RESEARCH PAPER

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Long-term monitoring of two snake species reveals immune–endocrine interactions and the importance of ecological context

Austin R. Spence[1] | Susannah S. French[2] | Gareth R. Hopkins[3] |
Andrew M. Durso[4] | Spencer B. Hudson[2] | Geoffrey D. Smith[5] | Lorin A. Neuman-Lee[6]

[1]Department of Ecology and Evolutionary Biology, University of Connecticut, Storrs, Connecticut, USA

[2]Department of Biology, Ecology Center, Utah State University, Logan, Utah, USA

[3]Department of Biology, Western Oregon University, Monmouth, Oregon, USA

[4]Department of Biological Sciences, Florida Gulf Coast University, Fort Myers, Florida, USA

[5]Department of Biological Sciences, Dixie State University, St. George, Utah, USA

[6]Department of Biological Sciences, Arkansas State University, Jonesboro, Arkansas, USA

Correspondence
Austin R. Spence, Department of Ecology and Evolutionary Biology, University of Connecticut, Storrs, CT 06269, USA.
Email: austin.reid.spence@gmail.com

Abstract

While there is huge promise in monitoring physiological parameters in free-living organisms, we also find high amounts of variability over time and space. This variation requires us to capitalize on long-term physiological monitoring to adequately address questions of population health, conservation status, or evolutionary trends as long-term sampling can examine ecoimmunological and endocrine interactions in wild populations while accounting for the variation that often makes ecophysiological field studies difficult to compare. In this study, we tested how immune efficacy and endocrinology interact while accounting for ecological context and environmental conditions in two snake species. Specifically, we measured bacterial killing ability, steroid hormones, and morphological characteristics in multiple populations of the Western Terrestrial Gartersnake (*Thamnophis elegans*) and Common Gartersnake (*T. sirtalis*) for multiple seasons over 6 years. Leveraging this long-term dataset, we tested how a broad immune measure and endocrine endpoints interact while accounting for individual traits, sampling date, and environmental conditions. Across both species, we found bacterial killing ability to be directly related to corticosterone (CORT) and temperature and greater overall in the spring compared to the fall. We found CORT and testosterone yielded relationships with individual sex, sampling temperature, and time of year. Wild populations can exhibit high amounts of variation in commonly collected physiological endpoints, highlighting the complexity and difficulty inherent in interpreting single endpoints without taking ecological and environmental conditions into account. Our study emphasizes the importance of reporting the environmental conditions under which the sampling occurred to allow for better contextualization and comparison between studies.

KEYWORDS
bacterial killing ability, body condition, corticosterone, gartersnake, reptiles, testosterone, *Thamnophis elegans*, *Thamnophis sirtalis*

1 | INTRODUCTION

It is increasingly clear that physiological monitoring of wild free-living animals is important for understanding the health (Wikelski & Cooke, 2006) and physiological underpinnings of behaviors such as migration or hibernation (Cornelius et al., 2013) in wild populations. Because measuring the physiology of an organism is a complex endeavor, a clear a priori selection process must be engaged to determine which physiological metrics will be examined. Researchers have increasingly focused their attention on the immune system (Graham et al., 2011; Martin et al., 2006), which provides insight into populations' ability to withstand infectious disease (Brock et al., 2014). In addition, examining the interaction of immunity with other commonly collected physiological measurements, such as hormones (Neuman-Lee and French, 2016), can be valuable to assessing life-history tradeoffs (French et al., 2007; Sanz et al., 2004).

Since the advent of our ability to use minimally invasive techniques to assess organismal physiology, we have discovered common metrics to be highly context-dependent. In wild free-living animals, this presents significant practical challenges. Individuals within a population may show different physiological patterns because they engage in multiple critical processes that are energetically costly (Wingfield, 2005) and must balance these integral aspects of their health and life history among the limited amount of energetic resources available to them (Cox et al., 2010). For example, when resources are limited, animals undergo tradeoffs between self-maintenance (e.g., immune system) and reproduction (e.g., sex hormones) across different environmental conditions or life-history stages (French et al., 2007). It is clear that these tradeoffs are connected and modulated at least partly through the endocrine system, with energy-mobilizing glucocorticoids (e.g., corticosterone [CORT] in reptiles; Moore & Jessop, 2003) playing an important role (Martin et al., 2008). For example, organisms may have to "decide" whether to place their available energy towards current reproduction or towards the immune system, which may increase chances of future reproductive events (Berger et al., 2005; Crossin et al., 2016; Gao et al., 2017). These tradeoffs are context-dependent, especially when accounting for resource availability (French et al., 2007) or previous experiences of the organism (Cyr & Romero, 2007; Rich & Romero, 2005), and it is clear that these physiological metrics must be examined simultaneously to make meaningful inference.

Assessments of the immune and endocrine systems are also largely dependent upon a suite of extrinsic factors (Wingfield, 2013), which further compounds the complexity of physiological analysis in free-living animals. Examining multiple extrinsic factors and their effects alongside underlying physiology often requires long-term studies that involve several logistical constraints. In fact, most work with immunity in wild organisms examines individual response to specific acute or chronic actions (McMahon et al., 2011) or focuses on free-living organisms in one population at a single time point (Vleck et al., 2000), despite the fact that extrinsic factors, including immediate environmental conditions during sampling or phenological differences associated with sampling date, are implicated in changing the physiology of an animal and its tradeoffs among physiological systems (Chown & Gaston, 2016; Krause et al., 2016; Wingfield, 2013). While much work has focused on interactive effects of glucocorticoids and condition across seasons and years (Lind et al., 2018; Palacios et al., 2012), only recent studies are examining immune function in a similar light (Meylan et al., 2010; Titon et al., 2018, 2019). To achieve a more comprehensive understanding of endocrine–immune interactions across different environmental conditions, this study presents a long-term analysis of multiple physiological endpoints, including immune function, while accounting for intrinsic and extrinsic environmental factors simultaneously.

Ectothermic reptiles, such as wild snakes, serve as appealing model organisms for long-term monitoring to test for physiological interactions while accounting for extrinsic factors. Many studies have detected physiological tradeoffs in non-avian reptiles, including snakes and lizards (e.g., Durso & French, 2018; French et al., 2007; Husak et al., 2016; Veiga et al., 1998). In addition, many temperate snake species, such as gartersnakes (*Thamnophis* spp.; Colubridae), are found predictably and at high abundance in the wild when leaving and arriving back at their hibernacula (Gregory, 1974) providing a chance to study a large number of snakes that have recently experienced similar environmental conditions and life-history changes (i.e., emergence from hibernation). In addition to having predictable capture opportunities, these two sampling points are interesting to biologists because as snakes emerge, they often have depleted energetic reserves but are reproductively active. Following emergence, mating occurs, after which both sexes disperse from the hibernacula and females give birth later in the summer (Gregory, 1974). In northern populations, when snakes return in the northern hemisphere autumn, males have high testosterone, spermatogenesis is occurring, and energy stores are greater (Rossman et al., 1996). These seasonal changes in energetic stores are important because lower body condition, a metric of energetic potential, is often associated with reduced immune function (Titon et al., 2018) and increased glucocorticoids (Kitaysky et al., 1999; Naulleau & Bonnet, 1996). Long-term monitoring has helped distinguish between the effects of body condition per se and the effects of sampling across seasonal life-history stages (Palacios et al., 2013), but few studies have investigated the interaction between immunity, body condition, and glucocorticoids simultaneously across seasons and years.

In this study, we explore the long-term physiological monitoring of two species of natricine snakes—the Western Terrestrial Gartersnake (*Thamnophis elegans*) and the Common Gartersnake (*T. sirtalis*). These two species are the most well-studied members of the genus *Thamnophis* (Carpenter, 1952; Moore, Lemaster et al., 2000; Palacios et al., 2013; Sparkman & Palacios, 2009) and provide an excellent opportunity to address long-standing questions of how immune and endocrine metrics interact in wild populations while accounting for variation in individual and environmental characteristics. Specifically, we sampled *T. elegans* and *T. sirtalis* populations over a 6-year period, during their emergence from hibernacula in the spring before/during copulation and in the fall while in preparation for hibernation. While accounting for environmental conditions

(season, daily mean temperature), we assessed variation in bacterial killing ability, steroid hormones (CORT, testosterone), and body condition among individual snakes. Our metrics were chosen because they represent commonly collected physiological endpoints that provide a broad and comparable understanding of immunity, endocrinology, and body condition. Bacterial killing ability is a comprehensive measure of innate immunity that includes a variety of immunological components, including complement, antimicrobial peptides, and natural antibodies (French & Neuman-Lee, 2012). CORT—an energy-mobilizing glucocorticoid—is often used as an indicator of stress and is known to affect immunity in a variety of ways. Acute elevation of CORT is immunoenhancing (McCormick & Langkilde, 2014) while chronic elevation, often caused by stress, is associated with immunosuppression (Dhabhar, 2000). We have additionally included body condition and testosterone to account for energy availability and male reproductive investment, respectively.

We hypothesized that bacterial killing ability would be positively related to CORT, as these snakes were not subjected to chronically stressful or experimental conditions. We expected a positive relationship between bacterial killing ability and body condition, as snakes with higher energy reserves can likely allocate more resources towards immune function (Titon et al., 2018). We also expected that there would be a positive relationship between bacterial killing ability and daily mean temperature, as snake immunocompetence increases as they near their preferred body temperature (Stahlschmidt et al., 2017; Wright & Cooper, 1981). In terms of endocrine measures, we hypothesized CORT would be negatively related to body condition (Lind et al., 2018), as well as daily mean temperature because CORT decreases near preferred temperature (Dupoué et al., 2013). Finally, we expected that testosterone, a measure of reproductive investment, would also be influenced by CORT and body condition, and predicted a negative relationship with CORT and a positive relationship with body condition (Moore et al., 1991).

2 | METHODS

2.1 | Study species

We hand-captured 416 Western Terrestrial Gartersnakes (*T. elegans*) near three different hibernacula and 122 Common Garternakes (*T.*

sirtalis) near two of these three hibernacula in Cache County (Table 1). We captured snakes between 2011 and 2016 in either the spring (March–May) or the fall (September–October). Approximately three-quarters of the sampling occurred in the spring. Across both species, 80.4% of the snakes we captured were male. All three populations are located to the west of the city of Logan, at an elevation of approximately 1350 m above sea level, and are adjacent to waterways that are part of the Bear River Watershed. All snakes were captured under Utah Division of Wildlife Resources Permit 1COLL8382, and all methods were approved under Utah State University Institutional Animal Care and Use Committee Protocol #1508.

2.2 | Morphology and blood collection

We captured snakes and took a baseline blood sample within 3 min (typically less than 1 min) of capture (Tylan et al., 2020). We then placed snakes individually in a breathable cloth bag for 30 min and took a second stress-induced blood sample (Neuman-Lee et al., 2015). We recorded mass, snout–vent length (SVL), and sex. We calculated body condition using the residuals of an ordinary least squares regression of mass on SVL (Schulte-Hostedde et al., 2005). To account for differences between species and sexes, we calculated body condition within each species-sex combination (i.e., male *T. elegans*, female *T. elegans*, male *T. sirtalis*, female *T. sirtalis*). As per Schulte-Hostedde et al. (2005), we tested if the residuals fit the appropriate assumptions of linearity and equal variance. All relationships were linear (Pearson correlation coefficient > 0.88, $p < 0.001$) and did not violate the homoscedasticity assumption (Breusch-Pagan test: $p > 0.05$). If snakes were unmarked, we cauterized their ventral and lateral scutes in an individual pattern using a medical cauterizer (Winne et al., 2006) and released them at the site of capture. We collected approximately 30–60 µl for both the baseline and stress-induced blood samples. All samples were kept on ice and were transported back to Utah State University where they were centrifuged (6000 rpm for 10 min) to separate the red blood cells from the plasma. Plasma and the buffy coat (a layer that includes leukocytes and platelets) were removed and frozen at −80°C.

TABLE 1 Total samples sizes of *Thamnophis elegans* and *T. sirtalis*, followed by female and male in parentheses, collected in the spring and fall of each year

Species	Season	2011	2012	2013	2014	2015	2016	Total
T. elegans	Spring	46 (22, 24)	45 (7, 38)	52 (4, 48)	45 (8, 37)	96 (11, 85)	24 (0, 24)	308 (52, 256)
	Fall	27 (14, 13)	7 (4, 3)	0	45 (19, 26)	29 (11, 18)	0	108 (48, 60)
	Total	73 (37, 38)	52 (12, 41)	52 (8, 48)	90 (27, 63)	125 (23, 104)	24 (0, 24)	416 (100, 316)
T. sirtalis	Spring	1 (0, 1)	11 (0, 11)	17 (2, 15)	22 (5, 17)	22 (2, 20)	19 (1, 18)	92 (10, 82)
	Fall	11 (2, 9)	0	0	13 (3, 10)	6 (1, 5)	0	30 (6, 24)
	Total	12 (2, 10)	11 (0, 11)	17 (2, 13)	35 (8, 27)	28 (3, 25)	19 (1, 18)	122 (16, 106)

2.3 | Bacterial killing assay

We completed all bacterial killing assays within 6 weeks of sampling. The bacterial killing assay measures an integrative immune response that includes the action of natural antibodies, complement, and opsonizing proteins. This approach measures an animal's capability to eliminate an actual pathogen via bacterial killing ability, providing an ecologically relevant assessment of organismal immunity (Demas et al., 2011; Tieleman et al., 2005). We performed bacterial killing assays on both baseline and stress-induced plasma samples in duplicate in a sterile laminar flow hood following French and Neuman-Lee (2012) using *Escherichia coli* (E$^{power™}$ Microorganisms #0483E7, ATCC 8739; Micro-BioLogics). Using 6 μl of plasma, we made 1:5 dilution in CO_2 independent media (Gibco #18045) and combined it with 10^5 CFU/ml *E. coli* in phosphate-buffered saline. We calculated bacterial killing ability as the mean absorbance for each sample, divided by the mean absorbance of positive controls (six replicates per plate—containing only phosphate-buffered saline and bacteria), and multiplied by 100 (i.e., % bacteria killed relative to the positive control). We also included negative controls (media only, no bacteria added) on each plate to ensure no background contamination occurred. We ran species together across the same plates. We calculated stress reactivity of bacterial killing ability by subtracting the baseline from stress-induced measurements. The coefficient of variation (CV) within all plates less than 2.4%, with an average within-plate CV of 1.1%. Between the years, the CV between plates less than 1.7%, with an average between-plate CV of 1%.

2.4 | Radioimmunoassay

We measured levels of CORT and testosterone using a radio-immunoassay following Neuman-Lee and French (2017). We assayed plasma samples in duplicate for CORT (Ab: MP Biomedicals; # 07-120016) or testosterone (Ab: Fitzgerald #WLI-T3003-01916). Each sample was extracted with 30% ethyl acetate: isooctane. Individual recoveries were measured, and final concentrations were corrected. We assayed CORT and testosterone separately (direct assays), and testosterone was only assayed in male snakes. The average CV within assays for CORT and testosterone was 11.3% (five assays) and 6.5% (two assays), respectively. Overall, the CV was 15.2% between the five CORT assays and 9.7% between both testosterone assays. We calculated these using independent standards to ensure accuracy and precision. Each assay had a minimum of six controls, with a maximum of 14, depending upon the number of samples in each individual assay. The minimum level of detection for CORT was 0.3 and 0.4 ng/ml for testosterone. We calculated stress reactivity of CORT by subtracting baseline from stress-induced measurements.

2.5 | Meteorological data collection

We acquired daily weather information from the National Oceanic and Atmospheric Administration (NOAA) Online Weather Database

in partnership with the National Weather Service Forecast (NOAA Online Weather Data, 2019). We used average daily temperature (°C) measured across three local weather stations (Utah State University, Logan Radio KVNU, and Logan Experimental Farm) that spanned the area of all populations. Due to occasional inconsistencies in measurements between stations (e.g., proximity discrepancies, missing values), data were consolidated into average values. Average daily temperatures ranged from 2.41 to 19.17°C during the spring and 6.38–19.17°C during autumn, with means of 8.07 and 13.16°C, respectively.

2.6 | Statistical analysis

We built generalized linear mixed-effects models examining the intrinsic and extrinsic factors associated with body condition, CORT, bacterial killing ability, and testosterone within a Bayesian framework. We completed all analyses with both species while accounting for species-specific differences in the modeling framework. While species-specific physiological differences are common (Klein, 2000), our hypotheses for how immune and endocrine metrics are related are directionally the same for *T. elegans* and *T. sirtalis* as these metrics are basal and highly preserved physiological traits. Therefore, it was imperative to include species as a covariate when testing for immune and endocrine interactions to account for any species-specific difference in magnitude while analyzing the general physiological relationships across the *Thamnophis* genus. In addition, we treated samples from recaptured snakes as independent samples due to the relatively low number of recaptured snakes compared to the total dataset (16%). Baseline bacterial killing ability was modeled as a Beta-distributed random variable. We used a generalized linear mixed-effect model with a zero-one inflated beta regression using the package "zoib" (Liu & Kong, 2015), with baseline CORT, body condition, the interaction of body condition and baseline CORT, sex, season, the interaction of sex and season, mean daily temperature, and species as fixed effects. We modeled reactive bacterial killing ability as a Gaussian-distributed random variable with a linear mixed-effect model with baseline CORT, reactive CORT, body condition, the interaction of body condition and baseline CORT, sex, season, the interaction of sex and season, daily mean temperature, and species as fixed effects. We modeled baseline CORT as a Gaussian-distributed random variable with a log-link and reactive CORT as a Gaussian-distributed random variable. We then used generalized linear mixed-effect models and linear mixed-effect models, respectively, with body condition, sex, season, the interaction of sex and season, daily mean temperature, and species as fixed effects. We modeled body condition as a Gaussian-distributed random variable with a linear mixed-effect model including sex, season, the interaction of sex and season, and species as fixed effects. Finally, we modeled testosterone as a Gaussian-distributed random variable with a log-link, and we used a generalized linear mixed-effects model with the same fixed effects as baseline bacterial killing ability. For all models, we used vague priors on all parameters (normal: $\mu = 0, \tau = 0.001$) and year of sampling

and site (hibernacula) as random effects with vague priors (gamma: $\alpha = 0.001, \beta = 0.001$). We ran three chains of 10,000 iterations thinned by 5 and a burn-in of 5000, resulting in a posterior sample of 3000 per model. We evaluated parameter convergence by visual inspection of trace plots and requiring a Gelman-Rubin statistic less than 1.1 (Gelman et al., 2013). We considered parameters as strong predictors if the 95% Bayesian credible interval did not overlap zero. We fit all models using JAGS via the R programming language version 3.6.1 (R Core Team, 2019) and the package "R2jags" (Su & Yajima, 2015).

3 | RESULTS

3.1 | Bacterial killing ability

We found baseline bacterial killing ability had a positive relationship with baseline CORT (Figure 1; Table 2) and temperature (Figure 2a; Table 2). Moreover, bacterial killing ability had a positive interaction term between CORT and body condition, indicating snakes with higher body condition have a stronger positive effect of additional CORT than expected (Table 2). Baseline bacterial killing ability showed a relationship with the season and was higher in the spring ($\mu = 30.338\%$ and $SE = 1.968\%$) than in autumn ($\mu = 15.714\%$ and $SE = 2.478\%$; Figure 3a; Table 2). We did not find a strong relationship between baseline bacterial killing ability and body condition, sex, the interaction between sex and season, or species. We did not find any relationship between reactive bacterial killing ability and any tested covariate (Table 2).

3.2 | CORT and body condition

Baseline CORT had a negative relationship with daily mean temperature (Figure 2b; Table 2), indicating lower baseline CORT levels at higher temperatures. We found a strong relationship between baseline CORT and the interaction of sex and season. Males and females showed different patterns in CORT levels between the spring and fall. Males in the spring showed higher CORT than females in the spring and males in the fall but was not different than females in the fall (Figure 4; Table 2). We did not observe a relationship between baseline CORT and body condition or species. Mean reactive CORT in both species was positive, indicating increased CORT after 30-min handling. There was a difference in magnitude of reactive CORT between species (Table 2), with *T. elegans* exhibited higher reactive CORT than *T. sirtalis* ($\mu = 26.438$ ng/ml and $SE = 2.854$ ng/ml; $\mu = 16.237$ ng/ml and $SE = 5.469$ ng/ml, respectively) with substantive variation in both species. Reactive CORT did not show a relationship with any other tested covariate. We did not find relationships between body condition and season, sex, or their interaction (Table 2), meaning there was no predictive capacity of any tested covariate to explain variation in body condition.

3.3 | Testosterone

We found a strong relationship between testosterone and the season in which the snake was sampled (Figure 3b; Table 2), with male snakes sampled in the fall exhibiting higher testosterone ($\mu = 67.979$ ng/ml and $SE = 7.566$ ng/ml) than those sampled in the spring ($\mu = 26.176$ ng/ml and $SE = 1.901$). We observed a minimal difference between species, but we found no relationship between CORT, body condition, the interaction between CORT and body condition, and temperature.

4 | DISCUSSION

We undertook this study to investigate how the immune system interacts with other commonly collected physiological metrics and how it is influenced by intrinsic and extrinsic ecological factors. Our results provide one of the few long-term ecoimmunological analyses of wild free-living reptiles, while simultaneously accounting for extrinsic factors including environmental conditions at the time of sampling and sampling season. Our data, collected over 6 years, found two relationships between a broad immune metric—bacterial killing ability—and endocrine measurements: (1) snakes with higher baseline CORT generally had a stronger immune response, and (2) the positive relationship between CORT and bacterial killing ability was stronger if snakes were in a higher body condition. Most importantly, these relationships were consistent across years when accounting for different seasons and environmental conditions. All of the other relationships we found that intrinsic factors, such as sex, and extrinsic factors, such as sampling date or temperature, affect some, but not all, commonly collected physiological metrics. Overall, our results highlight the high level of integration between the immune system and CORT, as well as the responsiveness of the immune system and glucocorticoids to the environment at multiple temporal scales, from daily temperatures to seasonal changes.
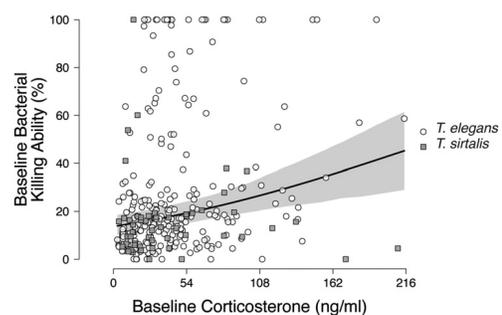
FIGURE 1 *Thamnophis elegans* (circle) and *T. sirtalis* (square) baseline bacterial killing ability as a function of baseline corticosterone. Baseline bacterial killing ability had a positive relationship with baseline corticosterone. Fitted response shows mean (black line) and 95% credible interval (gray)

TABLE 2 Sample size and parameter estimates for statistical models of baseline and reactive bacterial killing ability and corticosterone, body condition, and testosterone across both *Thamnophis elegans* and *T. sirtalis*

Test	Sample size (*T. elegans, T. sirtalis*)	Parameter	Coefficients (95% credible interval [CI])
Baseline below-knee amputation (BKA)	N = 305 (250, 55)	*Intercept*	*−2.378 (−3.032, −1.739)*
		Season (spring)	*0.668 (0.183, 1.180)*
		Sex (male)	−0.050 (−0.509, 0.402)
		Species (*T. sirtalis*)	−0.211 (−0.495, 0.073)
		Temperature	*0.081 (0.048, 0.114)*
		Body condition	0.050 (−0.059 0.158)
		Baseline corticosterone	*0.133 (0.016, 0.249)*
		Body condition × baseline corticosterone	*0.153 (0.046, 0.269)*
		Season × sex	0.329 (−0.243, 0.914)
Reactive BKA	N = 272 (225, 47)	Intercept	0.308 (−0.283, 0.889)
		Season (spring)	−0.195 (−0.513, 0.121)
		Sex (male)	−0.220 (−0.545, 0.109)
		Species (*T. sirtalis*)	0.016 (−0.088, 0.119)
		Temperature	−0.001 (−0.012, 0.009)
		Body condition	0.010 (−0.023, 0.045)
		Baseline corticosterone	−0.013 (−0.051, 0.024)
		Reactive corticosterone	−0.008 (−0.044, 0.027)
		Body condition × baseline corticosterone	−0.013 (−0.045, 0.017)
		Season × sex	0.119 (−0.074, 0.307)
Baseline corticosterone	N = 407 (328, 79)	*Intercept*	*5.185 (3.841, 6.600)*
		Season (spring)	*−1.485 (−2.247, −0.708)*
		Sex (male)	*−0.766 (−1.507, −0.708)*
		Species (*T. sirtalis*)	−0.125 (−0.379, 0.123)
		Body condition	0.070 (−0.016, 0.164)
		Temperature	*−0.235 (−0.372, −0.105)*
		Sex × season	*0.722 (0.267, 1.169)*
Reactive corticosterone	N = 374 (302, 72)	Intercept	18.400 (−25.836, 60.613)
		Season (spring)	14.985 (−11.903, 40.564)
		Sex (male)	14.189 (−11.852, 40.924)
		Species (T. sirtalis)	*−19.797 (−35.489, −3.764)*

(Continues)

TABLE 2 (Continued)

| Test	Sample size (T. elegans, T. sirtalis)	Parameter	Coefficients (95% credible interval [CI])
		Body condition	0.173 (−4.826, 5.235)
		Temperature	−1.224 (−7.350, 4.914)
		Sex × season	−7.816 (−22.992, 7.801)
Body condition	N = 538 (416, 122)	Intercept	−4.944 (−29.583, 20.012)
		Season (spring)	5.188 (−9.244, 20.256)
		Sex (male)	−5.332 (−19.796, 8.925)
		Species (T. sirtalis)	−0.814 (−5.656, 3.926)
		Sex × season	1.735 (−6.675, 10.381)
Testosterone	N = 259 (198, 61)	*Intercept*	*5.380 (3.567, 7.241)*
		Season (spring)	*−1.107 (−1.833, −0.340)*
		Species (T. sirtalis)	*−0.569 (−1.072, −0.072)*
		Temperature	0.012 (−0.274, 0.302)
		Body condition	−0.062 (−0.382, 0.262)
		Baseline corticosterone	0.001 (−0.006, 0.006)
		Body condition × baseline corticosterone	0.001 (−0.005, 0.005)

Note: 95% CIs on parameter estimate that did not overlap 0 are bolded and italicized.

4.1 | Immunological and endocrine interactions

While the immune and endocrine systems have long been known to work in conjunction in wild organisms (Zuk, 1996), long-term monitoring of these populations reveals that the relationship between bacterial killing ability, a broad measure of innate immunity, and CORT is maintained across years, seasons, and environmental conditions. Baseline bacterial killing ability had a positive relationship with baseline CORT, confirming patterns that elevated baseline CORT is often positively associated with bacterial killing ability in non-avian (*Amblyrhynchus cristatus*; Neuman-Lee & French, 2017) and avian reptiles (*Agelaius phoeniceu*; Merrill et al., 2014), as well as leukocyte abundance in mammals (*Rattus norvegicus*; Dhabhar et al., 2012). As immune function is costly (Uller et al., 2006), snakes with higher energetic stores, measured through body condition, experienced an increased positive effect of CORT, meaning CORT positively correlates with immune function to a greater extent in snakes with high body condition. We did not observe a similar relationship in reactive bacterial killing ability. In fact, we found no relationship between reactive bacterial killing ability and any tested covariate, suggesting that while baseline CORT is associated with baseline bacterial killing ability, baseline or reactive CORT may not predict how this specific measure of immunity changes after a stressful event. Other studies have also reported similar patterns where there is little ability to predict reactive bacterial killing ability in lizards or turtles (Neuman-Lee & French, 2014; Refsnider et al., 2015), and other allostatic mediators and energy metabolites, such as glucose, may play a more important role (Hudson et al., 2020). Our predictions were based on studies of endotherms, and this suggests there is an unrealized understanding of immuno-endocrine complexity (*e.g.*, energetic tradeoffs) in reptiles, potentially due to the reptilian reliance on the innate immune system (Neuman-Lee et al., 2015; Neuman-Lee et al., 2019; Zimmerman, Paitz, et al., 2010; Zimmerman, Vogel, et al., 2010).

Surprisingly, we found no relationship between body condition and sex, season, or endocrine measures, although these results may be influenced more by snake-specific feeding and metabolic habits (Secor & Diamond, 1998) rather than the absence of a general relationship. Because body condition is a proxy for the true energetic state, many studies have found relationships between body condition and reproduction, CORT, and immunity (Fokidis et al., 2011; Kitaysky et al., 1999; Moore, Lerner, et al., 2000; Naulleau & Bonnet, 1996; Palacios et al., 2013). Other studies have been more equivocal (Alonso-Alvarez & Tella, 2001; Neuman-Lee et al., 2015; Neuman-Lee & French, 2017), possibly because organisms were not energetically challenged enough to exhibit strong tradeoffs. Alternatively, a
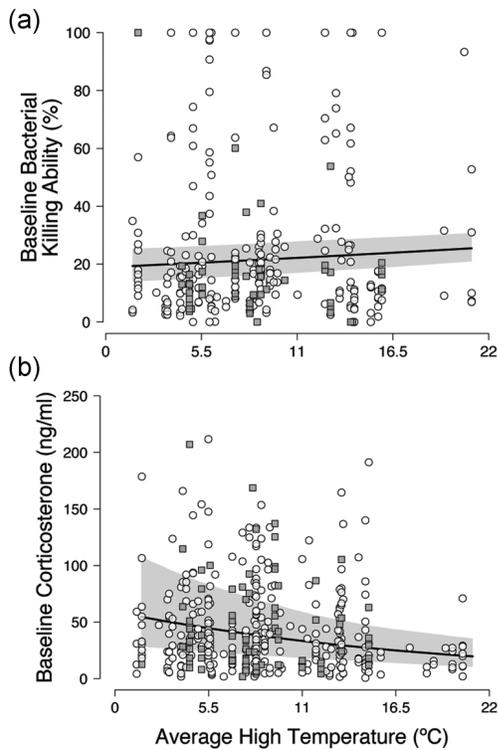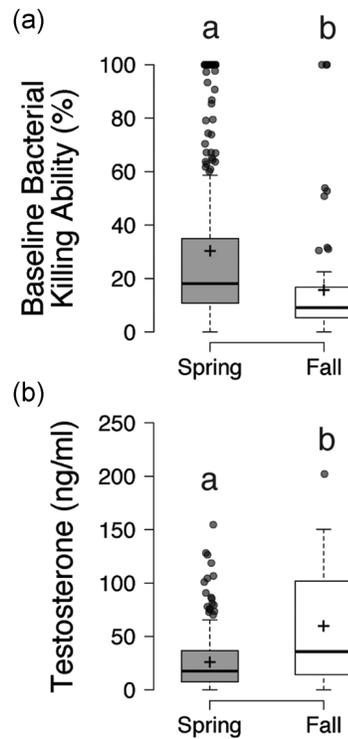
FIGURE 2 (a) Baseline bacterial killing ability and (b) baseline corticosterone as a function of daily mean temperature in *Thamnophis elegans* (circle) and *T. sirtalis* (square). Baseline bacterial killing ability had a slight positive relationship with daily mean temperature and baseline corticosterone had a negative relationship with daily mean temperature

FIGURE 3 (a) Baseline bacterial killing ability and (b) testosterone in *Thamnophis elegans* and *Thamnophis sirtalis* as a function of season. Boxplots encompass the 25th and 75th quartiles and the whiskers extend 1.5 times the interquartile length. The horizontal line indicates median, a plus (+) indicates mean, and points indicate outliers. Letters denote significance
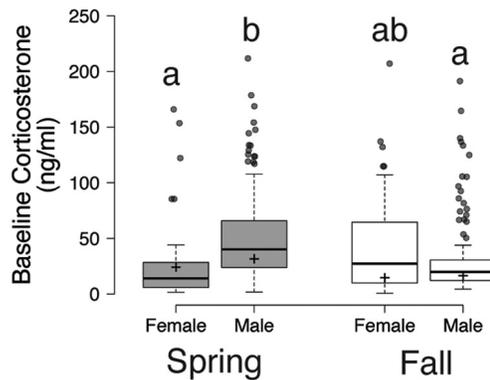
significant challenge in using body condition as a proxy for energy stores in snakes is that many snakes are infrequent eaters of large meals and can increase or decrease organ mass and physiological processes in response to food availability (McCue, 2007) to maximize energy assimilation and efficiency (McCue et al., 2012; Neuman-Lee et al., 2015). Moreover, their activity patterns and habitat use also change during digestion (Siers et al., 2018), which may affect physiology as well. We are unable to account for the feeding history of our snakes, and as such, they may be up- or downregulating biological functions in preparation for, or in response to, feeding. As our measurements indicate immediate physiological state metrics, we may not have fully captured relationships between body condition and other physiological measurements in an individual over time.

4.2 | Individual characteristics

As both species of gartersnakes in this study are sexually dimorphic, we wanted to account for potential differences that may extend to physiological measurements as well. Male and female gartersnakes showed different patterns in CORT across seasons. Males in the spring exhibiting higher CORT than spring females and fall males, but not fall females. This is potentially due to the breeding strategy of

FIGURE 4 Baseline corticosterone of both sexes of *Thamnophis elegans* and *T. sirtalis* across spring and fall sampling. Boxplots encompass the 25th and 75th quartiles and the whiskers extend 1.5 times the interquartile length. The horizontal line indicates median, a plus (+) indicates mean, and points indicate outliers. Letters denote significance

gartersnakes. Males often wait outside hibernacula and engage in intraspecific competition with other males to before mating with emerging females (Garstka et al., 1982). High intraspecific competition for mates is costly and increases glucocorticoid levels in those defending territories (Arnold & Dittami, 1997). A study examining the

physiology of *T. elegans* after the mating season (May–July) found no difference between male and female baseline CORT (Palacios et al., 2012), which may indicate that high male CORT levels drop after the reproductive season. While we did not find sex-related differences in any other measurement, the literature to date indicates sex may not be a strong predictor for differences in reactive CORT or immunity (Moreno et al., 1998; Neuman-Lee & French, 2017).

Species differences are important to account in multispecies studies (Klein, 2000). While we incorporated species into our statistical framework, we are not attempting to provide inference from our models regarding adaptation as to why there may be differences in magnitude, as two species comparisons do not yield phylogenetically interpretable results (Garland & Adolph, 1994). For example, reactive CORT for both species was positive, but there was a difference in the magnitude of the response. The direction of the response of both species is informative, as it indicates *T. elegans* and *T. sirtalis* both respond to a stressful situation with increased CORT, as expected (Moore, Lemaster, et al., 2000). Alternatively, while there is a difference in magnitude of the response between *T. sirtalis* and *T. elegans*, this result is not phylogenetically or evolutionarily informative.

4.3 | Environmental conditions

Temperature is an important environmental factor that influences snake physiology due to their ectothermic nature (Rossman et al., 1996), and the mean daily temperatures during our sampling period affected multiple physiological metrics. We found a weak positive relationship between temperature and bacterial killing ability, with higher temperatures boosting immune function. This pattern is not uncommon; ectotherms often increase immune capacity when closer to their preferred or optimal temperatures (Demas & Nelson, 1996; Wright & Cooper, 1981). In addition, we found a negative relationship between baseline CORT and temperature, which may be indicative of a lower need for energy-mobilizing hormones as metabolic rates increase with temperatures (Brischoux et al., 2016). Higher CORT at lower temperatures is consistent with other studies, where animals exposed to chronic periods of decreased temperature (e.g., winter dormancy Lutterschmidt & Mason, 2009) or nonoptimal body temperatures (Dupoué et al., 2013) had increased CORT concentrations. Future studies should aim to measure body temperatures along with ambient temperatures as snakes can behaviorally thermoregulate and subsequently affect CORT levels (Dupoué et al., 2013). Regardless, these relationships indicate that gartersnakes, like other species, have higher immune function and lower CORT while at preferred temperatures (Demas & Nelson, 1996; Romero et al., 2000). While significant, the patterns we observed were generally weak. For *T. elegans*, this is potentially because our study populations are at the geographic center of their distribution. There are documented physiological differences in populations from disparate geographical regions (Gangloff et al., 2017), which suggests that there is landscape-scale variation in how individuals respond to

environmental conditions. Snakes in our populations may be well-adapted to the full range of local temperatures and less likely to react severely to slight deviations in environmental conditions (Sorte & Hofmann, 2004).

Similar to how immediate environmental conditions affect physiological metrics, organisms also experience changes in their immune and endocrine system seasonally. We found that snakes had lower bacterial killing ability in the fall compared to the spring. This result is consistent with many other organisms, including other snakes, that alter their immune and endocrine system seasonally to enable shifts into migratory or overwintering stages, such as hibernation or migration (Dayger & Lutterschmidt, 2017; McCoy et al., 2017; Walker et al., 2015). Male snakes also had higher testosterone in the fall, consistent with seasonal changes in testosterone in other snakes (Taylor & DeNardo, 2011). This is likely because male gartersnakes' testosterone-dependent period of reproductive investment is spermatogenesis, which, in high latitude snakes, takes place in the fall. As the transition into breeding condition is costly (Alonso-Alvarez et al., 2004; Calow, 1979; Harshman & Zera, 2007) and increased reproductive effort can reduce immunocompetence (French et al., 2007; McKean & Nunney, 2005), future research should investigate a potential tradeoff between seasonally high testosterone and immunocompetence.

5 | CONCLUSION

When attempting to measure physiological responses in free-living organisms, it is increasingly important that the most informative metrics are used, as well as clearly communicating the context around which those measurements took place. This study supports the ever-growing body of literature that measuring single physiological metrics, such as immunity or CORT, is not enough, especially within a restricted sampling time (Bonier et al., 2009; Breuner et al., 2012). Rather, a suite of physiological metrics should be measured that are important for understanding the physiology of wild populations, including innate immunity (Beechler et al., 2012; Zimmerman, Vogel, et al., 2010), reproductive investment (Brannelly et al., 2016; Reedy et al., 2015), and stress-responsiveness (Bókony et al., 2009; DuRant et al., 2015). Moreover, it is imperative to report the environmental conditions under which the organism was sampled to allow better comparisons among studies. Our study found clear relationships between bacterial killing ability and CORT that are maintained across time, but additionally emphasizes that numerous factors, including sex, season, and temperature during sampling, influence variation in physiological parameters and should be accounted for when trying to understand how physiology changes over time and space. We also acknowledge that our models did not explain the majority of variation in physiological patterns that we observed, and thus many other factors likely influenced our findings, including feeding history and internal metrics such as changes in steroid binding proteins (e.g., corticosteroid binding globulin; Breuner & Orchinik, 2002), changes in receptor expression (Liebl & Martin,

2013), and maternal programming (Crino et al., 2013). Long-term field studies incorporating physiological metrics are complicated and require a multifaceted approach that incorporates fine-scale spatial and temporal monitoring. We must conduct these studies if we are to achieve a true understanding of how physiology interacts with fitness and evolutionary processes in the future.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available at https://github.com/austinspence/longterm_snake_monitoring

ORCID

Austin R. Spence (iD) http://orcid.org/0000-0002-1015-632X
Susannah S. French (iD) http://orcid.org/0000-0001-8923-9728
Andrew M. Durso (iD) http://orcid.org/0000-0002-3008-7763

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