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RESEARCH ARTICLE

Sex-specific immune component contribution to *Escherichia coli* killing ability in *Trachemys scripta elegans*

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ABSTRACT

Organisms are resource limited and allocation toward physiological processes may shift with context; for example, reproductive investment and stress. The immune system comprises a myriad of components such as leukocytes and immune proteins. The costs of innate immune components are likely not uniform because of differences in roles and composition, and it may be beneficial to prioritize specific components through within-immune system tradeoffs. We investigated the differential contribution of innate immune components in wild adult red-eared slider turtles (*Trachemys scripta elegans*) during the beginning of the nesting season in Arkansas, USA. We collected blood samples at 0, 120 and 240 min of capture (a standardized stressor). Enzyme-linked immunosorbent assays verified that corticosterone was elevated following capture. To assess the immune component contribution, we systematically removed immune components from the isolated serum and buffy layer (serum+BL) to assess the contributions of immune cells, complement and non-specific, heat-labile proteins (including antimicrobial peptides and natural, non-specific antibodies). After manipulating the serum+BL, we conducted functional microbial killing assays using *Escherichia coli*, a Gram-negative bacterium. Our findings suggest that complement and heat-labile proteins are upregulated during the acute stress response and the duration of upregulation differs by sex during the nesting season. Females were unable to maintain an increased protein-based immune response for as long as males following a stressor, indicating that females may be more energetically limited during the nesting season. This work provides evidence of tradeoffs within the innate immune system and enhances understanding of immunity in reptiles.

KEY WORDS: Ecophysiology, Reptile, Complement, Tradeoffs, Stress response

INTRODUCTION

Organisms have access to finite resources and must allocate these resources to all physiological processes (Stearns, 1989). Allocation toward each process may shift based on life history events and/or increased priority (i.e. sickness, wounds, environmental stressors) within individuals (Dayger and Lutterschmidt, 2017; Zimmer et al., 2019). Tradeoffs, the upregulation of one process in favor of another, may occur as a result (van Noordwijk and de Jong, 1986). These tradeoffs and prioritization patterns, thought to be mediated at least in part by glucocorticoids (Sapolsky et al., 2000; MacDougall-Shackleton et al., 2019), are often examined across broad system processes. Tradeoffs across taxa are frequently measured by examining single or a few metrics of complicated physiological processes such as immune function, stress physiology and reproduction (Bowers et al., 2015; Peck et al., 2016; Neuman-Lee and French, 2017; Palacios and Bronikowski, 2017). However, the use of a limited number of metrics may explain why broad patterns of tradeoffs across taxa and contexts have been difficult to find (Neuman-Lee et al., 2019; Spence et al., 2020; Garland et al., 2022). For example, the immune system is multifaceted (Medzhitov and Janeway, 1998) and myriad components respond differentially to extrinsic, stress-inducing factors such as predation risk or low food availability (Sapolsky et al., 2000; Dhabhar et al., 2012). Because the costs of immune components are likely not uniform in constitutive and induced roles (Martin et al., 2008; Sandmeier et al., 2025), it may be beneficial for an organism to prioritize specific components through within-immune system tradeoffs when possible (Zuk and Stoehr, 2002; Lee, 2006; Neuman-Lee and French, 2014; Judson et al., 2020).

The innate immune system (constitutive and induced function of germline-encoded components) is of particular interest in ecophysiological studies as it is highly conserved across vertebrates and can be measured using minimally invasive sampling techniques that do not require extended captivity of wild animals (Ohmer et al., 2021; Bronikowski et al., 2023; Cumplido et al., 2023). The circulating reptilian innate immune system is composed of several components: leukocytes, cytokines, antimicrobial peptides (AMPs), complement, natural antibodies (NAbs) and other protein-based components such as acute-phase proteins (Bonneaud et al., 2003; Lee, 2006; Zimmerman et al., 2010a). Leukocytes include heterophils (putative neutrophil analog), lymphocytes, eosinophils, basophils, monocytes and azurophils (found in some reptiles) that act individually and synergistically through phagocytosis, degranulation and cytokine release (Stacy et al., 2011). Phagocytosis occurs when leukocytes, such as heterophils (Stacy et al., 2011), monocytes (Jones, 2015) and B-1 cells (Zimmerman et al., 2010b; Slama et al., 2022), engulf pathogens. Degranulation occurs when toxic granules are released by granulocytes (heterophils, eosinophils, basophils, azurophils) to help clear a pathogen (Genovese et al., 2013). In conjunction with direct pathogen-clearing mechanisms, leukocytes release cytokines that stimulate further responses by other cells and molecules (Zimmerman et al., 2014). AMPs are a diverse group of protein-based molecules released from circulating leukocytes and tissues that disrupt pathogen membranes (Hancock and Scott, 2000; van Hoek, 2014). Complement proteins tag or clear pathogens by opsonizing or lysing, respectively, and their activation occurs following the activation of one of three cascade pathways: classical (by recognition of antigen–antibody complexes), alternative (by C3 hydrolysis and binding) and lectin (by mannose residue binding;

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Adamovicz et al., 2020). NAbs are germline-encoded antibodies released by B-cells that clear pathogens by opsonization and activating the classical complement cascade (Matson et al., 2005). Thus, because immune components have distinct structures and roles, conducting multiple assays can provide a more comprehensive understanding of innate immune function (Claunch et al., 2022; Field et al., 2022). However, immune metrics examined in a given study may be constrained because of limited sample volume, time and financial resources (e.g. Millet et al., 2007; Zimmerman et al., 2010a; Palacios and Bronikowski, 2017).

Sample manipulation directly influences which circulating immune components are assayed. Anticoagulant use during sample collection also impacts innate immune function components, as agents such as heparin and EDTA may deactivate complement and thus exclude it from any immune function analysis at certain concentrations (Weiler et al., 1992; Baker et al., 2019). These samples are referred to as plasma, whereas samples collected without anticoagulant are called serum. When fresh serum and buffy layer (hereafter referred to as 'serum+BL') are collected without an anticoagulant, the sample contains all potential innate immune components (Demas et al., 2011; Hartzheim et al., 2022), including leukocytes, complement, AMPs and NAbs. Freezing kills cellular components, so leukocyte function is eliminated from freeze-thawed serum+BL (Jacobs and Fair, 2016; Adamovicz et al., 2020). A mild heat treatment (56°C, at least 30 min) can inactivate complement activity (Merchant and Britton, 2006; Adamovicz et al., 2020) and some heat-labile NAbs (Matson et al., 2005). This heat treatment denatures smaller proteins but not the more heat-stable NAbs and AMPs (Matson et al., 2005; Akazawa-Ogawa et al., 2018). Differential serum+BL or plasma manipulations have been applied to examine differences between species (e.g. complement activity: Adamovicz et al., 2020; complement/non-complement pathways: Hartzheim et al., 2022) and to characterize components in a given species (e.g. Merchant et al., 2010). Few studies have compared differences in immune contribution due to sample treatment in a functional context, such as examining within-innate immune system tradeoffs.

Freshwater turtles provide a unique opportunity to examine within-innate immune system tradeoffs as they depend largely on innate immune function (Zimmerman et al., 2013, 2017). Many species exhibit sexual dimorphism (Lovich et al., 1990) and sex differences in the timing of heightened energy expenditure (e.g. reproduction costs; Shelby et al., 2000; Terry et al., 2023) that may influence immune function. For example, female red-eared sliders (*Trachemys scripta elegans*) have elevated baseline corticosterone and progesterone concentrations, as well as decreased protein-based microbial killing ability (MKA) at the height of egg development during the nesting season (Terry et al., 2023). Males exhibit higher corticosterone and testosterone concentrations and increased protein-based MKA prior to autumn spermatogenesis and mating (Terry et al., 2023). Red-eared sliders reach body sizes (Ernst and Lovich, 2009) that allow for the humane collection of relatively large blood sample volumes for use across multiple assays. Further, red-eared sliders are ubiquitous in native and invaded ranges (Mota et al., 2021) and thus are appropriate free-living organisms to examine patterns and variations in resource allocation to increase understanding of ecophysiology.

We aimed to assess the relative circulating innate immune component contribution across the stress response in wild adult red-eared sliders. We used a standardized stressor, capture, to elicit the acute stress response and collected blood samples at 0, 120 and 240 min of capture (Romero and Reed, 2005; Terry et al., 2023). Serum+BL samples were subjected to three manipulations (i.e. fresh, frozen, frozen+heat) to systematically remove innate immune components and were used in MKA assays. We predicted that (1) corticosterone would increase after capture, as it would stimulate an acute stress response; (2) bacterial killing ability should vary by treatment, as components likely contribute differentially to removing the pathogen; (3) the innate immune component contribution would vary across the acute stress response; and (4) the innate immune component contribution and overall response (i.e. microbial killing ability) would differ by sex.

MATERIALS AND METHODS

Sample collection

We captured adult red-eared sliders, *Trachemys scripta elegans* (Wied-Neuwied 1838), using baited hoop traps (nets) and by hand in May 2022 (46 females, 43 males) and May 2023 (50 females, 32 males) at Bearitage Farms (35.44105°, −90.80491°) in Cross County, AK, USA. We placed nets in various water bodies on the property accessible by wading and checked them daily (08:30–11:00 h). We recorded the time of removal from the net/capture as capture and restraint stimulate the acute stress response in wild organisms (Romero and Reed, 2005; Terry et al., 2023). To determine whether net capture elicits the acute stress response, we placed five turtles in a closed net overnight after their initial capture and blood collection. There was no statistical difference between their initial response and the response after containment in the net when assessed with paired Wilcoxon signed rank tests (V=7, P=1.00). We used a 26-gauge needle to collect blood (~1 ml) from the caudal vein within 3 min, 120 and 240 min of removal from the net/capture. Blood sampled within 3 min of capture (0 min time point) represents baseline levels whereas blood sampled at 120 and 240 min represents stress-induced levels. We stored blood in labeled 1.5 ml microcentrifuge tubes on ice until processing (approximately 1–3 h) and then centrifuged samples for approximately 5 min at 10,000 rpm. We removed the serum and buffy layer. Samples were then stored as described below.

This project (FY20-21-80) was approved by the Arkansas State University Institutional Animal Care Use Committee. Arkansas scientific collection permits from the Arkansas Game and Fish Commission (022320222, 022120231) were obtained before sampling.

Corticosterone assay

To test whether the acute stress response is incurred following capture in hoop nets and following removal from the net, we conducted corticosterone (CORT) enzyme-linked immunosorbent assays (ELISAs; item 501320, lot 0710040, Cayman Chemical, Ann Arbor, MI, USA). Before conducting ELISAs, we compared results between ELISA and radioimmunoassay (RIA) methods as RIAs were previously conducted to assess CORT in this study population (Terry et al., 2023; corticosterone rabbit antiserum, cat. no. 07-120016, lot 3R3-PB17, MP Biomedicals, Santa Ana, CA, USA; H3-labeled CORT, NET300250UC, PerkinElmer, Shelton, CT, USA; Fig. S1). We aliquoted four pooled serum+BL samples (two for 0 min, two for 120 min) into two replicates to test for parallelism (equivalent to assessment of endogenous hormones in samples and standard hormones provided in the ELISA kit), linearity (reliability of detection across sample dilutions) and interference (presence of components that may falsely elevate quantified hormone concentrations) between unextracted, raw serum+BL and serum+BL extracted with 30% ethyl acetate:iso-octane (Terry et al., 2023) across RIAs and ELISAs. When we conducted a Wilcoxon signed rank test, we found no difference (V=1, P=1.0) between extracted and unextracted serum+BL CORT

from samples assessed with RIA at the 1:33.3 dilution previously validated for this species (Terry et al., 2023). For the samples assessed with ELISA, we conducted Pearson's correlations to assess linearity in extracted (r=−0.826) and unextracted serum+BL (r=−0.862) across four dilutions (1:10, 1:20, 1:40, 1:80) in ELISAs (Fig. S1). Further, we used spiked samples with the 1:10 and 1:80 dilutions to test for parallelism and interference. As observed−expected ratios were in the range 80–100% for both unextracted and extracted samples across dilutions, we proceeded by running samples in duplicate using unextracted serum+BL at 1:20 or 1:40 dilution. Detection of the kit was in the range 8.2–5000 pg ml^{-1}, with sensitivity of 30 pg ml^{-1}. The intra-assay coefficient of variation was: 7.7%, 12.4% and 12.6% for each ELISA assay.

Serum+BL manipulation

To systematically inactivate immune components across a single sample, we subjected serum+BL to three manipulations, 'fresh', 'frozen' and 'frozen+heat', for use in MKA assays (Fig. 1). Immediately following centrifugation, we aliquoted each serum+BL sample into two 0.6 ml microcentrifuge tubes. One tube was stored at 4°C and the serum+BL was used in MKA assays within 4 days ('fresh' manipulation). The other tube was stored at −20°C to kill leukocytes and thawed after 1 month ('frozen' manipulation). We took an aliquot from the frozen–thawed serum+BL on the same day of thawing and incubated it at 56°C for 60 min ('frozen+heat' manipulation) in 2022. In 2023, we reduced the incubation time to 30 min as mild heat treatment for 30 min denatures complement proteins and heat-labile NAbs (Merchant and Britton, 2006; Graham et al., 2012a). Frozen–thawed serum+BL was not further manipulated and was returned to the freezer for future use after MKA assays. All frozen serum+BL underwent one thaw cycle.

MKA assay

To assess MKA of constitutive and induced immune components against Gram-negative *Escherichia coli*, we conducted MKA assays in 96-well plates using a modified protocol from French and Neuman-Lee (2012). Gram-negative bacteria lack a peptidoglycan wall and thus are found in aquatic environments and activate complement pathways as well as phagocytes and NAbs (Rautemaa and Meri, 1999; French and Neuman-Lee, 2012). Specifically, *E. coli* is most commonly used in MKA assays (Millet et al., 2007; French and Neuman-Lee, 2012). We used 1:10 diluted serum+BL across manipulations based on validations with fresh serum+BL to attain roughly 50% killing (Hartzheim et al., 2022; Davis et al., 2025; Terry et al., 2025). Use of this dilution for both fresh and frozen-manipulated serum+BL samples allows for direct comparison of killing ability. We used *E. coli* (ATCC 8739, Microbiologics, St Cloud, MN, USA) in 1× phosphate-buffered saline (PBS; concentration: 1.0×10^5 colony forming units). We added 9 µl diluted serum+BL, 9 µl 1× PBS and 6 µl *E. coli* and incubated for 30 min at 37°C. We then added 125 µl 3% tryptic soy broth (TSB) to all wells, read plates at 340 nm (BioTek Synergy2, Winooski, VT, USA; French and Neuman-Lee, 2012; Terry et al., 2023; Davis et al., 2025), and incubated plates at 37°C for 12 h. We read plates again at 12 h. We calculated percentage killing (MKA) by subtracting 1 from the quotient of the change in absorbance between readings and the positive wells (six replicates per plate of 1× PBS, bacteria, TSB). The positive wells were used to measure 100% growth, and thus represent 0% killing. Subtracting the 0 h absorbance from the 12 h absorbance removes background variability across samples. We also included negative controls (six replicates with 1× PBS and TSB, no bacteria) to ensure no background contamination and to represent 100% killing (no bacterial growth). We adjusted values <0% or >100% killing to 0% and 100%, respectively, to represent biological relevance (Rogers et al., 2022; Terry et al., 2023, 2025). Further, binding the MKA values by 0% and 100% prevents extrapolation, given that the positive and negative controls act as standards for each plate. We then rescaled all values from a 0–100 scale to a 0.0001–0.9999 scale for analyses with a beta distribution. Intra-assay coefficient of variation was in the range 0.08–4.60%, with an overall inter-assay coefficient of variation of 12.44%.

Statistical analyses

We conducted all analyses in the statistical program R (http://www.R-project.org/). We included all individuals in analyses as they are representative of the variation in natural populations. Sample sizes varied across metric and time point based on sample availability and relevance (see details on the generation of cell-dependent killing variables below; Table 1). All means are reported ±1 s.e.m. We interpreted results at a significance level of α=0.05. We constructed a single model for each response variable. We conducted *post hoc* pairwise comparisons and derived means and s.e.m. using the package 'emmeans' (https://CRAN.R-project.org/package=emmeans).

To assess whether CORT shifted over time following capture, we constructed a generalized linear mixed model with a Gamma error distribution using the package 'lme4' (Bates et al., 2015). The interaction of time post-capture (i.e. 0, 120, 240 min) and sex was a fixed effect and individual ID and year were random factors.

To understand how the immune component contribution shifted across the stress response, we used three types of response variables in the analyses: MKA data, cell-dependent killing (CDK) and proportion of cell-dependent killing (PCDK). The MKA variables

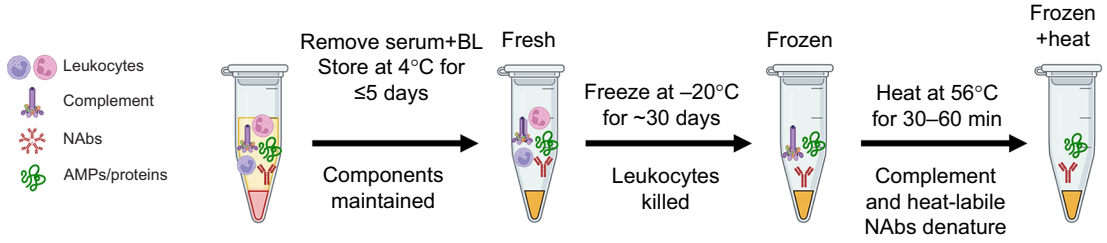

Fig. 1. Summarized serum+buffy layer (BL) treatment protocol. Serum+BL from red-eared slider turtle (*Trachemys scripta elegans*) blood was removed to a tube, vortexed and split between two tubes. One tube was stored at 4°C and used in microbial killing ability (MKA) assays within 5 days. The second tube was immediately stored at −20°C for 30 days to kill leukocytes. After thawing, an aliquot from the frozen sample was subjected to a mild heat treatment of 56°C for 30 or 60 min, denaturing complement proteins and heat-sensitive natural antibodies (NAbs). Created in BioRender by Terry, J. (2025) https://BioRender.com/j99k640. This figure was sublicensed under CC-BY 4.0 terms.

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Table 1. Mean±1 s.e.m. corticosterone (CORT) microbial killing ability (MKA), cell-dependent killing (CDK) and proportion cell-dependent killing (PCDK) against *Escherichia coli* across time (0, 120, 240 min) after capture in wild red-eared sliders (*Trachemys scripta elegans*) captured in 2022 and 2023

	Serum+BL treatment	0 min	120 min	240 min
Females				
CORT (ng ml^{-1})		1.24±0.15	1.98±0.29	2.20±0.37
		n=48	*n*=44	*n*=35
MKA (%)	Fresh	41.8±3.5	43.2±3.8	41.1±5.3
		n=95	*n*=74	*n*=40
	Frozen	32.8±3.1	42.8±3.8	37.0±5.1
		n=93	*n*=78	*n*=39
CDK (%)		35.9±3.4	28.2±3.3	33.4±5.2
		n=81	*n*=66	*n*=31
PCDK (%)		65.5±3.9	49.6±4.9	64.3±6.9
		n=63	*n*=50	*n*=20
Males				
CORT (ng ml^{-1})		1.62±0.22	3.16±0.73	3.03±0.68
		n=35	*n*=33	*n*=24
MKA (%)	Fresh	52.4±4.0	55.3±4.3	65.2±5.3
		n=82	*n*=63	*n*=34
	Frozen	36.2±3.7	50.1±4.4	61.7±5.8
		n=71	*n*=63	*n*=31
CDK (%)		38.0±3.7	28.9±3.7	28.3±5.1
		n=64	*n*=51	*n*=26
PCDK (%)		61.3±4.4	42.8±5.2	39.3±6.4
		n=55	*n*=43	*n*=26

Serum+buffy layer (BL) treatment is indicated. All values were generated from generalized linear mixed models with gamma (CORT) or beta (Immune variables) error distribution. Sample sizes (*n*) are indicated below the data.

represent killing ability from either fresh or frozen serum+BL against *E. coli*. CDK was calculated by subtracting frozen serum+BL MKA from fresh serum+BL MKA within individuals and represents the cell-dependent contribution relative to total (fresh serum+BL) MKA such that CDK includes cellular killing that may be facilitated by protein components, such as opsonization by NAbs (Zhou et al., 2013). We removed CDK values (11.63% of values removed; *n*=42) in which there were discrepancies (i.e. negative values) beyond 15% because they cannot be reasonably explained by artifacts of the assay. However, some frozen serum+BL MKA may be higher than the fresh serum+BL MKA of the same sample as a result of leukocyte inhibition of complement (Bernard et al., 1976), but this is beyond the scope of this study. We arbitrarily selected 15% based on biological relevance of differences in the relative MKA assay. CDK values <0 to −14.9 were changed to 0, following the same logic. PCDK was derived by dividing CDK by fresh serum+BL MKA within individuals, such that differences in overall killing ability between individuals was controlled. Any individual (18.6%, *n*=27) with 0 fresh serum+BL MKA was removed from PCDK variables such that 0 represented zero cellular contribution to observed killing. Therefore, we could assess whether the cell-dependent contribution relative to the cell-independent contribution to killing varies by sex and time regardless of individual differences in overall killing ability.

To assess immune component contribution and killing across time, we constructed a single generalized linear mixed model with a beta error distribution using the package 'glmmTMB' (Brooks et al., 2017) for each variable: fresh serum+BL MKA, frozen serum+BL MKA, CDK and PCDK. For each model, we included an interaction of time post-capture and sex and an additive factor of year as fixed effects and individual ID as a random factor.

RESULTS

CORT increased following capture in both male and female red-eared sliders (Table 1, Fig. 2). Males had higher CORT than females at 0 (P=0.037) and 120 min (P=0.005), but not at 240 min (P=0.055; Table 1). Females increased CORT at 120 min (P<0.001) and 240 min (P<0.001) relative to that at 0 min (Fig. 2). There was no difference in female CORT between 120 and 240 min (P=0.397). Males exhibited the same pattern, though at higher magnitude (Fig. 2), with higher CORT at 120 min (P<0.001) and 240 min (P<0.001) relative to that at 0 min. There was also no difference in male CORT between 120 and 240 min (P=0.864; Fig. 2).

There was 0% *E. coli* killing across heat-treated samples (n_0=114, n_{120}=97, n_{240}=31). Therefore, we excluded heat-treated serum+BL from further analyses. Fresh serum+BL MKA did not differ by year for either sex (both P=0.128) or at any time point (all P=0.128). We removed the additive factor of year to assess the interaction of time post-capture and sex. Fresh serum+BL MKA from males was consistently higher than that of females across 0 (P=0.049), 120 min (P=0.037) and 240 min (P=0.002; Table 1). However, there were no significant differences across time post-capture in female (all P>0.942) or male (all P>0.135) fresh serum+BL MKA (Fig. 3). Frozen serum+BL MKA did not differ by year for either sex (both P=0.726) or at any time point (P=0.726). After removing the additive factor of year, frozen serum+BL MKA by males was only higher than that of females at 240 min (P=0.002), but not at 0 min (P=0.465) or 120 min (P=0.206; Table 1). There was no significant difference across time post-capture in female frozen serum+BL MKA (all P>0.099; Fig. 3A). However, male frozen serum+BL MKA increased by 120 min (P=0.044) and 240 min (P=0.001) relative to that at 0 min, but there was no difference between 120 min and 240 min frozen serum+BL MKA (P=0.254; Table 1, Fig. 3B).

CDK was not different by year for either sex (both P=0.309) or at any time point (all P=0.309), so we removed year from the model. There was no difference between males and females in CDK at any time point (all P>0.479; Table 1, Fig. 4A). CDK did not vary across time in females (all P>0.210) or males (P=0.205; Fig. 4A).

PCDK was not different by year for either sex (both P=0.872) or at any time point (all P=0.872), so we removed year from the model. Females only had higher PCDK than males at 240 min (P=0.012), but not at 0 min (P=0.467) or 120 min (P=0.339; Table 1). Female PCDK decreased by 120 min (P=0.032; Fig. 4B). There was no significant difference in female PCDK between baseline and 240 min (P=0.988) or 120 and 240 min (P=0.214). Male PCDK was decreased by 120 min (P=0.022) and 240 min (P=0.018; Fig. 4B). There was no significant difference between 120 and 240 min male PCDK (P=0.907).

A conceptual summary of the results is provided in Table 2.

DISCUSSION

Our study compared MKA across serum+BL manipulations to understand within-immune system dynamics in a free-living organism (Table 2). Red-eared slider turtles, like other reptiles, are thought to depend on a robust innate immune system (Zimmerman et al., 2010a), making them a suitable model to investigate tradeoffs amongst components of the innate immune system. Our results highlight how serum+BL manipulations in immune assays such as the MKA assay maintain or inactivate immune cells and proteins, which provides a greater ability to interpret biological processes. Comparing MKA data across serum+BL manipulations provided a relatively simple way to

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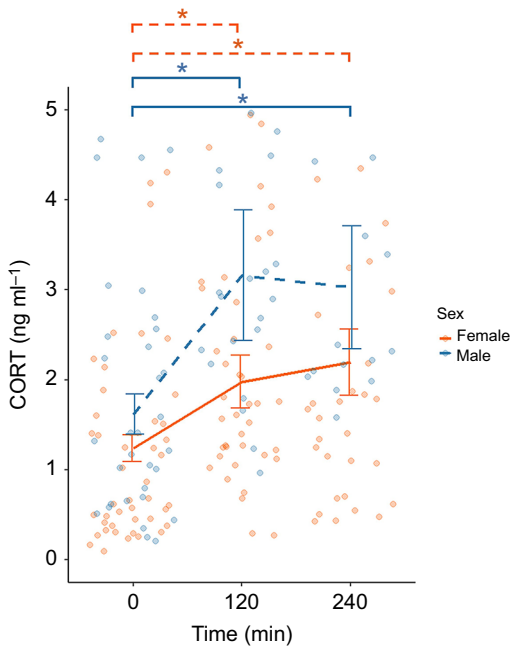

Fig. 2. Corticosterone (CORT) levels of female and male adult red-eared slider turtle serum+BL at 0, 120 and 240 min following a standardized stressor, capture. Means±s.e.m. were derived from a generalized linear mixed model with a Gamma error distribution. Significant relationships (P<0.05) between time points within sex are denoted with lines and asterisks above data. Female CORT was higher at 120 min (P<0.001) and 240 min (P<0.001) relative to that at 0 min. Male CORT was higher at 120 min (P<0.001) and 240 min (P<0.001) relative to that at 0 min. Points represent raw values for each individual (females: n_0=48, n_{120}=44, n_{240}=35; males: n_0=35, n_{120}=33, n_{240}=24).

tease innate immune system dynamics apart. Fresh, frozen and frozen+heat serum+BL manipulations revealed that the relative contribution of leukocytes, complement, NAbs and AMPs against a pathogen is not uniform. Frozen+heat serum+BL did not clear *E. coli*, whereas killing was observed when fresh and frozen serum+BL was used in MKA assays. MKA of frozen serum+BL from males significantly increased following the stressor and CDK and PCDK metrics suggest that these changes are largely due to an increase in heat-labile proteins in individuals that mounted a response. The proportion of CDK decreased, suggesting an increased contribution of these heat-labile proteins. Further, females did not sustain a heightened contribution of heat-labile proteins for as long as males. Because females were investing heavily in reproduction during the sampling period, this result suggests that reproductive investment may influence within-immune dynamics.

Given that the innate immune system comprises many components, we predicted that MKA would vary by serum+BL treatment. Although we did not explicitly identify components in samples, observed MKA differed by treatment, suggesting that immune components were systematically inactivated by freezing and heating manipulations. Thus, clearance of the pathogen, *E. coli*, involves both cellular and protein-based immune function. The remaining immune components in the heat-treated samples did not kill *E. coli*, which suggests that heat-stable proteins such as AMPs at this dilution contribute little to the observed MKA (although AMPs are present in this species; Kaplinsky et al., 2013; Gray et al., 2020). Previous research has shown that complement is deactivated by the relatively mild heat treatment (56°C) used in this study (Merchant and Britton, 2006), but some AMPs, NAbs and other acute-phase proteins might also denature at this temperature (Matson et al., 2005; Akazawa-Ogawa et al., 2018). Thus, complement and heat-labile proteins such as NAbs are likely the primary immune protein components against *E. coli* at this dilution in frozen serum+BL, with NAbs likely activating complement by the classical pathway. Lipopolysaccharides on Gram-negative bacteria such as *E. coli* also activate complement through the alternative pathway (O'Hara et al., 2001). Therefore, complement is likely the primary actor in the observed killing in frozen samples. We observed overall differences in killing ability of fresh and frozen samples, suggesting that innate immune components contribute differentially to the overall immune response. Observed killing using fresh serum+BL is likely due to synergistic action by innate immune components. For example,

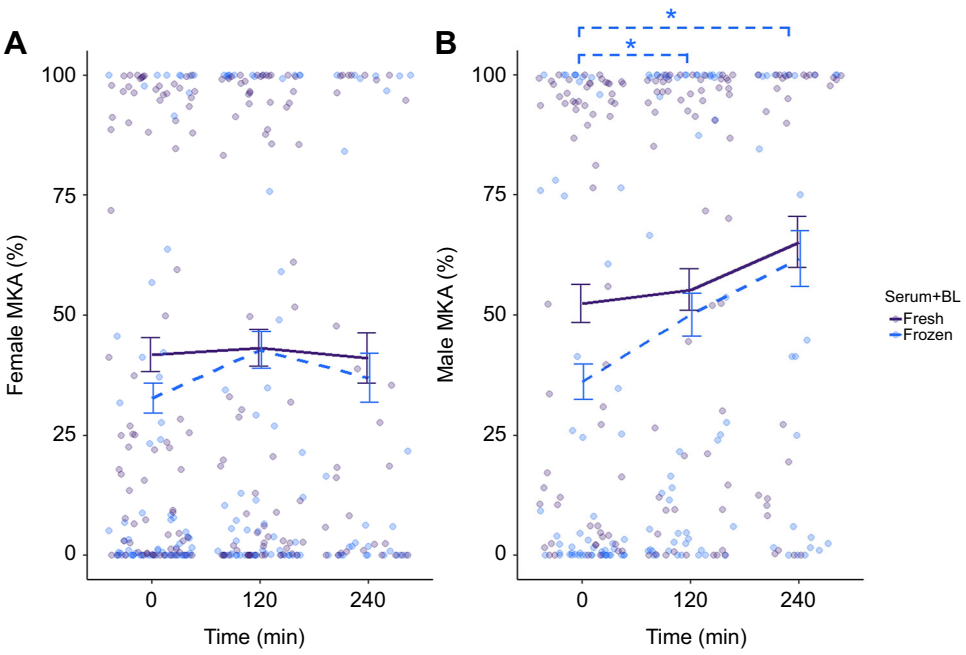

Fig. 3. MKA of female and male adult red-eared slider turtle serum+BL against *Escherichia coli* at 0, 120 and 240 min following capture. (A) Female and (B) male serum+BL MKA. Heat-treated serum+BL MKA results are not shown as there was 0% killing across all individuals. Means±s.e.m. were derived from generalized linear mixed models with beta error distribution. Significant relationships (P<0.05) between time points within sex are denoted with lines and asterisks above data. Male frozen serum+BL MKA increased by 120 min (P=0.044) and 240 min (P=0.001). Points represent raw values for each individual (female fresh: n_0=95, n_{120}=74, n_{240}=40; frozen: n_0=93, n_{120}=78, n_{240}=40; male fresh: n_0=82, n_{120}=63, n_{240}=34; frozen: n_0=71, n_{120}=63, n_{240}=34).

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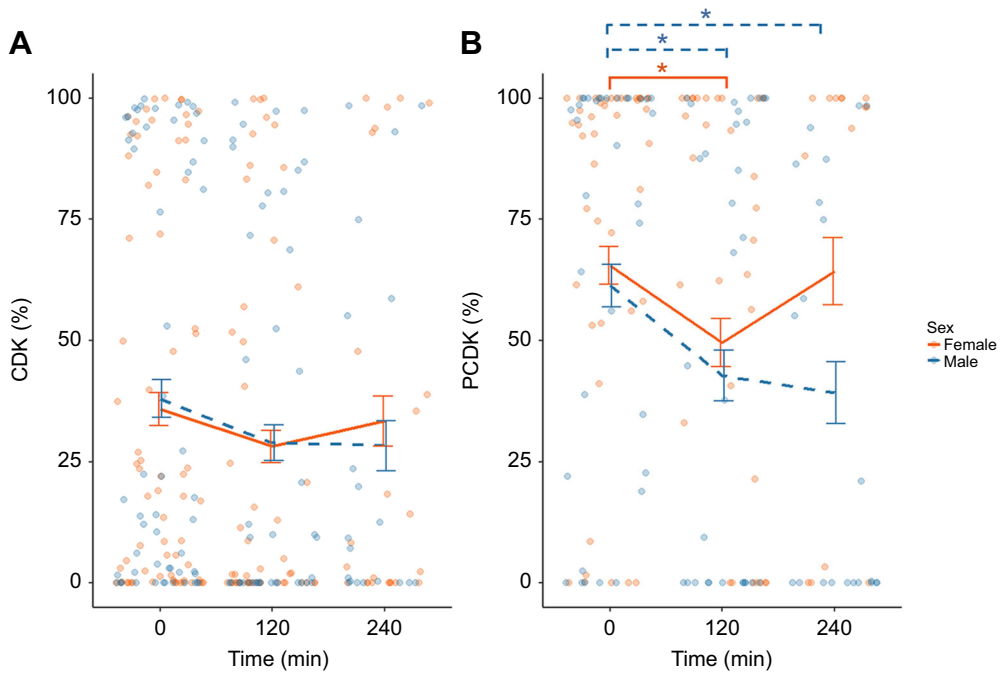

Fig. 4. Cell-dependent killing (CDK) and proportion of cell-dependent killing (PCDK) of female and male adult red-eared slider turtle serum+BL against *E. coli* at 0, 120 and 240 min following capture. (A) CDK was calculated by subtracting frozen serum+BL MKA from fresh serum+BL MKA within individuals and represents the cell-dependent contribution relative to total (fresh serum+BL) MKA. (B) PCDK was calculated by dividing CDK by fresh serum+BL MKA within individuals and represents the proportion of cell-dependent killing relative to the total (fresh serum+BL) MKA. Means±s.e.m. were derived from generalized linear mixed models with beta error distribution. The significant relationships at α=0.05 between time points within sex are denoted with lines and asterisks above data. Females had lower PCDK at 120 min relative to 0 min (P=0.032). Males had lower PCDK at 120 min (P=0.022) and 240 min (P=0.018) relative to 0 min. Points represent raw values for each individual (female CDK: n_0=81, n_{120}=66, n_{240}=31; PCDK: n_0=63, n_{120}=50, n_{240}=20; male CDK: n_0=64, n_{120}=51, n_{240}=26; PCDK: n_0=71, n_{120}=63, n_{240}=34).

NAbs opsonize pathogens, enhancing complement activity and phagocytosis (Matson et al., 2005; Rungelrath et al., 2020), and granulocytes release AMPs and other toxic granules upon encountering a pathogen (Stacy et al., 2022). In addition, the lack of killing in heat-treated serum+BL suggests that the presence of proteins such as AMPs in serum+BL is not substantial enough to clear pathogens; instead, concentrated release at the pathogen membrane by degranulation may facilitate killing (Hancock and Scott, 2000). However, we acknowledge that sample manipulations do not capture all interactions amongst components. For example, opsonization by complement proteins and NAbs for phagocytosis by leukocytes is not observable in frozen samples (Matson et al., 2005). Further, leukocytes may inhibit complement in fresh samples (Bernard et al., 1976) but the same inhibition would not occur in the frozen manipulated sample. This may explain, in part, the >15% difference in the 42 CDK data values we removed before analysis. By generating our CDK and PCDK variables, we aimed to estimate the contribution of cellular processes that we cannot infer from either fresh or frozen serum+BL data alone, although we recognize statistical artifacts may occur in proportion-derived variables such as PCDK. Therefore, our results are not an absolute measurement of immune component contributions *in vivo*. Immune function metrics based on sample treatment should be interpreted with care. Researchers may be limited based on sampling logistics, sample volume and time, so it may not be possible, or the priority, to examine nuances in innate immune function. In such cases, interpretations should be careful to specify immune components based on methodology, including how blood samples are processed.

We further predicted that the innate immune component contribution would vary across the acute stress response. Activation of the hypothalamic–pituitary–adrenal (HPA) axis causes physiological changes to increase the chances of survival during an acute stress event (Sapolsky et al., 2000). We verified that capture stimulated the acute stress response, as CORT was elevated in turtles 120 and 240 min post-capture. Increases of CORT in the context of an acute stress response are linked to a swath of physiological shifts (Sapolsky et al., 2000; Neuman-Lee and French, 2017). One expected change is an overall increase in innate immune function (Sapolsky et al., 2000; Neuman-Lee and French, 2017). We created two new variables from MKA fresh and frozen serum+BL data: CDK and PCDK. These variables examine protein dynamics through the lens of the cell-dependent contribution; fresh and frozen serum+BL MKA increased following the acute stressor (though non-significantly in females), yet CDK did not change, and PCDK decreased in both sexes by 120 min post-stressor. Our results suggest that red-eared sliders upregulate complement and heat-labile proteins following an acute stressor. This study provides further evidence (Neuman-Lee and French, 2017; Field et al., 2023; Terry et al., 2023) that protein-based immunity may be upregulated with stress (but see Graham et al., 2012b; Strobel et al., 2015, Gao et al., 2017). Previous research also found that leukocytes, except neutrophils, are mobilized away from the bloodstream following an acute stressor and toward tissues (Dhabhar et al., 2012; Claunch et al., 2022).

Overall MKA between sexes corroborates earlier findings in this population that female red-eared sliders modulate immune function to accommodate reproduction (Terry et al., 2023). This study found this pattern holds for both cellular and protein-based killing. We predicted that the innate immune component contribution and overall response (i.e. killing ability) would differ by sex. Generally,

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Table 2. Summary of the main findings of the CORT response and MKA in red-eared sliders

Response variable	Prediction	Differences?			Biological implication
		Year	Sex	Time	
CORT Energy mobilization	1	No	Yes	Females: increase 0–120, 0–240 min Males: increase 0–120, 0–240 min	CORT mobilized following capture in both sexes but males show higher increase
Frozen+heat serum+BL MKA AMPs, NAbs, other heat-stable proteins	2	No	No	No	No contribution of heat-stable immune proteins
Frozen serum+BL MKA Complement, AMPs, NAbs, other proteins	2,3,4	No	Yes	Males: increase 0–120, 0–240 min	Contribution of heat-labile complement and NAbs increases following acute stressor in males
Fresh serum+BL MKA Leukocytes, complement, AMPs, NAbs, other proteins	2	No	Yes	No	Higher than frozen serum+BL MKA but increases not due to substantial increase in leukocyte contribution
CDK Fresh–frozen serum+BL MKA Relative contribution of cellular components	3	No	No	No	Paired with observed increases in frozen serum+BL MKA, no increase in cellular components following acute stressor
PCDK CDK/fresh serum+BL MKA Contribution of cellular components regardless of overall MKA	3,4	No	Yes	Females: decrease 0–120 min Males: decrease 0–120, 0–240 min	Duration of increased contribution of heat-labile immune components varies by sex

Response variables and associated immune components [leukocytes, complement, antimicrobial peptides (AMPs), natural antibodies (NAbs) and other proteins] were tested for differences between years (2022/2023), sexes (male/female) and time points (0, 120, 240 min) after capture. Prediction 1: CORT increases after capture; prediction 2: killing ability varies by treatment; prediction 3: component contribution varies across the stress response; prediction 4: response and contribution vary by sex.

males exhibited stronger CORT and immune responses than females. Even though protein-based immunity is upregulated following the acute stressor in both males and females, the duration of protein mobilization varies by sex and suggests that within-immune system tradeoffs occur when resources are limited, such as during heightened reproductive investment. Females mobilize protein-based killing following an acute stressor but cannot maintain this mobilization as long as males following the stress event. This may suggest that the sexes have different timings of negative feedback of the HPA axis during this period (Lattin and Kelly, 2020). Based on these differences and already-known immune–endocrine patterns during reproduction (French et al., 2007; Kerr et al., 2010; Neuman-Lee and French, 2017), mobilization of complement or heat-labile proteins may be critical to the acute stress response. The interpretation of the immune component contribution should be examined in light of where these components are produced and turnover time, as patterns may be time dependent.

Relatively little work has been done to understand immune mechanisms in reptiles, but the larger swath of work in mammals and other taxa provides insight into time-sensitive patterns that may follow an acute stress event. Previous work indicates that a redistribution of immune components occurs following acute stress and thus circulating leukocyte profiles may shift in the following hours (Dhabhar et al., 2012; Poller et al., 2022). Further, these patterns vary by leukocyte type; while neutrophils remain in circulating blood, other leukocytes migrate from the bloodstream to other tissues, including bone marrow, spleen and skin (Dhabhar et al., 2012; Poller et al., 2022). Total migration occurs over several hours; thus, circulating leukocyte counts may vary across this time period as migration occurs (Poller et al., 2022). Immune cell responses may also be influenced by stress duration and generation time. Heightened levels of neutrophils or heterophils are associated with long-term stressors (Davis et al., 2008), which suggests that increased leukocytogenesis occurs over a longer time scale. We did not assess leukocyte profiles in our study and future work should examine nuances in specific cellular contribution and mode of action following stress. Complement proteins, however, are continuously produced primarily in the liver and circulate in the bloodstream in inactive forms (Kolev and Kemper, 2017; Lubbers et al., 2017). Following activation, complement proteins are degraded or recycled for further use (Elvington et al., 2017). These proteins may take a shorter time to produce, readily occur in higher relative volume and be recycled more than leukocytes. However, these processes and mechanisms have not been examined in detail in reptiles.

Our results suggest that immune components are differentially regulated and likely have different costs. Maintaining immune defenses is energetically costly (Lochmiller and Dereenberg, 2000) and tradeoffs within the innate immune system may allow organisms to maintain defenses while modulating investment according to context such as reproduction. For example, female garter snakes exhibited component-specific costs of fecundity, in which the T-lymphocyte response, an adaptive immune component, was lower in gravid females than in non-gravid females (Palacios and Bronikowski, 2017). However, there is an overall lack of research investigating the presence of tradeoffs within ecoimmunological processes; we suggest that research focuses on nuances in organism survival strategies by delving into within-process tradeoffs.

Conclusions

We found that innate immune components are differentially prioritized rather than uniformly modulated. Red-eared slider turtles may mobilize heat-labile immune proteins following a standardized stressor, capture. However, the magnitude and duration of heightened investment in these proteins may be resource dependent and strategies between sexes may vary. Our study also highlights the importance of considering sample condition when making conclusions about immune function, as sample collection protocols influence activation/inactivation of immune components. The use of several sample manipulations in studies allows for a relatively direct quantification of the immune component contribution in functional assays. Relatively little is known about within-innate immune system tradeoffs and our

findings provide evidence of immune component-specific investment. Understanding nuances within immune function may inform questions in ecophysiology and conservation and provide insight into the evolution of the immune system across taxa.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: J.T., L.A.N.-L.; Data curation: J.T.; Formal analysis: J.T., V.R., L.A.N.-L.; Investigation: J.T., L.A.N.-L.; Methodology: J.T., L.A.N.-L.; Resources: V.R., L.A.N.-L.; Supervision: V.R., L.A.N.-L.; Writing – original draft: J.T.; Writing – review & editing: J.T., V.R., L.A.N.-L.

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Data and resource availability
Data are available upon reasonable request from the corresponding author.

ECR Spotlight
This paper has an ECR Spotlight interview with Jennifer Terry.

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