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RESEARCH ARTICLE

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Immune and stress physiology of two captively-housed tortoise species

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Abstract

Ecoimmunology affords us the ability to better understand immunological processes through consideration of external factors, such as the thermal microenvironment. This consideration is imperative when examining the immunological processes of ectothermic organisms like reptiles. Reptiles uniquely rely heavily on their innate immune function but remain poorly understood in immunological studies. In this study, we examined innate immunity in two zoo-housed tortoise species, the Indian star tortoise (*Geochelone elegans*, Schoepff, 1795) and northern spider tortoise (*Pyxis arachnoides brygooi*, Vuillemin & Domergue, 1972). Bacterial killing assays (BKAs) were optimized and used to assess the monthly immunocompetence of these tortoises to three different bacteria: *Escherichia coli*, *Salmonella enterica*, and *Staphylococcus aureus*. We evaluated differences in blood biochemistry values (lactate and glucose) among months and species as well as fecal corticosterone (CORT) between species. Lastly, we examined the potential influences of individual thermal microenvironments on bactericidal ability. Both *G. elegans* and *P. a. brygooi* demonstrated immunocompetence against all bacterial challenges, but only bactericidal ability against *E. coli* varied over months. Optimal BKA serum dilutions, blood glucose levels, and fecal CORT concentrations differed between the two species. Finally, there was evidence that the thermal microenvironment influenced the tortoises' bactericidal ability against *E. coli*. Through use of nonmodel organisms, such as tortoises, we are given insight into the inner workings of innate immunity and a better understanding of the complexities of the vertebrate immune system.

KEYWORDS

bacterial killing assay, corticosterone, ecoimmunology, innate immunity, reptile, zoo

1 | INTRODUCTION

Physiological processes continually influence and modulate one another in an interconnected, complex network. We can understand how these processes interact through the lens of ecoimmunology (Downs et al., 2014; Sheldon & Verhulst, 1996). Ecoimmunology examines an organism holistically within its environment and considers extrinsic and intrinsic stimuli (Demas & Nelson, 2012). However, wild individuals can pose a challenge because of researchers' inability to accurately determine potentially confounding factors, such as sex, age, reproductive status (Millspaugh & Washburn, 2004), and pathogen exposure (Flies et al., 2015). The use of captive populations in zoos and other similar institutions is invaluable to controlling these factors, building a better understanding of these processes, and, in turn,

isolating specific physiological relationships. (Kleiman, 1985). Captive institutions hold a wealth of information within their animal collections (Conde et al., 2019; Poo et al., 2022) and can provide the opportunity to better understand the ecoimmunology of understudied non-model organisms, such as reptiles.

Compared with mammalian models, reptilian species rely more heavily on their innate immune system over their adaptive (Boehm, 2012; Zimmerman, 2018, 2020; Zimmerman et al., 2010). The innate immune system is responsible for the initial, nonspecific immune response; this branch of the immune system is found in all animals (Litman et al., 2005; Zimmerman, Vogel & Edwards et al., 2010). In contrast, the adaptive branch only exists in vertebrates; it responds slower but develops an immunological memory, which allows it to react to specific pathogens (Murphy & Weaver, 2017).

To date, immunological and ecoimmunological research has focused largely on birds and mammals (e.g., Behringer et al., 2021; Ramirez-Otarola et al., 2019; Scalf et al., 2019), leaving other taxa less understood. Non-avian reptiles remain vastly understudied regarding both functionality and characterizations of their immune system (Buchmann, 2014; Field et al., 2022; Zimmerman, Vogel, & Bowden, 2010) despite an apparent increase in the spread and severity of reptile-specific diseases (e.g., onygenalean fungus, Woodburn et al., 2019; snake fungal disease, Lorch et al., 2016; Ranavirus, Lesbarrères et al., 2012).

A valuable ecoimmunological tool when examining the life-history of an organism is the bacterial killing assay (BKA). Even without complete mechanistic knowledge of the reptilian immune system, researchers can examine the functional innate immune response. Assessing the response of immune components in a blood sample, such as leukocytes (present only in nonfrozen samples; Matson et al., 2006), complement, and natural antibodies (i.e., spontaneous and polyreactive antibodies; Ochsenbein & Zinkernagel, 2000) to a pathogenic threat (Demas et al., 2011; Millet et al., 2007) can provide insight into differential immune capabilities between individuals, populations, and species. BKAs are an optimal assay to use when assessing tortoises' immunity as they possess these immune components (Goessling et al., 2016, 2017; Sandmeier et al., 2012; Zimmerman, Vogel, & Bowden, 2010). The innate immune system is dynamic and can distinctly respond to different pathogenic threats, thus functional assessments can be modulated to test different components of the immune system. Innate immunity is influenced by various life-history traits such as life-stage (e.g., Evans et al., 2015), sex (e.g., Flies et al., 2016), diet (e.g., Odewahn et al., 2022), species (e.g., Irene Tieleman et al., 2005; Matson et al., 2006), stress-state (e.g., French et al., 2010), body condition (e.g., Spence et al., 2020), and reproductive status (e.g., Neuman-Lee & French, 2017).

The immune system is energetically costly and often siphons resources away from other physiological processes (Demas, 2004; French et al., 2007; Hegemann et al., 2013; Lochmiller & Deerenberg, 2000). To manage these allocations, organisms use trade-offs, a conflicting demand between energy-costly processes (Zera & Harshman, 2001), which are often modulated by glucocorticoids, especially during unpredictable events (i.e., stress; McEwen & Wingfield, 2003). The activation of the stress response often has profound effects on the immune response (Neuman-Lee & French, 2014; Sapolsky et al., 2000). Depending upon stress type, circulating glucocorticoids may enhance (i.e., acute stress) or impede (i.e., chronic stress) the organism's immune system (Dhabhar & McEwen, 1997; Sapolsky et al., 2000). Fecal glucocorticoids are useful when examining stress physiology (Sheriff et al., 2010). Unlike plasma levels, which only represent circulating glucocorticoid levels at discrete timepoint, fecal metabolite levels reflect average circulating glucocorticoid levels over a longer length of time (Harper & Austad, 2000; Touma & Palme, 2005). Other circulating biochemical parameters may also be considered in relation to glucocorticoid concentrations as indicators of energetic shifts. These parameters may include blood lactate (da Fonseca et al., 2020; Goessling & Mendonça, 2021), an intermediate product of anaerobic respiration (Allen & Holm, 2008) used in gluconeogenesis (Rui, 2014), and blood glucose (Neuman-Lee et al., 2020), a metabolic fuel (Rui, 2014). Glucocorticoid hormones influence physiological processes including lactate release from muscles (Neave, 2007) and circulating glucose concentrations (Sapolsky et al., 2000), making the measurement of these three factors important for providing insight into physiological energy allocations of the organism.

When examining reptilian physiological processes, including immune responses, the influences of the thermal environment must be considered due to their ectothermic nature. Most physiological processes have an optimal range of functioning, which are often species-specific (Butler et al., 2013) and impeded when the organism's body temperature lowers or raises beyond the boundaries of this range (Huey & Stevenson, 1979). This can cause the organism increased stress (Fabrício-Neto et al., 2019; Jessop et al., 2016; Telemeco & Addis, 2014). To account for this, ectotherms may either actively or passively adjust their body temperatures (thermoregulate or thermoconform, respectively) to remain in physiologically optimal ranges. Each of these strategies presents its own costs and benefits to the organism (i.e., predation risk, high ambient temperature, and resource competition; Huey & Slatkin, 1976). Reptilian thermoregulation may include strategies such as behavioral fever via basking (Goessling et al., 2017) or microhabitat selection (Smith, 1979). However, these mechanisms are dependent on the temporal and spatial variability within the organism's thermal environment (Stevenson, 1985). Previous studies have shown that the chelonian innate immune system has a relationship with temperature (Zimmerman et al., 2017) and has species-specific optimal ranges of functioning (Adamovich, Baker, Merchant, & Allender, 2020; Baker et al., 2019). This is likely because cellular production rates of many immune components must match the experienced thermal environment (Goessling et al., 2019).

The purpose of this study was to assess the differences between the immunocompetences of two understudied tortoises from a zoo collection, the Indian star tortoise (*Geochelone elegans*, Schoepff, 1795) and the northern spider tortoise (*Pyxis arachnoides brygooi*, Vuillemin & Domergue, 1972) to three bacteria: *Escherichia coli*, *Salmonella enterica*, and *Staphylococcus aureus*. The two tortoise

species are maintained under differing housing regimes; *G. elegans* is seasonally shifted between indoor and outdoor enclosures while *P. a. brygooi* is housed indoors year-round. *Geochelone elegans* is an IUCN Red List Vulnerable species (Choudhury et al., 2020) endemic to the dry regions (i.e., scrub forests, grasslands, and coastal scrublands) of south-eastern, southern, and north-western India; northern and eastern Sri Lanka; and the easternmost parts of Pakistan (Daniel, 1983). *Pyxis arachnoides brygooi* is an IUCN Red List Critically Endangered species (Leuteritz & Walker, 2020) endemic to the dry coastal forests of southern Madagascar (Pedrono, 2008). We predicted species-specific differences in the innate immune responses, fecal glucocorticoid metabolite concentrations, and blood biochemistry values as measured by blood lactate and blood glucose. Furthermore, we predicted immune capability would vary within each tortoise species in relation to bacterial challenge. Finally, we examined the influence of thermal patterns in the microenvironment at differing temporal resolutions on the tortoise's immunocompetence. We predicted that the thermal microenvironment in the week before the blood sampling would be the most influential time period for immunocompetence.

2 | MATERIALS AND METHODS

2.1 | Study species and site

Tortoises used in this study were adult *G. elegans* and *P. a. brygooi* housed at the Memphis Zoo (Memphis, Tennessee, USA). These tortoises were maintained in two separate off-exhibit housing groups: *G. elegans* (male = 8, female = 1) and *P. a. brygooi* (male = 9, female = 1). We identified the sex of individuals of both species by visually inspecting tail morphology. If the tail was larger and extended past the anal scute, it was classified as male. If the tail was smaller and did not extend past the anal scute, it was classified as female. One male from the *P. a. brygooi* group was transferred to another institution in July 2021 and thus has an incomplete data set. The *G. elegans* group seasonally moved between their off-exhibit outdoor and off-exhibit indoor housing spaces. In 2020, the *G. elegans* group was outdoors at the start of the study (August 2020), then moved indoors on September 30, 2020. They were moved back outdoors on May 16, 2021, and finally moved back indoors on September 23, 2021. The *P. a. brygooi* group was permanently housed off-exhibit indoors. The tortoise species in this study were selected due to their available sample size within the Memphis Zoo's herpetological collection. All work was conducted under Memphis Zoo IACUC #2020-3.

2.2 | Blood sample collection

We collected whole blood samples from all individuals at the beginning of each month from August 2020–December 2021 (average of 30.125 days between sample periods). Samples (<0.5 ml; <0.15% body weight for both species) were taken from the dorsal coccygeal vein using a non-heparinized 1 ml syringe outfitted with a 26 g × 5/8″ needle. Target sampling time was confined to within three minutes of removal from the enclosure to avoid confounding effects of the acute stress response (Romero & Reed, 2005). However, due to limited sample size, all samples were included (statistical tests revealed no effect on time of sampling on any metrics). Samples were centrifuged at 10,000 rpm until fractionation and serum was immediately decanted into separate 0.5 ml conical microcentrifuge tubes via pipette. Samples were then maintained on ice until arrival at Arkansas State University (<10 h later).

2.3 | Blood biochemistry

Blood biochemistry has been measured in other studies of chelonian physiology (Lewbart et al., 2018; Pereira et al., 2012; Reese et al., 2002). Parameters such as blood glucose and blood lactate levels are beneficial when looking at the health state of an organism (Chaffin et al., 2008; Lewbart et al., 2014) and can assist in building a more complete picture of immune function by allowing a glimpse into how energy may mobilize. Using the whole blood, lactate and glucose levels were read for each sample within 1 min of sample collection using a Nova Lactate Plus hand-held analyzer (Nova-Biomedical) and Accu-Chek® Performa hand-held analyzer (Roche Diagnostics), respectively.

2.4 | Sample storage

Following transport, all serum samples were stored at 4°C for short-term storage before the first round of BKAs within 6 days of collection (see below for details). The remaining portions of samples were then stored at −20°C until the second round of BKAs (within 30–60 days of collection).

2.5 | Bacterial killing assay optimizations

Complement is one of the initial defense mechanisms of innate immunity and is a cascade of proteins that facilitates pathogen removal via opsonization (Murphy & Weaver, 2017). Complement can be measured using different bacterial species (Demas et al., 2011). Complement-independent immune responses can be assessed through the use of Gram-positive bacteria, such as *Staphylococcus aureus* which have a thick peptidoglycan layer that hinders complement activation (Rautemaa & Meri, 1999). Conversely, through use of Gram-negative bacteria, such as *Escherichia coli* and *Salmonella* sp., complement-dependent pathways are assessed (Rautemaa & Meri, 1999). Gram-negative bacteria have outer membranes primarily composed of lipopolysaccharides that can activate complement (Rautemaa & Meri, 1999).

All assays were conducted following procedures established by French and Neuman-Lee (2012). BKAs were optimized for *S. aureus* in

TABLE 1 Optimization results outlining selected serum volumes and states for each tortoise species and months analyzed.

Species	Bacteria	Volume	Dilution[a]	Serum state	Months analyzed
Indian Star Tortoise (*Geochelone elegans*) 	*Escherichia coli* ATCC 8739	3 µl	1:5	Live	February–December 2021
	Salmonella enterica serovar Typhimurium ATCC 13311	5 µl	5:13	Live	February–December 2021
	Staphylococcus aureus ATCC 6538	6.5 µl	6.5:11.5	Frozen-thaw	August 2020–December 2021
Northern Spider Tortoise (*Pyxis arachnoides brygooi*) 	*Escherichia coli* ATCC 8739	3 µl	1:5	Live	February–December 2021
	Salmonella enterica serovar Typhimurium ATCC 13311	11 µl	11:7	Live	February–December 2021
	Staphylococcus aureus ATCC 6538	6.5 µl	6.5:11.5	Frozen-thaw	August 2020–December 2021

[a]Dilution of serum to either PBS (*S. aureus* assays) or CO_2-Independent media (*E. coli* and *S.* Typhimurium assays)

November 2020 and *E. coli* and *S.* Typhimurium from November 2020–February 2021 (Table 1). Assays were validated and optimized for each tortoise species for the bacteria *Escherichia coli* (EPower™ Microorganisms, ATCC 8739, MicroBioLogics), *Salmonella enterica enterica* serovar Typhimurium (EPower™ Microorganisms, ATCC 13311, MicroBioLogics), and *Staphylococcus aureus aureus* (EPower™ Microorganisms, ATCC 6538, MicroBioLogics). All bacteria suspensions were diluted to 1.0E + 05 CFU. We used increasing amounts of serum from randomly selected individuals to identify the serum volume(s) that resulted in an average of 50% killing for each bacterium and tortoise species. Initially, optimization assays were conducted using frozen-thawed serum samples and sterile phosphated buffer solution (PBS). Due to the lack of killing against *E. coli* and *S.* Typhimurium by the serum from both *G. elegans* and *P. a. brygooi*, the assays were modified to use live (unfrozen) serum and L-glutamine (Thermo Fischer Scientific) supplemented CO_2-Independent medium (Thermo Fischer Scientific) in place of PBS to further promote optimal growth. These optimizations led to identifying the serum dilution for each tortoise species and bacterial strain to provide the widest range of killing. The serum dilutions that achieved the closest average to 50% killing (thus allowing for individual variation) were selected for the sample assays (see below; Table 1).

2.6 | Sample bacterial killing assays

Samples were plated in triplicate in sterile round-bottom 96-well plates; if serum volume was insufficient, samples were plated either in singlicate or duplicate accordingly. Six positive (bacteria and broth with no serum to assess bacterial growth) and six negative control wells (only PBS or CO_2-Independent medium and broth to assess plate contamination) were included on each plate. Following the addition of serum and either PBS or CO_2-Independent media, 6 µl of bacteria suspension was added to all wells except negative controls. Plates were incubated at 37°C for 30 min and then 125 µl of sterile tryptic soy broth was added to all wells. Absorbance was read at 340 nm using an ELx 808 Microplate reader (Biotek Instruments, Inc.), and then plates were incubated for 12 h at 37°C before final absorbance was read. Immunocompetence for each sample was calculated as a percentage, all negative scores were adjusted to zero, and all positive scores over 100 were adjusted to 100.

2.7 | Fecal collection

Fecal samples were collected monthly, following the completion of blood sampling. Tortoises were placed in a shallow warm water bath for an average of 45 min. This procedure typically stimulates defecation in chelonian species (AH, personal observation). Samples were removed immediately after production to prevent degradation or coprophagy, drained of excess water, and placed in individual sterile tubes. Fecals produced before the water baths were caught by gloved hand and placed in an individual sterile tube. Samples were stored on ice during transportation (<10 h) and then stored at −80°C.

2.8 | CORT extraction

Corticosterone (CORT), the primary glucocorticoid in reptiles (Dickens & Romero, 2013), was extracted following the methods outlined by Shideler et al. (1994) and Bauman and Hardin (1998). Fecals were homogenized and any large particles of undigested food were removed. A 0.5 g sample was removed from the resulting homogenate and transferred to a sterile 15 ml conical tube. To extract the steroid, 5 ml of a 50% methanol and 50% PBS solution was added to each tube. Tubes were then agitated on a plate vortexer at 200 rpm for 17 h. All tubes were allowed 1 h to settle

before decanting the supernatant into a new 15 ml conical tube. These new tubes were then centrifuged for 1 h at 4000 rpm at 4°C. The resulting supernatant was decanted into 0.6 ml micro-centrifuge tubes and stored at −20°C until use in the radioimmunoassay (within 30 days). The remaining fecal samples were dried under a fume hood to be used in sample concentration corrections (see below).

2.9 | CORT radioimmunoassay

Fecal CORT metabolite concentrations were determined using radioimmunoassay protocols developed by Neuman-Lee et al. (2015). The assay was run using all samples at the end of the collection period to reduce assay variation. Individual samples of both tortoise species were selected to complete validations and assess binding-interference and parallelism before running all samples. A 10-point standard curve using known concentrations of CORT was run in triplicate. All samples were run in duplicate. Final CORT concentrations (hormone concentration per gram of fecal matter) were determined by correcting for dilution factor and dry fecal mass. Intra-assay variation was 6.1% and precision was 97.8%.

2.10 | Temperature loggers

Temperature loggers (iButtons; DS 192 1G-F5# Thermochron) can be used in a multitude of environments to assess changes in temperature by recording and storing data at regular intervals (Fawcett et al., 2019; Mittra et al., 2013) and have been successfully deployed in studies using small chelonian species (Converse & Savidge, 2003; Currylow et al., 2015). Loggers were deployed from June–December 2021. All loggers were wrapped in a single layer of sealing film and affixed using cloth medical tape to the right dorsal carapace of each tortoise. Readings were recorded every 30 min during deployment. Monthly, loggers were retrieved to download data, reset to avoid rollover, and redeployed onto the same individuals.

2.11 | Analysis

All statistical analyses were performed using R (R Development Core Team, 2021), and $\alpha = 0.05$ was used for all tests. Due to the small sample size of this study, we were unable to control for the differences between the species in relation to the difference in the housing conditions.

2.12 | Blood biochemistry analysis

To determine any species-specific differences in energy mobilization we compared the blood lactate and glucose between the two species. Normality of these data was initially assessed using a Shapiro–Wilk test. If data were normally distributed, a two-tailed two-independent-sample *t*-test was selected. If at least one variable was not normally distributed, a two-sided Mann–Whitney *U* test was selected. The Nova Lactate Plus hand-held analyzer's test range was 0.3 to 25.0 mmol/L, thus values that were under this range (i.e., a reading of "LOW") were arbitrarily adjusted to 0.01 mmol/L to be included in analysis. When the "LOW" values were all changed to 0.29 mmol/L, the final statistical outcomes were similar.

2.13 | Bacterial killing assays analysis

All data were initially assessed for homogeneity of variances using a Bartlett test. BKA data for all bacteria were analyzed separately for each tortoise species. Assay data for *E. coli* and *S.* Typhimurium were analyzed for the months February–December 2021 and *S. aureus* for the months August 2020–December 2021. To assess differences in bactericidal ability among months, a Kruskal–Wallis rank sum test paired with a Dunn's Multiple Comparison Test using package "FSA" (Ogle et al., 2022) was selected if data were homoscedastic, or a Welch's analysis of variance (ANOVA) paired with a Games-Howell post hoc test using package "rstatix" (Kassambara, 2020) if heteroscedastic.

2.14 | CORT radioimmune assay analysis

Because there were too few fecal samples across months to test for monthly differences, we instead combined all fecal CORT data to determine any general species-specific differences in CORT between *G. elegans* and *P. a. brygooi*. Normality of these data was initially assessed using a Shapiro–Wilk test. If data were normally distributed, a two-tailed two-independent-sample *t*-test was selected. If at least one variable was not normally distributed, a two-sided Mann–Whitney *U* test was selected. To examine if housing environment, indoors versus outdoors, affected *G. elegans* fecal CORT levels, normality of these data was initially assessed using a Shapiro–Wilk test. If data were normally distributed, a two-tailed two-paired-sample *t*-test was selected. If at least one variable was not normally distributed, a two-sided Wilcoxon Signed-Rank test was selected.

2.15 | Temperature loggers analysis

Logger data were analyzed based on day (0700h–1830h) and night (1900h–0630 h) periods for the timeframes of Week 1, Weeks 1–2, and Month. Weeks were established working backward in 7-day increments from the logger retrieval dates. All logger data were visually assessed for disruptions in recording (i.e., logger removal, data rollover, etc.). All data with disruptions were removed from analysis. Data collected following the 1200 h timepoint on the day before retrieval were also removed to create even 24-h day periods. For ease of modeling, BKA results were adjusted by adding 0.001 to remove all zeros. BKA results were selected as the response variable.

To examine the relationship between temperature and immunocompetence, BKA and logger data were incorporated into generalized linear mixed models (Gamma error distribution, log link) and run using R package "lme4" (Bates et al., 2014) for each of the bacteria and each tortoise species for the time periods: Week 1, Weeks 1–2, and Month, to examine the temporal resolution of microenvironment temperature data and immunocompetence. Week 1 Day Maximum ($W1D_{max}$), Week 1 Day Minimum ($W1D_{min}$), Week 1 Day Average ($W1D_{avg}$), Week 1 Night Maximum ($W1N_{max}$), Week 1 Night Minimum ($W1N_{min}$), Week 1 Night Average ($W1N_{avg}$), Week 2 Day Maximum ($W2D_{max}$), Week 2 Day Minimum ($W2D_{min}$), Week 2 Day Average ($W2D_{avg}$), Week 2 Night Maximum ($W2N_{max}$), Week 2 Night Minimum ($W2N_{min}$), Week 2 Night Average ($W2N_{avg}$), Month Day Maximum (MD_{max}), Month Day Minimum (MD_{min}), Month Day Average (MD_{avg}), Month Night Maximum (MN_{max}), Month Night Minimum (MN_{min}), Month Night Average (MN_{avg}) were scaled and then included as fixed effects after removal of highly correlating variables, and tortoise individual ID were included as a random effect. Before model construction, the covariance of the fixed effects was assessed for each of the two tortoise species' model sets (i.e., Week 1, Weeks 1–2, and Month) using a correlation matrix. Correlations greater than 0.7 were considered highly correlated and the variable with highest correlations was removed from each highly correlated pair (Lehmann, 1989).

Multicollinearity between fixed effects was assessed again through calculation of the variance inflation factor (VIF) for each model predictor (James et al., 2013) using R package "car" (Fox & Weisberg, 2019) before model fitting. Fixed effects with VIFs higher than four were removed beginning with the highest, the model was re-run and repeated until all VIFs were under four (James et al., 2013). Final fixed effects were selected for each model following the removal of all collinear variables.

A null model including no fixed effects was included for comparison in each analysis. Models were evaluated and ranked by means of Akaike Information Criterion correct for small samples (AIC_c; Burnham & Anderson, 2010) using package "wiqid" (Meredith, 2019) with lower AIC_c indicating a model better balancing data description and complexity (Symonds & Moussalli, 2011). We calculated the ΔAIC_c by subtracting the lowest overall AIC_c value from each AIC_c value in the analysis set and then calculated the Akaike weights (ω_i) of each model (Symonds & Moussalli, 2011). All models with a ΔAIC_c of less than or equal to two were considered parsimonious. Model validity was assessed using diagnostic plots to view residuals and the influence of outliers.

3 | RESULTS

3.1 | Blood biochemistry

All blood chemistry data failed Bartlett's test of homogeneity of variances ($p < 0.002$) and none of the data were normally distributed ($p < 0.001$), thus Welch's ANOVAs were used.

There was no difference in lactate among months in *G. elegans* ($F_{16,46.30} = 1.24$, $p = 0.274$) or in *P. a. brygooi* ($F_{16,\ 39.97} = 1.85$, $p = 0.058$). A Mann–Whitney *U* test indicated there were no differences ($W = 8717.5$, $p = 0.429$) between the blood lactate levels of the two species. There was no difference in glucose between months in *G. elegans* ($F_{16,47.42} = 1.39$, $p = 0.187$) or in *P. a. brygooi* ($F_{16,39.80} = 1.65$, $p = 0.101$). A Mann–Whitney *U* test ($W = 14910$, $p < 0.001$) indicated *G. elegans* glucose levels (median = 70 mg/dL) were higher than *P. a. brygooi* (median = 49.5 mg/dL).

3.2 | Bacterial killing assays

A serum dilution of 1:5 (serum to CO_2-Independent Media) and of 6.5:11.5 (serum to PBS) were selected for assays using *E. coli* and *S. aureus*, respectively, for both tortoise species. Serum dilutions of 5:13 and 11:7 (serum to CO_2-Independent Media) were selected for assays using *S.* Typhimurium for *G. elegans* and *P. a. brygooi*, respectively (Table 1).

Geochelone elegans serum killing of *E. coli* and *S. aureus* failed Bartlett's test of homogeneity of variances ($p < 0.001$). Bactericidal ability against *E. coli* ($F_{10,28.75} = 152.92$, $p < 0.001$) and against *S. aureus* ($F_{16,28.75} = 42.13$, $p < 0.001$) differed among months. Post hoc analysis for *E. coli* revealed the following months were from one another: February–April ($p = 0.005$), March–April ($p = 0.045$), April–May ($p < 0.001$), April–July ($p = 0.004$), April–August ($p = 0.007$), April–September ($p < 0.001$), April–November ($p < 0.001$), and April–December ($p = 0.009$). Post hoc analysis for *S. aureus* revealed no differences among months ($p > 0.068$ for all pairwise comparisons) when accounting for the adjustment of *p*-values. *Geochelone elegans* serum killing of *S.* Typhimurium passed Bartlett's test of homogeneity of variances ($p = 0.139$). There was no difference in bactericidal ability against *S.* Typhimurium among months ($\chi^2_{10} = 17.91$, $p = 0.057$; Figure 1).

Pyxis arachnoides brygooi serum killing of *E. coli* and *S.* Typhimurium failed Bartlett's test of homogeneity of variances ($p < 0.001$). There was a significant difference in bactericidal ability against *E. coli* ($F_{10,\ 26.79} = 42.13$, $p = 0.004$) and against *S.* Typhimurium ($F_{10,26.79} = 25.67$, $p = 0.002$) among months. Post hoc analysis for *E. coli* revealed the months April–May were different from one another ($p = 0.023$). Post hoc analysis for *S.* Typhimurium revealed no differences among months when accounting for the adjustment of *p*-values ($p > 0.060$ for all pairwise comparisons). *Pyxis arachnoides brygooi* serum killing of *S. aureus* passed Bartlett's test of homogeneity of variances ($p = 0.703$). There was no difference in bactericidal ability against *S. aureus* among months ($\chi^2_{10} = 18.47$, $p = 0.297$; Figure 2).

3.3 | CORT radioimmune assays

Geochelone elegans CORT concentrations were not normally distributed ($W = 0.318$, $p < 0.001$) while *P. a. brygooi* CORT

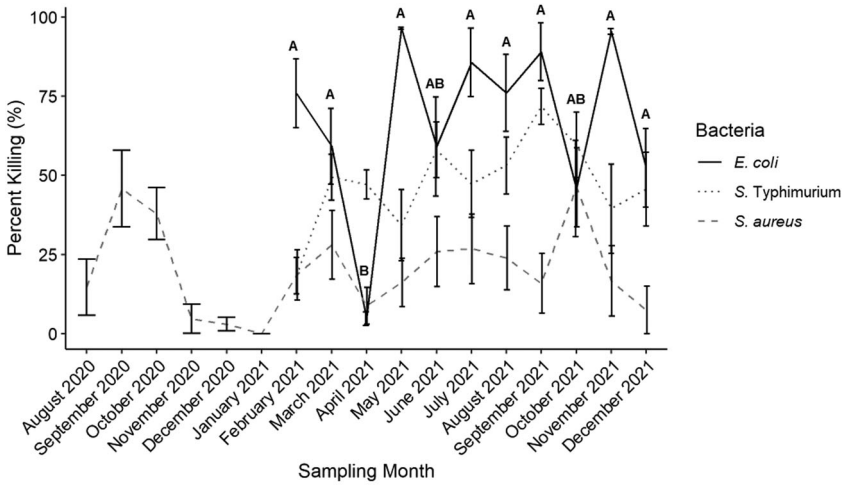

FIGURE 1 Bactericidal ability of *Geochelone elegans*. Bactericidal ability against *E. coli* varied monthly. Bactericidal ability did not vary monthly for either *S.* Typhimurium or *S. aureus*. Months significantly different from one another are indicated with different letters.

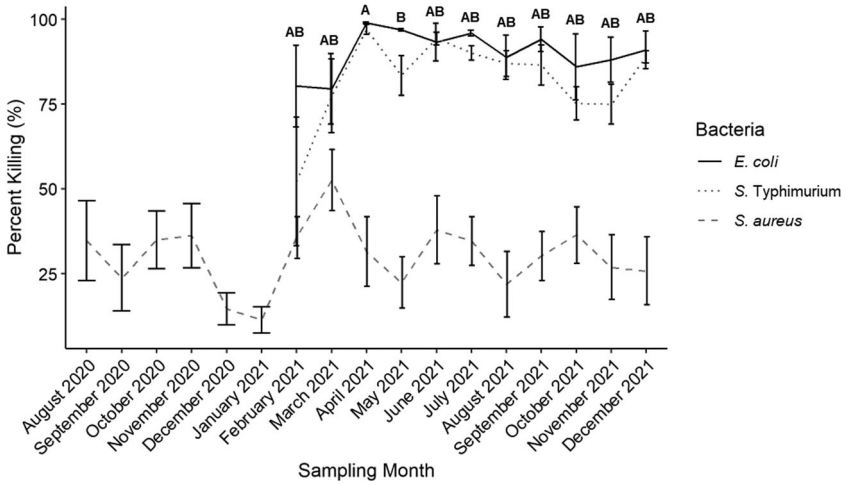

FIGURE 2 Bactericidal ability of *Pyxis arachnoides brygooi*. Bactericidal ability against *E. coli* varied monthly. Bactericidal ability did not vary monthly for either *S.* Typhimurium or *S. aureus*. Months significantly different from one another are indicated with different letters.

concentrations were normally distributed ($W = 0.946$, $p = 0.200$). A Mann-Whitney U test indicated *G. elegans* fecal CORT metabolite levels (Median = 0 ng/ml) were lower than *P. a. brygooi* fecal CORT levels (Median = 0.675 ng/ml; $W = 115$, $p < 0.001$). *Geochelone elegans* CORT concentrations were not normally distributed for either the indoor ($W = 0.374$, $p < 0.001$) or outdoor ($W = 0.273$, $p < 0.001$) months. Fecal CORT metabolite levels for the indoor months were not different from the outdoor months ($V = 35$, $p = 0.4755$).

3.4 | Temperature logger model selection

Fixed effects selected for each model following the removal of co-linear variables are outlined in Table 2. For the models *G. elegans* (*S.* Typhimurium; *S. aureus*) and *P. a. brygooi* (*S.* Typhimurium; *S. aureus*), the NULL model was the best fit model. For the *G. elegans* (*E. coli*) model, the NULL and Month models were parsimonious and selected as the best fit models. For *P. a. brygooi* (*E. coli*) the Month model was the best fit model; within this model, the daily max temperature was influential ($p = 0.001$).

4 | DISCUSSION

The present study examined physiological differences between two species of tortoise and the variation of those differences across a year in a captive environment. Species-specific differences were noted in both blood glucose levels and fecal CORT metabolite levels. Moreover, *G. elegans* experienced no difference in stress, as measured by fecal CORT, between their indoor and outdoor housing environments. Both tortoise species were able to exhibit immunocompetence against all bacteria tested; however, only bactericidal ability against *E. coli* significantly varied by month. The bactericidal abilities of *G. elegans* against *S. aureus* and *P. a. brygooi* against *S.* Typhimurium were moderate and there were no monthly differences. Despite experiencing different thermal microenvironments, similar monthly bactericidal trends were observed for both tortoise species. However, models examining bactericidal ability against *E. coli* and thermal microenvironments over a monthly period showed a potential relationship.

Both the *E. coli* and *S. enterica* BKAs produced no adequate bactericidal ability for either tortoise species when using frozen-thaw

TABLE 2 Model and Akaike Information Criterion corrected for small samples (AIC_c) information for the relationship between immunocompetence and temperature variables for all six model groupings

Species	Bacteria	Model	Fixed effects[a]	AIC_c	ΔAIC_c	ω_i[b]
Indian Star Tortoise (*Geochelone elegans*) *n* = 9	*Escherichia coli* ATCC 8739	**NULL**[c]	**Intercept**	**473.77**	**0.00**	**0.6351**
		Month[c]	**MD_{max} + MN_{max} + MN_{min}**	**475.34**	**1.57**	**0.2893**
		Weeks 1–2	$W1D_{min}$ + $W1D_{avg}$ + $W1N_{max}$ + $W2D_{avg}$	479.17	5.41	0.0426
		Week 1	$W1D_{max}$ + $W1D_{min}$ + $W1N_{max}$ + $W1N_{min}$	479.68	5.92	0.0330
	Salmonella enterica serovar Typhimurium ATCC 13311	**NULL**[c]	**Intercept**	**426.35**	**0.00**	**0.9489**
		Month	MD_{max} + MN_{max} + MN_{min}	433.14	6.79	0.0318
		Week 1	$W1D_{max}$ + $W1D_{min}$ + $W1N_{max}$ + $W1N_{min}$	435.47	9.12	0.0099
		Weeks 1–2	$W1D_{min}$ + $W1D_{avg}$ + $W1N_{max}$ + $W1N_{min}$	435.57	9.23	0.0094
	Staphylococcus aureus ATCC 6538	**NULL**[c]	**Intercept**	**109.53**	**0.00**	**0.7729**
		Week 1	$W1D_{max}$ + $W1D_{min}$ + $W1N_{max}$	113.49	3.96	0.1067
		Month	MD_{max} + MN_{max} + MN_{min}	114.48	4.95	0.0651
		Weeks 1–2	$W1D_{min}$ + $W1D_{avg}$ + $W1N_{max}$ + $W1N_{min}$	114.81	5.27	0.0553
Northern Spider Tortoise (*Pyxis arachnoides brygooi*) *n* = 10	*Escherichia coli* ATCC 8739	**Month**[c]	**MD_{max} + MD_{min} + MN_{avg}**	**388.77**	**0.00**	**0.7407**
		NULL	Intercept	391.29	2.52	0.2101
		Week 1	$W1D_{min}$ + $W1N_{max}$	395.18	6.41	0.0301
		Weeks 1–2	$W1D_{min}$ + $W1N_{max}$ + $W2D_{max}$	396.08	7.32	0.0191
	Salmonella enterica serovar Typhimurium ATCC 13311	**NULL**[c]	**Intercept**	**373.94**	**0.00**	**0.7312**
		Week 1	$W1D_{min}$ + $W1N_{max}$	376.73	2.79	0.1815
		Weeks 1–2	$W1D_{min}$ + $W1N_{max}$ + $W2D_{max}$	379.37	5.43	0.0483
		Month	MD_{max} + MD_{min} + MN_{avg}	379.80	5.86	0.0390
	Staphylococcus aureus ATCC 6538	**NULL**[c]	**Intercept**	**323.87**	**0.00**	**0.8655**
		Week 1	$W1D_{min}$ + $W1N_{max}$	328.61	4.74	0.0809
		Weeks 1–2	$W1D_{min}$ + $W1N_{max}$ + $W2D_{max}$	330.61	6.74	0.0298
		Month	MD_{max} + MD_{min} + MN_{avg}	331.05	7.18	0.0238

Note: All models were generalized linear mixed models (Gamma error distribution, log link). Groupings were arranged with the best fit model presented first and all bolded rows within a grouping indicate the best overall model and all parsimonious model(s) within 2 ΔAIC_c values (Difference between AIC_c value and overall lowest group AIC_c).

[a]Fixed effects chosen after adjusting for collinearity.

[b]AIC_c weight.

[c]Best model(s) of the grouping including parsimonious models.

serum. When live serum was used instead of frozen-thawed serum for the *E. coli* and *S. enterica* assays, bactericidal ability increased indicating the reliance on live cell immune function in this context. BKAs in other chelonian species have successfully used frozen-thawed serum (e.g., Beck et al., 2017). The use of frozen-thaw samples appears to have species-specific effects, with freezing being more detrimental to the bactericidal ability of some species than others (Jacobs & Fair, 2016), as freezing serum samples results in samples that are acellular (Tan et al., 2019) but the integrity of immune proteins remains (Demas et al., 2011). Our results demonstrate that the leukocytes (e.g., monocytes, azurophils, and heterophils in reptiles; Jurd, 1994) are likely the primary innate mechanism responsible for combatting Gram-negative pathogens, such as *E. coli* and *S. enterica*, in *G. elegans* and *P. a. brygooi*. In contrast, frozen-thaw serum displayed adequate bactericidal ability against Gram-positive *S. aureus* for both tortoise species. It appears likely that the noncellular components (e.g., antimicrobial peptides) present in these tortoises' serum appear highly competent at clearing *S. aureus* infections.

In reptilian species, infections caused by Gram-positive bacteria are typically less common than those caused by Gram-negative bacteria (Jacobson, 1987), although very little research has been conducted in wild populations. There is evidence that different reptiles may have differential immunocompetence against Gram-positive and Gram-negative bacteria. LaVere et al. (2021) noted the reduced bactericidal ability of American alligators (*Alligator*

mississippiensis) against *Salmonella typhimurium* may be caused by the lack of exposure to this pathogen under natural conditions. In our current study, prior pathogen exposure does not impact our results because BKAs are a measure of innate immunity (Demas et al., 2011). However, a lack of exposure to a particular pathogen over time may influence responses as the organism's immune response evolves in a manner that prioritizes removing commonly encountered pathogenic threats. Resulting in reduced immune ability against more infrequently encountered threats.

A study by Di Ianni et al. (2015) found Gram-negative bacteria are the predominant isolates from the conjunctiva of aquatic turtles. Aquatic species most likely encounter Gram-negative bacteria frequently due to their common presence in aquatic environments (Rhodes & Kator, 1988). Consequently, these chelonian species may have prioritized immune defenses against these bacteria. Reduced ability to effectively clear a Gram-positive bacterial challenge has been documented in freshwater turtle species such as the alligator snapping turtle (*Macrochelys temminckii*) and common snapping turtle (*Chelydra serpentina*; Baker et al., 2019) as well as the eastern box turtle (*Terrapene carolina carolina*; Adamovich et al., 2020). Though not a fully aquatic species, *T. c. carolina* still relies on wet environments (e.g., ephemeral ponds and wetlands) for its life-history (Donaldson & Echternacht, 2005) and is phylogenetically related to many aquatic species. In comparison, Gram-positive isolates were more common conjunctiva isolates in tortoises (Di Ianni et al., 2015). Gram-positive bacteria can survive in a variety of dry environments (Chaibenjawong & Foster, 2011). Consequently, terrestrial reptiles such as tortoises may encounter Gram-positive bacteria like *S. aureus* more frequently. More research involving non-model species such as reptiles can be instrumental when determining the impact of environment on innate immunity and can allow greater accuracy when attempting to isolate mechanisms or understand the root of observed variation in immune response.

The volume of serum required to achieve adequate killing of *S. enterica* was much higher for *P. a. brygooi* than *G. elegans*. A study by Matson et al. (2006) noted significant variation in bactericidal ability against *E. coli* among five different bird species. The authors hypothesized that the variation may suggest differing degrees of innate immune engagement when faced with *E. coli* infections and may result from compositions of circulating innate components being dictated on a species-specific basis. This hypothesis offers a possible explanation for why the serum volume required for *P. a. brygooi* was more than twice that needed for *G. elegans*. We propose antimicrobial peptides may differ along with serum concentration between the species. These peptides can kill Gram-positive bacteria (Hancock & Scott, 2000) and do not denature in the initial freeze-thaw process. The use of more mechanism-specific immune assays (Reviewed in Demas et al., 2011) may be more helpful in creating a more precise analysis of the difference in bactericidal ability between *G. elegans* and *P. a. brygooi* with respect to innate mechanism composition. These findings also underscore the need to optimize BKAs for every species.

Neither blood lactate nor blood glucose varied monthly for either species. However, there are apparent species-specific differences in energy use. Blood glucose levels varied between species, with median blood glucose levels of *G. elegans* higher than those of *P. a. brygooi*. Because CORT can induce gluconeogenesis (Kuo et al., 2015; Lin et al., 2004), we might expect that elevated fecal CORT would be associated with elevated glucose levels. We saw the opposite relationship when comparing the species-specific relationships. However, because we did not measure blood CORT, which may have provided a better comparison. It is unlikely that this difference in glucose is in response to different diets as both species are fed the same diet on the same schedule. Regarding fecal CORT metabolite levels, the median level for *G. elegans* was lower than that of *P. a. brygooi*. The relationship between CORT and glucose levels is not direct (Hudson et al., 2020; Neuman-Lee et al., 2020). Rises in CORT induced by acute stress have been shown to produce a variety of trends in glucose levels in other reptilian models (Hudson et al., 2020; Neuman-Lee et al., 2020). In response to chronic stress, daytime glucose did not change in an avian model (*Sturnus vulgaris*; Cyr et al., 2007). Furthermore, CORT and glucose relationship is nuanced and is impacted by factors such as energetic stores (Neuman-Lee et al., 2020).

CORT is a likely mediator of physiological trade-offs, the extent to which is dependent on available energy (French et al., 2007). Moreover, it likely influences vertebrate life-history strategies (Hau et al., 2010). More research as to the effects of endocrine control mechanisms, such as CORT, on organismal response to the environment must be done to better understand the differences among life-history strategies (Ricklefs & Wikelski, 2002). Though there was no observed difference in CORT between the indoor and outdoor months, it is possible that levels of CORT were lower in *G. elegans* due to their husbandry regime that includes outdoor naturalistic housing, but we were unable to observe this due to our limited data set. However, the more likely explanation is an innate species-specific difference on average basal CORT production between *G. elegans* and *P. a. brygooi* as both species and context-specific effects can influence results (Romero & Beattie, 2022). These results illustrate the need for establishing physiological baselines on a species-by-species basis and further emphasized the importance of evaluating multiple species and optimizing physiological assays when working with non-model species. It is a critical step that must be initially established otherwise determination of fluctuations in hormone levels, such as elevations, will be challenging (Millspaugh & Washburn, 2004). More specifically, generalizations regarding physiological baselines across similar taxa kept under comparable conditions must be made with caution.

The thermal microenvironment that the tortoises experienced did not appear to influence the immunocompetence metrics that we selected. Reptiles' physiological processes are often influenced by multiple environmental factors such as drought (Combrink et al., 2021) or resource availability (Smith et al., 2017). In the tegu lizard (*Salvator merianae*), differential leukocyte counts, another measure of innate immunity, positively correlated with the average body

temperature of the organism the week before blood sampling (Madelaire et al., 2021). In contrast, body temperature had no effect on the bactericidal ability of *S. merianae* to *E. coli*, regardless of the timeframe of body temperature averages (Madelaire et al., 2021). The authors theorize this difference in response may show differences in the stabilities of immune traits, where some are more stable across a broader temperature range.

In our current study, we included both short (i.e., the week prior) and long (i.e., the month prior) exposure period models to ascertain if the species displayed a correlation between immune function and thermal microenvironment experienced. Because none of the models regarding bactericidal ability against *S.* Typhimurium or *S. aureus* were better fit than the NULL model, it is likely that the current differences in thermal microenvironment (i.e., seasonally appropriate outdoor housing of *G. elegans* vs. constant indoor housing of *P. a. brygooi*) experienced by the two tortoise species at the resolution tested do not elicit differences in innate immunity. Interestingly, only models concerning *E. coli* showed potential effects. The *E. coli* models were either equal to the NULL (*G. elegans*) or better fit than the NULL (*P. a. brygooi*). It is possible that the thermal microenvironment variables we selected may influence the tortoises' bactericidal ability of *E. coli*. However, this influence may only be noticed after prolonged exposure to different temperatures or may experience a lag-like effect as demonstrated by the models incorporating month-long data being better fit than or comparable to the NULL models. Because we only assessed one aspect of immune function, bactericidal ability, we may have missed relationships between the thermal microenvironment and other innate metrics. A study in snakes did note that bactericidal ability was most strongly associated with other innate immune metrics including lysis (Neuman-Lee et al., 2019). In the future, the addition of other immune assays and metrics will be imperative to building a more comprehensive assessment of the effects, or lack thereof, of thermal microenvironment on the immune response of *G. elegans* and *P. a. brygooi*.

During the late fall, winter, and early spring periods, both tortoise species were housed indoors at constant summer-like temperatures. Consequently, this absence of exposure to significantly decreasing temperatures may have influenced the observed lack of effect of the thermal microenvironment on immune function. This study did not examine other environmental variables that may have influenced immunocompetence. For example, in a study by Combrink et al. (2021), spring precipitation was found to be the most plausible predictor of immune function as measured by bactericidal capacity and natural antibody levels for two species of gartersnakes (*Thamnophis elegans* and *Thamnophis sirtalis*). More specifically, negative response in immune function in reaction to drought-like conditions. Additionally, Currylow et al. (2015) documented the relationship between extreme weather events and behavior in free-living *P. arachnoides*. In association with higher cloud cover and presence of precipitation, *P. arachnoides* shifted from being buried to emerging as the weather event, a cyclone, progressed. Future studies should include other environmental variables such as precipitation, hours of direct sunlight, photoperiod, and barometric pressure.

Escherichia coli was the only bacterium for which there was a possible relationship with the selected thermal variables. In this instance, the mechanisms responsible for the removal of *E. coli* are likely more profoundly influenced by the thermal microenvironment than other mechanisms. Identifying and quantifying specific immune metrics using only BKAs (Demas et al., 2011; Neuman-Lee et al., 2019) is a delicate task because this assay is primarily a functional measure of serum immunocompetence. There are multiple innate immune mechanisms used to combat Gram-negative bacterial infections (Ferrandon et al., 2007), so it is difficult to use this functional approach to precisely identify the specific mechanism. It is possible that natural antibodies may play a substantial role in the defense against Gram-negative bacteria in chelonian species (Zimmerman et al., 2013) as they have also been shown to be temperature-dependent (Butler et al., 2013).

5 | CONCLUSION

In this study we demonstrated the diversity of bactericidal ability of two threatened tortoise species and highlighted potential explanations for these observed differences in innate immune function. We additionally explored the differences in physiological energy allocations of the two species. Lastly, we laid the groundwork for examining the potential relationships between innate immunity and temperature through the inclusion of multi-season sampling and exploratory modeling. To continue to expand our understanding of the immune system, ecoimmunologists must further diversify and optimize techniques (Downs et al., 2014). One major way to do this is through non-model species. Though immunity in reptilian species has largely been ignored, reptiles offer a unique system to study innate immunity due to their reduced reliance on the adaptive response (Zimmerman, Vogel, & Bowden, 2010). In these nontraditional model species, we are granted the ability to better investigate and comprehend how particular immune qualities may have emerged and determine whether they are phylogenetically or life-history motivated. Ultimately, this affords us a more complete understanding of vertebrate immunity.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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