Context-dependent immunity in Gopher Tortoises, *Gopherus polyphemus*

by

Jeffrey Michael Goessling

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Approved by

Mary T. Mendonça, Co-chair, Professor, Biological Sciences
Craig Guyer, Co-chair, Professor, Biological Sciences
Geoffrey E. Hill, Professor, Biological Sciences
Lora L. Smith, Associate Scientist, Joseph W. Jones Ecological Research Center

Abstract

Infectious diseases in wildlife are a significant threat to biological diversity, especially In light of recent anthropogenic alterations to the environment. North American tortoises (genus *Gopherus*) have experienced declines as a result of an upper respiratory tract disease (URTD) which can be caused by *Mycoplasma agassizii* and *Mycoplasma testudineum* infection. Recent evidence suggests that URTD is a result of multiple environmental contexts, beyond simply pathogen presence. The objective of this dissertation was to examine contexts in which the environment directly affects immunity in Gopher Tortoises, *Gopherus polyphemus*. Herein, I tested four hypotheses related to the environmental contexts of disease resistance in *G. polyphemus*: (1) population-level differences in URTD exist across *G. polyphemus* populations and disease state is positively related to diagnostic markers of mycoplasmosis; (2) natural seasonal acclimation causes seasonally-altered disease resistance; (3) rapid temperature change causes a lag of optimal baseline immune function, during which disease resistance is increased; (4) *G. polyphemus* alters thermoregulatory set-point and immune function as a response to acute bacterial infection. Across seven populations of *G. polyphemus,* I found that the frequency of external symptoms of disease varied considerably, and that this variation is directly related to the year in which a population is sampled. However, traditional diagnostic markers of mycoplasmosis failed to positively identify individuals with external symptoms of URTD. Antibody titers to *M. agassizii* were positively related to the presence of nasal scarring consistent with chronic URTD. Experimental manipulations of *G. polyphemus* seasonal acclimation state

demonstrated that winter causes a significant reduction in immune function. Moreover, rapid temperature increase during winter does not cause an increase in immune function, suggesting that acclimation states constrain immunity. Furthermore, rapid temperature decreases during the summer significantly reduced immune function, suggesting a direct mechanism by which aberrant weather may increase disease frequency. While immunity was strongly temperature dependent in *G. polyphemus*, I failed to find support for a predicted seasonal lag of immunity. Lastly, I found that when acutely stimulated with lipopolysaccharide, a bacterial endotoxin, *G. polyphemus* has an immunological response consistent with fever. This fever response included an increase in body temperature, a reduction in plasma iron, and an increase in innate immunity. The fever response was not directly related to acclimation state, as both winter-acclimated and summer-acclimate tortoises consistently displayed fevers. As a whole, this dissertation provides a strong link between the environmental context of thermoregulatory potential and disease resistance in *G. polyphemus*. Increased environmental change, especially as a result of a changing climate, will likely exacerbate the negative effects of disease in this taxon.

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Chapter 1: General introduction

ABSTRACT

Here, I provide a general background on current understanding of ecoimmunology in ectothermic vertebrates. Furthermore, I relate the fields of ecoimmunology and thermal biology. A model organism, the Gopher Tortoise (*Gopherus polyphemus*), is introduced to investigate multiple environmental contexts for disease and the four research objectives of this dissertation are outlined. The four objectives of this dissertation are framed to test falsifiable hypotheses that isolate how the environment directly affects biomarkers of immunity, and ultimately disease susceptibility, in a taxon of high ecological value and conservation need.

BACKGROUND

Global biodiversity loss has accelerated at an alarming rate as both direct and indirect consequences of a growing global human population (Pimm et al. 1995). Combined anthropogenic environmental effects, including habitat alteration and degradation, population fragmentation, invasive species transmission and global climate change have been collectively described as global change (Wingfield 2008). While the processes of global change are diverse, the outcome of such environmental modification is an overall loss of biological diversity. Ectothermic vertebrates may be especially vulnerable to negative effects of global change as a result of their physiologies, life-histories, habitat requirements and susceptibility to disease, (Gibbons et al. 2000). While the loss of any species from an ecosystem is ethically and aesthetically immeasurable, the loss of keystone species from ecosystems magnifies a biodiversity loss through further negative ecosystem effects (Ernest and Brown 2001).

North American tortoises (genus *Gopherus*) have experienced population declines and are the focus of intense conservation efforts; populations of two species of *Gopherus* are

federally protected under the Endangered Species Act (i.e. Mojave Desert Tortoises, *Gopherus agassizii*, and the western population of Gopher Tortoises, *Gopherus polyphemus*, US Fish and Wildlife Service). Declines of *Gopherus* have been attributed to many aspects of global change, including disease (Jacobson et al. 2014). Specifically, much research in this taxon has focused on the pathology and transmission of an upper respiratory tract disease (URTD) that is caused by *Mycoplasma agassizii* (Brown et al. 1994) and *Mycoplasma testudineum* (Brown et al. 2004). Specific diagnostic assays to these pathogens have been developed that both characterize the presence of the pathogens using molecular techniques (Brown et al. 1995; Braun et al. 2014) and titrate the pathogen-specific antibodies using an enzyme-linked immunosorbant assay (ELISA, Wendland et al. 2007). However, as a result of such intense pathogen-focused research efforts, the relationships between seroprevalence, pathogen prevalence and disease are still unknown, especially at population levels. Sandmeier et al. (2013) investigated inter-annual patterns of URTD in Mojave Desert Tortoises (*Gopherus agassizii*) and found that markers of URTD were more prevalent following severe winters. Sandmeier et al. (2013) thus concluded that URTD is a context-dependent disease, largely dependent upon environmental thermal parameters. Further, Jacobson et al. (2014) have suggested that the environment has direct effects on disease susceptibility in *Gopherus* populations, and the presence or absence of mycoplasma pathogens in a population does not solely determine disease state. Tracy et al. (2006) outlined several mechanisms of global change including climate change, disease and human habitat modification that collectively render *G. agassizii* susceptible to population extirpation.

ECOIMMUNOLOGY AND THERMAL BIOLOGY OF ECTOTHERMS

The recently emerging field of ecoimmunology utilizes immunological assays to isolate the effects of ecological parameters on disease susceptibility and resistance. Profound effects of

the environment on immunity have been demonstrated in ectothermic vertebrates, specifically amphibians (Raffel et al. 2006, Raffel et al. 2015) and reptiles (Seebacher 2005, Zimmerman et al. 2010). Past studies have demonstrated that immunity is both temperature and season dependent, which is hypothesized to optimize immune function while balancing energetic costs of the immune system (Pxytycz and Jozkowicz 1994). The overall pattern of seasonal acclimation of immunity is a reduction in baseline function during cool seasons when pathogen pressure is low (Raffel et al. 2006). There is a distinct risk to acclimation however, as seasonal acclimation may constrain individuals when temperature rapidly changes (Raffel et al. 2006). This risk is demonstrated by increased disease in amphibians when temperature is highly variable (Raffel et al. 2015). Rapid temperature changes are thus predicted to cause short-term differences between realized and optimal immunity, known as a seasonal lag of immunity (Raffel et al. 2006). A conceptual framework of seasonal acclimation and seasonal lag isolates how thermal environments, and changes in thermal environments, can ultimately drive patterns of disease. This conceptual framework includes specific predictions of immunity across acclimation states and temperatures, by which immunity is reduced below optimal levels when temperature rapidly decreases and by which immunity is increased above optimal levels when temperature rapidly increases.

While the vertebrate immune system is a complex and inner-connected network of processes, there is tight regulation of sequential order by which the immune system responds to antigenic stimuli (herein referred to as immune responses). The immune system is broadly organized into two branches: the innate immune system and the adaptive immune system. Differences in the function of these branches are rooted in the innate immune system's responsibility for "first-line defenses" and the adaptive immune system's responsibility for long-

term survival and the acquisition of features (such as antibodies and memory cells) that prevent re-infection by previously encountered pathogens (Lee and Klasing 2004). One of the first effects of immune responses following acute infection is fever, which is a systemic inflammatory response driven by the innate immune system (Lee and Klasing 2004). Although many processes, such as cellular redistribution, are seen in animals expressing fever, the main effector of fever is an increase in the hypothalamic thermoregulatory set-point (Boulant 2000). As a result, the expression of fever is defined as an increase in set body temperature (T_{set}) which is usually due to infection and is manifested as an increase in body temperature (T_b, Bernheim et al. 1979, Kluger et al. 1998). Febrile responses are first driven by activation of specific toll-like receptors (TLRs) by antigenic compounds, such as a bacterial endotoxin, lipopolysaccharide (Beutler 2004, Dinarello 2004). A cascade of endogenous pyrogens follows the activation of TLRs that target the hypothalamic thermoregulatory center (Dinarello 2004). The cascade of febrile effects following TLR activation is thought to be responsible for increasing the host's defenses against pathogenesis (Bernheim et al. 1979). The adaptive benefit of fever includes changes in the metabolic efficiency of the host, temperatures that compromise pathogen performance and reduced plasma iron which further compromises pathogen growth (Kluger and Rothenburg 1979).

Endothermic vertebrates, specifically mammals, have been used as traditional models of fever responses, as these organisms possess the physiological ability to increase T_b (Dinarello 2004). While the expression of this response to inflammatory antigens is highly conserved within endotherms, a consensus of data is still lacking regarding whether this response is equally present across ectothermic vertebrates. Because their T_bs are directly related to environmental temperature, the generation of fever in ectotherms is due to behavioral processes (such as

alterations of basking) and not direct physiological processes. Thus, behavioral fever and the associated immune responses may be further constrained by season in ectotherms when thermoregulatory potential is reduced. Among ectothermic vertebrates, fever responses have been demonstrated in amphibians (Bicego-Nahas et al. 2000), squamates (Ortega et al. 1991), turtles (Monagas and Gatten 1983) and crocodylians (Merchant et al. 2007). However, the presence of fever has not been shown in all species of turtle in which it was investigated. Fever was demonstrated in the Box Turtle and the Painted Turtle (*Terrapene carolina* and *Chrysemys picta*, Monagas and Gatten 1983) but was not observed in the Leopard Tortoise (*Geochelone pardalis*, Zurovsky et al. 1987). Additionally, while broad generalizations have been made (for example, lizards display fevers, as demonstrated in *Agama agama* and *Sceloporus orcutti* (Ortega et al. 1991), individual species may show very different patterns, as demonstrated by a photophasic hypothermic response of *Anolis carolinensis* (Merchant et al. 2008). Because fever in ectotherms is dependent upon environmental temperature, and environmental temperature is driven by season, one of the most important aspect when considering fever in ectotherms integrates seasonal changes in immune strategy.

MODEL ORGANISM

The Gopher Tortoise (*Gopherus polyphemus*) is a keystone species of the lower Southeastern US Coastal plain (Guyer and Bailey 1993) from South Carolina to Louisiana and has experienced significant declines across its range (Auffenberg and Franz 1982, Smith et al. 2006, Tuberville et al. 2009). *Gopherus polyphemus* is a habitat specialist for open-canopy fire-adapted habitats, including Longleaf Pine (*Pinus pallustris*) and Turkey Oak (*Quercus laevis*) ecosystems containing deep sandy soils for adequate burrowing (Diemer 1986). Populations of

Gopher Tortoises from south-central Alabama and west to Louisiana have been considered peripheral and are hypothesized to be more vulnerable than core populations to disease, climatic factors and anthropogenic habitat degradation (Tuberville et al. 2009).

Several studies have documented URTD across eastern populations of *G. polyphemus*. Diemer Berish et al. (2000) found clustering of Gopher Tortoise populations that were seropositive to URTD in northern and central Florida. In Georgia, McGuire et al. (2014) found that mycoplasmal URTD was present in seven out of eleven sampled populations. However, while Diemer Berish et al. (2000) and McGuire et al. (2014) are among the most extensive surveys of URTD in Gopher Tortoises, these two studies found relatively different epidemiological patterns of URTD as indicated by seroprevalence. Specifically, Diemer Berish et al. (2000) found that *Mycoplasma* were present at low seroprevalence, while McGuire et al. (2014) found that at the population level, sites usually had either very high or very low seroprevalence, with very few sites intermediate. Additionally, McGuire et al. (2014) found that the rate of seroprevalence to *M. testudineum* was higher (73% of sites were seropositive) than previously reported rates of prevalence of this pathogen from sites in northeastern Florida (27%; Wendland 2007). Lastly, Diemer Berish et al. (2010) found that seroprevalence of URTD varied significantly among years, suggesting that URTD has the ability to have cycles of health and disease recrudescence.

RESEARCH APPROACH AND HYPOTHESES

The goals of this dissertation were to understand direct links between aspects of global change and a declining keystone species of the southeastern US, the Gopher Tortoise (*Gopherus polyphemus*). This research utilized combined approaches of hypothesis testing in a field study and experimental manipulations to isolate multiple environmental contexts of disease in *G.*

polyphemus. The objectives of the field study were to characterize disease prevalence across seven populations of Gopher Tortoises in Alabama and test the hypothesis that diagnostic markers of URTD indicate individuals with external symptoms of URTD. The objectives of the experimental studies were to test hypotheses of seasonal acclimation of immunity, seasonal lag of immunity and seasonal components of fever presence and immune responses.

Field study

No systematic survey has been conducted for URTD in Alabama populations of Gopher Tortoises, yet several populations of this species have been the focus of important, long-term studies on movement and population demographics (e.g. Guyer et al. 2012, Tuberville et al. 2014). Additionally, numerous populations of Gopher Tortoises exist in Alabama that have not been studied or systematically surveyed. This study involved surveying disease in three populations of Gopher Tortoises that have been included in the aforementioned long-term studies, as well as the inclusion of four new study populations of Gopher Tortoises. None of these seven sites had been previously sampled for URTD. This study tested two hypotheses related to presence of diagnostic markers of URTD and external symptoms of URTD. The first prediction was that there is a significant positive relationship between diagnostics of URTD (antibodies to *M. agassizii*, *M. testudineum*, or the molecular presence of either *M. agassizii* or *M. testudineum*) and active symptoms of URTD consistent with acute infection (including presence of nasal exudate, conjunctivitis or periocular edema). The second prediction was that there is a positive relationship between diagnostics of URTD (antibodies to *M. agassizii*, *M. testudineum*, or the molecular presence of either *M. agassizii* or *M. testudineum*) and the presence of nasal scarring (including eccentric nasal asymmetries or erosion of the nares or upper beak) consistent with either past URTD or chronic URTD. The null hypothesis for each of these

predictions was no positive relationship between diagnostics of URTD and either acute symptoms of URTD or the presence of nasal scarring. The null hypotheses are falsified by identifying a significant positive relationship between any of the diagnostics and the presence of external symptoms of URTD. In addition to testing specific diagnostic hypotheses, goals of this study included gaining a better understanding of disease prevalence across these populations of Gopher Tortoises that will be important in future conservation efforts.

Experimental studies

Experimental studies focused on understanding the nature of seasonal physiological acclimation and how acclimation alters immunity by both temperature-dependent and season-dependent mechanisms. The first experimental study tested the seasonal acclimation hypothesis, which predicts an overall long-term reduction in immunity following acclimation to winter conditions. This study involved experimentally inducing winter conditions in adult Gopher Tortoises under semi-natural housing conditions and comparing immunity across four seasons (i.e. spring, summer, fall and winter). The null hypothesis for this study is no difference in immunity among seasons, which is falsified by finding significant differences in immunity among seasons.

The second experimental study isolated temperature-dependent immunity from season-dependent immunity by testing the seasonal lag hypothesis. To test this hypothesis, adult Gopher Tortoises were exposed to rapid temperature changes which reciprocated body temperatures for two days between winter and summer acclimation states (e.g., dormant tortoises at 12.5 °C were warmed to 32.5 °C and active tortoises at 32.5 °C were cooled to 12.5 °C). The predictions of the seasonal lag hypothesis are that dormant tortoises will show immunity increased above active-season levels when brought to active body temperatures and that active tortoises will show

immunity below dormant-season levels when brought to winter body temperatures. The null hypothesis for this experiment is that there are no effects of rapid temperature change on immunity, or that differences are opposite of those predicted. This hypothesis is falsified by finding that immunity changes following rapid temperature changes according to the above specific predictions.

The third experimental study addressed the presence of fever as an immune response in Gopher Tortoises and if immune response is affected by seasonal acclimation state. To address these questions, tortoises were stimulated with a bacterial endotoxin, lipopolysaccharide (LPS); thermoregulation and immunological parameters were measured in response. These experiments were conducted in both winter-acclimated tortoises and summer-acclimated tortoises. There were two predictions in this study. The first prediction is that LPS causes a hyperthermia, which is associated with acute immune responses. The null hypothesis for this prediction is that LPS does not affect thermoregulation or causes hypothermia. The second prediction is the presence of differences in the magnitude of the LPS immune response between winter and summer acclimation states. The null hypothesis for this prediction is no difference in the immune response across acclimation states.

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Chapter 2: Upper respiratory tract disease and associated diagnostic markers of mycoplasmosis in Alabama populations of Gopher Tortoises, *Gopherus polyphemus*

FORMAT FOR JOURNAL OF WILDLIFE DISEASES

Running head: Goessling et al. URTD in Gopher Tortoises from Alabama

Upper respiratory tract disease and associated diagnostic markers of mycoplasmosis in Alabama populations of Gopher Tortoises, *Gopherus polyphemus*

Goessling, Jeffrey M.[1]*, Craig Guyer[1], James C. Godwin[2], Sharon M. Hermann[1], Franzisca C. Sandmeier[3], Lora L. Smith[4],and Mary T. Mendonça[1]

[1] Dept. of Biological Sciences, 331 Funchess Hall, Auburn University AL 36849

[2] Alabama Natural Heritage Program, 1090 S Donahue Dr, Auburn University AL 36849

[3] Division of Natural Resources, Lindenwood University, Old Main Hall M220, Belleville IL 62226

[4]Joseph W. Jones Ecological Research Center, 3988 Jones Center Dr, Newton GA 39870

*corresponding author: goessling@auburn.edu; Phone: (859) 462-2547; Fax: (334) 844-9234

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ABSTRACT

Upper respiratory tract disease (URTD) in North American tortoises (*Gopherus*) has been the focus of numerous laboratory and field investigations, yet the prevalence and important of this disease remains unclear across tortoise populations. Furthermore, much research has been focused on understanding diagnostic biomarkers of two known agents of URTD, *Mycoplasma agassizii* and *Mycoplasma testudineum*, yet the importance of these diagnostic biomarkers at population levels is unclear. Gopher Tortoise (*Gopherus polyphemus*) populations have experienced significant declines and they are currently protected rangewide. Geographically, Alabama represents an important connection for Gopher Tortoise populations between the core and periphery of this species' distribution. Herein, we systematically sampled 197 Gopher Tortoises for URTD across seven sites in south-central and southeastern Alabama. We assayed plasma samples for antibodies to *M. agassizii* and *M. testudineum*; we also assayed nasal lavage samples for the presence of viable pathogens as well as pathogen DNA. Lastly, we scored animals for the presence of external symptoms and nasal scarring consistent with URTD. We found that external symptoms of URTD were present in *G. polyphemus* in all sites sampled in Alabama. There was no relationship between active symptoms of URTD and *Mycoplasma* antibodies, however we did find that the presence of URTD nasal scarring was positively related to *M. agassizii* antibodies ($P = 0.032$). For a single site that was sampled in three sequential years, seroprevalence to *M. agassizii* significantly varied among years ($P < 0.0001$). We isolated *M. agassizii* DNA from four of the seven sites using quantitative PCR, yet none of the samples was culture positive for either of the pathogens. An analysis of disease status and condition indicated that there was a significant, positive relationship between the severity of URTD symptoms and relative body mass ($P < 0.05$), which may indicate reduced digestive function in

diseased animals. This study highlights the need for continued monitoring of disease in *Gopherus*, with a specific focus on identifying other pathogens and relevant biomarkers that may be important drivers of URTD. Consideration should be given to environmental processes that may cause populations of *Gopherus* to develop disease symptoms.

KEY WORDS: global change, threatened and endangered species, turtle, biomarker, diagnostic

INTRODUCTION

Infectious diseases are of increasing risk to the survival of ectothermic vertebrates (Raffel et al. 2015) especially in light of recent global change (Wingfield 2008). Upper respiratory tract disease (URTD) in North American tortoises (*Gopherus*) is among the most intensively studied diseases in free-ranging ectotherms. Two pathogens have been identified as causative agents of URTD in *Gopherus*, *Mycoplasma agassizii* and *Mycoplasma testudineum* (Brown et al. 1994, Brown et al. 2004), and enzyme-linked immunosorbant assays (ELISA) have been developed to diagnose the presence of antibodies that are specifically reactive to each (Wendland et al. 2007). While much research has focused on transmission, pathology and diagnostics of URTD in *Gopherus* (Jacobson et al. 2014), we still lack an understanding of the presence of this disease across the geographic range of this genus, and the nature by which this disease may impact population viability.

While *M. agassizii* and *M. testudineum* are often considered invasive pathogens, they are present across multiple sites within the range of Gopher Tortoises (*Gopherus polyphemus*). Diemer Berish et al. (2000) found that evidence of URTD was present across many populations of Gopher Tortoises in central and northern Florida. McGuire et al. (2014) sampled eleven populations of Gopher Tortoises in Georgia and found that evidence of URTD was present in seven of these sites. However, while Diemer Berish et al. (2000) and McGuire et al. (2014) are

the most extensive state-wide surveys of URTD in Gopher Tortoises, these two studies found relatively different epidemiological patterns of URTD as diagnosed by antibody titers to the pathogens. Specifically, Diemer Berish et al. (2000) found that *Mycoplasma* were present at generally low levels of seroprevalence, while McGuire et al. (2014) found that at the population level, sites typically had either very high or very low rates of seroprevalence, with very few sites intermediate in this marker of disease prevalence. Additionally, McGuire et al. (2014) found that the rate of seroprevalence to *M. testudineum* was higher (73% of sites were seropositive) than previously reported rates of prevalence of this pathogen from sites in northeastern Florida (27%; Wendland 2007). As a result of the enigmatic nature of diagnostic markers, disease presence and mortality events, URTD in *Gopherus* has been described as context-dependent (Sandmeier et al. 2013).

Gopher Tortoises have experienced extensive range-wide population declines (Auffenberg and Franz 1982, Tuberville et al. 2014) and disease has exacerbated these declines (Jacobson et al. 2014). Gopher Tortoises are currently listed as federally threatened in the western portion of the species' range, west of the Mobile River in Alabama (US Fish and Wildlife Service, 1987). Moreover, the Gopher Tortoise is listed as a candidate species for federal protection throughout the remainder of its range (US Fish and Wildlife Service, 2011). Populations in the eastern range of Gopher Tortoises, in Alabama and northwestern Florida, are considered peripheral, and therefore may be at an increased risk of population extinction (Tuberville et al. 2009). While intense conservation efforts are underway for Gopher Tortoises in Alabama, no systematic study has been conducted in this region of the species' range to identify the nature of URTD in these important populations of tortoises.

Herein, our goals were to survey for the presence of URTD and its associated diagnostic markers in seven populations of Gopher Tortoises in Alabama. The seven populations include the largest populations of Gopher Tortoises on public lands in the state, and are thus important for long-term management and conservation efforts. Beyond simply assessing disease prevalence, we were also interested in testing the hypothesis that diagnostic markers of URTD are consistent with external disease symptoms. This goal provides a better understanding of the epidemiology of this disease, and provides a context for how to best monitor disease in free-ranging *Gopherus*.

MATERIALS AND METHODS

Study sites

Tortoises were sampled at seven sites in Baldwin, Clarke, Covington and Geneva counties of Alabama (Fig. 1). Two of the sites are long-term Gopher Tortoise study sites and are located in Conecuh National Forest (Conecuh National Forest Site 1, CNF-1, and Conecuh National Forest Site 3, CNF-3). Three of the sites are located on state-owned lands that are maintained as wildlife management areas: the Rayonier Tract, RT, and Geneva State Forest, GSF, which are both maintained under the Geneva Wildlife Management area, and the Perdido River Wildlife Management Area, PWMA. One state-owned site, Stimpson Wildlife Sanctuary (SWS), is closed to the public and is maintained as a wildlife sanctuary. The seventh site, Solon Dixon Forestry Education Center (SDFEC) is privately owned by Auburn University School of Forestry and Wildlife Sciences. All seven sites are actively managed for multi-use, including timber production and wildlife management. Each site was sampled once in either 2013, 2014 or 2015, with the exception of SDFEC, which was sampled in all three years.

Tortoise capture, sampling and assays

Tortoises were trapped using custom-ordered Tomahawk® live traps (Hazelhurst Wisconsin USA) placed at the entrance of each occupied burrow. Adult tortoises (> 18 cm carapace length) were targeted for this study. Traps were shaded using either a piece of burlap or vegetation from around the burrow, and traps were checked twice daily. In most cases, a blood sample (0.3 – 1.0 ml) was collected upon capture from the femoral vein of each tortoise using a 25 gauge needle affixed to a 1 ml syringe that had been previously heparinized with a sterile 0.5% sodium heparin solution diluted in ultrapure water (Sigma Aldrich, St. Louis Missouri USA). In the rare cases in which blood could not be collected from the femoral vein, whole blood was collected either from the brachial vein or subcarapacial venous sinus. A sample was not used in immune assays if it appeared to be contaminated with lymphatic fluid. Prior to venipuncture, the site was sanitized with an isopropyl alcohol wipe to prevent contamination or site infection. Whole blood samples were temporarily stored on ice, centrifuged and plasma separated and stored in liquid nitrogen within one hour of collection. Samples were thawed once prior to diagnostic submission to make serial aliquots of plasma samples for later assays. Immediately upon capture, tortoises were weighed and later measured for morphometric (e.g. relative condition) calculations.

Each tortoise was scored, visually, for external symptoms of URTD according to established guidelines (Wendland et al. 2009). Specifically, we scored URTD symptoms from 0 – 3 (none – severe) at five focal locations: nares, eyes, conjunctiva, eyelids, periocular region (Wendland et al. 2009). In addition to scoring active signs of disease (e.g. nasal drip, mucous build up or inflammation), we classified the presence or absence of nasal scarring consistent with URTD. This scarring included obvious nares asymmetries, erosion of the nares and/or erosion of the upper beak. The same person (JMG) scored all animals to reduce sampler bias in disease

symptoms. Relative condition (Stevenson and Woods Jr. 2006) was calculated by dividing each animal's mass by its predicted mass. Predicted masses were calculated from the slope of an ordinary least squares regression between all ln-transformed lengths and ln-transformed masses.

Nasal lavage samples were collected following the guidelines outlined in Wendland et al. (2009) and per guidance from the staff at the University of Florida Mycoplasma Research Laboratory. Six milliliters of sterile saline was flushed through the paranasal sinuses using a sterile 22 gauge intravenous catheter, with the stylet removed, attached to a 10 ml sterile syringe. The lavage sample was collected as it dripped out of the nares into a sterile specimen collection cup. Immediately upon lavage collection, one milliliter of sterile SP4 mycoplasma culture medium (University of Florida Mycoplasma Research Laboratory, Gainesville FL) was added to the sample. Lavage samples were immediately divided into aliquots in two milliliter cryogenic vials and were flash frozen in liquid nitrogen. Lavage samples were stored during each field season at -80 C and were never thawed; following each field season, all samples were sent as a batch to the University of Florida Mycoplasma Research Laboratory. Lavage samples were submitted for PCR/culture diagnostic assays of *M. agassizii* and *M. testudineum*. Plasma samples were also submitted to the University of Florida Mycoplasma Research Laboratory for diagnostic assays of antibodies to both *M. agassizii* and *M. testudineum*.

In addition to PCR/culture assays, we assessed the presence of *M. agassizii* and *M. testudineum* DNA using a quantitative PCR (qPCR) technique according to the protocol by Braun et al. (2014). Genomic DNA was extracted from 500 ul of the nasal lavage sample using a DNEasy blood and tissue extraction kit (Qiagen, Redwood City CA) and re-suspended in 100 ul of assay buffer. qPCR reaction conditions were according to Braun et al. (2014). We considered

a sample to be positive if any one of three triplicated samples were below the critical cutoff value.

RESULTS

Antibody presence across sites and disease markers

We found that every site had animals present that were at least suspect for exposure to *M. agassizii* (Fig. 2). The site with the highest frequency of animals with positive titers to *M. agassizii* was CNF-1, which was 63% positive and 37% suspect. Otherwise, we found that the prevalence of antibodies to *M. testudineum* was very low; in two sites, CNF-3 and SWS, 100% of the animals we sampled were negative for antibodies to this pathogen.

At SDFEC, which was sampled in three consecutive years, we found that the prevalence of antibodies to *M. agassizii* varied significantly among sampling years (G-test: $G^2 = 36.88$, df = 2, $P < 0.0001$). Specifically, we found a very high seroprevalence in 2013 (42% positive, 42% suspect, 16% negative), very low seroprevalence in 2014 (0% positive, 8% suspect, 92% negative) and low seroprevalence in 2014 (0% positive, 25% suspect, 75% negative).

We found that neither antibody titer nor relative condition significantly predicted the presence of disease symptoms consistent with URTD (Multiple logistic regression, n = 174: *M. agassizii* P = 0.365; *M. testudineum* P = 0.060; relative condition P = 0.176). However, we did find a significant positive relationship between antibodies to *M. agassizii* and the presence of nasal scarring, but there was not a significant relationship between antibodies to *M. testudineum* and presence of nasal scarring (Multiple logistic regression, n = 174: *M. agassizii* P = 0.032; *M. testudineum* P = 0.391; relative condition P = 0.364).

We found that there were no differences in antibody titers between male and female for either *M. agassizii* (G-test: $G^2 = 0.019$, df = 1, P = 0.89) or *M. testudineum* (G-test: $G^2 = 0.007$,

df = 1, P = 0.93). Similarly, male and female *G. polyphemus* did not differ in the proportion of animals showing active disease symptoms (G^2 = 0.0008, df = 1, P = 0.97) or the presence of scarring consistent with past infection with URTD (G^2 = 0.0007, df = 1, P = 0.98).

We identified a positive relationship (Model 2 linear regression: $P < 0.05$, $R^2 = 0.084$, Fig. 3) between total disease score in animals with clinical signs of URTD and relative condition.

Pathogen presence across sites

None of the lavage samples was culture positive for either *M. agassizii* or *M. testudineum*. However, we identified the presence of *M. agassizii* DNA using the qPCR at four of the sites (RT, CNF-3, SDFEC and SWS; table 1 for pathogen survey results and sample sizes by site). None of the animals sampled were qPCR positive for *M. testudineum*.

DISCUSSION

Evidence of URTD was present in all sampled sites in Alabama further adding to the body of literature that indicates this disease is of widespread geographic extent, and that it may be of importance to affect wild populations of tortoise. However, our data find a weak link between the traditional diagnostic markers of *M. agassizii* and *M. testudineum* and external symptoms expressed by animals likely infected with URTD. In fact, no association between diagnostic markers for *M. testudineum* and disease symptoms was found and, of these symptoms, only nasal scarring was significantly correlated with *M. agassizii* antibodies.

Significant variation was present in *M. agassizii* antibody titers among years at SDFEC. Previous research (Diemer Berish et al. 2010) has found a similar pattern in which seroprevalence to *M. agassizii* varies across years. An epidemiological model of URTD in tortoises has emerged that suggests populations are generally either positive or negative (e.g. McGuire et al. 2014) for this disease, and such extreme inter-annual variation would not be

predicted within diseased populations. Our data indicate that caution should be applied when interpreting these diagnostics to infer disease status at the population level, as this specific marker of disease, antibody titers, varied significantly within populations among years. Furthermore, as has been previously suggested (Brown et al. 2002) because of the diversity of potential pathogens of URTD, populations should not be considered "positive" based solely on the presence of *Mycoplasma* antibodies.

We isolated *M. agassizii* DNA from four of the seven sites using a qPCR technique. Interestingly, the site with the highest seroprevalence to *M. agassizii* was among the sites from which we failed to isolate this pathogen's DNA. Furthermore, we isolated DNA from the three sites with the lowest seroprevalence to *M. agassizii* (SWS, CNF-3 and RT). Many of the extant tortoise populations in Alabama are relatively small (15 – 30 individuals), thus while larger sample sizes may clarify patterns between pathogen presence and seroprevalence, many sites simply lack adequate numbers of tortoises to further resolve this discrepancy.

Mycoplasma testudineum has been considered the less pathogenic of two known URTD pathogens in *Gopherus* (Jacobson and Berry 2012). Our data suggest that this pathogen is seropresent in at least three (e.g. PWMA, SDFEC and RT) and maybe five (including two suspect sites: GSF and CNF-1) sites in Alabama. We commonly observed animals with symptoms of URTD, yet this pathogen was detected very infrequently, which further indicates this pathogen may be of relatively minor importance to cause morbidity at the population level.

We expected a negative relationship between relative condition and URTD severity. However, we observed a significant positive relationship between these two parameters. We had predicted a negative relationship between relative condition and disease because we expected that either poor-condition animals become susceptible to disease, or that diseased animals are

sickened to poorer condition. One potential interpretation of this positive relationship is related to either increased food or water retention in diseased animals. Tortoises are hindgut-fermenting herbivores, thus processes that reduce digestive passage rates (causing increased digesta retention) have the ability to increase parameters of condition (such as relative mass), even if an animal is actually in poorer physiological condition. Hind-gut fermenting mammals have been shown to reduce digestive efficiency in response to heat stress (Lippke 1975), although, to our knowledge, know studies have specifically addressed mass gain in diseased hind-gut fermenting herbivores. It is possible that in response to the physiological stress of acute disease, digestive passage rates are reduced thereby increasing condition parameters. It is also possible that true relative condition is increased in response to disease, as a compensatory mechanism in animals fighting infection. We do not have the ability to fully address this problem with our data, but future studies should investigate the response of condition to acute infection in this taxon.

It has been suggested that other pathogens (such as pathogenic viruses, Jacobson et al. 2014) may be important sources of disease in North American tortoises. Given that we found the disease diagnostic assays for *M. agassizii* and *M. testudineum* were not good indicators of current disease state, our data suggest that other undetected pathogens may be important agents of URTD in Gopher Tortoises. Further research should focus on identifying additional pathogens of URTD, as well as focus on pathogens that may be more important in driving rapid population die offs observed elsewhere in the range of Gopher Tortoises.

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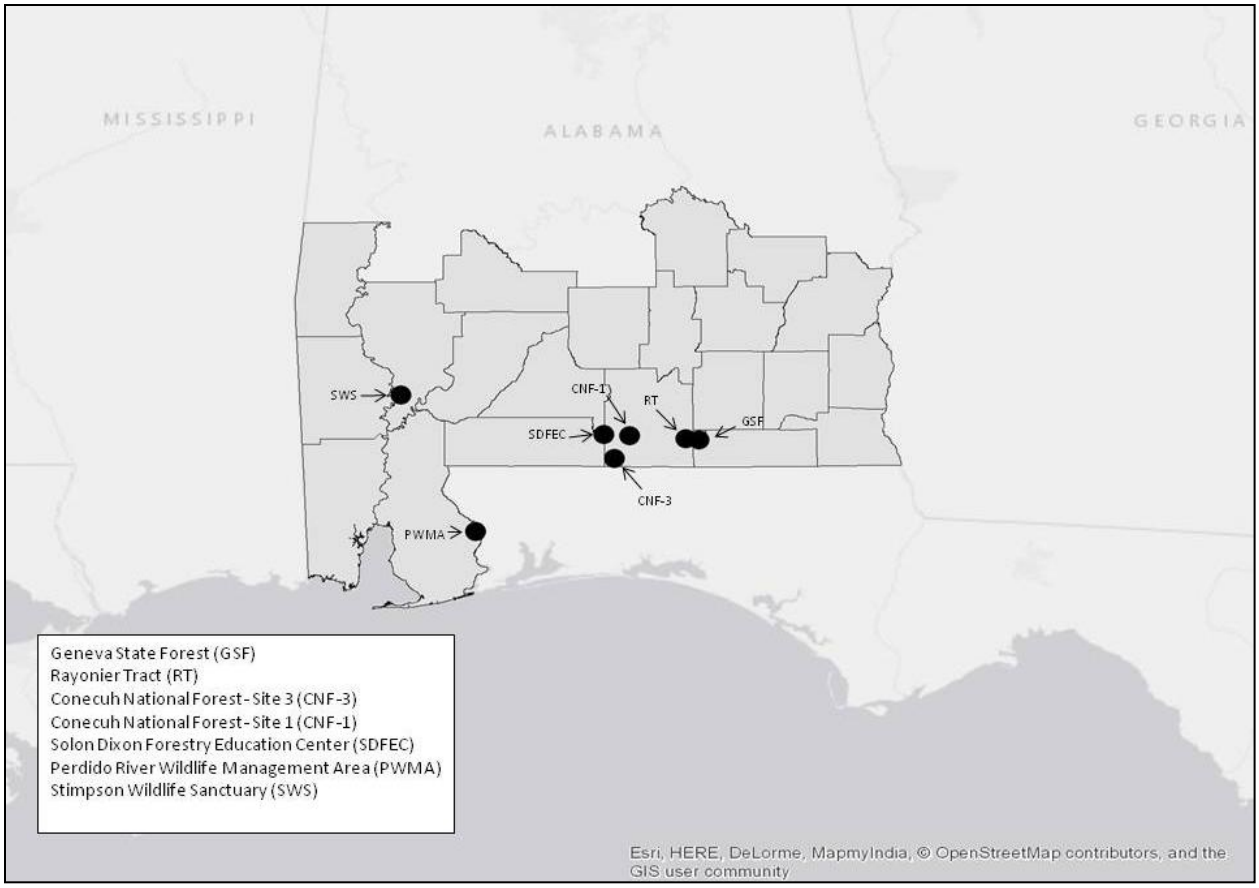

Figure 1. We sampled upper respiratory tract disease in populations of Gopher Tortoises from seven sites in Alabama. Counties in gray indicate Gopher Tortoise distribution in Alabama.

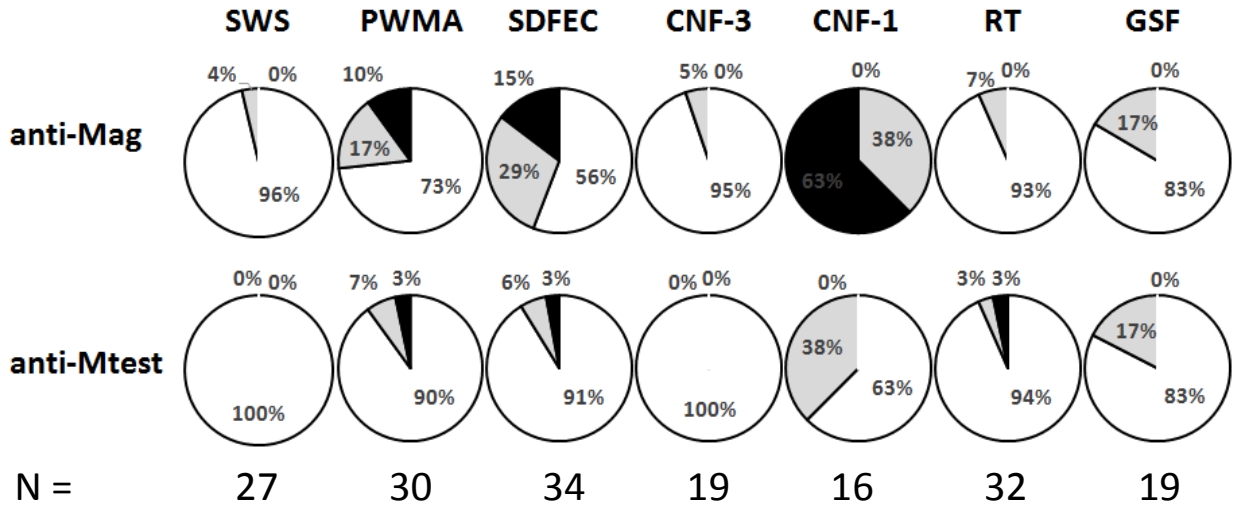

Figure 2. Assays of antibody titers to *Mycoplasma agassizii* (Mag) and *Mycoplasma testudineum* (Mtest) in *Gopherus polyphemus* varied across sites sampled in Alabama. Positive titers are indicated in black, suspect titers are indicated in gray and negative titers are indicated in white.

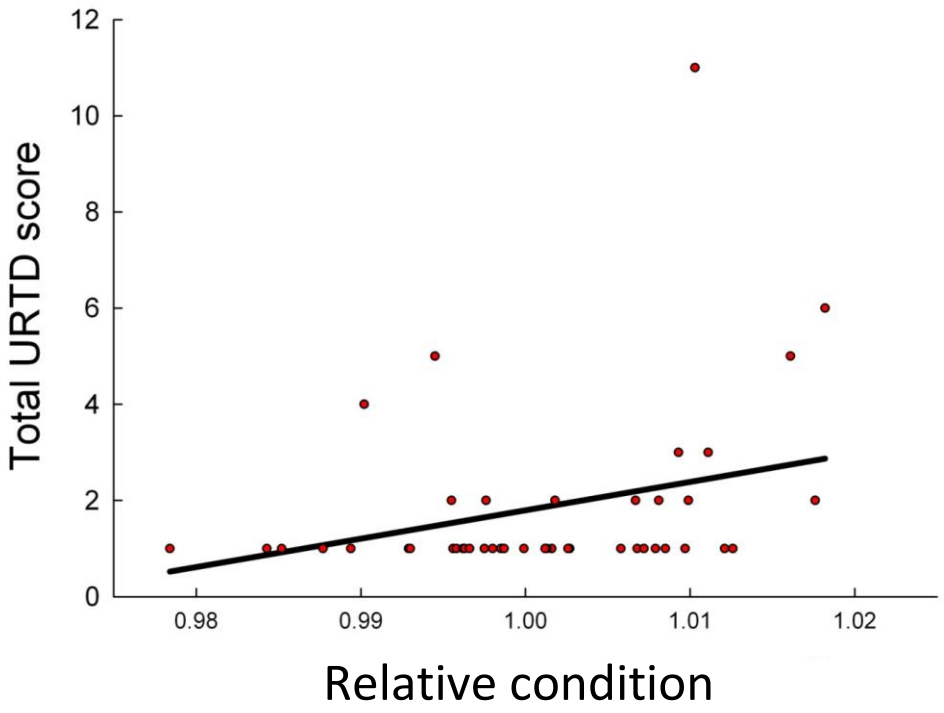

Figure 3. We found a positive relationship between relative condition and total upper respiratory tract disease score in Gopher Tortoises from Alabama (Model 2 regression: $P < 0.05$, $R^2 = 0.084$).

Table 1. URTD in Gopher Tortoise in Alabama separated by year, number of animals qPCR positive for *Mycoplasma agassizii*, number of animals with active symptoms of URTD and number of animals with nasal scarring consistent with URTD.

Site	**Year sampled**	**No. sampled**	**No. symptomatic**	**No. with scarring**	**No. qPCR positive/total no. qPCR assayed for *M. agassizii***
GSF	2014	24	5	3	0/18
RT	2015	33	8	14	1/30
CNF-1	2013	24	2	1	0/12
CNF-3	2015	20	7	14	1/19
SDFEC	2013, 2014, 2015	39	13	16	2/33
PWMA	2015	29	7	17	0/26
SWS	2015	28	5	17	1/27

Chapter 3: Seasonal acclimation of baseline immunity in Gopher Tortoises, *Gopherus polyphemus*

FORMAT FOR *PHYSIOLOGICAL AND BIOCHEMICAL ZOOLOGY*

Jeffrey M. Goessling,[*1], Craig Guyer[1] and Mary T. Mendonça[1]

[1.] Department of Biological Sciences, Auburn University, Auburn, AL 36849

[*] Corresponding author: goessling@auburn.edu

Guyercr@auburn.edu

mendomt@auburn.edu

Short title: Seasonal immunity in Gopher Tortoises

Key-words

Ecoimmun-, bactericidal ability, bacterial killing assay, differential leukocyte, heterophil: lymphocyte ratio, global change, glucocorticoid

What is already known

Immune function is generally related to the thermal environment of ectothermic vertebrates. However, no studies have directly examined the role of seasonal acclimation during dormancy on baseline tortoise immune function.

What this study adds

This study demonstrates a strong seasonal pattern among several immune parameters, with generalized reduction during dormancy. Additionally, we pair the immune data to baseline glucocorticoid concentration, which documents that there is no pattern between these two physiological systems.

Abstract

Natural seasonal change can drive patterns of disease, especially among ectothermic vertebrates. In light of recent climate change, it is important to understand baseline disease resistance in a seasonal context to further understand the role that changes in seasonal weather patterns may have on disease frequency. Herein we found support for the seasonal acclimation hypothesis in *Gopherus polyphemus* (Gopher Tortoise), which indicated that natural seasonal variation causes differences in baseline immune function across seasonal acclimation states. We found that an innate immune parameter, bactericidal ability (BA), was significantly elevated in the summer ($P < 0.00001$). We found that circulating leukocyte profiles varied significantly among seasons, with increased heterophils and monocytes ($P = 0.00019$ and $P = 0.0001$, respectively) and decreased lymphocytes during winter ($P < 0.00001$). We assayed baseline glucocorticoid concentration (*i.e.* corticosterone, CORT) across seasons and sampling conditions to test whether CORT drove the seasonal pattern in immunological acclimation. Our sampling included free-ranging animals and animals maintained under semi-natural conditions for seasonal manipulations. We found that CORT was lowest during winter and in animals maintained in semi-natural conditions. These changes in CORT occurred independently of the immunological adjustments, suggesting that the immunological adjustments were not mediated by CORT secretion. The reduction in lymphocytes and BA suggest that seasonal acclimation is likely a restraint on energetic output when temperature is low and physiological performance is thermally constrained. While these parameters were reduced in winter, the increase in heterophils and monocytes may indicate a compensatory immune adjustment to increase the number of innate phagocytic cells.

Introduction

Global change has been described as a combination of anthropogenic environmental parameters including climate change, habitat loss and degradation, population fragmentation and invasive species that threatens the fitness of wild populations (Wingfield 2008). In light of global change, wildlife disease is predicted to be of increasing importance as it relates to seasonal changes in immune defenses. Specifically, diseases are predicted to increase in frequency both at the individual and population levels when winter conditions are harsh (Nelson and Demas, 1996; Nelson et al. 2002). Furthermore, normal physiological function may be compromised when individuals experience shifts in allostatic load that occur in response to seasonal change (McEwen and Wingfield 2003). Ectothermic vertebrates represent a group that is potentially most at risk to global change, and especially climate change; many critical physiological processes in these organisms are directly dependent on thermal parameters of the environment, changes in which could increase susceptibility to disease. Numerous diseases in ectothermic vertebrates are hypothesized to be linked to recent anthropogenic environmental change, including upper respiratory tract disease in North American tortoises (Jacobson et al. 2014), chytridiomycosis in amphibians (Fisher et al. 2009) and, recently, snake fungal disease in North American snakes (Sleeman 2013).

The seasonal acclimation hypothesis (Raffel et al. 2006) was generated and tested in amphibians to explain how natural seasonal changes in the immune system could account for increased disease susceptibility as a response to changing climate. In this case, seasonal acclimation is considered an adaptive shift in baseline immune performance to match the most appropriate level of immune function for the seasonal environment in which an individual is acclimated. Specifically, this hypothesis states that immune function is reduced in response to

natural seasonal shifts from warm-acclimation states to cold-acclimation states; this shift is hypothesized to be driven by the high energetic costs of maintenance of high immune function (Bonneaud et al. 2003) when the intrinsic growth rate of pathogens is low. Thus, maintenance of continuous and robust immune function when pathogen burden is low is an energetic sink for ectotherms during seasonally low temperatures. Moreover, the process of seasonal acclimation is described as a physiological restraint, rather than a temperature-direct constraint because cold-acclimated amphibians retain the ability to facultatively produce robust immune responses when stimulated (Pxytycz and Jozkowicz 1994). While under natural contexts, seasonal acclimation is considered a restraint selected as a means of energetic conservation, alterations in thermal environment have the potential to cause a change in immune function that drives an increase in disease susceptibility. Specifically, thermal shifts during seasonal transitions have the potential to cause disequilibrium between the optimal and the realized level of immune function while individuals are acclimating to a new thermal environment. This disequilibrium between optimal and realized immune performance has thus been term an "acclimation effect" (Raffel et al. 2006); this has been further supported in amphibians as thermally variable environments have been shown to directly increase disease susceptibility (Raffel et al. 2015).

Reductions in innate immune parameters of winter-acclimated animals have been detected in amphibians (Raffel et al. 2006) and mammals (reviewed in Demas et al. 2009). While seasonal differences in immune parameters have been shown within the active season in turtles (Zimmerman et al. 2010), the seasonal acclimation hypothesis of immune function has not been tested in ectothermic reptiles (see Seebacher 2005 for a review of seasonal and thermal acclimation in other physiological functions). Specifically, this hypothesis has not been tested across acclimation states including winter dormancy (when immune performance is hypothesized

to be most reduced). Most studies that have found seasonal variations in turtle immune responses have focused on induced changes (such as allograft rejection [Borysenko 1969] and immune cell proliferation assays, Munoz et al. 2001), which take longer to respond and do not represent baseline parameters (*e.g.* Borysenko 1969, Munoz et al. 2001). Our goal was to test the seasonal acclimation hypothesis across four seasons (*e.g.* spring, summer, fall and winter) using physiological parameters that represent constitutive function.

The Gopher Tortoise (*Gopherus polyphemus*) is a long-lived burrowing species of North American tortoise characterized by low juvenile recruitment and high adult survival (Tuberville et al. 2014). This species has experienced both long-term historical declines due to habitat loss and modification (Auffenberg and Franz 1982) as well as recent population declines attributable to disease outbreaks (Jacobson et al. 2014). Declines of North American tortoises (genus *Gopherus*) have been attributed to several pathogens including herpesvirus (Jacobson et al. 2012), ranavirus (Johnson et al. 2010) and most notably, mycoplasmal upper respiratory tract disease (URTD; caused by *Mycoplasma agassizii* and *Mycoplasma testudineum*, Jacobson et al. 2014). Direct links have been established between pathogen presence and population decline in *G. polyphemus* (e.g. Siegel et al. 2003), yet populations of *G. polyphemus* persist despite the presence of these pathogens (McGuire et al. 2014b). It has thus been hypothesized that disease patterns in North American tortoises are linked to environmental parameters that trigger susceptibility and not solely the presence of pathogens (Jacobson et al. 2014, Sandmeier et al. 2013).

Using the seasonal acclimation hypothesis, we were interested in testing if seasonal variation in constitutive immune parameters could account for changes in disease susceptibility in *G. polyphemus*. We used a functional assay, bactericidal ability (BA), to measure innate

immune function in Gopher Tortoises. Bactericidal ability is a commonly-used assay of the innate immune system that integrates humoral immunity and the complement system (Zimmerman et al. 2010). The second set of immune data we collected was both total and relative differential circulating leukocytes. Specifically, total leukocyte counts estimate how many white blood cells are circulating (and are counted relative to erythrocytes), while relative differential leukocyte counts indicate the proportion of each leukocyte class circulating (*e.g.* heterophils, lymphocytes, eosinophils, basophils and monocytes) relative to leukocytes as a single population. Differential leukocyte counts indicate differences in acquired verses innate immunity, as lymphocytes are solely considered cells of the adaptive immune system (Owen and Moore 2006), while heterophils, eosinophils, basophils, and monocytes perform innate functions of phagocytosis and driving inflammatory processes (Davis et al. 2008). Lastly, we measured baseline plasma corticosterone (CORT) across groups to test for a direct link between baseline immune function and baseline CORT concentration.

To test the hypothesis of seasonal immune acclimation, we used both field-collected samples and samples from animals maintained under semi-natural conditions. Using semi-natural housing conditions and hibernating animals in environmentally-controlled chambers is a well-established technique for studying hibernation physiology when dormant animals would otherwise be inaccessible. Similar experimental approaches have been used to study hibernation physiology in the Yellow Mud Turtle (*Kinosternon flavescens*) (Seidel 1978), the Argentine Black and White Tegu (*Tepinambis merianae*) (Andrade and Abe 1999) and the Golden-mantled Ground Squirrel (*Spermophilus lateralis*) (Prendergast et al. 2002).

We predicted that, if seasonal acclimation of immune parameters is present in *G. polyphemus*, a decrease in BA in the winter-acclimated individuals would occur. Because of the

temporal nature of acclimation, we predicted that individuals transitioning between acclimation states would show intermediate BA. Additionally, we hypothesized that total and differential leukocyte counts would vary among seasons. Under the context of the seasonal acclimation hypothesis, our prediction was that relative leukocyte populations would vary seasonally due to differential pathogen pressures among seasons, and that total leukocyte counts would be reduced in dormancy.

Methods

Animals, captive care & maintenance

Free-ranging adult *Gopherus polyphemus* (n=85) were trapped from private and public properties in Covington and Geneva Counties, Alabama USA. Tortoises were trapped using custom-ordered Tomahawk® live traps (Hazelhurst Wisconsin USA) placed at the entrance of each burrow. Traps were shaded using either a piece of burlap or vegetation from around the burrow. Upon capture, a blood sample was collected from either the femoral or brachial vein of each tortoise using a 25 gauge needle affixed to a 1 ml syringe that had been previously heparinized with a sterile 0.5% sodium heparin solution diluted in ultrapure water (Sigma Aldrich, St. Louis Missouri USA). Prior to venipuncture, the site of venipuncture was sanitized with an isopropyl alcohol wipe to prevent contamination of the blood sample and to minimize risk of infection to the tortoise. Ott et al. (2000) found that CORT was not affected by duration spent in the trap below 12 hours and was only marginally affected by durations greater than 12 hours. Thus, to ensure we collected baseline parameters and to minimize stress to the tortoise, we checked our traps at least twice daily.

Blood was stored on ice, and within one hour of collection the sample was centrifuged for approximately eight minutes at 1200 rpm, and the plasma fraction was removed and frozen in liquid nitrogen. Prior to centrifugation a 3-5 ul sample of whole blood was used to make blood smears.

Two different sub-populations (n=12 in 2013, n=11 in 2014) of adult *G. polyphemus* were placed into an on-campus animal facility at Auburn University in August 2013 and 2014 to serve as samples for seasonal manipulations. These tortoises were housed under semi-natural conditions in 10 x 5 m outdoor pens constructed using one meter tall hardware cloth with 0.635

cm x 0.635 cm mesh size buried approximately 20 cm below the surface to keep animals from digging out. Tortoises were housed individually within each pen and 45.7 cm black silt fencing was placed around the lower perimeter of the pen as a visual barrier between neighboring tortoises. Each pen contained a 45.7 cm x 10.16 cm aluminum pan sunk approximately 10 cm into the ground and filled with water, as well as an artificial burrow constructed using a 3 m section of 45.7 cm diameter plastic culvert that was longitudinally bisected to have the physical configuration of a *G. polyphemus* burrow. Artificial burrows were placed on the surface of the ground and were filled with sand to resemble natural tortoise substrate; a mound of sand was made at the entrance of each culvert to mimic the apron of a natural burrow. Burrows were partially shaded to prevent excessive solar heating. Pens were located in an open field that contained ample forage of grasses and forbs, thus we did not provide additional food. When overnight low temperatures were less than 6°C, tortoises were removed from the outdoor pens and brought into a thermostatically-controlled environmental chamber. The thermostat in the chamber was set to 12.5°C, which is the mean carapacial temperature of *G. polyphemus* overwintering in South Carolina (DeGregorio et al. 2012). To mimic the natural photoperiodic regime of an overwintering tortoise within a burrow, there was no light source within the chamber, but the room surrounding the chamber had unshaded windows that allowed a natural photoperiod within the room. The door entering the chamber had a small window that allowed scattered light to pass into the chamber.

Immediately prior to maintenance in the environmental chamber (12 November 2013) blood was collected from all tortoises in 2013 at approximately 1000h. Tortoises were removed from the outside pens and placed into the environmental chambers following the final sample collection. At that point, the tortoises were consistently inactive, and remained in their burrows,

even during the day. All tortoises were brought into the environmental chambers when they were inactive, so as to not interrupt normal activity patterns. Moreover, DeGregorio et al. (2012) found that, once adult Gopher Tortoises have initiated dormancy in November, under normal conditions, they remain cool (~12.5 °C) and below ground in an inactive state. Following the long-term maintenance in the environmental chamber, blood was again collected on 7 March 2014 at approximately 1000h. While in the winter-acclimated state, we placed tortoises in a bath filled with approximately 2 cm of water at room temperature for approximately four hours every four weeks to maintain proper hydration. Following all experimentation during summer 2014, the first group of captive tortoises was released at its exact point of capture. In August 2014, a second group of tortoises was brought into the same semi-natural conditions as the first group. Beginning in November 2014, this second group of tortoises was maintained throughout dormancy in the animal facility similarly to the first group. In March 2015, these animals were transferred back to the outside pens and were housed outside under semi-natural conditions until June 2015, when a blood sample was collected from each animal. These animals thus represent the immune state of tortoises sampled during the summer, but maintained under our semi-natural conditions throughout dormancy.

BLOOD SMEARS AND LEUKOCYTE COUNTS

Once blood was collected, a smear was immediately made by streaking approximately 3-5 ul of whole blood across a glass microscope slide using another microscope slide held at approximately a 45° angle. Blood samples were allowed to completely air dry, and were fixed within 24 hours in Hema 3 ® fixative (Fisher Scientific Company LLC, Kalamazoo, Michigan USA). Slides were later stained with a Hema 3 ® stain set (Fisher Scientific Company LLC, Kalamazoo, Michigan USA) according to the manufacturer's instructions.

We performed two different counts to approximate the number of each leukocyte type (*i.e.* heterophils, lymphocytes, eosinophils, basophils and monocytes). First, we identified leukocytes out of 3000 total blood cells, including leukocytes and erythrocytes, referred to from here as total blood differential counts. We also classified the proportion of each leukocyte type out of 100 total leukocytes, referred to from here as leukocyte differential counts. Heterophil: lymphocyte ratios (HLR) were calculated from the leukocyte differential counts. Cells were examined using a light microscope at 1000X magnification with oil immersion and were classified by visual inspection according to cell-specific morphological characters (Fig. 4) from Alleman et al. (1992).

BACTERICIDAL ABILITY

We used plasma samples to perform an assay of the bactericidal ability (BA) of plasma. This is a functional assay of the constitutive innate immune system mediated by the complement cascade (Zimmerman et al. 2010). Samples were frozen and stored in the field in liquid nitrogen and then stored in a -80°C freezer until the assay was performed. Bactericidal assays were performed within two weeks of plasma collection to prevent temporal degradation of samples. To perform the assay, 3 ul of freshly thawed plasma was added to 137 ul of CO_2-independent/L-Glutamine medium. This medium was made by adding 40ul of L-Glutamine to 200 ml of Gibco ® CO_2-independent medium (Thermo Fisher Scientific LLC, Waltham Massachusetts USA). Ten microliters of an *Escherichia coli* stock solution suspended in phosphate-buffered saline was then introduced to the plasma/medium solution. The stock *E. coli* suspension was diluted from stored suspensions so that negative controls contained approximately 200 colony forming units (CFU) per 50 ul of final assay suspension. The final plasma dilution for this assay was 1:50. Within each assay were embedded two negative controls, which received 140 ul ofCO_2/L-

Glutamine medium and no plasma sample. The assay was incubated at room temperature for 1 hour, following which 50 ul of each sample were plated using sterile technique on sterile culture plates pre-made with Tripticase® Soy Agar (catalog number 221283, VWR International, Atlanta Georgia USA). Each culture sample was plated in duplicate and the mean of the pairs was used for statistical analyses. Plates were incubated at 32°C for approximately 16 hours, or until individual CFU were visible. Bactericidal ability was calculated as the percent bacteria killed, which was the mean CFU of both negative controls minus the mean CFU of the sample, divided by the mean CFU of the negative controls, and multiplied by 100.

CORTICOSTERONE ASSAY

Plasma steroids were extracted using a single anhydrous diethyl ether extraction. The protein fraction was separated by freezing, and the ether supernatant containing the steroids was decanted, dried under nitrogen gas stream, and then resuspended in 300 uL of phosphate-buffered saline (PBS) gel, thus yielding a final plasma steroid dilution of 1:20. Known CORT standards were extracted in parallel with unknowns to determine extraction efficiency, which averaged 79% for this assay. Baseline corticosterone was assayed from the extracted samples using 100 uL of sample on a corticosterone Enzyme Immunoassay kit according to the manufacturer's instructions (Enzo Life Sciences, Farmingdale New York USA, cat. #: ADI-900-097). Manufacturer's instructions were followed to yield a 1:20 plasma dilution. Corticosterone was assayed using one kit and the intra-assay variation was 3.7%. Corticosterone levels were calculated and then adjusted to compensate for extraction efficiency.

SEASONAL CLASSIFICATIONS AND STATISTICAL ANALYSES

We found no effect of sex on BA (T_{66} = 1.71, P = 0.25), CORT (U = 361, P = 0.67), or HLR (U = 445, P = 0.31) for each parameter, thus we combined data for all individuals in each seasonal sample.

Because our housing conditions may have effects on immune parameters, we used sequential Bonferroni p-value adjustments (Holm 1979) to determine significant family-wise differences under either free-ranging or semi-natural conditions. Fall samples were directly compared to winter samples (using a paired t test), winter samples were directly compared to captive summer samples (using a t test), wild summer samples were directly compared to captive summer samples (using a t test) and free-ranging spring samples (from animals collected in the wild upon their first emergence) were directly compared to free-ranging summer samples (using a t test).

Results

BACTERICIDAL ABILITY

We found that there was a significant effect of seasonal acclimation state on BA (Table 2, Fig. 5), with a significant reduction in winter, relative to animals in semi-natural conditions during the summer ($t_{20} = 5.633$, $P < 0.00001$). There was also a significant reduction in BA in fall relative to summer ($t_{18} = 3.565$, $P = 0.002$). There was a tendency for lower BA in free-ranging animals first emerging from dormancy relative to free-ranging animals during the summer, although this difference ($t_{60} = 2.212$, $P = 0.0308$) was not significant at the sequentially-adjusted p value (0.017).

TOTAL BLOOD COUNTS

We found significant differences in the total number of circulating leukocyte types across seasons (Table 3, Fig. 6). These differences resulted from a significant increase in heterophils during winter relative to animals under semi-natural conditions in the summer ($t_{21} = 4.521$, $P = 0.0002$), a significant decrease in the number of lymphocytes in the winter relative to animals under semi-natural conditions during the summer ($t_{21} = 6.466$, $P < 0.00001$), and a significant reduction in the number of lymphocytes during the fall relative to the summer ($t_{19} = 6.913$, $P < 0.00001$). We did not find any significant differences in the number of eosinophils or basophils. We found that the total number of monocytes was significantly elevated in winter, relative to fall ($t_9 = -6.26$, $P = 0.0001$) and summer ($t_{21} = 4.324$, $P = 0.0003$). In free-ranging animals, we found that there was a significant increase in monocytes during spring relative to summer ($t_{58} = 3.911$, $P = 0.0002$).

DIFFERENTIAL LEUKOCYTE COUNTS

We found that, in general, differential leukocyte counts revealed similar patterns as the total blood counts. Differential leukocytes significantly varied between seasons (Table 4, Fig. 7), and heterophils were significantly elevated in fall (t_{18} = -6.563, P < 0.00001) and winter (t_{22} = 11.575, P < 0.00001) relative to tortoises under semi-natural conditions during the summer. An opposite pattern was found for lymphocytes, which were significantly reduced in fall (t_{18} = 6.618, P < 0.00001) and winter (t_{22} = -12.901, P < 0.00001) relative to animals under semi-natural conditions during the summer. Additionally, we found that relative lymphocytes were significantly reduced in free-ranging animals during the summer when compared to animals under semi-natural conditions during the summer (t_{52} = 2.772, P = 0.0078). We found that relative eosinophils did not significantly vary. Relative basophils were significantly increased in animals under semi-natural conditions during winter (t_9 = 4.894, P = 0.0009) and fall (t_{18} = -2.792, P = 0.012) relative to summer. We found that relative monocytes were significantly increased in animals under semi-natural conditions during winter relative to fall (t_9 = -6.075, P = 0.0002) and summer (t_{22} = 3.795, P = 0.001).

BASELINE CORTICOSTERONE

We found that baseline CORT varied significantly among seasons, but the main effect on CORT was whether animals were free-ranging or under semi-natural conditions (Table 5, Fig. 8). Animals maintained under semi-natural conditions during winter had low CORT levels. There was a significant reduction in CORT during winter relative to fall (t_{10} = 3.881, P = 0.0031) and a significant reduction in CORT in animals during summer relative to fall (t_{18} = -3.13, P = 0.0058). Lastly, CORT was lower in animals maintained under semi-natural conditions during summer relative to free-ranging animals during the summer (t_{47} = 2.539, P = 0.0145). Linear regression

indicated no significant effect of CORT on either BA ($F_{1,54} = 0.67$, $P = 0.42$) or HLR ($F_{1,44} = 2.38$, $P = 0.13$).

Discussion

We found support for the seasonal acclimation hypothesis in *Gopherus polyphemus*. Derived from laboratory and field studies of amphibians (Raffel et al. 2006), this hypothesis has direct relevance to disease outbreaks in ectothermic vertebrates. Our results support that seasonal immunological patterns exist in testudines. Understanding how predicted seasonal change and, furthermore, unpredicted thermal instability (Raffel et al. 2015) affect disease susceptibility in ectothermic tetrapods is of great and increasing importance.

A seasonal pattern was present in an innate immune parameter, bactericidal ability (BA), which was reduced in winter dormancy, and was maintained at intermediate levels during transitional acclimation states in fall and spring (Fig. 5). Bactericidal ability was highest during the summer active season, in both free-ranging animals and those maintained under semi-natural conditions. Using a different innate immune assay, lysozyme activity, Raffel et al. (2006) described a strongly seasonal-dependent response in amphibians, in which lysozyme activity was greatest in the middle of summer, and decreased throughout the rest of the year to very low levels by late spring. We resolved a similar seasonal pattern for BA in *G. polyphemus*. Thus, this parameter of immune function enters a state of relative dormancy during winter, rather than displaying compensatory phenotypic flexibility in which a robust baseline is maintained independently of season (Seebacher 2005).

Interestingly, both parameters of circulating leukocytes (*i.e.*, leukocyte differential counts and total blood differential counts) produced similar results, and supported seasonal acclimation of cellular components of the immune system in *G. polyphemus*. Two different measures of leukocyte counts indicated that circulating lymphocytes are lowest during winter and highest during summer, with slightly intermediate levels during the spring and fall.

In contrast to our results for lymphocytes, heterophil counts were highest during winter (in both parameters of leukocyte counts) and lowest during summer. Thus, as a whole, our heterophil data indicate an increase in the absolute number of heterophils during winter that is not simply attributed to a relative decrease in the number of lymphocytes. Given the differences in the function of heterophils and lymphocytes (Davis et al. 2008), our data suggest that, as part of the process of seasonal acclimation, the immune system of *G. polyphemus* acclimates with a response that is largely dependent on innate, phagocytic cells (*e.g.* heterophils), and less dependent on slowly responding cells of the acquired immune system (*e.g.* lymphocytes). This acclimation to winter may represent a compensatory tradeoff in the immune system towards the most relevant and functional immune response.

We compared baseline CORT across seasonal treatments to test if glucocorticoid secretion may either regulate seasonal acclimation (Nelson et al. 2002) or immune function itself. We found that, while CORT varied among treatment groups, this variability did not explain the immune differences. Moreover, we found that CORT was lowered in animals that had been sampled in semi-natural conditions. We cannot determine whether or not these differences in CORT were a short-term result of handling in the field. However, Ott et al. (2000) found that trapping did not significantly elevate CORT in *G. polyphemus*. Additionally, Kahn, Guyer, and Mendonça (2007) found that tortoises removed from the wild had lower CORT than free-living individuals, although the differences were not statistically significant. We suspect that lowered CORT in penned animals resulted from some factor other than direct handling of the animals. We suggest that maintaining animals individually under semi-natural conditions may reduce stressors, such as predation and territory defense, which may increase CORT. Regardless of what caused the reduction of CORT in these short-term captive animals, the fact that the

immune parameters did not show the same pattern suggests that immune responses and glucocorticoid concentration are not always directly related. This has been shown elsewhere in a meta-analysis that demonstrated CORT and heterophil: lymphocyte ratios do not consistently correlate (Goessling et al. 2015). Both CORT and heterophil: lymphocyte ratio (HLR) have been used as indicators of baseline stress in vertebrates (Goessling et al. 2015, Davis et al. 2008), yet these related stressor-induced changes do not consistently co-vary with each other (Müller et al. 2012). Our data suggest that, in this case, the immunological changes that occur in response to seasonal acclimation are not mediated by a stressor-dependent response, but rather are thermally mediated physiological acclimation

Seasonal down-regulation of immunity has been considered a restraint on the immune system, selected for as resource conservation when intrinsic growth rates of pathogens are reduced (Bonneaud et al. 2003, Raffel et al. 2006). Our data suggest there is a seasonal pattern that might function to optimize performance of the immune system (such as through increasing circulating phagocytes), while compensating for thermally-constrained components of the immune system (such as reduced circulating lymphocytes and reduced bactericidal ability).

Under natural conditions, seasonal down-regulation of physiological performance has been considered a restraint on physiological process in ectotherms (Raffel et al. 2006), yet under recent anthropogenic conditions of global change, this process may become a constraint that reduces pathogen resistance in an organism during times when thermal stability varies (Raffel et al. 2015). During seasonal transitions or variable thermal environments, individuals may experience body temperatures to which they have not acclimated, and thereby suffer mis-alignment of optimal and realized immune function (*e.g.* an acclimation effect, Raffel et al. 2006). Moreover, climatic instability has been shown to increase disease susceptibility in

amphibians (Rohr and Raffel 2010, Raffel et al. 2015), thereby further indicating the potential importance of climate change in driving patterns of disease in ectotherms.

Understanding the temporal nature of shifts between seasonal acclimation states is crucial to understanding how individuals may be constrained to acclimation. Specifically, tests of a seasonal lag effect (Raffel et al. 2006) in *G. polyphemus* would further inform the role that climatic instability has in driving patterns of disease outbreak. Furthermore, examination of the temporal nature of temperature-dependent immunological acclimation will further understanding of behavioral changes, such as fever responses, associated with disease in this taxon (McGuire et al. 2014a).

While there are major differences in the taxonomic breath and thermal physiologies of ectothermic vertebrates, this study indicates the same processes that subject ectotherms to increased disease susceptibility may be shared across taxa. With increased negative effects of global change, especially climate change that threatens environmental and thermal stability (Raffel et al. 2015), it is crucial to test hypotheses related to ectotherm immune systems to better understand threats to population stability.

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Table 2. Statistical comparisons of bactericidal ability (Bonferroni-significant p values highlighted)

Comparison	t value	D.F.	p value	Bonferroni-adjusted significant p value
captive summer to captive winter	-5.633	20	<0.00001	0.01
captive summer to captive fall	3.565	18	0.0022	0.0125
free-ranging spring to free-ranging summer	-2.212	60	0.0308	0.016667
captive summer to free-ranging summer	1.656	62	0.103	0.025
captive fall to captive winter	1.633	8	0.141	0.05

Table 3. Statistical comparisons of total blood counts (Bonferroni-significant p values highlighted)

Comparison	t value	D.F.	p value	Bonferroni-adjusted significant p value
Differences in total heterophils				
captive summer to captive winter	4.521	21	0.000187	0.01
captive fall to captive winter	-3.068	9	0.0134	0.0125
captive summer to captive fall	-1.874	19	0.0763	0.0167
free-ranging spring to free-ranging summer	1.713	58	0.092	0.025
captive summer to free-ranging summer	-1.075	61	0.287	0.05
Differences in total lymphocytes				
captive summer to captive winter	-6.466	21	<0.00001	0.01
captive summer to captive fall	6.913	19	<0.00001	0.0125
captive summer to free-ranging summer	2.03	61	0.0467	0.0167
captive fall to captive winter	-0.391	9	0.705	0.025
free-ranging spring to free-ranging summer	0.082	58	0.935	0.05
Differences in total eosinophils				
captive fall to captive winter	-2.794	9	0.0209	0.01
captive summer to captive winter	1.506	21	0.147	0.0125
captive summer to captive fall	1.449	19	0.164	0.0167
captive summer to free-ranging summer	-0.638	61	0.526	0.025
free-ranging spring to free-ranging summer	0.28	58	0.78	0.05
Differences in total basophils				
captive fall to captive winter	1.175	9	0.27	0.01
captive summer to free-ranging summer	-0.823	61	0.414	0.0125
captive summer to captive winter	-0.462	21	0.649	0.0167
captive summer to captive fall	0.447	19	0.66	0.025
free-ranging spring to free-ranging summer	0.183	58	0.856	0.05

Differences in total monocytes				
captive fall to captive winter	-6.26	9	0.0001	0.01
free-ranging spring to free-ranging summer	3.911	58	0.0002	0.0125
captive summer to captive winter	4.324	21	0.0003	0.0167
captive summer to captive fall	1.115	19	0.279	0.025
captive summer to free-ranging summer	0.377	61	0.708	0.05

Table 4. Statistical comparisons of differential leukocyte counts (Bonferroni-significant p values highlighted)

Comparison	t value	D.F.	p value	Bonferroni-adjusted significant p value
Differences in relative heterophils				
captive summer to captive winter	11.575	22	<0.00001	0.01
captive summer to captive fall	-6.563	18	<0.00001	0.0125
captive summer to free-ranging summer	-2.243	52	0.0292	0.0167
free-ranging spring to free-ranging summer	1.691	49	0.0972	0.025
captive fall to captive winter	-1.551	9	0.155	0.05
Differences in relative lymphocytes				
captive summer to captive winter	-12.901	22	<0.00001	0.01
captive summer to captive fall	6.618	18	<0.00001	0.0125
captive summer to free-ranging summer	2.772	52	0.0078	0.0167
captive fall to captive winter	2.028	9	0.0732	0.025
free-ranging spring to free-ranging summer	-0.76	49	0.451	0.05
Differences in relative eosinophils				
free-ranging spring to free-ranging summer	-1.313	49	0.195	0.01
captive summer to captive fall	1.239	18	0.231	0.0125
captive fall to captive winter	-0.894	9	0.394	0.0167
captive summer to free-ranging summer	0.739	52	0.463	0.025
captive summer to captive winter	-0.235	22	0.816	0.05

Differences in relative basophils				
captive fall to captive winter	4.894	9	0.0009	0.01
captive summer to captive fall	-2.792	18	0.012	0.0125
captive summer to free-ranging summer	-1.313	52	0.195	0.0167
captive summer to captive winter	0.534	22	0.599	0.025
free-ranging spring to free-ranging summer	0.488	49	0.628	0.05

Differences in relative monocytes				
captive fall to captive winter	-6.075	9	0.0002	0.01
captive summer to captive winter	3.795	22	0.001	0.0125
free-ranging spring to free-ranging summer	0.54	49	0.592	0.0167
captive summer to captive fall	-0.472	18	0.642	0.025
captive summer to free-ranging summer	0.417	52	0.679	0.05

Table 5. Statistical comparisons of baseline corticosterone (Bonferroni-significant p values highlighted)

Comparison	t value	D.F.	p value	Bonferroni-adjusted significant p value
captive fall to captive winter	3.881	10	0.0031	0.01
captive summer to captive fall	-3.13	18	0.0059	0.0125
captive summer to free-ranging summer	-2.539	47	0.0145	0.0167
free-ranging spring to free-ranging summer	-0.521	45	0.605	0.025
captive summer to captive winter	-0.274	20	0.787	0.05

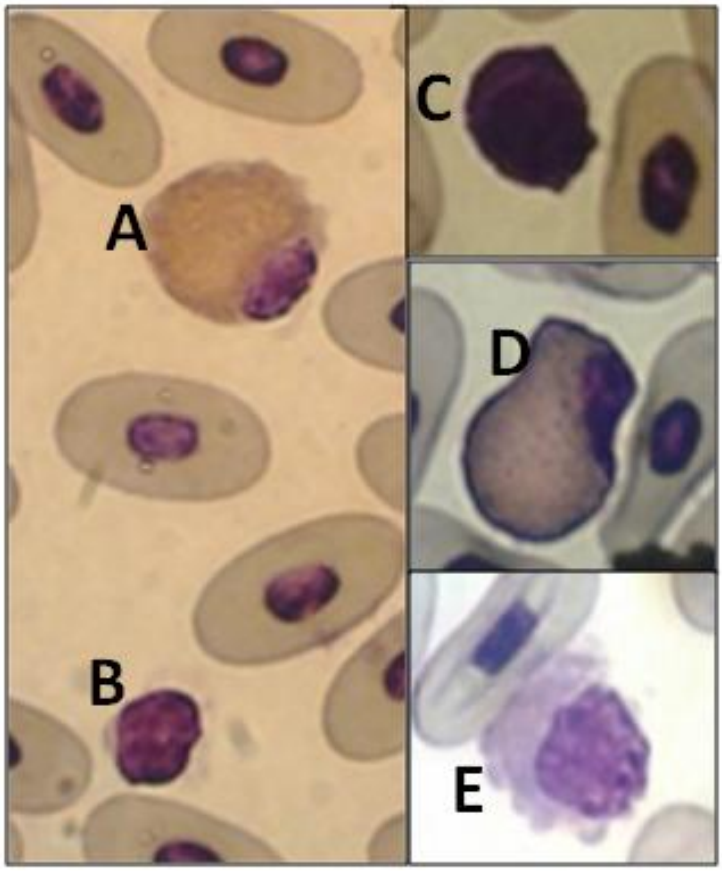

Figure 4. The five leukocyte morphological classes diagnosed in this study: (A) heterophil, (B) lymphocyte, (C) basophil, (D) eosinophil and (E) monocyte.

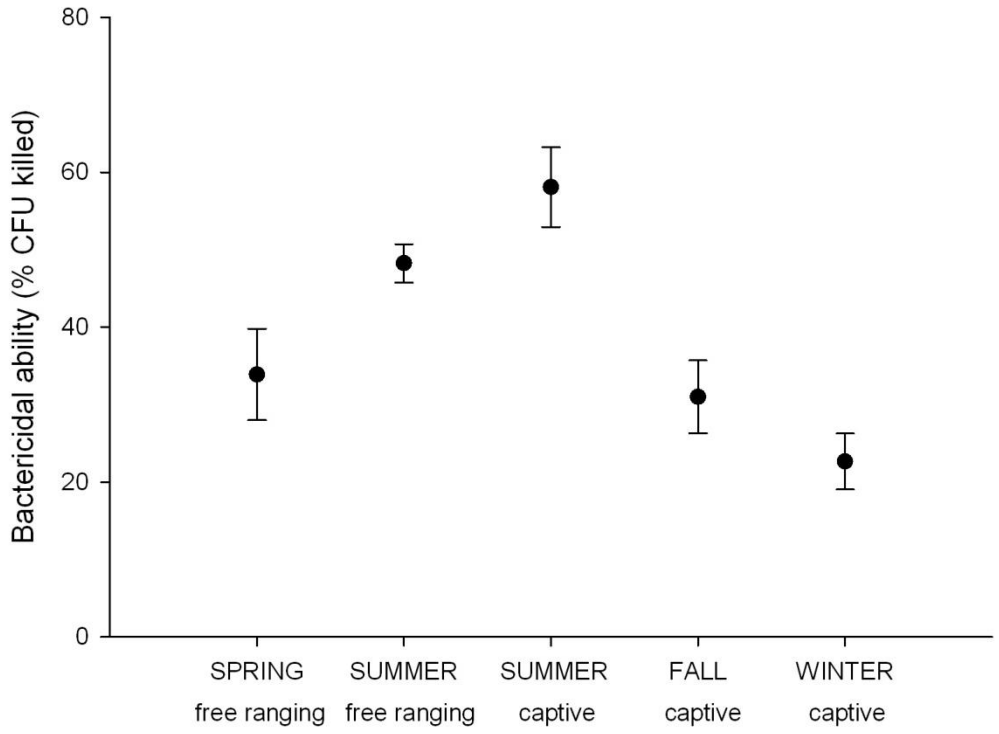

Figure 5. *Gopherus polyphemus* bactericidal ability (BA) varied significantly among seasons with the greatest function in the summer acclimation state and the least function in the winter acclimation state. See Table 1 for statistical comparisons.

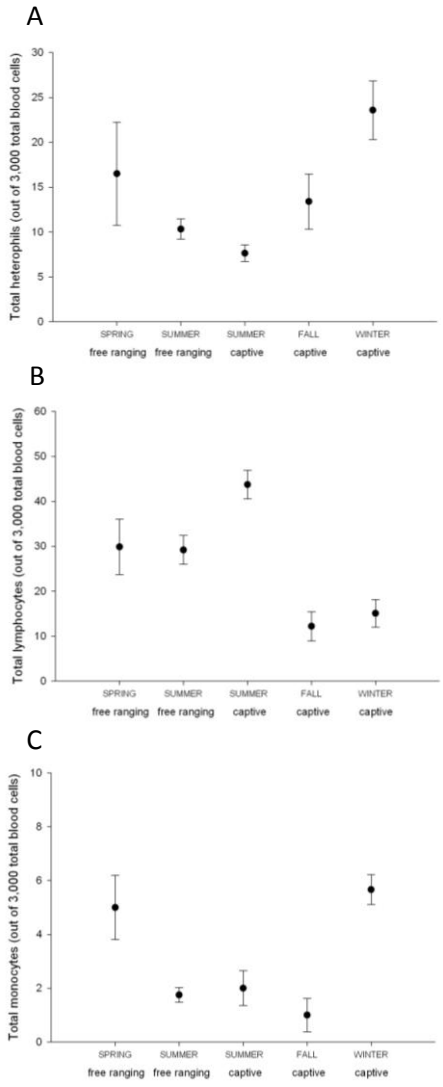

Figure 6. We performed total blood differential counts by identifying leukocytes out of 3000 total blood cells (*e.g.* erythrocytes, heterophils, lymphocytes, eosinophils, basophils, and monocytes). There was an increase in the number of heterophils and a decrease in the number of lymphocytes during dormancy. "Captive" animals were maintained for short-term captivity under semi-natural conditions. Significant differences were within (A) heterophils, (B) lymphocytes and (C) monocytes. See Table 2 for statistical comparisons.

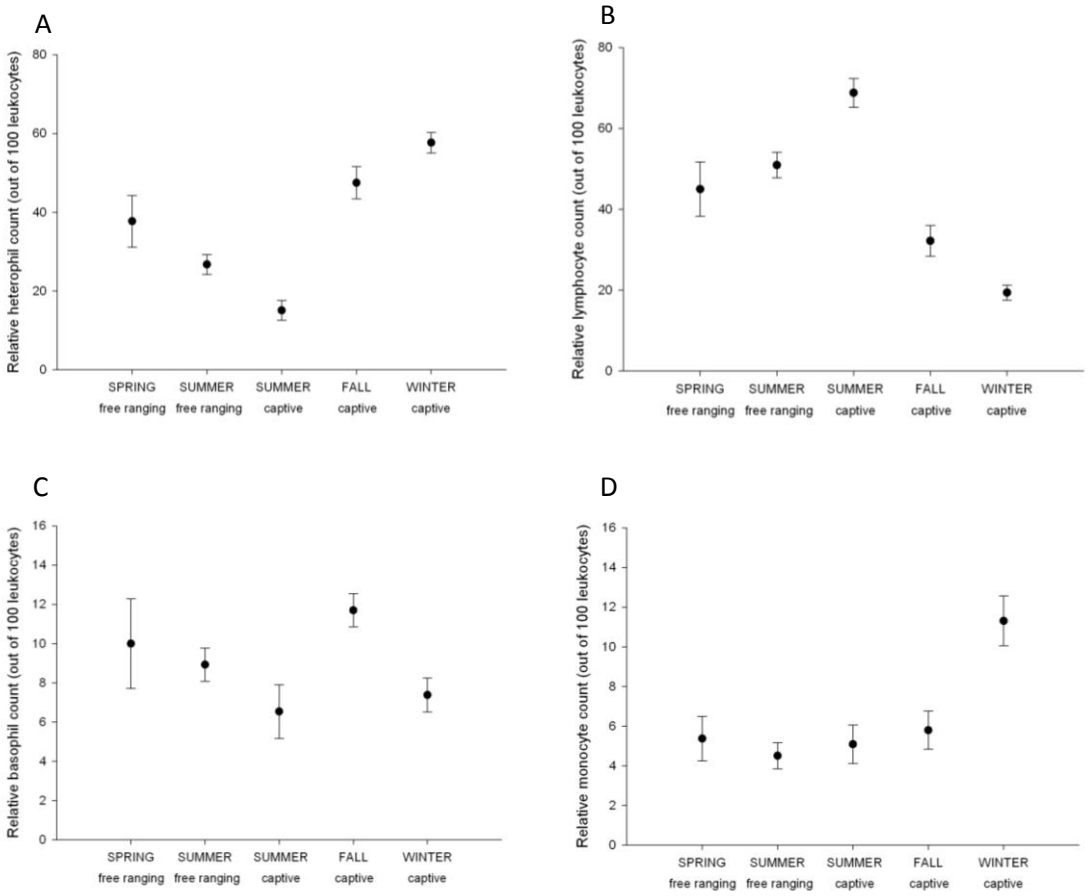

Figure 7. We performed differential leukocyte counts by counting the number of each leukocyte type (*e.g.* heterophil, lymphocyte, eosinophil, basophil, or monocytes) out of 100 leukocytes. The relative number of heterophils was increased during winter dormancy while the relative number of lymphocytes was decreased during winter dormancy. "Captive" animals were maintained for short-term captivity under semi-natural conditions. Significant differences were within (A) heterophils, (B) lymphocytes, (C) basophils and (D) monocytes. See Table 3 for statistical comparisons.

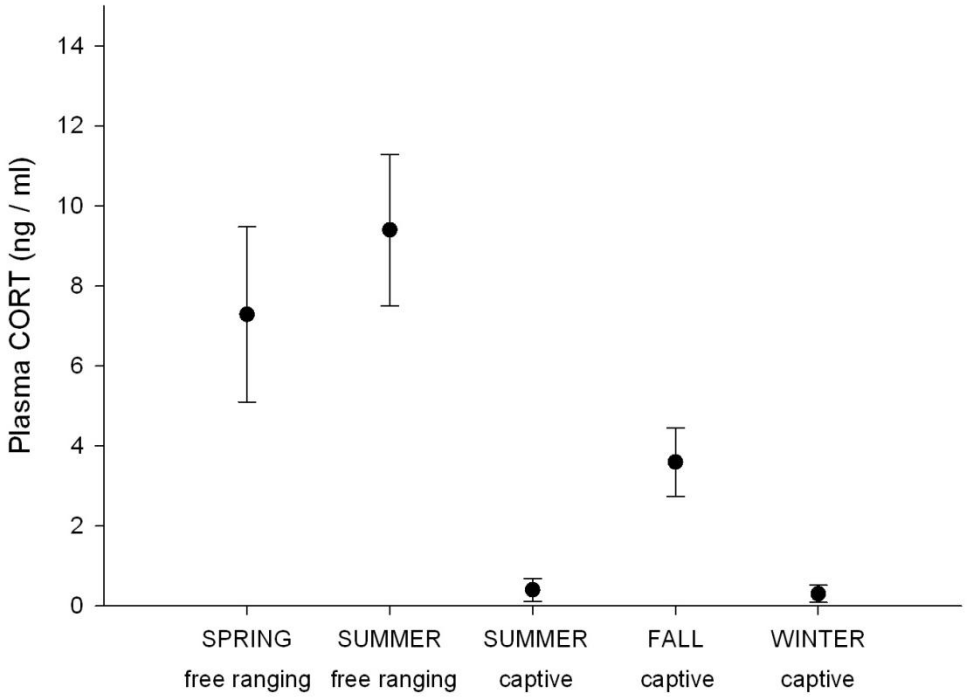

Figure 8. There were significant differences in baseline plasma corticosterone concentration (CORT) among treatments. Animals maintained under semi-natural conditions generally had reduced CORT. See Table 5 for statistical comparisons.

Chapter 4: Seasonal lag of immunity in Gopher Tortoises (*Gopherus polyphemus*)

FORMAT FOR JOURNAL OF EXPERIMENTAL BIOLOGY

Title: Seasonal lag of immunity in Gopher Tortoises (*Gopherus polyphemus*)

Running title: Immunity in Gopher Tortoises

Goessling, Jeffrey M.[1]*, Shane A. Koler[1], Brian D. Overman[1], Elizabeth H. Schwartz[1], Craig Guyer[1] and Mary T. Mendonça[1]

[1] Dept. of Biological Sciences, 331 Funchess Hall, Auburn University AL 36849

*corresponding author: goessling@auburn.edu

Key words: Ecoimmun*, ELISpot, bactericidal ability, global change, tortoise, *Gopherus*

Summary statement: We experimentally demonstrated strong thermal constraints on tortoise immune function, but failed to find support for the seasonal lag hypothesis.

ABSTRACT

Disease outbreaks are of increasing consequence to wild populations of ectothermic vertebrates as a direct result of global change. Anthropogenic climate change is predicted to increase climatic instability, thereby altering natural thermal environments. In this study we evaluated the direct effects of rapid temperature change on baseline immunity in Gopher Tortoises (*Gopherus polyphemus*). Specifically, we tested the seasonal lag hypothesis, which predicts severe misalignment of optimal and realized immunity when temperature rapidly changes. We assayed baseline innate immunity, cell-mediated humoral immunity and differences in relative innate and adaptive immunity in response to rapid temperature increase in animals acclimated to winter conditions and in response to rapid temperature decrease in animals acclimated to summer conditions. We found that during summer, rapid temperature reduction caused a series of changes in immunity, including reduced bactericidal ability ($P = 0.002$), reduced cell-mediated humoral immunity ($P < 0.0001$) and increased heterophil: lymphocyte ratios ($P < 0.00001$). During winter, we found that a temperature increase provided no benefit to immunity. Specifically, there was no increase in bactericidal ability as was predicted by the seasonal lag hypothesis. Cell-mediated humoral immunity was significantly reduced in response to rapid warming ($P = 0.011$) and the rapid warming caused a significant reduction in heterophil: lymphocyte ratios ($P < 0.00001$). Independent of temperature, we found a significant acclimation effect of winter relative to summer conditions on cell-mediated humoral immunity ($P < 0.001$) in which winter caused an overall increase in baseline cell-mediate humoral immune response. Our findings generally indicate that rapid temperature change is a constraint on immunity in ectothermic vertebrates. Furthermore, this study provides a proximate mechanism by which disease frequency may increase as a result of global change.

INTRODUCTION

Climate change and its associated thermal consequences have been shown to cause increased susceptibility to infectious diseases in amphibian populations (Raffel et al. 2006, Raffel et al. 2015). Because their physiologies are directly linked to the thermal environment, ectotherms represent a group that may be especially vulnerable to negative effects of climatic instability, including alterations to baseline immune function and disease susceptibility.

North American tortoises (genus *Gopherus*) have been the subject of intense study and conservation efforts, as this taxon is of both high conservation value and has experienced recent declines (Jacobson et al. 2014, Tuberville et al. 2014). Moreover, environmentally-linked disease outbreaks have been considered an important source of mortality in *Gopherus* populations (Jacobson et al. 2014). For example, Sandmeier et al. (2013) found a positive relationship between severe winter weather and markers of upper respiratory tract disease (URTD) in Mojave Desert Tortoises (*Gopherus agassizii*). Additionally, telemetry studies have indicated that disease may be an important constraint on Gopher Tortoises (*Gopherus polyphemus*) during winter, when diseased individuals were found to leave their burrows during cold weather (McGuire et al. 2014).

In light of recent climate change, there is increasing need to understand the mechanisms by which immune function may be constrained by thermal variability. The seasonal acclimation hypothesis (Raffel et al. 2006) describes natural seasonal variation in immunity as season-dependent acclimation in function. A prior study (Ch2 of this dissertation) found support for the seasonal acclimation hypothesis in *G. polyphemus*, which showed that baseline immune function is strongly season dependent. Bactericidal ability and circulating lymphocytes were found to be significantly reduced during winter dormancy in *G. polyphemus*. This study additionally found a

significant increase in the number of heterophils and monocytes in circulation during winter. While a seasonal pattern was found in immunity, the temporal mechanisms of immune acclimation remain unclear.

Raffel et al. (2006) tested the seasonal lag hypothesis to determine how immune function is affected by rapid temperature fluctuation within seasonal acclimation states. Seasonal lag of immunity in ectotherms has two temperature-dependent (and thus season-independent) predictions: (1) rapid increases in temperature cause over production of immune components above optimal levels and (2) rapid decreases in temperature cause under production of immune components below optimal levels (see Fig. 9). An important consideration of seasonal lag of immunity is that "optimal" immune state is defined as the seasonally-acclimated immune state. For example rapid reduction of temperature from that expected by animals during summer to that expected by the same animals during winter not only causes a reduction in immune function, but causes continued reduction to levels below the winter acclimation state. Thus, seasonal lag provides a prediction and explanatory mechanism by which thermal instability alters immune function from optimal levels and thus a direct explanation of how climatic instability can increase disease frequency.

Our previous work (Chapter 3) found support for the seasonal acclimation hypothesis in *G. polyphemus*. Here, we test the seasonal lag hypothesis of immunity in this species using a series of immune parameters to assay constitutive innate immune function, cell-mediated humoral immune function, and differential leukocyte counts to quantify relative innate verses adaptive immune parameters.

MATERIALS AND METHODS

Animals, captive care and maintenance

Wild adult *Gopherus polyphemus* (Daudin; n = 12 in 2013, n = 11 in 2014) were trapped in Covington Co AL and returned to a live-animal facility at Auburn University in August. Tortoises were individually housed in outdoor pens that were 5 x 10 m. Each pen contained an artificial burrow constructed using a 3 m section of culvert pipe that was longitudinally bisected and partially filled with sand to mimic tortoise burrow substrate. Each pen contained an aluminum water dish; tortoises did not receive supplemental food as the pens contained ample forage of grasses and forbs for *ad libitum* feeding. In November, when tortoises were consistently inactive, all animals were brought into thermostatically-controlled environmental chambers which were maintained at 12.5°C through the dormancy period. This temperature was selected as it represents the mean body temperature of wild dormant tortoises in South Carolina (Degregorio et al. 2012), the closest population to our study site for which such data exist. Tortoises were individually housed through dormancy in the environmental chambers in plastic bins filled with approximately 2 cm of sand from each tortoise's respective burrow. Tortoises were allowed to soak in a shallow pan of room temperature water approximately every three weeks to provide adequate rehydration and waste excretion. In early March, following maintenance through dormancy, the winter lag experiments were conducted. Following the winter lag experiments, each tortoise was returned to its pen. The July following the winter lag experiments, summer lag experiments were conducted. To conduct winter lag experiments, tortoises remained in their bins and were placed into an environmental chamber set to 32.5°C for 48 hours. Thus, winter experiments were conducted by applying a rapid temperature increase to normal summer body temperatures during dormancy. To conduct summer lag experiments, tortoises were brought into an environmental chamber set to 32.5°C (which was approximately the summer thermoregulatory setpoint for *G. polyphemus* (Goessling, unpubl. data) and were

allowed to acclimate for 24 hours to the environmental chamber. Following the initial 24 hours, the thermostat was changed to 12.5°C. Tortoises were maintained in the 12.5°C environmental chamber for 48 hours. Thus, the summer lag experiments represented a rapid temperature decrease from the normal summer temperature to the normal winter body temperature. During experiments, two blood samples were taken from each tortoise, representing pre- and post-temperature change conditions.

Approximately 500 ul of whole blood were collected from either the femoral or brachial vein using a heparinized syringe. Both pre- and post- samples were always collected from the same vein to prevent confounding treatment effects with potential effects of venipuncture site. Upon collection, a blood smear was made using ~5 ul of whole blood, which was temporarily stored on ice. The whole blood sample was centrifuged and the plasma fraction was stored at -80°C for later BA assays. In 2015, we used most of the blood sample for isolation of live circulating lymphocytes. Approximately 100 ul of the whole blood was used to isolate plasma for BA assays.

Assays

ELISpot assays were performed by isolating circulating lymphocytes and quantifying cell-specific antibody responses (Zimmerman et al. 2013). We performed these assays on samples collected from experiments in March and July 2015. Lymphocytes were isolated by spinning whole blood samples over a hystopaque density gradient (Sigma Aldrich, cat. 11191) and culturing the cells at ~50,000 per well on a multiscreen HTS IP sterile plate (Millipore, cat. MSIPS4W10). Twenty-four hours prior to adding cells, plates were hydrated and coated with capture antibody (University of Florida Monoclonal Laboratory, HL-673 anti-Desert Tortoise). Cells were cultured in RPMI medium supplemented with fetal calf serum,

penicillin/streptomycin, glutamine and β-mercaptoethanol. Cell cultures were divided into replicates of three different LPS treatments: no stimulation, which received 100 ul of additional culture media, and two LPS treatments, which received 100 ul of culture media supplemented with either 4 ug/ml LPS or 40 ug/ml LPS. Cell cultures were then incubated for three days in 5% carbon dioxide at 32.5 °C. After incubation, the supernatant was removed, and the immunospots were developed using a biotinylated tortoise antibody (University of Florida Monoclonal Laboratory, HL-673 anti-Desert Tortoise) and a streptavidin-horseradish peroxidase kit with an AEC substrate (BD Life Sciences, cat. 557630 and 551951). Once developed, membranes were individually photographed using a dissection scope and spots were counted and spot size was quantified in ImageJ (nih.gov). The stimulative fraction (SF) was calculated as the proportion of cells that produced a measurable response (i.e. number of spots out of the total cells added) and the stimulation index (SI) represents the average size of spots per each responding cell. In 2014, we did not perform ELISpot assays. Non-parametric statistics were used to compare non-normally distributed ELISpot responses across seasonal acclimation states.

Bactericidal assays were performed within two weeks of sample collection. The plasma was assayed at a 1:50 final dilution. We added plasma to CO_2-independent growth medium (Thermo Fisher Scientific, cat. 18045-088) and supplemented the growth media with L-glutamine. Stock *Escherichia coli* suspensions were made to challenge plasma samples with approximately 200 colony-forming units in negative controls. Bacterial suspensions were made in sterile phosphate-buffered saline. We added bacteria stocks to the plasma/medium solution, and this was incubated for 60 minutes at room temperature. Following incubation, the solutions were plated in duplicate on Tripticase soy agar plates (VWR cat. 221283) and were incubated

overnight at 32°C. Colonies were counted the following morning. Data were analyzed as the mean percentage of CFU killed in the suspension relative to negative (e.g. no plasma) controls.

Differential leukocyte counts were made by assigning all identifiable leukocytes to one of five morphological classes: Heterophils, lymphocytes, eosinophils, basophils and monocytes. The same person (JMG) performed all leukocyte counts to reduce surveyor variability and the counts were done blindly so as to reduce any surveyor bias. A total of 100 leukocytes were counted for each slide, thus the number of each cell type represents that cell type's circulating percentage.

RESULTS

Bactericidal ability (BA)

We found that, in winter, a rapid increase in temperature caused no significant change in BA (Paired t test: $t_{21} = 1.009$, $P = 0.325$; Fig. 10). However, during summer, we found that a rapid decrease in temperature caused a significant reduction in BA (Paired t test: $t_{16} = 3.610$, $P = 0.002$).

To test for the presence of a lag effect, we compared post-temperature increase BA in winter-acclimated animals to pre-temperature decrease BA in summer-acclimated animals. We failed to identify a significant lag effect in animals during cold conditions (Paired t-test: $t_{13} = -1.124$, $P = 0.281$). We tested for the opposite lag effect in warm animals by comparing BA in post-temperature-increase animals in the winter-acclimated state to BA in pre-temperature decrease in summer-acclimated animals. Under warm conditions, we also failed to identify a significant lag effect (Paired t-test: $t_{13} = -1.043$, $P = 0.316$).

In vitro *humoral response: Stimulative fraction (SF)*

During the winter experiments, we found that neither rapid temperature increase nor LPS concentration caused a significant change in SF (Two-way repeated measures ANOVA:

temperature $F_{1,67} = 0.003$, P = 0.954; LPS concentration $F_{2,67} = 0.001$, P = 0.999; Fig. 9). During the summer experiments, we found that there was a non-significant reduction in SF due to the rapid temperature decrease and that LPS had no significant effect on SF (Two-way repeated measures ANOVA: temperature $F_{1,45} = 3.405$, P = 0.084; LPS concentration $F_{2,45} = 2.113$, P = 0.142). When we compared the baseline level of secretion between the pre-temperature change levels for winter and summer, the reduction in SF of cells during summer approach statistical significance ($t_{49} = 1.947$, P = 0.057; Fig. 12A).

In vitro *humoral response: Stimulation index (SI)*

Under both seasonal acclimation states, we found that the rapid change in temperature caused a significant reduction in SI, and that LPS concentration had no effect on SI (Fig. 13). During winter, a temperature increase caused a significant reduction in SI (Two-way repeated measures ANOVA: temperature $F_{1,65} = 9.160$, P = 0.011; LPS $F_{2,65} = 0.465$, P 0.634). During summer, a rapid temperature decrease caused a significant reduction in SI (Two-way repeated measures ANOVA: temperature $F_{1,45} = 19.957$, P < 0.001; LPS $F_{2,45} = 0.097$, P = 0.908). When we compared the baseline levels of secretion between the pre-temperature change levels for winter and summer, we found that there was a significant reduction in SI in tortoises during the summer (Mann-Whitney rank sum test: $U_{18,34} = 49$, P < 0.001; Fig. 12B).

Differential leukocytes

We found that, across seasonal acclimation states, HLR was highest in animals at the colder temperature (e.g. 12.5 °C; Fig. 14). During winter, a rapid increase in temperature caused a significant decrease in HLR (Paired t-test: $t_{20} = -7.548$, P < 0.00001). Mean HLR for animals prior to the rapid temperature increase was 2.83 which was reduced to 0.56 following the two day temperature warming. During summer, the rapid cold treatment caused a significant increase

in HLR (Paired t-test: $t_{16} = -5.224$, $P < 0.0001$). Mean HLR prior to the rapid temperature decrease was 0.34, which increased to 0.60 following the cold treatment.

DISCUSSION

Climate change has been considered a significant risk to global biodiversity (Sala et al. 2000). Beyond increases in average temperature, the negative effects of anthropogenic climate warming include increases in the frequency of extreme weather events and increased climatic instability (Raffel et al. 2015). Understanding the mechanisms by which alterations to thermal stability affect organisms is a necessary step in understanding the risk that climate change imposes on populations.

Gopherus polyphemus is a keystone species of the Southeastern US Coastal Plains (Guyer and Bailey 1993), and its loss from the landscape is predicted to drive cascading ecological collapse. Recently, disease in North American tortoises (*Gopherus*) has been the source of intense study (e.g. Jacobson et al. 2014). Because a clear epidemiological model of pathogens has not been supported in *Gopherus* species, disease in this taxon has been described as "context-dependent" (Sandmeier et al. 2013). An important context of disease in this taxon includes the thermal environment during changes from winter periods of quiescence to spring, summer, and fall periods of activity (Sandmeier et al. 2013). We experimentally evaluated the role of rapid temperature change on immune function to understand a context in which climatic instability may alter disease prevalence.

The seasonal lag hypothesis (Raffel et al. 2006) predicts that when temperature rapidly changes, organisms lack the ability to instantaneously match realized immune function to optimal levels within the new thermal environment. In response to rapid temperature change, a resulting misalignment of realized and optimal immune state occurs. Predictions of the seasonal

acclimation hypothesis are that rapid temperature increase causes an over production of immune components, while rapid temperature decrease causes an under production of immune components (Fig. 9). Across seasonal acclimation states, temperature had a profound effect on immune function. However we did not find support for our specific predictions of a seasonal lag effect.

In winter-acclimated tortoises, we found no significant increase in BA following a 48 hour increase in temperature. Because we found that BA remained at the lower, winter-acclimated levels, this result indicates that winter acclimation is not only a restraint on immune function, but that it also is a significant constraint on the ability to increase baseline immune function. Moreover, this demonstrates that there is no gain in this immune parameter by increasing body temperature during winter dormancy. DeGregorio et al. (2012) found that adult *G. polyphemus* did not frequently arise from dormancy and increase body temperature during the winter, even when external ambient temperatures were high. Our data provide one explanation for the degree to which *G. polyphemus* remain dormant during winter, even when thermoregulatory potential is high. This result suggests that increasing body temperature without increasing immune function could be detrimental to individuals during dormancy because pathogen pressure may increase when temperature is increased, despite reduced immune function. McGuire et al. (2014) found that diseased tortoises tend to become active during the winter in an attempt to elevate body temperature. Interpretations from this study indicate that, while immune responses may drive an increase in body temperature during winter, baseline immunity is not benefitted by rapid temperature increases during dormancy.

We tested BA for a lag effect within temperatures and across acclimation states. We failed to identify a pattern in this immune parameter that matched our prediction of a lag effect.

We found that when temperature was constant across acclimation states, there were no significant difference in BA. Moreover, our prediction of a lag effect was that cold animals in a summer acclimation state would have significantly lower BA than cold animals in the winter acclimation state. While not significantly different, the differences in the means of these parameters were opposite the lag prediction, with cold summer-acclimated animals having a higher mean BA than cold winter-acclimated animals. Similarly, the prediction for warm animals was that winter-acclimated individuals would have higher BA than warm summer-acclimated animals. The mean differences for BA in warm animals across seasonal acclimation states were opposite our prediction of a seasonal lag and not significantly different. While the precise mechanisms of innate immunity in turtles are still generally unknown, BA is a measure of both plasma antibodies and complement proteins (Zimmerman et al. 2010). Failure to match our lag predictions indicates that these components of innate immunity likely have long half lives within circulation, and, therefore, while temperature change may alter their circulation, there is a minimum level of function maintained above the winter-acclimated level when temperatures are cold.

In vitro humoral responses were temperature dependent and varied across seasonal acclimation states, but we did not find support for our prediction of a seasonal lag in this parameter. In either acclimation state, the rapid temperature change caused a significant reduction in stimulation index. However, across temperatures, an overall increase was present in the humoral responses of animals acclimated to winter relative to animals acclimated to summer. Specifically, there was a significant increase in stimulation index during winter ($P < 0.001$), and a tendency for greater stimulative fraction in the winter ($P = 0.057$). Thus, we did not find support for a seasonally-reduced level of cell-mediated humoral function during dormancy.

During cold conditions, cells function less efficiently, and thus must adjust fundamental rates of output to maintain set levels. This level of cellular adjustment includes alterations in isozyme profiles, as well as adjustments in transcriptional rates to effectively match performance to environmental demands (Seebacher 2005). Because the humoral assays are performed *in vitro* at a common temperature (i.e. 32.5 °C), we interpret these data to indicate that the cellular rate processes are increased during winter as an adjustment to reduced body temperature.

We found that the cell-mediated humoral parameters were not affected by LPS presence or concentration. We are aware of only one other study that has quantified *in vitro* antibody production in a turtle species, which found a similar pattern in which LPS concentration did not affect antibody production, yet antibody production remained high (Zimmerman et al. 2013). Turtles likely possess an important population of lymphocytes that are responsible for secreting "natural antibodies" even when non-stimulated. Thus, these cells maintain high rates of antibody secretion independently of antigen presence.

The ratio of heterophils to lymphocytes (HLR) has been used as an indicator of baseline stress (Goessling et al. 2015). While the stability of this measure to indicate environmental stress has been demonstrated, the adaptive nature of this immune parameter is not well known. In general, increases in HLR are thought to indicate differential allocation of resources towards innate, rapidly-responding immune defense (thus an increase in heterophils) and energetic restraint on investment in adaptive, slowly-responding immune defenses (thus a reduction in lymphocytes, Davis et al. 2006). While used as an indicator of baseline stress, HLR is a parameter that indicates differential resource allocation within the immune system. We found that, across seasonal acclimation states, cold body temperature caused an increase in innate immune investment. There was a significant reduction in HLR in winter-acclimated animals

rapidly exposed to warm temperatures, and there was a significant increase in HLR in summer-acclimated animals rapidly exposed to cold temperatures. Across seasons, this indicates that it is likely adaptive for individuals to allocate energetics towards innate immune strategies when it is cold and to allocate energetics towards adaptive immune strategies under warm conditions.

There is a growing body of evidence indicating that global change, and specifically thermal instability, is a significant risk to wildlife populations (Raffel et al. 2015). Our data suggest that rapid temperature changes generally constrain immune performance in Gopher Tortoises, and further indicate that there are temporal patterns of temperature change (such as during seasonal transitions) that pose an increased risk for disease susceptibility. Specifically, these findings provide a mechanism by which late-season and early-season cold fronts may increase disease susceptibility in Gopher Tortoises. This process includes rapid reduction in body temperature of summer-acclimated animals, thereby experiencing reduced immunity. Lastly, this study indicates the importance of contexts in which organisms may become susceptible to disease in both a natural setting of seasonal change as well as in response to anthropogenic climate change.

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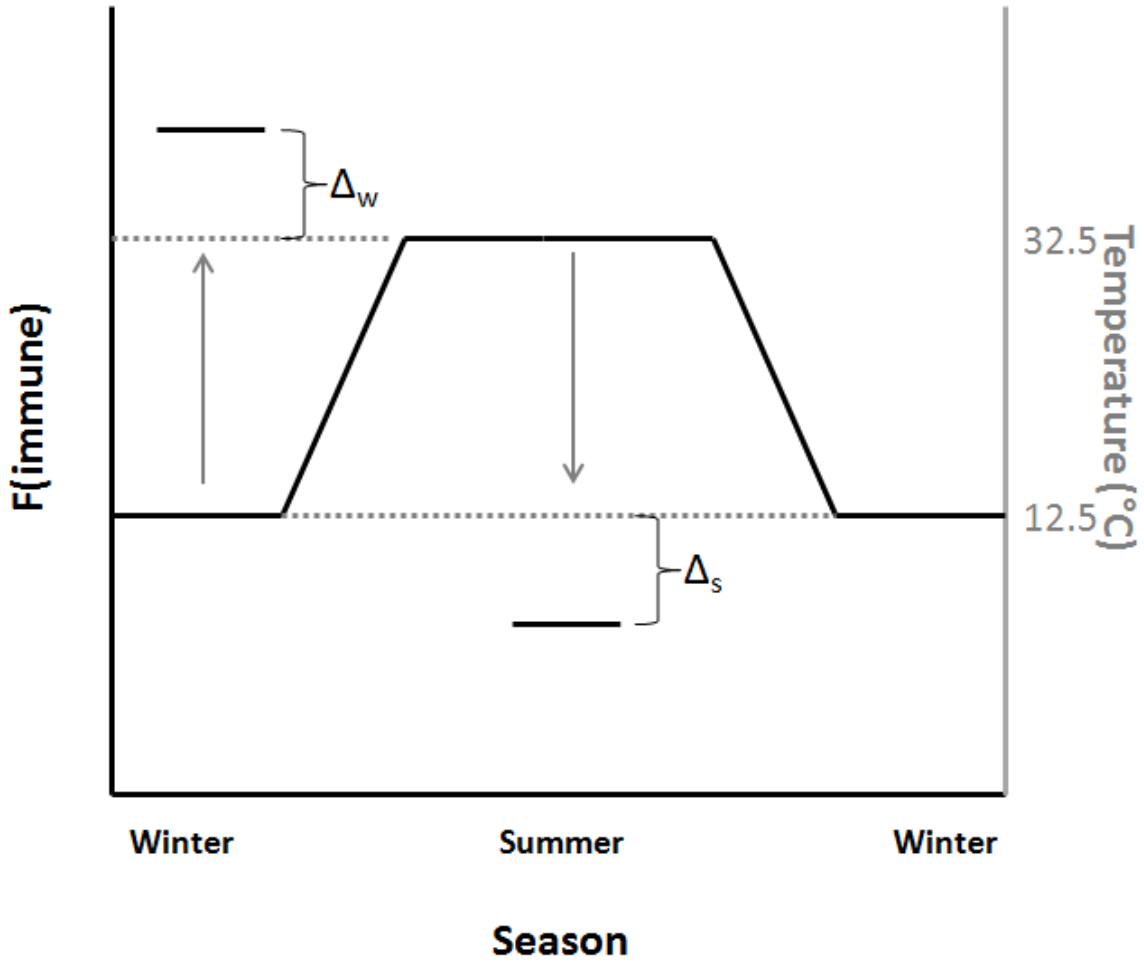

Figure 9. A hypothetical relationship between seasonal acclimation of immune parameters and seasonal lag predicts a misalignment of immunity between optimal and realized function. There is a predicted increase in immune function [F(immune)] during summer, and a reciprocal decrease in immune function during winter. The seasonal acclimation states of immune function are factors of both body temperature and season and are considered restraints to optimize immune investment. When temperature is rapidly changed, realized immune function (indicated by the solid line) is constrained by the temperature to which function is not already acclimated, thus yielding a misalignment of realized and optimal immune function (indicated by dashed line). The difference between the optimal and realized function (Δ) is predicted to be

negative when individuals are acclimated to summer conditions (Δ_s) and positive when animals are acclimated to winter conditions (Δ_w).

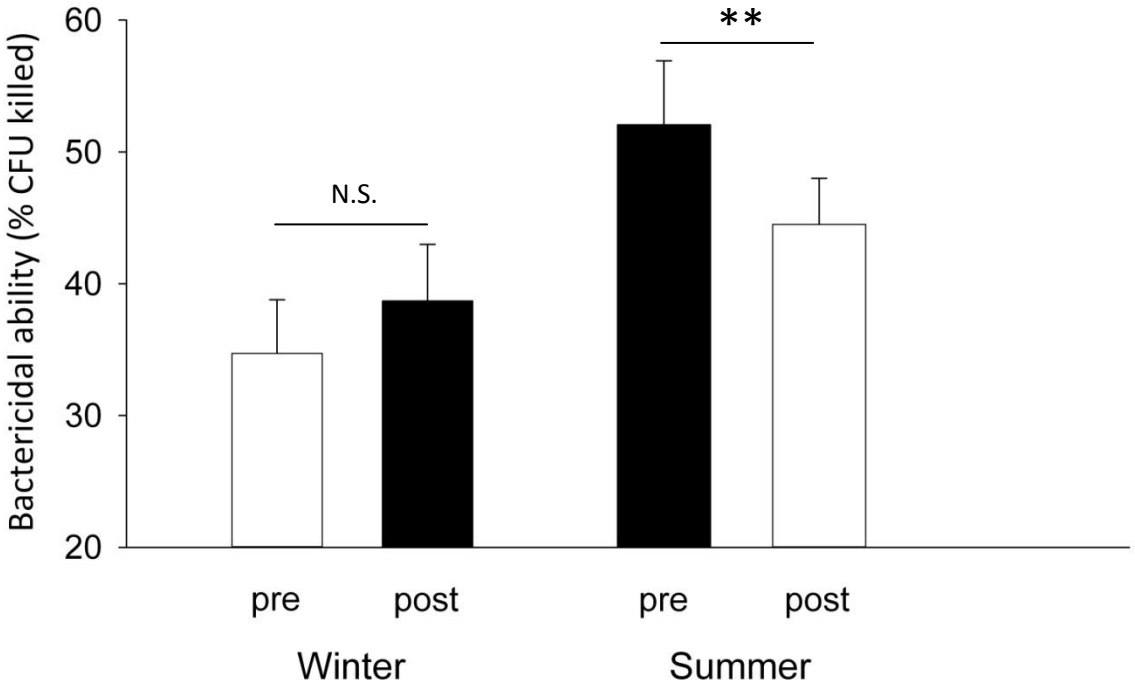

Figure 10. There was no significant effect of rapid temperature increase on *Gopherus polyphemus* bactericidal ability (BA) during the winter (P = 0.325), but there was a significant decrease in BA following a rapid temperature decrease during the summer (P = 0.002). White bars indicate animals at 12.5 °C and black bar indicate animals at 32.5 °C.

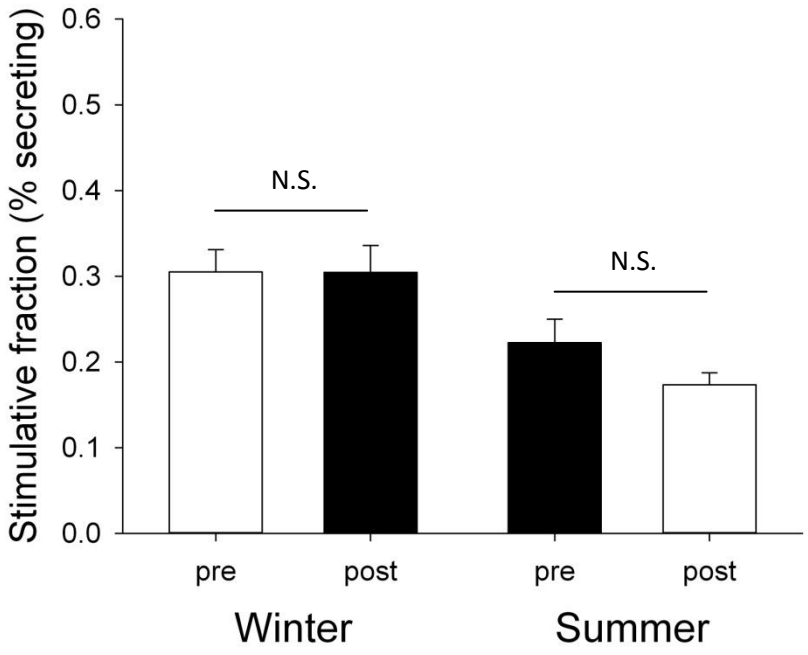

Figure 11. Rapid temperature change did not cause a significant change in the humoral stimulative fraction of *Gopherus polyphemus* lymphocytes in either summer (P = 0.084) or winter conditions (P = 0.954). White bars indicate animals at 12.5 °C and black bar indicate animals at 32.5 °C.

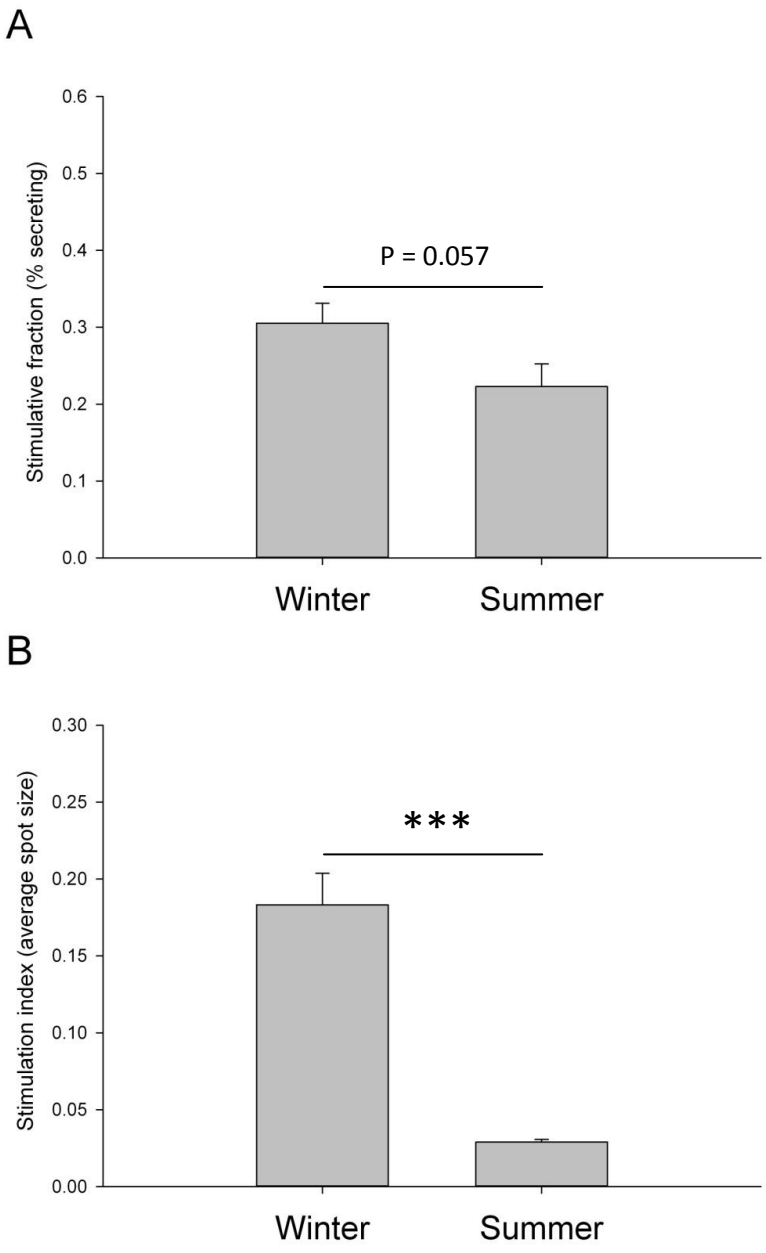

Figure 12. There was a significant effect of seasonal acclimation on cell-mediated humoral immunity. Stimulative fraction tended to be higher in *Gopherus polyphemus* acclimated to winter conditions ($P = 0.057$) and stimulation index was significantly higher in *G. polyphemus* acclimated to winter ($P < 0.0001$). Comparisons made are between animals prior to a rapid temperature change.

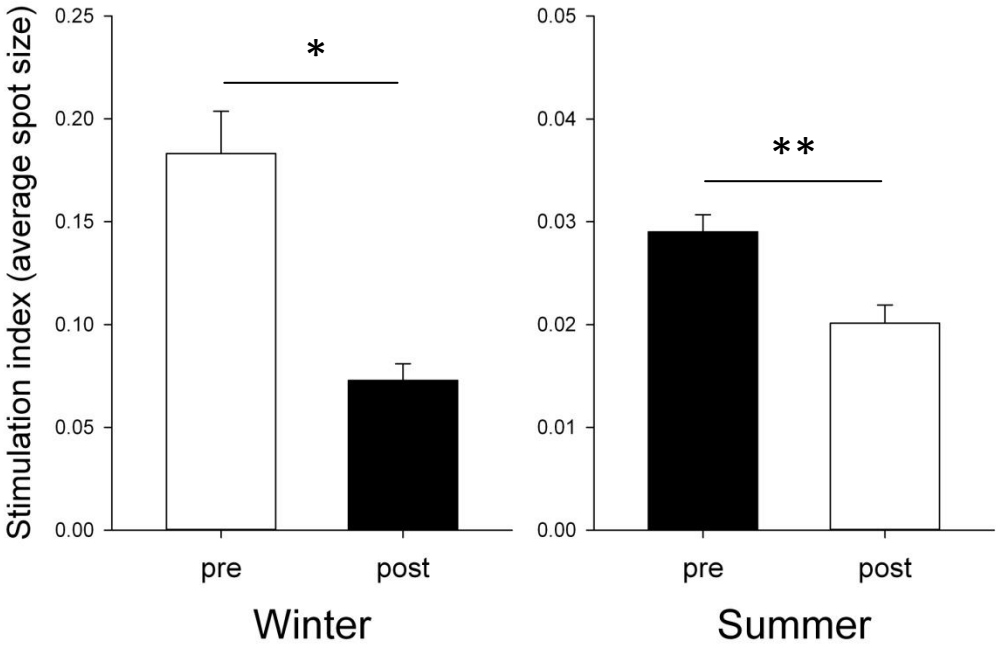

Figure 13. Rapid temperature change caused a significant reduction in stimulation index in both seasonal acclimation states (P = 0.011 during summer and P < 0.001 during winter). White bars indicate *Gopherus polyphemus* at 12.5 °C and black bars indicate *G. polyphemus* at 32.5 °C.

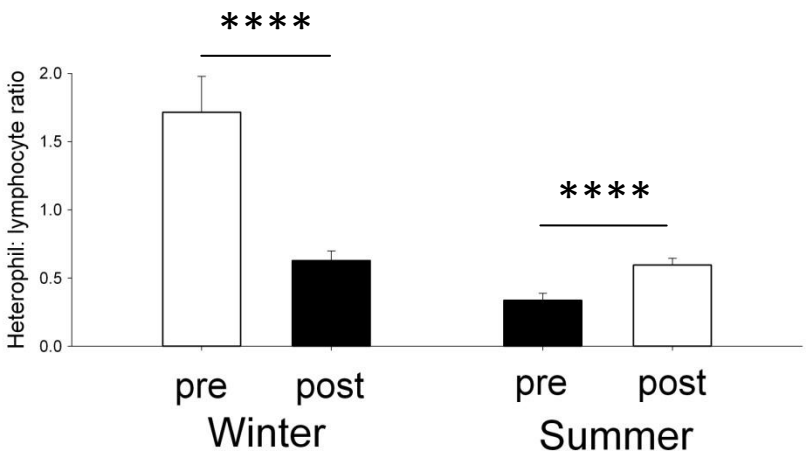

Figure 14. In both seasonal acclimation states, cold caused a significant increase in the heterophil: lymphocyte ratios of *Gopherus polyphemus* (P < 0.00001 in both seasons). White bars indicate animals at 12.5 °C and black bar indicate animals at 32.5 °C.

Chapter 5: More than fever: Thermoregulatory responses to immunological stimulation and consequences of thermoregulatory strategy on innate immune function in Gopher Tortoises (*Gopherus polyphemus*)

FORMAT FOR *JOURNAL OF ANIMAL ECOLOGY*

More than fever: Thermoregulatory responses to immunological stimulation and consequences of thermoregulatory strategy on innate immune function in Gopher Tortoises (*Gopherus polyphemus*)

Jeffrey M. Goessling[*a], Craig Guyer[a], and Mary T. Mendonça[a]

[a]Department of Biological Sciences, Auburn University, AL 36849

[*]Corresponding author: goessling@auburn.edu

Summary

1. Individuals possess a range of thermoregulatory responses to antigenic stimuli, which can subsequently cause a cascade of life-history consequences. Understanding the suite of responses to infection, including thermoregulatory responses, indicates basic costs and benefits of physiological function.

2. Herein, we were interested in measuring how Gopher Tortoises (*Gopherus polyphemus*) respond to acute antigenic stimulation across immune processes, including thermoregulation, innate immune function and biochemical response. We were also interested in identifying if thermoregulation is a determinant of functional immune differences among individuals. Lastly, we compared immune responses across seasonal acclimation states to test if seasonal context determines immune response.

3. We found that *G. polyphemus* increased body temperature (T_b) in response to lipopolysaccharide (LPS) injection compared to saline controls. We also found that LPS caused a significant increase in plasma bactericidal ability (BA) and a reduction in plasma iron concentration. Interestingly, we identified a very close, positive relationship between average T_b of non-stimulated tortoises and BA, which suggests that individuals have the ability to alter, facultatively, immune function. This relationship was not present in LPS-injected animals, which suggests that, once stimulated with LPS, BA is maximized and temperature-independent. We found that, in general, seasonal acclimation state does not influence immune responses to LPS, although baseline levels of plasma iron were significantly lower in animals acclimated to winter.

4. Our data suggest an important role for thermoregulation to determine immune function as a baseline measure of disease resistance. This study indicates a specific tradeoff of immune function for other physiological parameters, likely related to metabolic processes.

5. Understanding how immunological processes are integrated is fundamental to understanding constraints on individual responses, especially in ectothermic vertebrates. This study indicates that, while the ability to mount responses is present in individuals across seasonal contexts, the scope of responses is fine-tuned and the result of a physiological tradeoff. When infection risk is low, individuals take a conservative strategy to reduce T_b and subsequently reduce immune function.

Key-words conservation physiology, ecoimmun*, thermal ecology, ibutton, Lipopolysaccharide, acclimation, thermoregulation, thermocouple

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Introduction

All physiological processes are affected by temperature. Thus, how organisms (especially ectotherms) function in the environment is largely a result of environmental thermoregulatory potentials (Sartorius et al. 2002). One of the vital components of animal physiological maintenance is the immune system, which is known to vary in function as a result of temperature (e.g. Raffel et al. 2006).

While the vertebrate immune system is a complex and integrated network of processes, there is tight regulation of sequential order by which immune responses are evoked (Lee and Klasing 2004). Fever has been described as one of the first sequences of innate immune stimulation (Lee and Klasing 2004) and is driven by an increase in thermoregulatory setpoint (T_{set},Boulant 2000) within the pre-optic area of the hypothalamus (Bicego, Barros & Branco 2007). This increase in T_{set} is subsequently manifested as a temporary increase in body temperature (T_b; Bernheim et al. 1979, Kluger et al. 1998, Lee and Klasing 2004). Febrile responses are first caused by activation of specific toll-like receptors (TLR) by an antigenic compound, such as bacterial endotoxins (Beutler 2004, Dinarello 2004). In addition to the increase in T_{set}, fever responses are associated with physiological effects that better suit hosts to survive infection, such as reduction in plasma iron concentration, which reduces the intrinsic growth rate of pathogens (Kluger and Rothenburg 1979).

While the expression of the fever response to inflammatory antigens is highly conserved within endotherms, no consensus has emerged regarding the conservation of this response across ectothermic vertebrates. Because their body temperatures are directly related to environmental temperatures, the generation of fever in ectotherms is caused by behavioral changes (such as alterations in thermoregulatory behaviors) and not the direct physiological mechanisms by which

endotherms adjust body temperature. Thus, increases in thermoregulatory set-point in ectotherms are considered behavioral fevers. Behavioral fever responses have been demonstrated in teleosts (Reynolds, Casterlin & Covert 1976), amphibians (Bicego-Nahas et al. 2000), squamates (Ortega et al. 1991), turtles (Monagas & Gatten 1983), and crocodilians (Merchant et al. 2007). While fever is present among these taxa, differences remain within these groups. For example, while some turtles have been shown to generate fevers, the presence of fever has not been shown in all turtle species in which fever was investigated. Fever has been demonstrated in the Box Turtle (*Terrapene carolina*) and the Painted Turtle (*Chrysemys picta*) (Monagas and Gatten 1983) but a fever response was not demonstrated in the Leopard Tortoise (*Geochelone pardalis*) (Zurovsky, Mitchell & Laburn 1987).

While numerous studies have investigated thermoregulatory responses of ectotherms to antigenic stimuli, we have yet to gain a full understanding of the functional effects of altered thermoregulatory responses on immune function itself. For example, Raffel et al. (2006) found support for a seasonal lag in baseline immunity in an amphibian that was subjected to varying temperatures. In this study, individuals experienced rapid temperature changes and immunological function was measured in response. However, the ability of ectotherms to thermoregulate, thereby determining their own immunological state, has yet to be investigated. Studies that have looked at the immunological consequences of thermoregulation in ectotherms (and specifically, fever responses) have investigated survival of individuals which were injected with lethal antigenic doses (e.g., Kluger, Ringler, and Anver 1975). While such studies demonstrate links between temperature and survivorship, they may represent the extreme case of immunological challenge, and indicate the constraints and adaptations of thermoregulatory strategy on baseline immunological function.

Seasonality has been hypothesized as an important environmental component that drives observed patterns of baseline immunity (Raffel et al. 2006; Raffel et al. 2015; Goessling et al. in review), which in turn generates cyclical patterns of disease prevalence in wild populations (Nelson and Demas 1996, Nelson et al. 2002). The next step in understanding seasonal relationships of the immune system is to understand how seasonality contributes to immune responses, not just baseline immune function. Most studies investigating seasonal relationships of immune responsiveness have been performed in endotherms. The general pattern which has emerged suggests a reduced response of the immune system during hibernation in seasonally inactive species (Bouma et al. 2010). For example, Golden-mantled Ground Squirrels (*Spermophilus lateralis*) show no response to lipopolysaccharide (LPS; a known immune pyrogen) injection during hibernation, unless they are in a period of hibernation arousal (Prendergast et al. 2002). Because ectotherms can only acquire body heat from environmental sources, immune responsiveness is directly related to environmental temperature (T_e) and associated environmental thermoregulatory potential

To better understand the nature of fever responses in ectothermic vertebrates, the thermal contingency of immune function, and the effects of seasonal acclimation on immune responses, we used Gopher Tortoises (*Gopherus polyphemus*) as an experimental model. *Gopherus polyphemus* have suffered severe range-wide declines over recent decades (Auffenburg and Franz 1982; Tuberville et al. 2014), which has been partially attributed to disease outbreaks among populations (Jacobson et al. 2014). Additionally, McGuire et al. (2014) suggested that thermoregulatory patterns in *G. polyphemus* may be altered when individuals are expressing acute symptoms of disease, and that these individuals may utilize fever as an immune response to infection. Our interest in this study was rooted both in the conservation-specific questions of the

thermal consequences of infection in this species, as well as an interest to better understand basic thermal physiology of immune responses of ectothermic vertebrates.

The objectives of this study were to: 1) test the nature of a thermoregulatory response in *G. polyphemus* to stimulation with an antigen that has been shown to cause febrile responses in reptiles (Lipopolysaccharide, [LPS] Merchant et al. 2007; Merchant et al. 2008); 2) examine how thermoregulatory and immune responses vary by seasonal acclimation state; and 3) indentify how thermoregulation affects baseline immune function.

Materials and Methods

ANIMALS, CAPTIVE CARE, AND MAINTENANCE

Wild adult *Gopherus polyphemus* (n=27) were trapped from Covington County, Alabama USA, and transferred to an on-campus housing facility at Auburn University, where they were maintained individually in 5 x 10 meter pens under semi-natural conditions outside. Each pen contained a 45.7 cm x 10.16 cm aluminum pan that was sunk approximately 10 cm into the ground ; additionally, an artificial burrow having the physical configuration of a *G. polyphemus* burrow was constructed using a 3 m section of 45.72 cm diameter plastic culvert that was longitudinally bisected. Artificial burrows were then placed on the surface of the ground and filled with sand to resemble natural tortoise substrate, and a mound of sand was made at the entrance of each culvert to mimic the apron of a natural burrow. Burrows were partially shaded to prevent excessive solar heating. Pens were located in an open field that contained ample forage of grasses and forbs, thus we did not provide additional food. When overnight temperatures were less than 6°C and tortoises were consistently inactive, they were removed from the outdoor pens and brought into an environmentally-controlled chamber. The thermostat in the chamber was set to 12.5°C, which is the mean carapacial temperature of *G. polyphemus*

overwintering in South Carolina (DeGregorio et al. 2012). To mimic the natural photoperiod of an overwintering tortoise within a burrow, there was no light source within the chamber, but the room surrounding the chamber had un-shaded windows that allowed a natural photoperiod within the room. The door entering the chamber had a small window that allowed some light to pass into the chamber. While in the winter-acclimated state, we allowed tortoises to soak in a pan containing approximately 2 cm of water at room temperature (~20°C) for approximately four hours every four weeks to maintain proper hydration. Following all experimentation, all tortoises were released at their exact point of capture.

All experiments in this study were conducted on individual tortoises on a linear thigmothermal gradient (do Amaral, Marvin & Hutchison 2002). The thermal gradient was approximately 1 x 4 m (w x l) and was constructed by attaching a solid sheet of aluminum flashing to the underside of a plywood box. Below the aluminum flashing was a series of cross wires, to which hot plates and ceramic bulbs were attached to create the warm end of the thermal gradient. There was no heat source attached to the cool end of the gradient; the gradient was kept in an environmental chamber set to 10 °C for winter experiments and 19 °C for summer experiments. Thus the thermal gradient provided a range of temperatures from 10 – 40 °C in the winter and 19 – 40 °C in the summer. A high-spectrum UV light was installed over the gradient and was placed on a 10:14 light:dark cycle (photophase from 0700 h – 1700 h) for winter experiments, which simulated the local, natural photopheriod during winter. For summer experiments, the light:dark cycle was 14:10 (photophase from 0600 h – 2000 h) which simulated the local, natural photoperiod during the summer.

For summer experiments in 2014 and for the winter experiments, DS-1921 ibuttons (Maxim Integrated, San Jose CA, USA) were surgically implanted in tortoises and used to

measure body temperature. Ibuttons were first programmed with a delay to capture the scheduled experiments, and set to record a data point every 20 minutes. Following programming, ibuttons were double coated in bees wax, and were gas-sterilized in ethylene oxide (Auburn University College of Veterinary Medicine, Auburn University AL, USA). Ibuttons were surgically implanted subcutaneously in the space anterior to the left hind limb. To implant ibuttons, tortoises were first anesthetized according to the protocol of McGuire et al. (2014). The surgical site was prepared using standard veterinary techniques of a triple scrub of chlorhexidine solution / 70% ethanol. Following surgical preparation, a pre-sterilized drape was affixed to the anesthetized tortoise to reduce the risk of infection, and ibuttons were implanted using standard veterinary techniques. The implant incision was closed using 4-0 polydioxanone suture. The incision was treated with silver sulfadiazine to reduce the risk of site infection. Tortoises were kept inside and on dry paper towels for 24 hours following the surgical implantation. After this 24-hour recovery period, tortoises were returned to their individual pens. All implant surgeries were performed at least two months prior to the anticipated experiments to allow the incisions to completely heal. Following the LPS experiments, ibuttons were retrieved during the active season using the same procedure as in the implantation. Surgeries were directed under the supervision of the IACUC project veterinarian.

In summer 2014, four out of eleven, and in winter 2014, seven out of eleven implanted ibuttons failed to record data. Thus, to reduce the data loss caused by frequent ibutton failures, we used a cloacally-inserted thermocouple to record T_b for the summer 2015 experiments. The thermocouples were made using an approximately 20 cm long section of 22 gauge copper/constantan wire (Omega Engineering, Stamford CT, USA). The outer rubber wire insulation was removed from an approximately 1 cm section of wire, exposing the underlying

wires, which were spliced and sealed in an epoxy bulb (Deen and Hutchison 2001). The wire was attached to a Type T thermocouple attachment and data were recorded on an OM-EL-WIN-USB thermocouple data logger (Omega Engineering, Stamford CT, USA). Thermocouples were validated against known temperatures prior to use. For the experimental procedure, the thermocouple was inserted cloacally approximately 3 cm, the wire was wrapped around the posterior margin of the carapace, and the data logger was affixed to the tortoise's shell using duct tape. All thermocouples were attached to tortoises when the tortoises were placed onto the thermal gradient.

To begin the experiment, each tortoise was placed in the middle of the thermal gradient between 0800 – 0900 H. A 24 h acclimation period followed initial placement of the tortoise on the gradient. On the morning following the acclimation phase (between 0800 – 0900 H) each tortoise was injected intraperitoneally with either 1 mg / kg LPS (derived from *Escherichia coli* 0111:B4, Sigma-Aldrich Co., St. Louis MO, USA) dissolved to a concentration of 5 mg / ml in sterile, pyrogen-free saline, or with sterile, pyrogen-free saline at the same volume as LPS (negative control). A whole-blood sample was collected at the same time that the treatment was administered. Each tortoise was then returned to the thermal gradient for the 48 h experimental phase. Once the experimental phase was finished, a second whole-blood sample was collected, the thermocouple was removed (summer 2015), and individuals were returned to their enclosures.

As a backup for ibutton failures and to measure thermoregulatory response during the winter experiments, paired PlantCams (Ebsco Industries, Birmingham AL, USA) were attached to opposing inside walls of the thermal gradient and set to automatically take a no-flash photograph every five minutes during the photophase of the experiment. Thermoregulation was

classified dichotomously as the individual tortoise located on the heated or the unheated half of the thermal gradient. We classified time spent on the heated end of the thermal gradient as "basking". Pictures were analyzed following all experiments and a t-test was used to compare the proportion of time that LPS- and saline-injected animals spent on the heated half of the gradient.

BIOLOGICAL SAMPLE PROCESSING AND ASSAYS

Whole-blood samples were collected using sterile heparinized syringes, affixed with a 22 gauge needle, at two time points: pre-injection and 48 h post-injection. Most samples were collected from the femoral vein; if the femoral vein could not be accessed in the pre-injection sample, the blood sample was collected from the brachial vein. If the pre-injection sample was collected from the brachial vein, the post-injection sample was also collected from the brachial vein. Thus, the pre-injection and post-injection samples were always collected from the same venipuncture site. Whole-blood samples were temporarily stored on ice, centrifuged for 8 minutes at 1200 RPM, and the plasma fraction was isolated, and stored at -80 °C in a sterile micro-centrifuge tube until assays were performed. All plasma samples were frozen within 60 minutes of collection. At the time of sample collection, duplicate blood smears were made with approximately 5 – 10 ul of blood. Blood smears were air dried, and were fixed in Hema 3 ® fixative (Fisher Scientific Co, Kalamazoo MI, USA) within 24 hours of sample collection.

Bactericidal assays were performed within four weeks of plasma collection to prevent degradation of samples. To perform the assay, 3 ul of freshly thawed plasma was added to 137 ul of CO_2-independent/L-Glutamine medium. This medium was made by adding 40ul of L-Glutamine to 200 ml of Gibco ® CO_2-independent medium (Thermo Fisher Scientific LLC, Waltham Massachusetts USA). Ten microliters of an *E. coli* stock solution suspended in sterile phosphate-buffered saline was then introduced to the plasma/medium solution. The stock *E. coli*

suspension was diluted from frozen suspensions so that negative controls contained approximately 200 colony forming units (CFU) per 50 ul of final assay suspension. The final plasma dilution for this assay was 1:50. Two negative controls, which received no plasma but were supplemented with an extra 3 ul of media, were embedded within each assay. The assay was incubated at room temperature for 1 H, following which 50 ul of each sample were plated using sterile technique on sterile culture plates pre-made with Tripticase® Soy Agar (catalog number 221283, VWR International, Atlanta Georgia USA). Each culture sample was plated in duplicate and the mean of the pairs was used for statistical analyses. Plates were incubated at 32°C for approximately 16 hours, or until individual CFU could be visualized. Bactericidal ability was calculated as the percent bacteria killed, which was the mean CFU of both negative controls minus the mean CFU of the sample, divided by the mean CFU of the negative controls, and multiplied by 100.

Plasma iron concentrations were measured using Inductively Coupled Plasma with Optical Emission Spectroscopy (ICP-OES, PerkinElmer Life Sciences, Waltham MA, USA). For this measurement, 50 uL of plasma was diluted in 300 ul of ultrapure metal-free water. Iron concentration was determined by comparing emission intensities to a standard curve created from certified metal standards (SPEXcertiprep, Metuchen NJ, USA). Final concentrations were corrected by subtracting the average of five background measures from each sample.

STATISTICAL ANALYSES

We calculated hourly moving averages for the 48-hour experiment for each individual tortoise in the summer experiment. Additionally, we were interested in identifying if there were thermoregulatory differences at different temporal scales. Thus, we calculated moving averages divided into blocks of six hours post injection (e.g. 0 – 6 h post injection, 6 – 12 h post inject, 12

– 18 h post injection, etc) as well as photophasic/scotophasic blocks (Merchant et al. 2008). Following the calculation of the moving averages, a t-test was used to compare the mean T_bs between LPS- and saline-injected tortoises. Additionally, due to the high variability of the tortoise T_bs, we performed a non-parametric sign test of the median T_b within each treatment group to test the hypothesis that LPS caused an increase in T_b throughout the experiment.

We used a two-way repeated measures ANOVA to test for differences in BA and plasma iron as a result of treatment (i.e. LPS or saline), time of sample (i.e. pre-injection or post-injection) and the interaction of treatment and time. Using this statistical analysis, a significant effect of LPS would be indicated by a significant interaction of time and treatment.

To compare how seasonal-acclimation state affected the change in BA, we first calculated the change in BA during the experiment as the difference in the pre-injection and post-injection sample. We then compared the change in BA with a two-way ANOVA with the factors seasonal-acclimation state and treatment type. Because we predicted that T_b might have an effect on immune function, we used a linear regression to test for a relationship between T_b and BA. To address this question, we averaged the T_b of each individual tortoise throughout the experiment. We then regressed both post- and pre-injection BA against average T_b. We analyzed both time points of BA verses T_b because we wanted to be able to assess if the thermoregulatory behavior of the tortoise while on the thermal gradient caused a difference in BA.

We also compared how the change in plasma iron concentration was affected by seasonal acclimation state by calculating the percent change in iron, and subsequently tested for significance using a two-way ANOVA as above for BA. We tested for a seasonal effect of baseline plasma iron concentration by comparing the pre-injection plasma iron concentrations

between summer- and winter-acclimation states using a Mann-Whitney Rank Sum test. We used a non-parametric test for plasma iron because values were non-normally distributed.

Results

Effect of LPS on thermoregulation-summer

Overall, LPS caused an increase in the T_bs of summer-acclimated *G. polyphemus* compared to the saline control over the 48 hour experiment (Sign test, $P < 0.0001$). Within the 48 hour experiment, we found that LPS caused a significant elevation in body temperature above the saline control for the first hour of the experiment ($P = 0.041$). After that, the mean T_b for the LPS-injected animals remained greater than the mean T_b for the saline-injected animals at every hour, however none of the hourly means demonstrated a significant effect at the 0.05 alpha level (there were, however, P values < 0.10 at 2, 3, 16, 17, 18, 19, 20, 25, and 26 h post injection; Fig.13).

The six-hour moving average also indicated a general increase in T_b in animals injected with LPS (Sign test, $P = 0.004$), however none of the individual six-hour interval differences in T_b was significant at the 0.05 alpha level ($P < 0.1$ at 0 – 6 h, 18 – 24 h, and 24 – 30 h post injection; Fig. 16). When analyzed by photophase/scotophase, we found a similar pattern in which all mean values for LPS-injected animals were greater than saline-injected animals, yet none of these differences were significant.

In winter-acclimated tortoises, we found a significant increase in the proportion of time that tortoises injected with LPS spent basking (T-test, $t_8 = 2.856$, $P = 0.021$). Tortoises injected with LPS spent 95% of their time basking compared to saline-injected tortoises which spent 59% of time basking (Fig. 17)

Effect of LPS on innate immunity

In summer-acclimated *G. polyphemus* we found a significant effect of treatment (Two-way ANOVA, control vs. LPS: $F_{1,19} = 8.055$, $P = 0.011$), time (pre- vs. post-: $F_{1,19} = 6.171$, $P = 0.022$) and the interaction of treatment X time ($F_{1,19} = 8.153$, $P = 0.010$) on BA. In winter-acclimated *G. polyphemus*, we only found a significant effect of time (Two-way ANOVA, pre- vs. post-: $F_{1,10} = 5.408$, $P = 0.042$) on BA. We did not find a significant change in BA by treatment ($F_{1,10} = 0.777$, $P = 0.399$) or the interaction of time X treatment ($F_{1,10} = 1.908$, $P = 0.197$). However, when we analyzed the change in BA during the experiment (Fig. 18), we found a significant effect of treatment (Two-way ANOVA, $F_{1,29} = 8.193$, $P = 0.006$) and not seasonal-acclimation state ($F_{1,29} = 2.849$, $P = 0.102$) or the interaction of seasonal-acclimation state and treatment ($F_{1,29} = 0.101$, $P = 0.753$).

Effect of LPS on plasma iron concentration

LPS caused a significant reduction in plasma iron concentration (Two-way ANOVA, treatment: $F_{1,28} = 0.130$, $P = 0.722$; time: $F_{1,28} = 0.139$; $P = 0.712$; treatment X time: $F_{1,28} = 4.601$, $P = 0.041$, Fig. 19A). There was a significant difference in plasma iron between seasonal-acclimation states (Mann-Whitney Rank Sum Test: $T_{12,18} = 108.00$, $P = 0.001$ Fig. 19B), but the change in plasma iron as a result of LPS was not affected by seasonal acclimation state (Two way ANOVA of percent change in plasma iron: treatment $F_{1,26} = 4.297$, $P = 0.048$; seasonal acclimation state $F_{1,26} = 0.100$, $P = 0.754$, interaction of treatment X seasonal acclimation state $F_{1,26} = 0.093$, $P = 0.763$).

Effect of thermoregulation on innate immunity

We found that average T_b during the 48-hour experiment affected the BA of tortoises injected with the saline control, but not LPS (control: $t_{1,6} = 5.844$, $P = 0.001$, $R^2 = 0.851$; LPS: $t_{1,7} = 2.157$, $P = 0.068$ Fig. 20). To confirm that body temperature during the experiment caused the

change in immune performance, and not that immune function was reduced prior to the experiment, we also compared the pre-treatment BA to the average T_b during the 48-hour experiment. We found that pre-treatment BA was not related to average T_b (control: $t_{1,6} = 0.0743$ $P = 0.943$; LPS: $t_{1,7} = -0.0665$, $P = 0.949$, Fig. 20).

DISCUSSION

Thermal heterogeneity is present in all environments on earth. How species utilize this variability is a function of environmental constraints driving adaptations to a thermal niche within an environment (Huey and Slatkin 1976). Seasonal thermal change has been considered a selective process on populations and it has the potential to drive cyclical patterns of disease (Nelson & Demas 1996, Nelson et al. 2002). Because ectothermic vertebrates lack the physiological mechanisms of generating body heat, seasonal change may even play a larger role in affecting immune function in these taxa (Raffel et al. 2006; Goessling, Guyer & Mendonça, in review). While seasonal variability in baseline immune function is informative, understanding how organisms respond to immune stimuli can better indicate the constraints imposed on individuals when risk of infection may increase. Additionally, immune responses may indicate restraints that individuals express in eliciting a response (Bouma et al. 2010) if the benefit of the response does not outweigh the cost of the response itself. Thus, understanding how individuals respond to immune stimuli is fundamentally important to understanding their basic ecology and the thermal niches and selective pressures under which they exist. Moreover, individuals are constantly threatened with potential pathogens and the thermoregulatory decisions they make in response to these threats indicate the basic tradeoff of competing physiological needs.

We found that behavioral fever was present in *Gopherus polyphemus* and that this response was present in both seasonal-acclimation states of summer and winter. Across seasons,

G. polyphemus increased body temperature and basking (T_b, Fig. 15 and Fig. 17), increased bactericidal ability (BA, Fig. 18) and reduced plasma iron concentration (Fig. 19) in response to lipopolysaccharide (LPS) injection. All three of these responses can be considered primary immune defenses against acute bacterial infection. First, elevating T_b both increases host defense and reduces pathogen performance (Kluger, Ringler & Anver 1975). Second, reducing iron concentration, especially when coupled with increased temperature, reduces bacterial growth (Kluger & Rothenburg 1976, Merchant et al. 2007). Lastly, our data suggest that increased BA is a response to innate immune stimulation that increases host defense.

While we did not detect a significant interaction of treatment and time on the BA of winter-acclimated tortoises, BA was more variable in winter-acclimated tortoises and the overall pattern of increasing BA in response to LPS was similar to summer-acclimatized tortoises. Furthermore, when we analyzed the relative change in BA during the experiment across seasons, we found that treatment only affected the change in BA, and that this change was not affected by seasonal-acclimation state or the interaction of seasonal-acclimation and treatment. Taken together, these data suggest that individuals retain the ability to mount an immune response, regardless of season.

To our knowledge, this is the first study to confirm a behavioral fever response in any testudinid, although behavioral fevers have been suggested as a response to mycoplasmosis in free-ranging *G. polyphemus* (McGuire et al. 2014). We found that the behavioral fever was an immediate response to LPS injection, as the significant time interval for elevated T_b was found at one hour post injection. While this response to LPS was immediate, both experimental groups showed a gradual decrease in T_b, which has been interpreted in other studies (do Amaral et al. 2002) as acclimatization to the thermal gradient itself.

We found significant functional effects of thermoregulation on the immune system. While BA was elevated in tortoises injected with LPS, we also found an interesting effect of temperature on immune function in the saline-injected tortoises. Specifically, there was a close relationship between BA and average T_b of animals that were not injected with LPS (Fig. 20C). This temperature-dependent BA response was not present in LPS-injected tortoises. We interpret this finding to indicate that the immune responses were maximized in LPS-injected animals, and that non-stimulated animals have the ability to drive immune function specifically through thermoregulation. Specifically, reducing T_b reduces BA.

We did not predict that BA would decrease in as little as two days (which was the duration of each experiment) as a result of thermoregulatory behavior because this parameter is driven by antibodies and complement (Zimmerman et al. 2010). Johnson et al. (2010) have shown that humoral immune components of *G. polyphemus*, specifically antibodies, may take 1 – 3 months for full seroconversion, and that antibody titers likely remain elevated for much longer following seroconversion. A significant reduction in BA in two days likely suggests that circulating complement has either a very short half life, or that tortoises have a mechanism of reducing the circulating complement proteins. Future research should specifically address the mechanisms of humoral immune function and complement production in the *G. polyphemus* and other reptiles.

Reduction of immune function through thermoregulation in the saline-injected control tortoises likely indicates a tradeoff that balances immune function with energetics. Specifically, we hypothesize a discrete energetic cost to maintaining elevated T_b when there is a less defined risk of infection. Thus, the immune function maintained by elevating T_b does not compensate for the energy loss due to the increased metabolic rate of warming T_b (Litzgus & Hopkins 2003).

Adult *G. polyphemus* have been shown to remain cool and in dormancy even during short durations of warm winter weather (DeGregorio, Buhlmann & Tuberville 2012). This maintenance of reduced T_b when thermoregulatory potential is high seems paradoxical in a simple model in which the immune system is the only function driving thermoregulatory decisions. However, immune systems, and the suite of thermoregulatory decisions that hosts make, are evolved within host taxa, and are thus a product of the thermal ecology of the host. When energetic costs of elevating T_b are high (such as warm, winter days when food remains scarce), there may be a distinct immunological benefit to warming, yet a clear metabolic cost to becoming active. Specifically, thermoregulation is described as a metabolic cost when specific microhabitats have reduced potential for food acquisition (Huey and Slatkin 1976). In this case, the microhabitat limiting food is temporal in nature when winter food resources are less abundant. Ultimately, elevation of winter T_b could thus be interpreted as a metabolic cost. Moreover, as has been described by Huey and Slatkin (1976), thermoregulatory decisions may be the result of conflicting ecological and physiological optima. Thus, when non-antigenically stimulated, the resultant thermoregulatory decision is one driven by maintenance of an ecological optimum by remaining cool and conserving energy. However, when injected with LPS, tortoises selected warm temperatures, likely at an energetic cost, thereby maintaining a physiological optimum.

This study suggests a mechanism by which disease in North American tortoises may be environmentally context-dependent. Sandmeier et al. (2013) found that tortoises were more likely to express physiological parameters of URTD following unusually cold winters. Our study suggests that thermoregulation, and specifically fever, is an important component of the immune response of Gopher Tortoises. Therefore, pathogen success, and thus disease, may be a more

likely result following pathogen exposure when the thermal environment does not support elevations in T_b.

We found that LPS significantly reduced plasma iron concentration, and that this reduction in plasma iron was not directly related to seasonal-acclimation state. Reductions in iron concentration in response to infection are considered to be a host-protective strategy (Merchant et al. 2007) that reduces bacterial growth (Kluger & Rothenburg 1979). This experiment supports prior findings that fever responses integrate physiological responses across multiple scales, and that iron is an important component to fever responses. We also found that seasonal-acclimation state had a strong effect on baseline plasma iron concentration, and that plasma iron concentration was lower in winter-acclimated tortoises than summer-acclimated tortoises. While the reduction in plasma iron in winter-acclimated tortoises may be caused by the seasonal acclimation, it likely provides an additional level of host protection when baseline immune function is reduced during dormancy (Goessling et al. in review). Additionally, Goessling et al. (in review) found that winter acclimation in *G. polyphemus* includes significant increases in heterophils, which may be compensatory for other reductions in immune function. It is possible that the reduction in plasma iron during winter also functions as an additional host-protective strategy during dormancy.

Previous work has focused on understanding isolated physiological responses to antigenic stimuli (e.g. Merchant et al. 2008, do Amaral et al. 2002). However, few studies have investigated the functional effects of and responses to antigenic stimuli and behavioral thermoregulation, as well as determining how these parameters are directly related. This study furthers our understanding of life history theory because it indicates that neither thermal ecology nor immune function acts as a single determinant of the other; rather, both are vital components

of a shared feature. Ultimately, the process of physiological maintenance through immune function in ectotherms can be seen as the nexus between biochemical, cellular and thermoregulatory costs and benefits. Future investigations should focus on quantifying the discrete metabolic cost of immune responses, and how these costs, and associated benefits, vary across environments.

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Data accessibility

If accepted, data for this manuscript will be archived in the Auburn University Museum of Natural History online database.

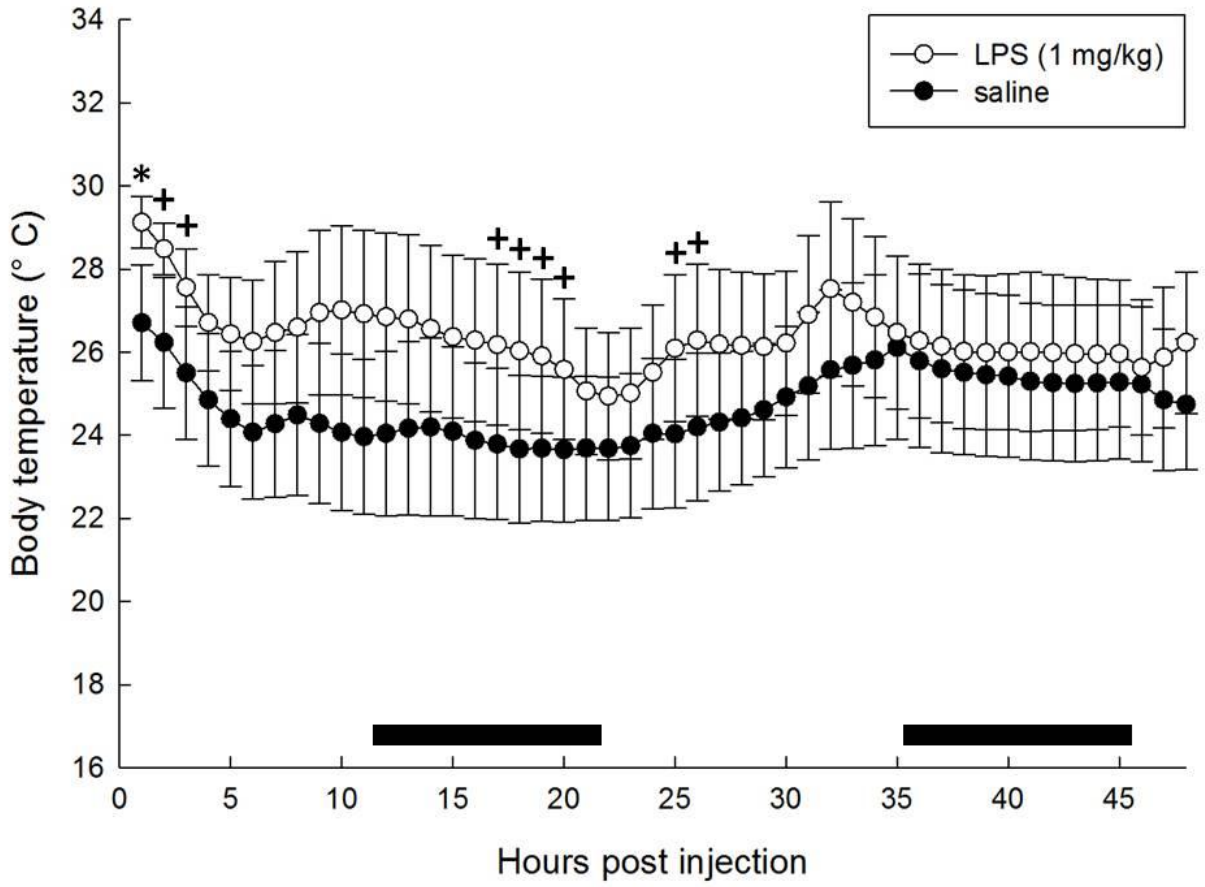

Figure 15. Comparison of hourly moving averages indicated that there was a significant increase (P = 0.04) in body temperatures of *Gopherus polyphemus* one hour post injection with lipopolysaccharide (LPS) relative to saline controls (indicated with an asterisk). Hourly comparisons marked with a plus sign indicate differences with P-values less than 0.10. Horizontal black bars indicate scotophase.

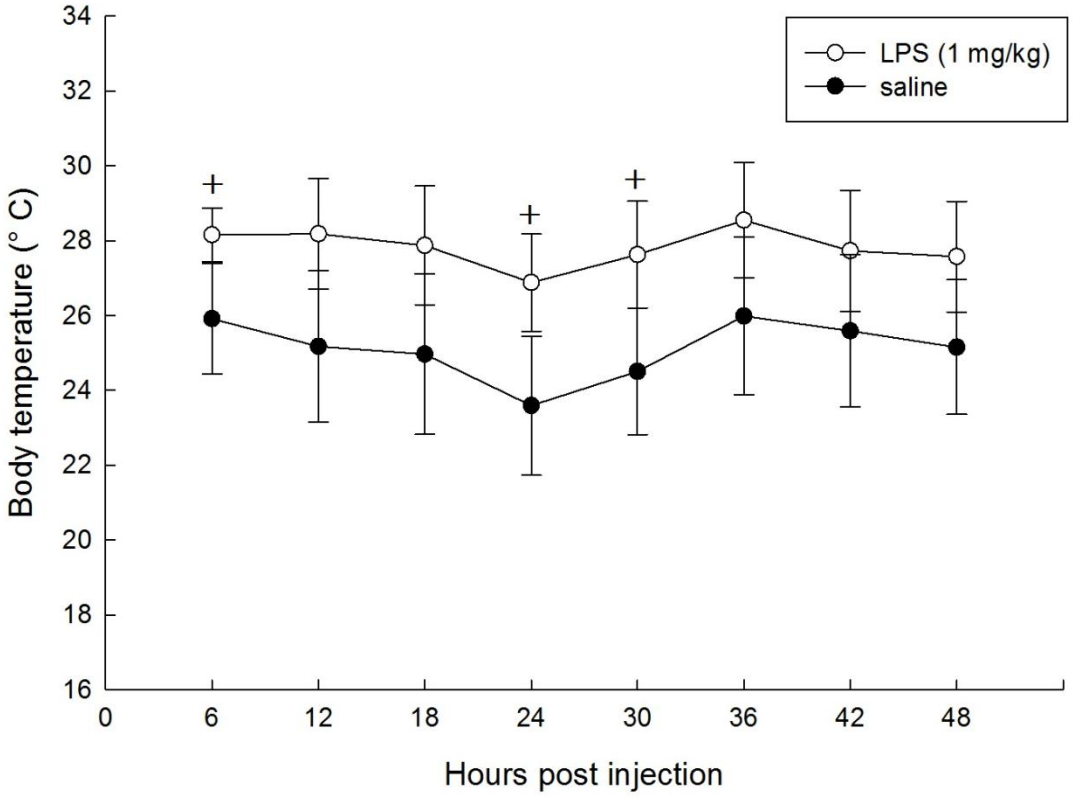

Figure 16. Six-hour moving averages depicting a trend for lipopolysaccharide-injected *Gopherus polyphemus* to maintain higher body temperatures than saline-injected control *G. polyphemus*. However, none of these comparisons was significant at an $\alpha = 0.05$ level. Differences where p-values were < 0.10 are marked with a plus sign.

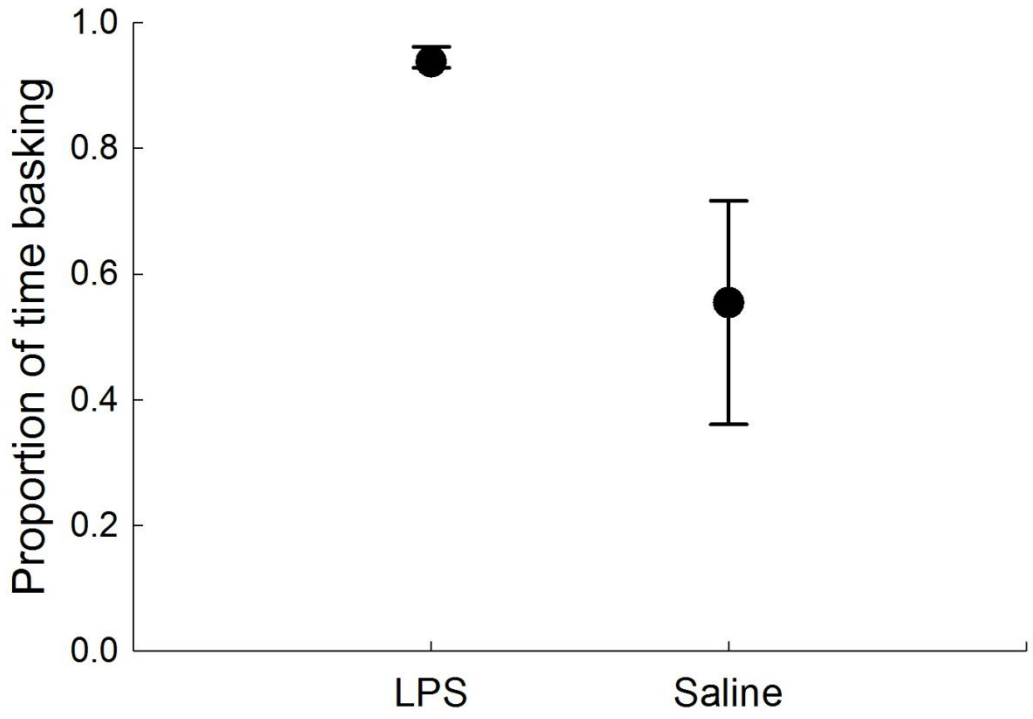

Figure 17. Proportion of time that winter-acclimated *Gopherus polyphemus* spent basking on the thermal gradient between animals injected with lipopolysaccharide (LPS) and the saline control. LPS-injected animals spent a significantly greater amount of time basking than saline-injected animals ($P = 0.021$).

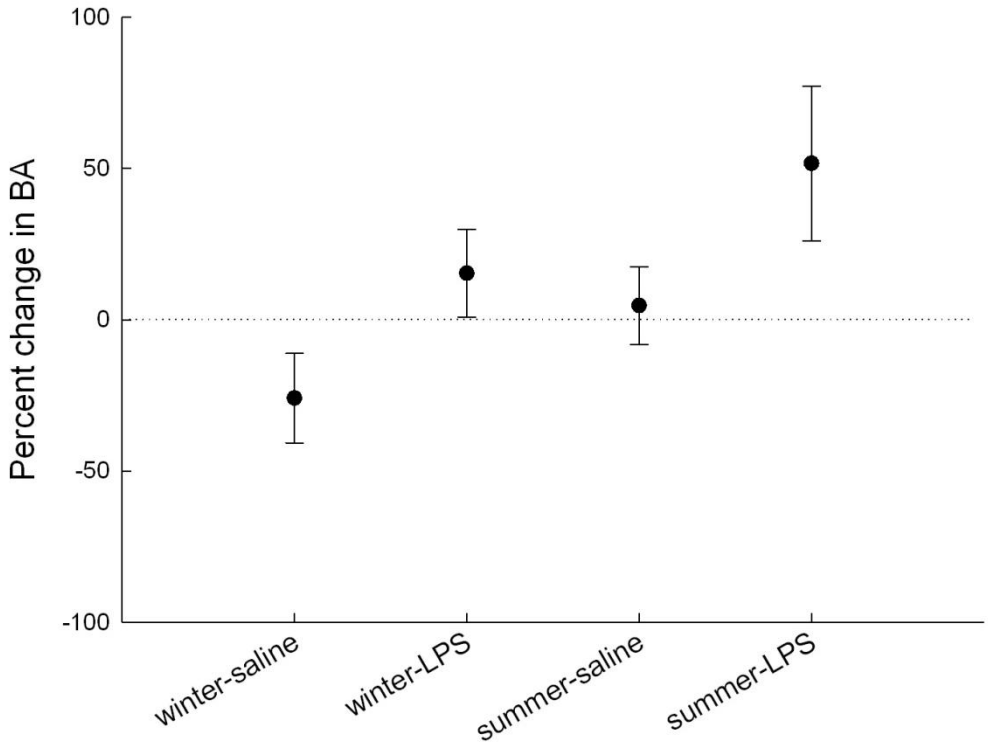

Figure 18. *Gopherus polyphemus* injected with lipopolysaccharide (LPS) showed a significant increase in plasma bactericidal ability (BA; P = 0.006), which was not directly related to seasonal acclimation state (P = 0.102) or the interaction of acclimation state and LPS treatment (P = 0.753).

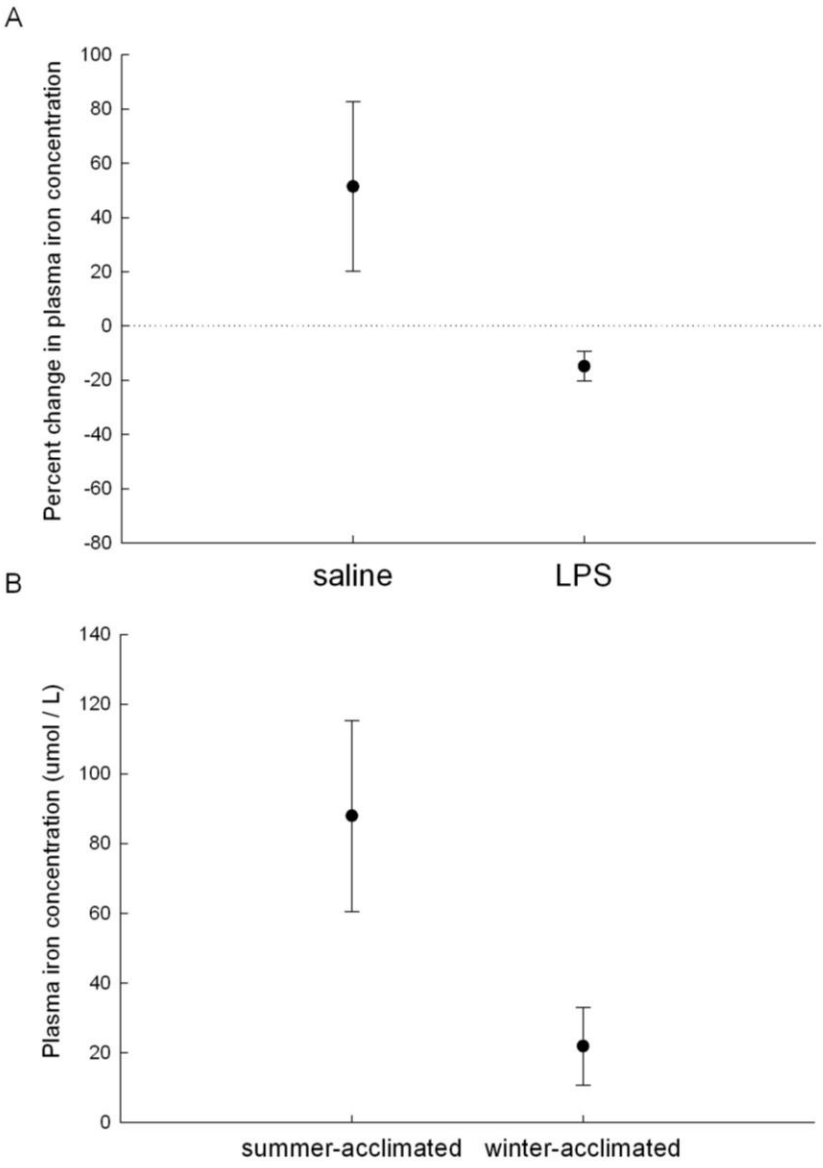

Figure 19. Injection with lipopolysaccharide (LPS) caused a significant reduction in plasma iron concentration of *Gopherus polyphemus* (A, P = 0.041). Also, seasonal acclimation state had a significant effect on baseline plasma iron concentration (B, P = 0.001), but this effect did not alter the change in plasma iron as a response to LPS injection.

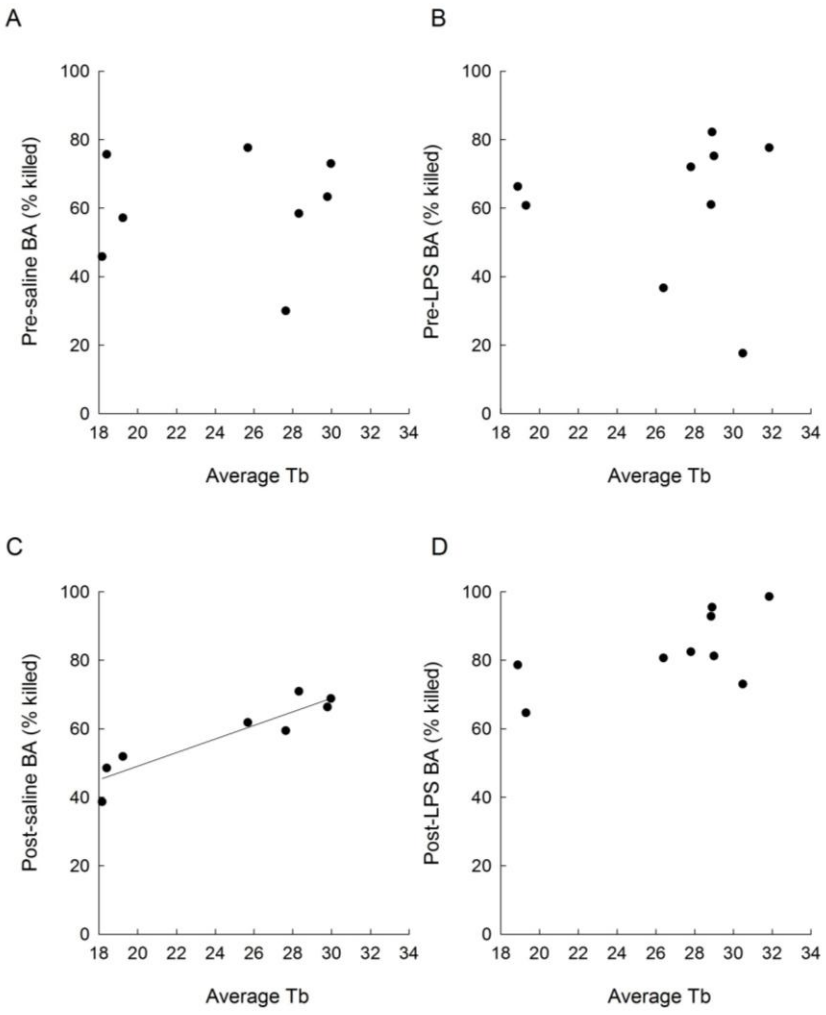

Figure 20. Thermoregulatory behavior of *Gopherus polyphemus* had a significant effect on bactericidal ability (BA). There was no relationship between the immune measure prior to the experiment and average *G. polyphemus* body temperature (T_b; A and B, P = 0.943 and P = 0.949, respectively). However, following the two-day experiment on the thermal gradient, we found that T_b was a good predictor of BA in animals injected with saline (C, P = 0.001, R^2 = 0.851). This strong relationship between average T_b and BA was not present in animals injected with LPS (D, P = 0.068).

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