.166 | – | – |
| Treatment (META) \times time (30 min) | 0.619 (0.34–1.127) | 0.134 | – | – |
| Treatment (vehicle injection) \times time (60 min) | 1.726 (0.948–3.142) | 0.089 | – | – |
| Treatment (CORT) \times time (60 min) | 3.638 (1.999–6.622) | <0.00001 | – | – |
| Treatment (ACTH) \times time (60 min) | 1.414 (0.777–2.574) | 0.278 | – | – |
| Treatment (META) \times time (60 min) | 0.478 (0.263–0.87) | <0.05 | – | – |
| Random effect | | | | |
| Individual identity | 1.376 (1.237–1.614) | | 1.678 (1.491–1.954) | |

Results from multivariate mixed models on the effects of *in situ* and manipulation experiments on \log_e -transformed physiological variables (CORT and glucose). Included are beta coefficients, 95% confidence intervals (CI) and P -values for fixed-effect parameters, and standard deviations and 95% CI for the random-effect parameter. Values are exponentiated to account for \log_e -transformation. Significant P -values are highlighted in bold.

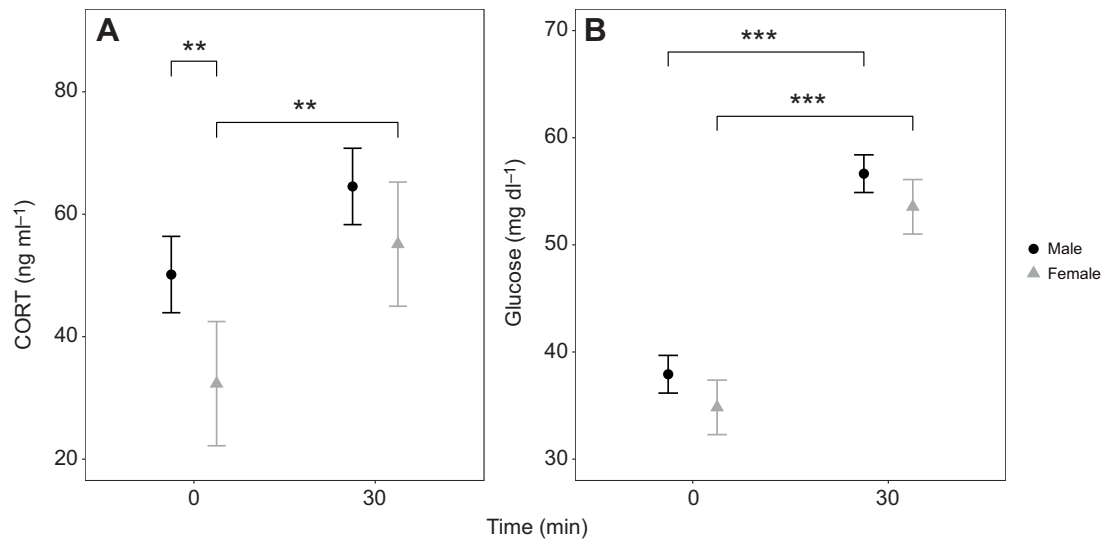


Fig. 3. Multiple comparisons of sex effects on CORT and glucose levels in wandering gartersnakes. (A) Baseline and stress-induced CORT levels. (B) Baseline and stress-induced glucose levels. Symbols represent untransformed least squares mean (\pm s.e.m.) concentrations at 0 min (baseline) and 30 min (stress induced). Asterisks represent the degree of significance between comparisons (** P <0.005, *** P <0.0005), whereas a lack thereof indicates no significant relationship. These data were gathered by using individuals as replicates in a field setting.

prediction, baseline glucose was positively related to baseline CORT, but this did not hold true for the stress-induced levels of glucose and CORT, although both did increase. Other physiological factors may be more influential than CORT in increasing glucose during acute stress. Baseline or stress-induced glucose levels were not related to either season or sex.

Both glucose and CORT concentrations increased during acute stress. However, the absolute values of these increases were not correlated in this study. While many other studies have shown an increase in both CORT and glucose in response to stress, they typically did not explicitly test to see whether the values are indeed correlated (e.g. Aguirre et al., 1995; Hunt et al., 2016). While contrary to our initial predictions that stress-induced glucose would be correlated directly with CORT, the lack of a direct correlation is consistent with another study in gartersnakes (Gangloff et al., 2017). This study showed a correlation between baseline CORT and glucose, as in our study, but a lack of correlation at 3 h post-stress. After 3 days, there was a weak correlation again between the two physiological measures, indicating a possible return to baseline.

There was no direct relationship between stress-induced CORT and glucose (other than both were elevated after the acute stress); thus, it is possible that the elevation in glucose is also under the control of catecholamine release. Quantifying catecholamines in wild organisms is extremely difficult because of the near-impossibility of obtaining a true baseline blood sample (Hart et al., 1989). Further, the characterization of catecholamines in reptiles is still in its infancy. We do know that reptiles respond physiologically to exogenous catecholamines (Woolley et al., 2004), have measurable concentrations of circulating catecholamines (Hart et al., 1989; Lance and Elsey, 1999; Matt et al., 1997) and catecholamine-secreting cells (Brauth, 1988; Smeets and González, 2000), but we still do not understand the intricacies of release and feedback control.

While catecholamines may be responsible, it is possible that other physiological mechanisms are involved. In mammals, studies have shown that GCs play an important role in glucose regulation by reducing insulin sensitivity and thereby increasing the amount of glucose in circulation (Clare and Thurby-Hay, 2009; Warne et al., 2009). However, non-mammalian vertebrates, including snakes,

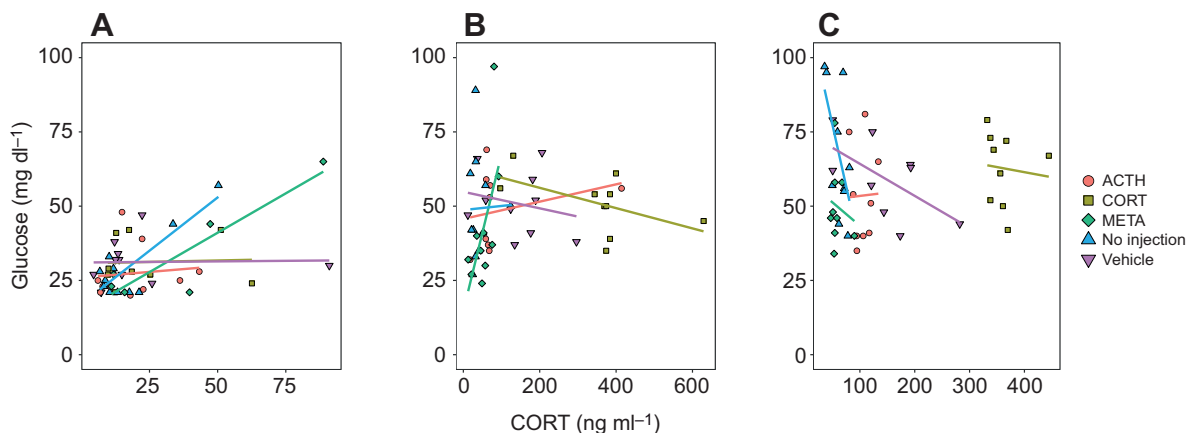


Fig. 4. Covariation between CORT and glucose by treatment at different time points in wandering gartersnakes. (A) 0 min (baseline), (B) 30 min and (C) 60 min after treatment with CORT, adrenocorticotropic hormone (ACTH), the CORT-blocker metyrapone (META), saline vehicle or no injection. Values represent untransformed data. These data were gathered by using individuals as replicates in a field setting.

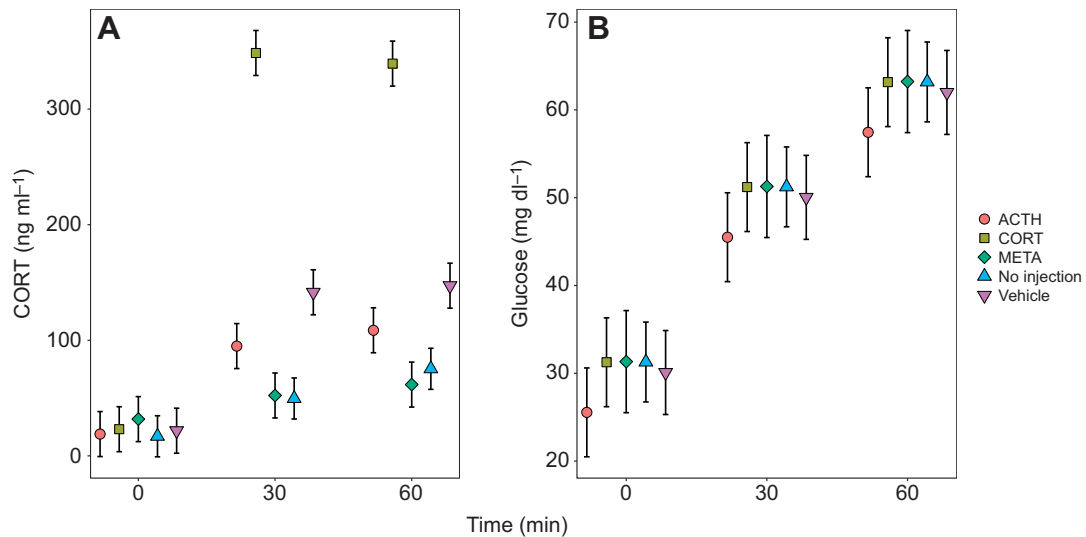


Fig. 5. Multiple comparisons of treatment effects on CORT and glucose levels in wandering gartersnakes. (A) CORT and (B) glucose levels at 0 min (baseline) and 30 and 60 min following treatment with ACTH, CORT, META, vehicle or no injection. Points represent untransformed least squares mean (\pm s.e.m.) concentrations at 0, 30 and 60 min. These data were gathered by using individuals as replicates in a field setting.

seem to have a different role for insulin with regards to carbohydrate metabolism (del Sol Novoa et al., 2004; Miller, 1960), potentially resulting in the lack of a correlation between stress-induced levels of CORT and glucose that we found. Although insulin, CORT and glucose increase linearly with increasing temperature in gartersnakes (Gangloff et al., 2016), the proximate effect of each of these components on each other has not been definitively shown. Several experiments with European starlings (*Sturnus vulgaris*) have shown that there may be links between elevated CORT, insulin and glucose, but these are dependent upon multiple factors, such as the time of day, stress state (chronic versus acute) and photoperiod (Cyr et al., 2007; Remage-Healey and Romero, 2001). These findings also reveal a much more complex relationship with CORT and glucose. Indeed, metabolic processes may be more tightly controlled by glucagon in fish and reptiles (del Sol Novoa et al., 2004; Miller, 1960), and the effect of GCs on glucagon is not well understood. CORT does have a role in gluconeogenesis and lipolysis, which is another way that energy can be mobilized (Xu et al., 2009). Because we only measured glucose, it is possible that CORT was primarily involved in controlling other metabolic processes such as lipolysis.

One finding in this study was that glucose levels did not vary between spring and autumn. The snakes were sampled in the spring as they emerged from hibernation and they are typically anorexic during this period. Snakes will typically not have eaten since the previous year and will not eat until they have completed mating (O'Donnell et al., 2004). Snakes returning to their hibernaculum in the autumn should have had more recent meals. However, because snakes are preparing to overwinter, it is possible that they are fasting to avoid having food items in their gut and/or prey items have become unavailable (Aleksiuk and Stewart, 1971). Webb et al. (2017) found that 15 days of food deprivation had no effect on glucose levels in another natricine, *Nerodia sipedon*. This is consistent with a review of fasting literature of snake physiology that reveals multiple strategies for coping with food deprivation (McCue et al., 2012). It is also unknown what responses we might have seen if we had sampled snakes during peak activity seasons (mid-summer) or while overwintering.

GC action is also modulated by differential binding to two receptor types. GCs have a higher affinity to mineralocorticoid receptors and

will saturate those receptors before binding to the lower-affinity GC receptors (de Kloet et al., 2008; Landys et al., 2006; Sapolsky, 2000). Because of these properties, mineralocorticoid receptors are often considered to regulate baseline energy mobilization, while GC receptors regulate the stress response (Busch and Hayward, 2009). Additionally, this study did not examine corticosteroid binding globulins, which are critical for transporting and controlling binding of CORT (Breuner et al., 2013). When considering glucose mobilization, it is important to recognize that glucose is likely regulated by CORT's differential affinity of the two receptor types.

We also expected a difference between sex and glucose levels because males and females have different energetic requirements during the mating and reproductive season. In a study conducted by Crews et al. (1987) with a species of the same genus, red-sided gartersnakes (*Thamnophis sirtalis parietalis*), there were differences in oxygen consumption and glucose concentration between males and females after emergence in the spring. However, this population is located on the extreme northern edge of any snake geographic range in Manitoba, Canada. Further, CORT levels were not measured in that study. A study analyzing differences in males and females during non-breeding time periods in birds did, however, demonstrate that there were no differences between male and female baseline or stress-induced glucose levels (Remage-Healey and Romero, 2000).

In our study, CORT concentrations were not different between spring and autumn, but males had consistently higher levels. Further, males had a dramatically increased CORT level after being subjected to the stressor as compared with females. Red-sided gartersnakes from the Manitoba population have also been examined in this context and have demonstrated a different pattern (Dayger and Lutterschmidt, 2016). Unlike in this northern population, in Utah, there was a higher CORT response in the spring versus autumn. This may be because these are different species, but could also be due to differences in time spent overwintering. We know that many of these traits can be highly specialized and plastic in gartersnakes and specific populations can demonstrate different life histories even when geographically close (Bronikowski and Arnold, 1999).

In the second (laboratory) experiment, only males were examined. Unexpectedly, we found that the ACTH treatment did not elevate CORT significantly above control levels. One possibility

is that this could be due to a rapid inhibition of CORT through negative feedback (Walker et al., 2015). If rapid inhibition of CORT was occurring, then we would expect elevated CORT prior to our 30 min sampling time in the ACTH group that was then suppressed by 30 min. However, based on other studies showing longer time courses for CORT release than 30 min in reptiles (Klukowski, 2011; Telemeco and Addis, 2014), and because our CORT group did not show any evidence of negative feedback inhibition, this seems unlikely in the present study. More likely, the porcine ACTH used did not specifically bind snake receptors well enough to exert a proper response above that of control (vehicle) injected animals, although it does effectively induce a response in amphibians (Neuman-Lee et al., 2015). Our other exogenous treatments (CORT, no injection and vehicle) elevated CORT in the bloodstream as expected, with levels in the CORT group being highest, followed by the vehicle control and ACTH groups, and finally no-injection controls. While META application did not completely block CORT secretion, the concentrations remained low in the bloodstream compared with those in the no-injection and vehicle groups. There was an increase in glucose over time, as seen in the *in vivo* study, but glucose was not related to CORT concentration. This may be because the exogenous CORT treatment could not elevate any response beyond a physiological limit, which was met by the restraint stress (Remage-Healey and Romero, 2001). It is possible that sampling at different time points could have allowed us to clarify a potential relationship.

Combining physiological measurements with ecological systems is clearly beneficial for understanding the health and status of individuals and populations. However, it is also apparent that these physiological mechanisms are highly complex and context dependent. As studies continue to incorporate stress state, energetics must be considered. This study allows us to further determine the role of the commonly used metric of CORT and its role in glucose regulation in a reptilian species. Future studies must consider the mechanisms behind these physiological responses to truly understand the ecological implications.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: L.A.N.-L., S.S.F.; Methodology: L.A.N.-L., A.C.W., S.S.F.; Validation: S.S.F.; Formal analysis: S.B.H.; Investigation: L.A.N.-L., A.C.W.; Resources: L.A.N.-L., S.S.F.; Data curation: L.A.N.-L., S.B.H.; Writing - original draft: L.A.N.-L., S.B.H., A.C.W.; Writing - review & editing: L.A.N.-L., S.B.H., A.C.W., S.S.F.; Visualization: L.A.N.-L., S.B.H., A.C.W.; Supervision: L.A.N.-L., S.S.F.; Project administration: L.A.N.-L., S.S.F.; Funding acquisition: L.A.N.-L., S.S.F.

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