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Assessment of glucocorticoids, sex steroids, and innate immunity in wild red-eared slider turtles (*Trachemys scripta elegans*)



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ABSTRACT

When access to resources is limited, organisms must shift energy investment among physiological processes to survive, reproduce, and respond to unpredictable events. The shifting of these limited resources among processes may result in physiological tradeoffs, often mediated by glucocorticoids. We assessed relationships among the physiological processes of immunity, reproduction, and the stress response in wild adult red-eared slider turtles (Trachemys scripta elegans). Red-eared sliders exhibit a multi-clutching reproductive strategy that requires high energetic investment in reproduction at the beginning of the nesting season in females. Males mate in spring and undergo spermatogenesis and mating in late summer/early fall. We expected to observe tradeoffs when investment toward reproductive processes was particularly demanding. To test this, we subjected 123 individuals to a standardized acute stressor and collected blood to measure innate immunocompetence and circulating steroid hormone concentrations. Tradeoffs between female reproduction and immunocompetence occurred early in the nesting season. This high reproductive investment was evident by heightened circulating progesterone and reduced baseline innate immunity. Corticosterone (CORT) was also high during this period, indicating a role in facilitating allocation of energy. Tradeoffs were not as evident in males, though males upregulated innate immune function, baseline CORT, and testosterone prior to fall spermatogenesis and mating. Throughout the entire sampling period, both males and females increased CORT and immunocompetence following the acute standardized stressor. Taken together, we concluded that reproduction requires shifts in energy allocation in during the highest reproductive period for females but all individuals in this population remain able to respond to the standardized stressor even during increased reproductive investment. These findings reinforce the continuing evidence that physiological relationships are context-dependent and resource demands are dynamic across the reproductive season.

1. Introduction

All wild organisms typically have limited access to resources (Stearns 1989; Zera and Harshman, 2001). Individuals must allocate energy toward basal processes, including the immune response (Zera and Harshman 2001; Schneider 2004; Lee et al., 2013). However, energetic investment toward basal physiological processes may shift to accommodate activities such as responding to unpredictable factors (i.e., the stress response) and predictable life history events (i.e., reproduction; Romero 2002; Nunes et al., 2006). When shifts in investment occur such that one process is compromised in favor of another due to limited energy, these patterns are considered physiological tradeoffs (van Noordwijk and de Jong 1986; de Jong 1993; Zera and Harshman 2001; Holden

et al., 2019) and are likely mediated by glucocorticoids (i.e., cortisol and corticosterone; CORT; Sapolsky et al., 2000; MacDougall-Shackleton et al., 2019). Since energetic investment in the immune system is costly (Lochmiller and Deerenberg, 2000), tradeoffs among immune function and reproduction are commonly observed across taxa (French et al., 2007; Kerr et al., 2010; Hegemann et al., 2013; Rafferty et al., 2014).

Reproduction requires extensive energetic investment and influences changes in an individual's physiological state. Reproductive investment includes courtship and mating efforts, gamete production, vitellogenesis, gestation/gravidity, birth/oviposition, and parental care and are associated with sex steroid hormones (Congdon and Gatten, 1989; Harshman and Zera, 2007; Hayward and Gillooly, 2011; Jones, 2011).

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In oviparous females, estradiol (E_2) progesterone (P_4), and testosterone (T) concentrations correlate with clutch development and oviposition, with P_4 highest during gravidity (Ho et al., 1982; Jones, 2011). In many species, including turtles, male T peaks in late spring and late summer/early fall, corresponding to spermatogenesis and mating (Callard et al., 1976; Licht et al., 1985; Currylow et al., 2013). Thus, these activities and corresponding sex steroid circulation vary by sex and do not come without energetic cost, with female reproductive processes considered more costly than male processes (Reeder et al., 2004; Hayward and Gillooly, 2011).

Glucocorticoids are constantly secreted by the hypothalamic–pituitaryadrenal axis, but individual circulating concentrations vary by context (i.e., circadian rhythms, seasonal variation; abiotic factors; Reeder et al., 2004; Romero et al., 2009; Baldan et al., 2021; Bókony et al., 2021; (Kolonin et al., 2022)). Considered energy mobilizing hormones, increased baseline glucocorticoid concentrations are associated with reproductive investment across taxa (Taylor et al., 2004; Nunes et al., 2005; Selman et al., 2012; Love et al., 2014). Measuring the baseline physiological state (i.e., sex steroid hormones, CORT, immunocompetence) provides critical information, but examining the stress response allows for a more thorough understanding of physiological patterns and overall condition (Neuman-Lee and French, 2017; Assis et al., 2019; Shidemantle et al., 2022).

To assess tradeoffs in organisms among basal processes (e.g., immune ability) and other energetically expensive processes (e.g., reproduction), measuring the stress response can prove useful. Often the overall health state of the animal can be inferred through its ability to invest energy in the stress response. A slough of physiological changes follows a stress event, including increased circulating glucocorticoids within minutes (Romero and Reed, 2005; Neuman-Lee et al., 2015) and upregulated innate immunity (Neuman-Lee and French, 2017; Assis et al., 2019). Within hours, negative feedback of glucocorticoids on the hypothalamus is expected to return the individual to a baseline state. The physiological changes from baseline state to stress state (and the recovery to baseline, if possible) allows for quantification of the magnitude of change in physiological metrics and provides a more complete scope than measuring one of these metrics or baseline physiology alone (Jessop et al., 2002; Romero and Wikelski 2010; Schoech et al., 2013). Shifts in magnitude of stress responsiveness (i.e., through innate immunity and glucocorticoids) with respect to expected energetic shifts (i.e., reproduction) provide insight toward how organisms physiologically accommodate changes in resource investment (Neuman-Lee and French, 2014; Neuman-Lee and French, 2017). Dysregulation (e.g., inability to return to a baseline state once the stressor is removed) or a complete lack of a response may indicate chronic stressors in the environment (Romero et al., 2009; Wingfield, 2013; Neuman-Lee et al., 2015).

In this study, we aimed to investigate metrics of reproduction, innate immunity, and the stress response to an acute stressor in wild adult redeared sliders to assess how individuals physiologically manage the cost of reproduction. We measured baseline circulating sex steroids (E2, P4, T) and the presence of eggs and developing follicles to assess reproductive investment. We also measured bacterial killing ability (BKA), an integrative functional innate immune metric, and circulating CORT concentrations at three time points (within three minutes [0], 30 min post-stress, 60 min post-stress). We predicted that investment in these physiological processes would change with respect to reproductive investment and individual condition (e.g., body condition and age via plastron length (Litzgus et al., 2008; Virgin, et al., 2022; Bronikowski et al., 2023). Increased reproductive investment, indicated by higher E₂ and P₄ concentrations in females and T concentrations in males, would coincide with decreased innate immune function and CORT response to acute stress, indicating tradeoffs were required. Overall, we predicted that CORT would increase following the acute stressor of capture (Cash et al., 1997; Cash and Holberton, 2005). We expected non-cellular BKA to increase because innate immune function is often prioritized

following an acute stressor (Dhabhar, 2014; Neuman-Lee et al., 2015; Neuman-Lee and French, 2017; Hudson et al., 2020). Examination of both baseline and stress-induced BKA and CORT with sex steroid concentrations allowed for investigation of physiology patterns throughout the major life history event of reproduction.

2. Methods

2.1. Study species

Red-eared slider turtles (*Trachemys scripta elegans*) have a robust innate immune system, exhibit sexual dimorphism, and females can develop multiple clutches within a given reproductive season (Trauth et al., 2004; Zimmerman et al., 2010b). Red-eared sliders exhibit indeterminate growth, therefore shell size, measured by the length of the lower shell (plastron length) can be a proxy for age (Trauth et al., 2004).

2.2. Capture and sampling

We captured red-eared sliders (Trachemys scripta elegans) using baited hoop traps, box traps, and by hand between May-September 2019 at Bearitage Farms (35.44105°, -90.80491°) in Cross County, Arkansas, USA. We placed nets in various water bodies on the property accessible by wading or boat and checked nets twice daily, once in the morning (0800-1100 h) and once in the late afternoon (1500-1800 h). We recorded the time of removal from net or capture, as capture and restraint stimulate the acute stress response (Cash et al., 1997; Romero and Reed, 2005). Cash et al. (1997) found that capture in nets does not elicit a HPA response based on low CORTO concentrations from baseline blood samples following removal from nets. We used a 1-mL, 26-gauge need to collect blood (~0.5 mL) from the caudal vein within 0-3, 30, and 60 min of removal (hereafter referred to as "bleed time") from the net or capture. Blood sampled at 0-3 min represents baseline levels whereas blood sampled at 30 and 60 min represent stress-induced levels (Romero and Reed, 2005; Sheriff et al., 2011; Tylan et al., 2020). We stored blood in labeled 1.5-mL microcentrifuge tubes on ice until processing (approx. 1–3 h) and then centrifuged samples. We stored frozen plasma at -20 °C at the field site and $-80\ ^\circ C$ at the Arkansas State University-Jonesboro campus until analysis. Following blood sampling, we kept animals for further physical assessments and measurements (described below).

This project was approved (IRB#: FY17-18–76) by the Arkansas State University Institutional Animal Care Use Committee. An Arkansas scientific collection permit (100320181) from Arkansas Game and Fish Commission was obtained prior to sampling.

2.3. Reproductive assessments

We assessed all adult females for ovarian status using a portable ultrasound and MC6-A micro convex transducer (ECO3, CHISON USA Inc, Bellevue, WA), using water as a conductor fluid. We recorded images from the left and right prefemoral acoustic windows of the animal. We categorized turtles as "gravid" if shelled eggs were present and "nongravid" if no shelled eggs were present. We also noted if vitellogenic follicles, characterized by large or medium follicles were present (Shelby et al., 2000). Shelled eggs can be distinguished from vitellogenic follicles because of a thin, white oval casing (shell) surrounding a gray spherical shape (yolk; Rostal et al., 1990).

2.4. Morphometrics

We measured carapace and plastron lengths and widths (curved and straight; cm), recorded mass (g), and determined sex (male, female, unknown – juveniles) by the presence of secondary sex characteristics in adults. We used straight plastron length (PL) in analyses as a proxy for age and to represent body size because red-eared sliders exhibit indeterminate growth (Schwanz et al., 2011; Zimmerman et al., 2010a). We

marked novel animals with a unique code using a file or drill bit along the marginal scutes for future identification (Nagle et al., 2017). We released animals at their original capture location.

2.5. Body condition

Internal energy sources are stored in lipid form and are thought to be used for mostly reproduction (Price 2017). We calculated a body condition index (BCI), an estimate of energy reserves, by using the residuals from the simple linear regression of straight carapace length and the cube root of body mass (Cash et al., 1997; Schulte-Hostedde et al., 2005; Newman et al., 2019). We analyzed by sex because clutch development bias female measurements, and many females showed evidence of stages of clutch development between May and July (Litzgus et al., 2008).

2.6. Bacterial killing assays

We conducted bacterial killing assays using a modified protocol to assess bacterial killing ability (BKA) by non-cellular components of the innate immune function: natural antibodies, antimicrobial peptides, and complement following French and Neuman-Lee (2012). We used 5 μ l thawed plasma and 6 μ l of *Escherichia coli* (ATCC 8739; Microbiologics) in 1X PBS (Concentration: 1.0×10^5 colony forming units) after validating with thawed plasma to attain roughly 50 % killing. We calculated percentage killing (BKA) using the change in absorbance (read at 340 nm using BioTek Synergy2; ELx808) between readings and 100 % growth of the positive wells (six replicates per plate). We also included negative controls (six replicates with media, no bacteria) to ensure no background contamination. We ran assays within seven months of collection and all samples thawed twice prior to use. We adjusted all values below 0 or above 100 percentage killing to 0 and 100, respectively.

2.7. Hormone radioimmunoassays

We conducted radioimmunoassays (RIAs), in duplicate, using a modified protocol to analyze CORT concentrations (Ab: MP Biomedicals, Cat: 07–120016, Lot 3R3-PB17; H³-labeled CORT: Perkin Elmer, NET300250UC), the primary GC in reptiles, E₂ (Ab: BioRad Cat: 7010–2650, Lot: 0113; H³-labeled E₂: Perkin Elmer, NET517250UC), P₄ (Ab: Fitzgerald, Cat: 20R-PR053W, Lot: 01,719 A-Z; H³-labeled P₄: Perkin Elmer, NET381250UC, and T (Ab: Fitzgerald. Cat: 20R-TR018W, Lot: 01916; H³-labeled T: Perkin Elmer, NET370250UC; Moore, 1986; Neuman-Lee and French 2017). We used individual recovery values to correct final sample concentration for each sample. CORT was analyzed from samples across bleed time and sex steroids were analyzed from baseline samples. For all CORT assays, intra-assay coefficient of variation ranged 11.0–14.3 % and inter-assay coefficient of variation was 14.5 %. Intra-assay coefficient of variation for E₂ (female assay) was 9.7 %, P₄ (female assay) was 12.7 %, and T was 8.8 %.

2.8. Statistical analyses

We conducted all analyses in statistical program R (R Core Team, 2018). We excluded all recaptures and juveniles from analyses because there was not a high enough sample size to draw meaningful conclusions. We combined data from September and October 2019 (post-reproductive months) for a more robust sample size. Then, we included all individuals in analysis as they are representative of the variation in natural populations. All means are reported with \pm standard error.

We calculated the magnitude of change between BKA or CORT concentration between each bleed time ($\Delta 0$ -30, $\Delta 0$ -60, $\Delta 30$ -60). All response variables except CORT60 and Δ CORT variables by sample were not normally distributed despite transformation efforts; thus, we proceeded with non-parametric tests. Cube root-transforming CORT60 and Δ CORT variables allowed for parametric tests. We validated models by

assessing diagnostic plots for normality of residuals and influence of outliers.

We conducted Spearman correlations between BKA, hormone concentrations, PL, and BCI variables for each sex and interpreted at a significance level of $\alpha = 0.05$ prior to further analyses. We also assessed if gravidity varied by month using a two-sided Fisher's exact test. All analyses were conducted by sex, except for Δ CORT and Δ BKA, in which we also conducted Wilcoxon Rank Sum tests to assess differences in the stress response between sexes by month.

We conducted Kruskal-Wallis tests and post-hoc Dunn's tests with Bonferroni correction (package 'dunn.test'; Dinno, 2017) to assess if BCI, CORT0, CORT30, and sex steroids varied by month and if CORT or BKA varies across bleed time within month. Due to the binary nature of the BKA data, we transformed all 0 s to 0.01 and 100 s to 99 before transforming to decimals on a 0–1 scale to construct beta regression models (package 'betareg'; Cribari-Neto and Zeileis 2010) to assess BKA0, BKA30, and BKA60. We used package 'emmeans' to compare months post-hoc (package 'emmeans'; Lenth et al., 2022). For all other variables (cube root transformed-CORT60, cube root transformed- Δ CORTs, and Δ BKAs), we conducted ANOVAs with a post-hoc Bonferroni correction.

To assess if changes in CORT, sex steroid, or BKA across the active period is influenced by age, body condition, or gravidity, we constructed models examining the significance of the interaction of month and age, body condition, and gravidity variables. For CORT0, CORT30, and sex steroid response variables, we constructed generalized linear models with a Gamma distribution with log link. For BKA0, BKA30, and BKA60 variables, we constructed beta-regression models with logit link. For cube-root transformed-CORT60, cube root transformed- Δ CORT variables, and Δ BKA variables, we constructed linear models. We conducted all linear and generalized linear models with a Bonferroni correction. We conducted beta-regression models with a p-value adjustment using function (function glht; package 'multcomp'; Hothorn, 2021). We deemed interactions meaningful based on adjusted p-values of interaction effects.

3. Results

3.1. Sample collection

We captured 123 turtles over the sampling period in which at least the baseline blood sample was collected. We omitted September/ October from male analyses due to low (n = 1) sample size. Because sample sizes varied based on ability to get blood samples in subsequent bleeds or plasma volume availability, specific sample sizes are indicated for each metric below.

3.2. Females

There was a significant association between gravid status and month (P = 0.001; Table 1). Shelled eggs were present May–July (Fig. 1). No shelled eggs were detected in individuals August–October. Vitellogenic follicles were detected in May and June, indicating that eggs detected in

Table 1

Gravidity status (gravid and nongravid) and vitellogenic follicle presence by month. We considered females gravid if shelled eggs were present on the sonogram during reproductive assessments. Females are reproductively active May–July. We considered August, September, and October post-reproductive periods, because of the lack of gravid individuals.

| | May | June | July | August | Sept/Oct |
|-----------------------------|-----|------|------|--------|----------|
| Gravid | 21 | 15 | 2 | 0 | 0 |
| With Vitellogenic Follicles | 21 | 15 | 0 | 0 | 0 |
| Nongravid | 12 | 12 | 6 | 6 | 6 |
| With Vitellogenic Follicles | 12 | 10 | 0 | 0 | 0 |



Fig. 1. Ultrasound imaging allowed for female reproductive assessment across sampling months. Arrows point to representative reproductive structures noted during assessments. A) Eggs were characterized by a solid thin white elliptical structure (shell) surrounding a smaller gray mass (yolk). B) We observed both shelled eggs (1) and developing vitellogenic follicles (2), characterized by a circular gray mass with defined boundaries, in multi-clutching individuals in May and June 2019. C) Atretic (regressing) follicles, characterized by a circular gray mass with defined boundaries and internal striations, were detected throughout sampling months of varying sizes. D) While small (non-vitellogenic) follicles could be detected throughout sampling months, the presence of small follicles and absence of eggs/vitellogenic follicles were more apparent in July–October, once nesting had concluded for the year.

July are the final clutch of the nesting season (Table 1).

Female body condition significantly shifted across months (Kruskal-Wallis $\chi_3^2 = 13.9$, P < 0.001; Supplementary Table 1). However, there were no significant pairwise comparisons when a conservative post-hoc Dunn test with Bonferroni correction was conducted (all pairwise P > 0.05).

There were significant negative correlations between female CORT30 and E₂ ($\rho = -0.277$, P = 0.027), $\Delta 0$ -30 CORT and E₂ ($\rho = -0.311$, P = 0.013), E₂ and PL ($\rho = -0.256$, P = 0.020), and T and P₄ ($\rho = -0.285$, P = 0.032). There was a significant positive correlation between CORT0 and P₄ ($\rho = 0.406$, P < 0.001). There were no significant correlations between any other combination of female CORT, sex steroid, BKA, or BCI variables (P > 0.050).

Female P₄ was significantly different across months ($\chi^2_4 = 23.8$, P < 0.001), with May P₄ higher than July (Z = 3.07, P = 0.011), August (Z = 2.98, P = 0.014), and Sept/Oct (Z = 4.26, P < 0.001; Fig. 2). Female E₂ did not significantly differ across months ($\chi^2_4 = 5.189$, P = 0.268; Table 2). Female T did not significantly differ across months ($\chi^2_4 = 1.94$, P = 0.747).

Female CORT0 significantly differed across months ($\chi_4^2 = 19.5$, P < 0.001; Table 3), with CORT0 higher in May than June (Z = 3.44, P = 0.003) and Sept/Oct (Z = 3.53, P = 0.002; Fig. 2). CORT30 ($\chi_4^2 = 0.878$, P = 0.928), CORT60 (F_(4,56) = 1.21, P = 0.317), and Δ CORT variables did not significantly differ across months (all P > 0.05; Fig. 3). There was a significant interaction between PL and CORT60 (P = 0.032) in which female CORT60 in Sept/Oct was lower with longer PL. All other interactions of month and PL, gravidity or BCI were not meaningful in CORT or sex steroid concentrations (all P > 0.050).

Female CORT in response to the acute stressor significantly differed across bleed time (0, 30, 60 min) within May (χ^2_2 = 16.2, P < 0.001), June (χ^2_2 = 34.3, P < 0.001), July (χ^2_2 = 11.3, P = 0.004), and August (χ^2_2 = 21.6, P < 0.001), but not in Sept/Oct (χ^2_2 = 4.27, P = 0.118; Fig. 3). Female CORT significantly increased between 0 and 30 min in May (Z = -3.29, P = 0.002), June (Z = -4.17, P < 0.001), July (Z = -2.84, P =



Fig. 2. A) Female red-eared slider (*Trachemys scripta elegans*) baseline corticosterone (CORT0) and progesterone (P_4) concentrations (ng/mL) across sampling months, May (CORT0: n = 19; P_4 : n = 19), June (n = 25), July (n = 8), August (n = 12), and Sept/Oct (n = 6). The gray box signifies the expected nesting period for multi-clutching females. Both CORT0 and P_4 were highest in May and decrease across months into the post-reproductive period. B) Male red-eared slider CORT0 and testosterone (T) concentrations across sampling months, May (CORT0: n = 17; T: n = 16), June (CORT0: n = 10; T = 9), July (n = 11), and August (n = 9). Testosterone is highest in August and CORT0 slightly increases from prior months. The gray box signifies the expected timing of spermatogenesis. All means include standard error bars. The increase in T concentration and slight increase in CORT by August suggests that males invest in spermatogenesis and fall mating effort in the late summer. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2

Mean \pm standard error sex steroid concentration (ng/mL) by sex and month in red-eared slider turtles (*Trachemys scripta elegans*), with sample sizes (n). Hormones were assessed using blood samples collected within 3 min of the standardized stressor.

| | Mean ± SE | | | | | |
|--------|----------------|---------------------|---------------------|---------------------|--|---------------------|
| | n | May | June | July | Aug | Sept/ Oct |
| Female | E ₂ | 0.361 ± 0.023 | 0.327 ± 0.043 | 0.297 ± 0.039 | $\begin{array}{c} 0.382 \pm \\ 0.072 \end{array}$ | 0.32 ± 0.023 |
| | | 22 | 26 | 8 | 12 | 6 |
| | Т | 0.103 + | 0.257 + | 1.12 ± 1.056 | 0.063 ± 0.001 | 0.172 |
| | | 0.038 | 0.194 | 1.050 | 0.001 | 0.108 |
| | | 14 | 21 | 7 | 10 | 6 |
| | P ₄ | 0.954 ± 0.065 | 0.694 ± 0.081 | 0.463 ± 0.080 | $\begin{array}{c} \textbf{0.53} \pm \\ \textbf{0.080} \end{array}$ | 0.354 ± 0.018 |
| | | 19 | 25 | 8 | 12 | 6 |
| Male | т | 0.411 | 3.894 | 5.405 | 17.201 | |
| | | $^\pm$ 0.287 | $^\pm$ 2.208 | ± 4.3 | ± 7.584 | |
| | | 16 | 9 | 11 | 9 | |

0.007) and August (Z = -2.77, P = 0.008). Female CORT also significantly increased between 0 and 60 min in May (Z = -3.48, P < 0.001), June (Z = -5.59, P < 0.001, July (Z = -2.95, P = 0.005), and August (Z = 4.61, P < 0.001). CORT did not differ between 0 and 30 or 0 and 60 in Sept/Oct (χ^2_2 = 4.27, P = 0.118). Female CORT did not differ between 30 and 60 min (all P > 0.050) within any sampling month.

 Δ 0–30 BKA was significantly different (F_(4,61) = 3.70, P = 0.009), with magnitude of change in June lower than in August (P = 0.014; Table 4). BKA0 (beta regression log-likelihood₆ = 83.6, all P > 0.152), BKA30 (beta regression log-likelihood₆ = 80.5, P = 0.157), BKA60 (beta regression log-likelihood₆ = 79.2, P = 0.541), Δ 0–60 BKA (F_(4,57) = 1.34, P = 0.266), and Δ 30–60 BKA (F_(4,54) = 0.187, P = 0.944) did not differ across months (Fig. 3). Interactions between month and PL, gravidity, or BCI were not meaningful in BKA response (all P > 0.05).

Female BKA significantly differed across bleed time in June (χ^2_2 = 19.0, P < 0.001), in which BKA significantly increased between 0 and 30 min (Z = -4.19, P < 0.001) and 0 and 60 min (Z = -3.14, P = 0.003), but not between 30 and 60 min (Z = 1.00, P = 0.473; Fig. 3). However, BKA did not significantly differ across bleed time in May (χ^2_2 = 4.57, P = 0.102), July (χ^2_2 = 0.069, P = 0.966), August (χ^2_2 = 0.129, P = 0.938), or September/October (χ^2_2 = 2.00, P = 0.368).

3.3. Males

Male body condition did not significantly change by month (χ_3^2 = 5.004, P = 0.172; Supplementary Table 1). Male T significantly differed across months (χ_3^2 = 19.2, P < 0.001; Table 2), with May T lower than August T (χ_3^2 = -4.29, P < 0.001; Fig. 2).

There were significant positive correlations between male CORT0 and BKA0 ($\rho = 0.323$, P = 0.028), CORT30 and $\Delta 30$ –60 BKA ($\rho = 0.382$, P = 0.019), BKA0 and PL ($\rho = 0.363$, P = 0.011), CORT0 and T ($\rho = 0.333$, P = 0.029), BKA0 and T ($\rho = 0.359$, P = 0.017), and BKA60 and T ($\rho = 0.383$, P = 0.018). There were significant negative correlations between male CORT0 and $\Delta 0$ –30 BKA ($\rho = -0.348$, P = 0.022), CORT0 and $\Delta 0$ –60 BKA ($\rho = -0.461$, P = 0.004), and $\Delta 0$ –30 BKA and T ($\rho = -0.348$, P = 0.026). There were no significant correlations between any other combination of male CORT, T, BKA, and BCI variables (P > 0.050).

All male CORT and Δ CORT variables did not differ across months (all P > 0.050; Figs. 2–3; Table 3). Interactions between month and PL or BCI

Table 3

Mean \pm standard error corticosterone concentration (ng/mL) by sex and month in red-eared slider turtles (*Trachemys scripta elegans*), with sample sizes (n). Blood samples were collected within 3 min of the standardized stressor for baseline (0) and 30 and 60 min following stress event.

| Corticosterone (ng/mL) by Month | | | | | | |
|---------------------------------|------------|------------|-------------|----------------------------|------------|--|
| Mean ± SE | May | June | July | Aug | Sept/Oct | |
| n | | | | | | |
| Females | | | | | | |
| 0 | $1.22~\pm$ | 0.50 \pm | $0.56 \pm$ | $0.68 \pm$ | $0.30 \pm$ | |
| | 0.24 | 0.06 | 0.16 | 0.12 | 0.01 | |
| | 23 | 25 | 8 | 12 | 6 | |
| 30 | $2.47 \pm$ | $2.70 \pm$ | $4.00 \pm$ | $2.43 \pm$ | $2.70 \pm$ | |
| | 0.36 | 0.45 | 0.72 | 0.69 | 1.45 | |
| | 18 | 23 | 8 | 12 | 6 | |
| 60 | 2.84 + | 3.78 + | 3.28 + | 4.47 + | 3.45 + | |
| | 0.45 | 0.48 | 0.78 | 0.67 | 1.45 | |
| | 15 | 21 | 7 | 12 | 6 | |
| A0-30 | 1.29 + | 2.08 + | , 3.45 + | 1.75 + | 2.40 + | |
| | 0.36 | 0.41 | 1 47 | 0.50 | 1 40 | |
| | 16 | 22 | 8 | 12 | 6 | |
| A0-60 | 1.80 + | 3 28 + | 2 68 + | 3 79 + | 315+ | |
| 10 00 | 0.43 | 0.49 | 0.77 | 0.69 | 1.45 | |
| | 13 | 20 | 7 | 12 | 6 | |
| 120 60 | 1.5 | 20 | 0.18 | 2.04 | 0.75 | |
| <u>430-00</u> | 0.98 ± | 0.90 ± | 0.18 ± | $2.04 \pm$ | 0.73 ± | |
| | 0.30 | 0.58 | 1.20 | 0.64 | 0.42 | |
| | 12 | 19 | / | 12 | 0 | |
| Males | | | | | | |
| 0 | $1.82 \pm$ | $1.53 \pm$ | $1.84 \pm$ | $4.08 \pm$ | | |
| | 0.35 | 0.43 | 0.64 | 1.13 | | |
| | 17 | 10 | 11 | 9 | | |
| 30 | 6.76 + | 5.47 + | 6.52 + | 8.06 + | | |
| | 1.22 | 1.65 | 0.96 | 1.67 | | |
| | 19 | 10 | 10 | 10 | | |
| 60 | 5.08 + | 6.34 + | 8.08 + | 7.16 + | | |
| | 0.77 | 1.03 | 1 39 | 1 42 | | |
| | 13 | 9 | 9 | 8 | | |
| A0-30 | 4 69 + | 3 94 + | 4 52 + | 2 47 + | | |
| 20-30 | 1.07 ± | 1.68 | 4.52 ± | 2. 1 /⊥ 1.35 | | |
| | 1.51 | 1.00 | 10 | 1.55 | | |
| 10 60 | 2 50 1 | 10 E 10 | 10 6 EE | 9 17 1 | | |
| 40-00 | 3.39 ± | $5.10 \pm$ | 0.35 ± | $3.17 \pm$ | | |
| | 0.75 | 0.91 | 1.30 | 2.08 | | |
| 100 (0 | 11 | 9 | 9 | 8 | | |
| Δ30-60 | 1.18 ± | $0.95 \pm$ | 2.89 ± | $0.44 \pm$ | | |
| | 0.46 | 1.55 | 1.53 | 1.13 | | |
| | 12 | 9 | 8 | 8 | | |

were not meaningful in CORT response (all P > 0.050).

Male CORT significantly differed across bleed time in May (χ^2_2 = 19.8, P < 0.001), June (χ^2_2 = 11.2, P = 0.004), and July (χ^2_2 = 15.1, P = 0.001), but not August (χ^2_2 = 5.13, P = 0.077; Fig. 2). Male CORT increased between 0 and 30 min in May (Z = -4.21, P < 0.001), and July (Z = -2.92, P = 0.005). Male CORT also significantly increased between 0 and 60 min in May (Z = -3.27, P = 0.002), June (Z = -3.27, P = 0.002), and July (Z = -3.62, P < 0.001). However, male CORT did not differ between 30 and 60 min (all P > 0.050) within any sampling month.

BKA0 significantly differed across months, with mean May BKA0 lower than August BKA0 (beta regression log-likelihood₅ = 44.7, P = 0.029; Fig. 3; Table 4). BKA30 (beta regression log-likelihood₅ = 46.5, all P = 0.602), BKA60 (beta regression log-likelihood₅ = 40.6, P = 0.726), and all Δ BKA variables (P > 0.050) did not differ across months. Interactions between month and PL or BCI were not meaningful in BKA response (all P > 0.050).

Male BKA did not significantly differ across bleed time within any sampling month (all P > 0.05; Fig. 3).

3.4. Sex comparisons of stress response

Female $\Delta 0$ -30 CORT was significantly lower than male $\Delta 0$ -30 CORT





Fig. 3. Female red-eared slider (*Trachemys scripta elegans*) A) $\Delta 0$ -30 corticosterone concentration (ng/mL; CORT) and B) bacterial killing ability (%; BKA) across sampling months, May (n = 16), June (CORT: n = 24; BKA: n = 22), July (n = 8), August (n = 12), and September/October (n = 6). Male red-eared slider C) $\Delta 0$ -30 CORT and D) BKA) across sampling months, May (CORT: n = 16; BKA: n = 15), June (CORT: n = 10; BKA: n = 9), July (CORT: n = 10; BKA: n = 11), and August (CORT: n = 9, BKA: n = 10). Blood samples were taken within 3 min of standardized stressor and 30 and 60 min following stress event. All means include standard error bars. The gray boxes signify the expected nesting period and expected timing of spermatogenesis for females and males, respectively.

in May (W = 56, P = 0.006), but differences in Δ 0–30 CORT in June (W = 102, P = 764), July (W = 25, P = 0.203), and August (W = 43, P = 0.464) were not significant. Female Δ 0–60 CORT was also significantly lower than male Δ 0–60 CORT in July (W = 7, P = 0.008), but not in May (W = 39, P = 0.063), June (W = 56, P = 0.116), or August (W = 45, P = 0.851). There were no significant differences in Δ 0–30 BKA or in Δ 0–60 BKA between sexes within any months (all P > 0.050), with the exception of July Δ 0–60 BKA in which females have a significantly lower change in BKA than males (W = 11.5, P = 0.038).

4. Discussion

There are disparities between the sexes in terms of reproductive investment and timing of processes (mating, spermatogenesis, oocyte development, oviposition; Callard et al., 1976; Ho et al., 1982; Licht et al., 1985; Shelby et al., 2000). Thus, we predicted that male red-eared

sliders invest less energy into reproduction across the activity season relative to females and that the expected heightened periods of energy expenditure toward reproduction would coincide with increased baseline glucocorticoid and sex steroid circulation. Female red-eared sliders had higher concentrations of P_4 and basal concentration of CORT (CORT0) in May, the beginning of the nesting period. Male red-eared sliders upregulated T circulation in August, which reflects expected timing of fall spermatogenesis (Callard et al., 1976; Licht et al., 1985). We also observed positive associations between T, baseline CORT, and baseline BKA, indicating that males may upregulate these processes in advance of spermatogenesis and mating. Finally, though we predicted that an increase in reproductive investment would correlate to a lowered stress response, both male and female adults maintained the ability to respond to an acute stress event, indicating that this population is likely not energetically challenged by chronic stressors or resource limitation in their environment.

Table 4

Mean \pm standard error bacterial killing ability (%) by sex and month in redeared slider turtles (*Trachemys scripta elegans*), with sample sizes (n). Blood samples were collected within 3 min of the standardized stressor for baseline (0) and 30 and 60 min following stress event. Bacterial killing ability results indicate immune function by non-cellular immune components.

| Bacterial Killing Ability (%) by Month | | | | | | | |
|--|-------------|-------------|-------------|-------------|--------------|--|--|
| Mean ± SE | Мау | June | July | Aug | Sept/ Oct | | |
| n | | | | | | | |
| Females | | | | | | | |
| 0 | 39.8 \pm | $24.9 \pm$ | 56.9 \pm | 76.0 \pm | 84.6 \pm | | |
| | 8.40 | 7.30 | 15.7 | 12.6 | 15.3 | | |
| | 23 | 25 | 8 | 12 | 6 | | |
| 30 | 70.3 \pm | 74.3 \pm | 52.6 \pm | 69.9 \pm | 100 ± 0 | | |
| | 9.70 | 7.90 | 17.9 | 13.0 | | | |
| | 18 | 25 | 8 | 12 | 6 | | |
| 60 | 54.8 \pm | 62.2 \pm | 57.2 \pm | 70.7 \pm | 100 ± 0 | | |
| | 11.8 | 9.40 | 19.20 | 12.50 | | | |
| | 15 | 24 | 7 | 12 | 6 | | |
| Δ0-30 | 15.7 \pm | 50.5 \pm | $-4.28~\pm$ | $-6.10~\pm$ | $15.3 \pm$ | | |
| | 12.1 | 10.3 | 20.0 | 13.0 | 15.3 | | |
| | 16 | 24 | 8 | 12 | 6 | | |
| Δ060 | 17.7 \pm | 33.9 \pm | $1.00 \pm$ | $-5.26 \pm$ | 15.3 \pm | | |
| | 16.8 | 10.0 | 4.20 | 17.8 | 15.3 | | |
| | 14 | 23 | 7 | 12 | 6 | | |
| Δ30-60 | $-4.80~\pm$ | $-12.2~\pm$ | $-1.10~\pm$ | $0.80~\pm$ | 0 ± 0 | | |
| | 13.7 | 9.30 | 21.6 | 19.0 | | | |
| | 11 | 23 | 7 | 12 | 6 | | |
| Males | | | | | | | |
| 0 | 20.2 + | 55 5 ± | 425+ | 87 0 ± | | | |
| 0 | 20.2 ± | 15 0 | $13.0 \pm$ | 10.1 | | | |
| | 17 | 10.0 | 13.0 | 10.1 | | | |
| 30 | 55.5 + | 71.0 + | 61.6 + | 85.6 + | | | |
| 00 | 10.9 | 14.9 | 14.2 | 9.3 | | | |
| | 18 | 9 | 11 | 10 | | | |
| 60 | 55.6 + | 67.1 + | 58.3 + | 79.5 + | | | |
| | 12.2 | 14.1 | 16.5 | 13.5 | | | |
| | 15 | 9 | 9 | 8 | | | |
| Δ0-30 | 38.3 + | 20.3 + | 19.1 + | -1.40 + | | | |
| | 12.0 | 19.8 | 17.5 | 15.0 | | | |
| | 15 | 9 | 11 | 10 | | | |
| Δ0-60 | 38.9 ± | 5.60 \pm | $28.6~\pm$ | $-4.20 \pm$ | | | |
| | 13.4 | 17.6 | 18.8 | 18.4 | | | |
| | 13 | 9 | 9 | 8 | | | |
| Δ30-60 | $-3.00 \pm$ | 7.30 \pm | 5.20 \pm | $-13.6 \pm$ | | | |
| | 8.60 | 19.0 | 17.0 | 15.0 | | | |
| | 13 | 8 | 9 | 8 | | | |

Females laid eggs in May, June, and July and thus reproductive processes related to clutch development, gravidity, and nesting concluded by mid-July. Females captured multiple times throughout the sampling period were observed with multiple clutches through the detection of shelled eggs and larger, vitellogenic follicles in May and June or the presence of shelled clutches a month apart. Few females were gravid in July, and eggs were not present in August, September, or October. Female P₄ concentrations were highest in May and June, which corresponds with the expected role of P₄ with maintaining gravidity (Taylor et al., 2004). We observed the highest circulating baseline CORT (CORT0) in May and a positive correlation with CORT0 and P₄, which suggests that females of this population may invest most energy toward reproductive activities in May (Shelby et al., 2000; Reeder et al., 2004; Love et al., 2014). This is likely due to the simultaneous development of all clutches (at different stages) to be laid within the nesting season as vitellogenesis and shelling requires high energy investment (Webb et al., 2019). We did not observe significant changes in E₂ or T across months. This is different from other studies in which E2 and T shifted during reproduction across taxa (Shelby et al., 2000; Taylor et al., 2004; reviewed in Szewjser et al., 2017). However, there may be a finer-scale difference of E2 investment and offspring investment in follicle/egg size

and clutch size in this population (Shelby et al., 2000). Females also were able to mount an increase in CORT following an acute stressor, but the magnitude varied by month. For example, the lower Δ 0–30 CORT in females compared with male counterparts Δ 0–30 CORT in May suggests the reproductive processes are prioritized over the stress response. We also predicted that female innate immunity would be compromised during reproduction, but we observed no significant differences across months except for the magnitude of change between 0 and 30 min. There were negative correlations between E2 and CORT variables, suggesting that E2 investment may be related to the ability to mount a glucocorticoid response during an acute stress event. Cartledge and Jones (2007) similarly observed that post-ovulatory female skinks (Egernia whitii) exhibited a lower CORT response following a stressor. Female body condition did not significantly shift across months, though a slight, though non-significant decrease in turtle body condition between May–July was observed. This suggests that female reserves may be compromised during clutch development and nesting,

By the post-reproductive portion of the active period, September and October, females likely regained energy stores from foraging and were not allocating as much energy toward a specific process (e.g., reproduction, foraging effort). Our findings are supported by a study that showed that female green sea turtles (*Chelonia mydas*) also had elevated baseline CORT concentrations related to follicular development and decreased baseline CORT concentrations in the post-reproductive period (Hamann et al., 2002). In our study, the relationship between baseline CORT and energy mobilization is further supported by the slightly higher average body condition in September and October.

Shifts in baseline BKA across months indicated when heightened energy expenditure in females occurred. Baseline BKA in June was lower than May (though not statistically significant), which may indicate that following heightened reproductive process prioritization, females may have less energy in June to mount heightened baseline immunity. We also observed a significantly lower $\Delta 0$ –30 BKA in June, suggesting that tradeoffs in innate immunity following an acute stressor occur to accommodate clutch development. Tradeoffs between female reproduction and immune function have been observed in tree lizards (*Urosaurus ornatus*; French et al., 2007), fish (reviewed in Szwejser et al., 2017), and other taxa (reviewed in Martin et al., 2008).

Within males, we predicted increased investment in T and baseline CORT would coincide with mating and spermatogenesis. However, male baseline CORT was not significantly different across months, although we did observe a slight increase in mean concentrations in August and we were unable to capture enough males in September/October (Lance et al., 2001). We also observed increased T in August, which suggests that males begin to mount energy toward spermatogenesis and fall mating effort during this time. Increasing T in summer and fall has been observed in yellow-blotched map turtles (*Graptemys flavimaculata;* late July with peaks in September/October; Shelby et al., 2000), male desert tortoises (*Gopherus agassizi;* July with peak in August; Lance et al., 2001), painted turtles (*Chrysemys picta;* August with peak in December; Callard et al., 1976), and Western Diamond-backed rattlesnakes (July with peak in August; *Crotalus atrox;* Taylor et al., 2004).

The differences in baseline immunocompentence in males (BKA0) across months suggest that energy investment in immunity is linked to reproductive effort. Exhausted from spring mating efforts, males may not be able to invest in baseline innate immunity in May, suggesting that male reproductive processes, like mating effort, are not energetically negligible (Friesen et al., 2015). Low baseline BKA in May relative to August suggests that spring mating may negatively impact baseline innate immune function, resulting in a tradeoff, until energy stores can be replenished, and males can mount energy toward a more robust baseline innate immunity. Increased baseline innate immunity in August suggests that males may prioritize heightened baseline innate immunity during initial mating efforts, and it is not compromised by spermatogenesis. Baseline BKA was high and remains high following an acute stressor, which suggests that tradeoffs between processes relating to

spermatogenesis and non-cellular circulating innate immune function do not occur. This is in contrast with studies relating to male reproductive activities, testosterone, and immune suppression (e.g., Kerr et al., 2010; Corlatti et al., 2012; Neuman-Lee and French, 2017). Previous studies have found that physiological tradeoffs may be component specific and differences in patterns observed may be limited to the metrics used or year-specific conditions (Buehler et al., 2009; French et al., 2017; Neuman-Lee et al., 2019).

While our study provides baseline information on investment toward immune function and stress response across the reproductive period of red-eared sliders, limitations exist. First, we did not observe the peak and subsequent decline of glucocorticoids following a stress event. Though the authors noted that the greatest increase in CORT was between baseline and 30 min, Cash et al. (1997) also observed a continued increase in CORT after 30 and 60 min and CORT responsiveness varies by population (Polich, 2016). Second, we found minimal interactions between individual characteristics (age via PL, BCI, gravidity). This may be due to limitations due to small sample sizes within months especially in the late summer and fall, lack of analysis of juveniles, and resolution of sonogram analysis. From correlation analysis of CORT60 and PL in females, there is a potential negative relationship between age and stress-induced CORT. Older females may invest less energy toward the glucocorticoid response than younger females. Third, the sole innate immune metric, BKA, used frozen-thawed plasma against one pathogen, E. coli. Though use of frozen-thawed plasma and E. coli is commonplace in the literature as a measure of integrative innate immune function (Nebel et al., 2013; Schneeberger et al., 2014; Neuman-Lee and French, 2017; Assis, et al., 2019; Caracalas et al., 2021), we acknowledge that freezing kills cellular components and freeze-thaw cycles or freeze duration may degrade protein components (Jacobs and Fair, 2016, but see Beck et al., 2017). However, all samples were treated similarly and were frozen for the same approximate amount of time. Last, this study is limited to one sampling year. Changes in resources, weather patterns, and other challenges such as emergent pathogens may reveal different physiological patterns across years (French et al., 2017; Spence et al., 2020). Further analysis is required to identify the scope of immune-endocrine interactions across the entire stress response in red-eared sliders.

5. Conclusion

This study provides baseline information on reproductive timing and endocrine-immune investment in red-eared sliders (Trachemvs scripta elegans). While sex differences are present in reproductive investment timing, changes in baseline CORT and sex steroids indicate that reproductive processes are dynamic and require shifts in energy allocation. This is evidence of a shifting of physiology to cope with reproduction. On a one-year scale, both male and females maintained the ability to increase both glucocorticoid mobilization and innate immune function following a stress event. However, we observed that during high reproductive investment, females cannot invest in the same magnitude of innate immune function following a stressor. Males may be exhausted of energy stores from spring mating effort and have compromised stress response until energy stores are regained. We also observed that males may prepare for fall spermatogenesis and mating by increasing T, baseline CORT, and innate immune function, a strategy that may prepare males for increased risk of infection during mating. Thus, tradeoffs occur between immune function, the stress response, and reproduction, but not so severely that individuals are unable to mount a positive stress response.

CRediT authorship contribution statement

Jennifer Terry: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Writing – original draft. Emily Field: Investigation, Writing – review & editing. Lorin A. Neuman-Lee: Funding acquisition, Supervision, Conceptualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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