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# Abiotic and Biotic Factors Influence Refuge Use at the Community and Organismal Level

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ABIOTIC AND BIOTIC FACTORS INFLUENCE REFUGE USE AT THE COMMUNITY  
AND ORGANISMAL LEVEL

by

GEORGE TODD

(Under the Direction of Zachary Stahlschmidt)

ABSTRACT

Animal behavior is influenced by a wide range of factors. One factor that can heavily influence behavior is the presence or absence of refuge (i.e., refuge provides a direct benefit to animals' fitness). Many animals seek refuge to avoid predation despite clear costs to other life processes. The decision to use refuge is complex and anthropogenic activities may alter the abundance of refuge. Artificial refuge structures can be successful in conservation efforts and are an effective means to measure biodiversity. Thus, I used cover boards to examine how habitat structure and season influence vertebrate abundance and diversity in the southeastern U.S. Vertebrate abundance was driven by proximity to roads where abundance was higher at sites that were further from roads. Season influenced the diversity of vertebrate classes where diversity was greater in the summer than in the fall and winter. My results provide evidence that anthropogenic, biotic, and temporal factors can influence vertebrate abundance and biodiversity. In addition to temporal and spatial factors, biotic factors can influence refuge use. These factors can create tradeoffs that are well-studied in some contexts of life history evolution. One such tradeoff that affects refuge use is the thermoregulation-predator avoidance tradeoff. This tradeoff may be plastic in response to environmental conditions such as pathogen exposure.

Thus, I examined the dynamics of a thermoregulation-predator avoidance tradeoff using the cornsnake (*Pantherophis guttatus*) in a controlled lab setting. Immune activation did not elicit behavioral fever or change shelter use when shelter was available across the entire thermal gradient. Although snakes strongly prioritized shelter use, their prioritization shifted during immune challenge. Snakes injected with LPS that were forced to choose between preferred temperature and shelter use maintained thermoregulation, but they spent up to 9-fold more time exposed relative to when they were injected with saline. These results demonstrate the plasticity of the widespread tradeoff between thermoregulation and shelter use.

INDEX WORDS: Ecological immunology, fever, shelter utilization, temperature, thermal preference

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GEORGE TODD

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## CHAPTER 1

### INTRODUCTION

Animal behavior is influenced by a wide range of factors, and understanding these factors can reveal the needs, preferences, and physiology of animals (Mench & Mason 1997). One such aspect that can heavily influence behavior is the presence or absence of refuge (shelter structures). Refuge provides a direct benefit to animals' fitness (e.g., animals utilize refuges to avoid predation) (Dayton 1971; Fox & Madsen 1997; Halpin 2000) and can play an integral role in interspecific competition for resources (Turchin & Kareiva 1989; White et al. 1996). Many animals seek refuge to avoid predation despite clear costs to other life processes, such as foraging (Koivula et al. 1995), thermoregulation (Weatherhead & Robertson 1992), and mate searching (Crowley et al. 1991). Thus, tradeoffs can manifest between shelter use and other behaviors (see below).

Decision-making regarding refuge use is influenced by the temporal and spatial dynamics of refuge availability in nature (Sih 1997). For example, habitat modification through anthropogenic activities may alter the abundance of refuge. As the number of natural refuge opportunities fluctuates, studies have shown that artificial refuge structures can be used to monitor biodiversity and abundance related to conservation efforts (Fitch 1992; Wilson et al. 2007; Arida & Bull 2008; Grillet et al. 2010). Thus, I used artificial refuge structures (i.e., cover boards) to examine how habitat structure and season influence vertebrate abundance and diversity in the southeastern U.S. These abiotic factors influence biodiversity (Dibaba et al. 2014; Matsuda et al. 2015), but it remains unclear how they affect animal communities in forest habitats of the southeastern U.S.

In addition to temporal and spatial factors, biotic factors can influence refuge use. For example, most animals behaviorally thermoregulate, which can influence refuge use (Downes 2001; Goldsbrough et al. 2006). Also, foraging time is typically inversely related to refuge use (Martín et al. 2003a; Cooper & Pérez-Mellado 2004; Cowlshaw et al. 2004). Further, phenotypic traits can influence refuge use (e.g., smaller and/or male animals tend to take refuge more readily: Dill & Gillett 1991; Shine et al. 2000). Thus, animals must integrate a number of biotic factors when making refuge use decisions, which can result in complex dynamics that require careful experimentation to understand.

Biotic factors create behavioral tradeoffs that are well-studied in other contexts of life history evolution (e.g., the tradeoff between mating effort and parental effort: Magrath & Komdeur 2003). One tradeoff that directly affects refuge use is the thermoregulation-predator avoidance tradeoff (Amo et al. 2004). This tradeoff may be plastic in response to environmental conditions, such as pathogen exposure. Although biologists understand how immune activation influences thermoregulation and predator avoidance independently (Otti et al. 2011; Stahlschmidt & Adamo 2013b), there is a lack of understanding in how it influences the tradeoff between thermoregulation and predator avoidance. Thus, I examined the dynamics of a thermoregulation-predator avoidance tradeoff using the cornsnake (*Pantherophis guttatus*) in a controlled lab setting.

The availability of refuges influences biological levels of organization ranging from organism to populations (Berryman & Hawkins 2006). Sources of variability in refuge use remain unclear (Parkos III et al. 2011) and my study address this lack of understanding. The two studies I performed also reveal how both abiotic factors (temperature and landscape features) and a biotic factor (immune activation) affect decisions to use refuge. I stress the importance of

studying factors that are experiencing dramatic shifts (e.g., temperature: Poirier et al. 2012 and landscape structure: McGarigal et al. 2001).

## CHAPTER 2

### EFFECTS OF HABITAT FEATURES AND SEASON ON VERTEBRATE COMMUNITIES IN SOUTHERN GEORGIA

#### INTRODUCTION

The southeastern U.S. exhibits high biodiversity (Stein 2002) and contains species that inhabit a wide range of habitats, including several types of forest (e.g., mangrove, oak-hickory, bottomland, and pine) (White & Wilds 1998; Stein 2002; Parks 2013). In addition to harboring many plant and animal species, this region is also increasingly the home of humans: the Southeast was the fastest growing region in the U.S. from 2000 to 2010 (Mackun & Wilson 2011). Humans are modifying ecosystems in complex ways, and these changes often coincide with observed changes in the biodiversity (Travis 2003; Buczkowski & Richmond 2012) and distribution of animal species (Pike 2011; Ordiz 2014; Sato 2014). Anthropogenic modifications of the landscape often take the form of habitat loss and fragmentation, which can affect animal behavior (Knopff et al. 2014) and greatly threaten species abundance (Zamfirescu et al. 2011). Therefore, examining habitat use in human-altered ecosystems provides insight into how animals will continue to respond to their rapidly changing environments (Kloskowski 2013; Pham et al. 2014; Vandavelde 2014).

Forests are a priority in studying human-habitat interactions because they provide habitat for more than half the world's terrestrial species (Groombridge & Jenkins 2000) and host numerous vulnerable species (Jenkins et al. 2015). Yet, fundamental information regarding the dynamics and distribution of animals within forest ecosystems is typically lacking (White & Wilds 1998). In forests, human activity can cause changes in landscape structure (Faulkner 2004; Nagy & Lockaby 2011), including changes in patch size (Kapos et al. 2000), canopy cover (Blouin-Demers 2001), edge density (Blouin-Demers & Weatherhead 2001), and carbon flow

(Liu & Taylor 2001). Because the diversity of animal taxa can be influenced by such changes (e.g., composition of forest: Mazurek & Zielinski 2004), it is important to account for a range of structural variables related to habitat (e.g., canopy cover, distance to edges, etc.) when studying human-impacted forests, including those in the southeastern U.S.

Several metrics of biodiversity may also vary seasonally (Grøtan et al. 2012; Liu et al. 2013; Shimadzu et al. 2013). Seasonal changes heavily influence species in several ways—from the life history stages of insects to the migratory patterns of birds (Rosenzweig 1995). Climate change may amplify seasonal effects, which may threaten forest-dependent species (Thomas et al. 2004). Further, surveys of biodiversity depend on our ability to observe species in their habitats, and it can be difficult to obtain accurate data as many taxa alter their activity based on the seasons (Houze & Chandler 2002; Paul et al. 2008; Rizkalla et al. 2015). Thus, it is important to account for seasonal variation when examining biodiversity (e.g., Houze & Chandler 2002; Brotherton et al. 2007; EIAO Note 10/2004; MacNeil & Williams 2014).

Therefore, I examined the effects of forest structure and season on several indices of biodiversity to test two hypotheses in forested sites in the southeastern U.S. that represented a range of management practices and proximity to human development indicative of the region. First, I hypothesized that landscape structure would influence vertebrate abundance, and I specifically predicted that the distance to the nearest edge would be the strongest indicators of vertebrate abundance patterns because of their thermoregulatory importance (e.g., Blouin-Demers & Weatherhead 2001). Second, I hypothesized that vertebrate diversity would be affected by season, and I specifically predicted that biodiversity indices would be higher during the more mild spring and fall months when vertebrates display more above-ground activity (Foá

et al. 1994; Bakkegard 2002; Brito 2003). The results of my study will give insight into the factors driving vertebrate diversity in forest ecosystems.

## METHODS

This study was carried out from September 2014 to September 2015 and used five forested sites across two locations in the sub-tropical climate of southeast Georgia (GA), U.S.A. Each site varied in land-management practice, as well as proximity to human development. One location was near Metter, GA, and contained three sites with different types of rural forests: sand hill (M-SH), mature pine (M-MP), and immature (3-4 years old) longleaf pine (M-IP). The distances between the sites ranged from 0.6 – 0.8 km. The second location was 24 km east of the Metter location and adjacent to human development in Statesboro, GA, a city with a population of approximately 30,000 residents (U.S. Census Bureau) surrounded by forest and agriculture. It contained two sites: mature pine with a modified sand hill on the campus of Georgia Southern University (S-GS) and a bottomland forest that abuts residential housing (S-RH). The distance between the two sites was 0.6 km.

Each site was comprised of an array of 20-25 cover boards (minimum convex polygons of arrays: 2.3 – 4.6 hectares), which were artificial cover objects consisting of two pieces of corrugated steel sheeting (1 m × 1.5 m) laid on the ground on top of each other (*sensu* Joppa 2009). Cover boards are an effective way of measuring vertebrate diversity (e.g., Grant et al. 1992; Houze & Chandler 2002; Costall & Death 2010; MacNeil & Williams 2014). Approximately twice per month, each cover board was overturned to document the presence of vertebrate genera, as well as ant colonies. Ants can influence vertebrate behavior, such as foraging (Orrock & Danielson 2004) and predator avoidance (Langkilde 2010; Long et al. 2014), and colonies of ants (*Camponotus floridanus*, *C. pennsylvanicus*, and *Solenopsis invicta* in my



study) use artificial cover objects and are easy to observe. For vertebrates, richness (R, the number of taxa present), Shannon's Diversity (H), and Shannon's Evenness (E) were calculated for each site during each season because R, H, and E are effective metrics for estimating biodiversity (Magurran 2004). Metrics of vertebrate abundance and diversity were compared across meteorological seasons (i.e., spring: March-May; summer: June-August; fall: September-November; winter: December-February).

To characterize the structural features of the forested sites, several abiotic and biotic variables were measured. The daily precipitation, minimum air temperature ( $T_{\min}$ ), and maximum air temperature ( $T_{\max}$ ) of each site during data collection was approximated using data from weather stations of National Oceanic and Atmospheric Administration that were 9 km from the Statesboro location and 5 km from the Metter location.

Several structural variables were used to characterize the habitats at each site (patch size) or near each cover board (all other variables: see below). Patch size, or an area of habitat surrounded by a border that constitutes a change in habitat type, was measured at each site and plays an integral role in ecosystem dynamics (Pickett & White 1986). Canopy cover, a measure of the degree to which foliage blocks sunlight from reaching the ground in a forest habitat, is a key variable when characterizing thermal quality of habitats (Blouin-Demers 2001) and was measured during the summer (July 2015) to estimate maximum yearly values. Canopy cover was determined at each cover board by measuring photographs taken through a fisheye lens using ImageJ software (version 1.48, National Institute of Health). The distance to the nearest snag (tree stump or log  $\geq 7.5$ cm), overstory tree, road, and building (modified from Blouin-Demers & Weatherhead 2001) were also measured. The distance to the nearest edge, a border between habitat types (e.g., the border between a forest and a habitat consisting of short

vegetation), was also measured. Edges can be natural or anthropogenic (e.g., created via mowing, burning, or other land practices) and are key to understanding thermoregulation because they represent a distinct shift in thermal characteristics within a habitat (Blouin-Demers & Weatherhead 2001).

All abundance values gathered from the sites were square root transformed and underwent nonmetric multidimensional scaling (NMDS) to ordinate the data and visualize resemblance patterns by site and season—that is, NMDS allowed visualization of the effects of site and season on vertebrate abundance (see below).

Analyses were performed in SPSS (v.22 IBM Corp., Armonk, NY) and Primer 7 (v.7.0.8 Lutton, UK). When necessary, data were either square root transformed or logarithmically transformed to achieve normality. Two-tailed significance was determined at  $\alpha < 0.05$ .

Several tests were performed to examine if weather or vertebrate diversity varied due to location (i.e., Metter, GA vs. Statesboro, GA). To determine if weather varied across locations, t tests comparing the weather data (daily precipitation,  $T_{\min}$ , and  $T_{\max}$ ) of the Statesboro and Metter locations during the entire sampling period (365 d) and on the days that sampling occurred (50 d) were used. To test for differences in abundance levels between sites and season, a permutational analysis of variance was used.

Due to relatively low observed abundances, observation data were pooled by season (e.g., for spring: the sum of all individuals observed under cover boards from March to May 2015). To determine correlations between abundance values and habitat variables (i.e., to address my first hypothesis that habitat variables influence vertebrate abundance), a distance-based linear model was used. To determine seasonal effects on R, H, and E of vertebrate class and vertebrate genera

(i.e., to address my second hypothesis that season influenced biodiversity), linear mixed models were used. Season was included as a fixed effect, the percentage of observations of ants was included as a covariate, site was included as a random effect, and diversity indices were included as dependent variables.

## RESULTS

Daily precipitation,  $T_{\min}$ , and  $T_{\max}$  did not differ near the two sites across the year (all  $P > 0.14$ ) or on the days that were sampled (all  $P > 0.70$ ).

A total of 3 classes, 16 genera and 20 species were observed under cover boards over the course of the study (Table 1).

Vertebrate abundance varied by season where fall/winter and spring/summer were the only season combinations that were similar to one another ( $F_{3,19}=8.0$ ,  $P=0.68$  and  $0.1$  respectively). Note the points in Figure 1 are clustered between spring/summer and fall/winter on the left and right sides respectively. Vertebrate abundance differed between sites M-SH and S-RH ( $F_{4,19}=3.5$ ,  $P=0.04$ ), M-MP and S-RH ( $F_{4,19}=3.5$ ,  $P=0.05$ ), and M-IP and S-RH ( $F_{4,19}=3.5$ ,  $P=0.04$ ) (Fig. 1). Note the points in Figure 1 also show that Site S-RH differed from all three Metter sites. The points for all four seasons of the S-RH are clustered at the top of the figure whereas the points for the Metter sites, although split by seasonal differences, are located closer to the bottom of the figure. The stress value is the degree to which the data is represented by the dimensions in the plot. The value for the plot in Figure 1 (0.12) is acceptable for an NMDS ordination (Buttigieg & Ramette 2014). The distance-based linear model explained 28% of the total variance in vertebrate abundance. The distance to the nearest road (d-road) significantly influenced vertebrate abundance ( $P=0.018$ ) where a higher number of vertebrates were observed

under cover boards far from roads. No other landscape variables influenced vertebrate abundance.

Season influenced H of classes ( $F_{1,13}=5.0$ ,  $P=0.016$ ) where Shannon's diversity index for vertebrates was greater in the summer than in the fall and winter (sequential Bonferroni-adjusted pairwise comparison) (Fig. 2). The H of classes positively covaried with the percentage of cover boards occupied by ants ( $F_{1,10}=5.6$ ,  $P=0.039$ ). Neither the presence of ants nor season were significantly associated with other metrics of biodiversity or abundance (all  $P>0.07$ ).

## DISCUSSION

Vertebrate abundance was influenced by the distance to the nearest road, which partially supported my first hypothesis that landscape structure would influence vertebrate abundance (Fig. 1). Season influenced Shannon's diversity of classes (Fig. 2) where H was greater in the summer than in the fall and winter. Yet, I predicted higher biodiversity in the milder spring and fall seasons for my second hypothesis that vertebrate diversity would be affected by season.

The distance to the nearest road (d-road) was the only structural variable that influenced vertebrate abundance where abundance was inversely related to d-road. Related, "road-ecology" has gained attention as the country's network of roads continues to rapidly expand (Fahrig & Rytwinski 2009). This is evidenced by the creation of the International Conference on Ecology and Transportation, as well as various road-ecology research centers. However, not all roads are created equally, and unpaved roads may impact wildlife less than paved roads because they experience less disturbance and traffic (Andrews et al. 2015). Yet, unpaved roads are not without costs to ecosystems as they are more vulnerable to erosion, can smother surrounding streams and vegetation, and generate significant dust during dry periods (Strayer 2012).

Roads often have negative effects on animal populations for several reasons, including direct mortality risk (Forman et al. 2003) and reduced accessibility to habitat and resources (Jaeger & Fahrig 2004). However, some species can experience neutral or positive road effects. Typically, these species are either (1) attracted to roads for food and can avoid mortality (e.g., vultures) or (2) avoid road contact but are not disturbed by traffic and easily avoid traffic (e.g., small mammals and birds) (Fahrig & Rytwinski 2009). The inverse relationship between vertebrate abundance and d-road that I found aligns with previous work demonstrating the susceptibility of herpetofauna to roads (Fahrig & Rytwinski 2009) given 86% of my observations were of amphibians and reptiles (Table 1).

The effects of roads on animal habitat use is made complex by the fact that, although roads can fragment a landscape, they can also increase the number of edges in a landscape (Murcia 1995; Mehmood & Zhang 2001). Ecological edges separate habitats and can be ecologically advantageous because they allow quick movement to adjacent habitats and facilitate thermoregulation (Murcia 1995; Blouin-Demers & Weatherhead 2002; but see Hanski et al. 1996; Kingston & Morris 2000). Despite these advantages for certain taxa, edges can be a double-edged sword as they can also allow easy access to habitats by predators, cause higher rates of nest predation, and parasitism (birds: Gates & Gysel 1978). Although I found no support for my prediction that proximity to edge would drive abundance, edge effects are complex and can vary in time and space (Laurence et al. 2007). One possible explanation for my negative result related to edge effects is that edge effects have been found to increase with habitat area (Ewers et al. 2007), and my sites were relatively small (<14 hectares). Edge effects also tend to be greater when a forest edge borders agricultural or pastureland as opposed to another forest patch (Mesquita et al. 1999). However, all five of my sites had at least some edges adjacent to

more forest patches. Although I did not find edge effects in my study, future studies should continue to account for edge density as it can have profound influence on biodiversity (Saunders et al. 1991; Laurence et al. 2007).

In contrast to my second prediction, vertebrate diversity in my study was greater in the summer than in the fall and winter, but there was not higher diversity in the milder spring and fall seasons (Fig. 2). This effect may be attributed to the seasonal activity patterns of the taxa observed in my study—most of the taxa (i.e., ectotherms) are largely inactive and fossorial during cooler months (Ultsch 1989; Halliday 1994). Beyond this generalization, understanding the biology of my commonly encountered taxa (Table 1) may provide insight into shifts in observed biodiversity. For example, amphibians have seasonal patterns of breeding (Watling & Donnelly 2002; Vignoli et al. 2007), and it is likely these patterns drove the observations of the genera *Bufo* and *Lithobates* (Table 1). *Bufo* species breed during the spring and early summer, and *Lithobates* species breed in the late fall and winter (Jenson et al. 2008). All of the amphibian genera I observed are more fossorial during the summer months, especially *Gastrophryne*, which begins breeding in early spring (Jenson et al. 2008). Certain genera in the class Reptilia also showed distinct seasonal patterns in their utilization of cover boards. *Anolis* was observed much more in the fall and winter, and this also corresponds with their breeding activity (Gorman & Licht 1974; Orrell et al. 2004). *Scincella* was observed more during the fall and winter in my study (Table 1). Although this does not correspond with their breeding activity (Jenson et al. 2008), they have been shown to thermoregulate much more effectively in the fall and winter (Parker 2014). *Eumeces* displays breeding activity in the spring or early summer, and this corresponds with my observations (Trauth 1994; Table 1). *Eumeces laticeps* even aggregates underground for hibernation during cold months (Jenson et al. 2008). Given the proportion of

reptiles I observed, it is likely that these seasonal patterns in Reptilia alone drove the seasonal variation in Figure 1. The cotton mouse (*Peromyscus gossypinus*) was observed many times during the fall and winter, but at no point during the spring and summer (Table 1). Although *P. gossypinus* can be active all year, its activity tends to increase during breeding (sometimes in spring but more often in fall), especially for females in estrus (O'Farrell 1974; Cushing & Cawthorn 1996). It is plausible that the presence of active-foraging snake species, such as *Coluber constrictor* (which was observed with much more frequency in the spring/summer: Table 1) after a short period of hibernation (Jenson et al. 2008) may have led to the disappearance of *P. gossypinus* under cover boards in the spring and summer. Future biodiversity studies should collect data across seasons to account for the seasonal activity patterns of the taxa involved.

My results indicate that vertebrate diversity may have been influenced by the presence of ants under cover boards. The red imported fire ant (*Solenopsis invicta*) is a dominant invasive species in the southeastern U.S. and tends to displace native ants and prey upon small mammals, reptiles, and amphibians (Porter & Savignano 1990; Allen et al. 1994; Todd et al. 2007). Although *S. invicta* can discourage the presence of vertebrates under cover boards (Heyer et al. 1994), it is likely that the positive covariation between class diversity and presence of ants found in my study was driven by the large proportion of lizards observed in my study. *Anolis*, *Eumeces*, and *Scincella* composed 58% of my observations throughout the study (Table 1). Although these species may be negatively affected by *S. invicta*, their primary diet is ants (including, *C. floridanus* and *C. pennsylvanicus* which were the other species of ants I observed) and other insects (Martof et al. 1989; Jenson et al. 2008). Future work could disentangle the positive (food source) and negative (predation risk) effects of ants on refuge use by vertebrates

by identifying specific ant species under cover objects or, better, excluding certain species and monitoring potential shifts in vertebrates' use of cover objects.

Although cover object surveys are a common method to sample biodiversity (e.g., Grant et al. 1992; Houze & Chandler 2002; Costall & Death 2010; MacNeil & Williams 2014), one of their limitations is that animals may use other, more thermally stable refuges (e.g., subterranean burrows) during periods of unfavorable surface temperatures (Houze & Chandler 2002). While cover objects are typically positioned on the ground (e.g., Grant et al. 1992; Hampton 2007), this method has been expanded to include arboreal cover boards (e.g., foam and cardboard strapped around a tree trunk), which can increase observations of both arboreal vertebrates (Nordberg & Schwarzkopf 2015) and invertebrates (Lettink & Patrick 2006; Hodge et al. 2007). The utilization of cover objects by animals can also be enhanced by designing cover objects to resemble natural refuges, which has been successful in restoring degraded habitats (Bowie et al. 2014) and vulnerable species (Souter et al. 2004). Thus, I recommend the continued use of artificial cover objects in studies of urban ecology because of their numerous advantages: low cost and labor intensity (Kjoss & Litvaitis 2001), properties that encourage utilization by animals (e.g., protection from predators: Fitch 1992), and less weather-dependent than other methods, such as pitfall traps (Wilson et al. 2007).

My results provide insight into how variation in forest structure and season alter the abundance and diversity of vertebrates in the southeastern U.S. Distance to the nearest road was the only structural variable I found to be an indicator of vertebrate diversity, and this may be due to two of my other variables (distance to nearest snag and overstory tree) being more important indicators in landscapes that have been more intensively altered by humans (e.g., agriculture or urban core) (Harvey et al. 2006). My results provide further insight into how activity patterns of



taxa can influence biodiversity surveys (Dodd 2009; Sung et al. 2011). Future studies should continue to quantify the role of these ecological drivers of biodiversity (e.g., via manipulations of land management).

## CHAPTER 3

### IMMUNE ACTIVATION INFLUENCES THE TRADEOFF BETWEEN THERMOREGULATION AND SHELTER USE

#### INTRODUCTION

The vast majority of animal species (99.9%) are ectothermic (Atkinson & Sibly 1997) and, thus, are particularly sensitive to changes in ambient temperature. Temperature affects many life processes, including locomotion (Kubisch et al. 2011), digestion (Wang et al. 2002), growth (Yagi & Litzgus 2013), and reproduction (Abde El-Hafez 2014). Because ambient temperature varies spatially and temporally, thermoregulation (i.e., regulation of body temperature,  $T_b$ ) is a crucial behavioral process for most animal taxa (Reinert 1993; Blouin-Demers & Weatherhead 2001; Sauter et al. 2000; Deban & Lappin 2010; Caillon et al. 2014; Hoecherl & Tautz 2015).

Several features of the environment promote thermoregulation, including shelters (refuge structures), which can exhibit different absolute temperatures and temperature variability than ambient conditions (Stahlschmidt et al. 2012; van den Berg et al. 2015). In addition to promoting thermoregulation and other behaviors (e.g., ambush-hunting: Bevelander et al. 2006; Clark 2007), shelters can promote predator avoidance for many animals—from worms (Dill & Fraser 1996) to primates (Cowlshaw 1997). Both facilitation of thermoregulation and predator avoidance can coincide (Schwarzkopf & Alford 1996; Roper et al. 2001), but shelters are not always thermally optimal (e.g., a log exposed to constant sunlight may be too hot to promote optimal growth for an animal: Downes 2001). Thus, a tradeoff can occur where an animal is forced to choose between thermoregulation and using a shelter for protection from predators (Amo et al. 2004; Stahlschmidt & Adamo 2013a). Although behavioral thermoregulation improves locomotive performance (Angilletta et al. 2002), predator avoidance is linked to

immediate survival and, thus, has been shown to be prioritized in thermoregulation-predator avoidance tradeoffs (Mabille & Berteaux 2014).

Like other important tradeoffs, the thermoregulation-predator avoidance tradeoff may be plastic in response to environmental conditions (Stahlschmidt et al. 2013a; Stahlschmidt et al. 2014; Dosmann et al. 2015). For example, pathogen exposure varies temporally and spatially (Raucher 2002, Suwanpakdee et al. 2015), and it can influence thermoregulation and predator avoidance independently. During immune activation, many animals putatively shift their  $T_b$  to create an undesirable thermal environment for pathogens (Kluger 1986; Bicego et al. 2007). This shift in  $T_b$  often presents as fever (increase in  $T_b$ ), such as in crayfish (Payette & McGaw 2003), insects (reviewed in Stahlschmidt & Adamo 2013b, but see Ballabeni et al. 1995), mice (MacDonald et al. 2012), and reptiles (Merchant et al. 2008, but see Zurovsky et al. 1987), but immune activation can have hypothermic effects on animals (Deen & Hutchison 2001; Almeida et al. 2006). Immune activation can also influence predator avoidance behavior (e.g., cause an infected individual to react slower to predator attacks even at preferred temperature) (Lefcort & Eiger 1993; Joop & Rolff 2004; Rantala et al. 2010; Otti et al. 2011). The role of immune activation in the tradeoff between thermoregulation and predator avoidance has been proposed (e.g., Otti et al. 2011; Nord et al. 2014) but never explicitly tested.

Thus, I examined the dynamics of a thermoregulation-shelter use tradeoff to test two hypotheses. I used the cornsnake (*Pantherophis guttatus*) to test my hypotheses because snakes utilize existing shelter (Hyslop 2009) and adjust their habitat selection to carefully regulate  $T_b$  (Blouin-Demers & Weatherhead 2001; McConnachie et al. 2011; Lorigou et al. 2012; Stahlschmidt et al. 2012; Aidam et al. 2013; Lourdaïs et al. 2013). Like other animals (Lefcort & Eiger 1993; Johnson 2002), snakes may exhibit a shift in  $T_b$  and increase shelter use due to

immune activation; thus, I first hypothesized that immune activation influences thermoregulation and shelter use independently (*sensu* Kluger 1986; Ottie et al. 2011). This hypothesis predicted that snakes would undergo fever and increase their shelter use during an immune activation. My second hypothesis was that immune activation affects the thermoregulation-shelter use tradeoff. I predicted that animals normally prioritize shelter use over thermoregulation (*sensu* Mabilie & Berteaux 2014) but that this prioritization is reduced during an immune challenge due to the strong effects of immune activation on thermoregulation (Deen & Hutchison 2001; Payette & McGaw 2003; Almeida et al. 2006; Merchant et al. 2008; MacDonald et al. 2012; reviewed in Stahlschmidt & Adamo 2013b, but see Zurovsky et al. 1987). The results of my study will give insight into how an important environmental factor (immune activation by pathogens) influences the tradeoff between two widespread behaviors (thermoregulation and shelter use).

## METHODS

*Pantherophis guttatus* is a member of the family Colubridae and is commonly found throughout the southeastern United States (Dorcas & Gibbons 2005). The experiment used 23 captive-born *P. guttatus* (1.5-2 years of age; 14 males and 9 females; body mass range: 119-486 grams) that were 1<sup>st</sup>-4<sup>th</sup> generation progeny of snakes caught in Beaufort County, SC, USA. Prior to trials, snakes were housed individually in translucent plastic enclosures (17 cm × 38 cm × 14 cm). To facilitate behavioral thermoregulation, sub-surface heat tape at one end of the enclosures created a temperature range of 24.5-33 °C, which accommodates the preferred  $T_b$  of *P. guttatus* (Roark & Dorcas 2000; Raske et al. 2012; Stahlschmidt et al. 2015). Snakes were fed frozen/thawed mice (10-20% of each snake's body mass) every 1-2 wks and provided water *ad libitum*. Digestion can invoke  $T_b$  shifts in *P. guttatus* (Sievert et al. 2013) so snakes were non-absorptive (>5 d post feeding; Crocker-Buta & Secor 2014) during trials. Throughout the study,

snakes exhibited no inflammation or evidence of trauma at the sites of injection and, as further evidence that treatments were not overly stressful, they readily accepted food shortly after trials (see details below). All procedures were approved by the Institutional Animal Care and Use Committee at Georgia Southern University (protocol #I14004).

Experiments were performed in thermal gradient lanes (length: 2.4 m; width: 0.3 m; height: 0.4 m) within an arena (Fig. 3). The temperature gradient was achieved with modified Flex-Watt heat tape placed below 2-3 cm of sand (a temperature stable substrate). Substrate temperature ranged from 20-35°C because *P. guttatus* exhibit a preferred  $T_b$  of 26-27°C (Roark & Dorcas 2000; Raske et al. 2012; Stahlschmidt et al. 2015). When trials were in session, the top of the arena was covered with acrylic glass to keep snakes inside and to help stabilize the temperature of the substrate.

To determine the role of immune activation on a thermoregulation-shelter use tradeoff in *P. guttatus* (n=23), a repeated measures  $2 \times 2$  factorial experimental design in the thermal gradient arena was used. Two treatments were used, each with two levels: shelter availability (full and partial) and presence of immune status (challenge or control) (described below).

For the shelter availability treatment, either shelter across the entire gradient (“full” shelter) or only at temperature extremes (“partial” shelter) was provided (Fig. 3). In both cases, shelter consisted of a corrugated steel sheet (0.15 m wide) placed 3-4 cm above the substrate (Fig. 3). The temperature extremes in the partial shelter level were at least 2-3 °C below and above preferred  $T_b$  (20-23 °C and 29-35 °C, respectively)—that is, the partial shelter level of treatment forced snakes to choose between finding shelter and preferred temperature.

For each snake, the temperature it selected ( $T_s$ ) in full and partial shelter treatments was determined in two immune states, the order of which was randomized. For each trial, snakes were given a subcutaneous injection (1 ml per kg body mass) of phosphate-buffered saline (PBS) solution (1x concentration) or lipopolysaccharide (LPS, 10 mg LPS dissolved in 1x PBS). Lipopolysaccharide is an endotoxin found on the membranes of Gram-negative bacteria that typically elicits a strong immune (and often febrile) response in many animals, including reptiles (Ortega et al. 1991; Burns et al. 1996; Alexander & Rietschel 2001; but see Zurovsky et al. 1987). The LPS was a lyophilized powder from *Escherichia coli* (Sigma-Aldrich, Rehovot, Israel, Lot # 044M4004V) and the 1xPBS was made by adding tablets (Fisher Scientific, Fair Lawn, NJ, Lot # 145850) to distilled H<sub>2</sub>O. Each snake was given an injection (LPS or PBS) at 20:00 and was then allowed to habituate to its own lane within the thermal gradient overnight. Six hourly measurements of temperature choice and shelter utilization were taken the next day from 8:00 to 13:00. Temperature measurements were taken using a factory-calibrated infrared thermometer (ProTemp 12, Jewell Instruments, Manchester, NH; range: -50–550 °C; accuracy: 1.5%; resolution: <0.1 °C). Because substrate temperature and  $T_b$  are highly related in reptiles (Lagarde et al. 2012), this method is useful in quiescent snakes and also because it is less invasive than direct methods such as cloacal probes. Snakes that were moving during an observation (<2% of observations) were not recorded for temperature. To avoid confounding olfactory signals among snakes, the sand was mixed and shelters cleaned with 70% ethanol between trials. Each snake's trials (n=4) were separated by  $\geq 2$  wks.

Analyses were performed in SPSS (v.22 IBM Corp., Armonk, NY), and two-tailed significance was determined at  $\alpha < 0.05$ . For shelter preference, a binary logistic generalized linear mixed model was used where shelter status (full or partial) and immune status (control or

LPS-challenged) were included as fixed effects, and each observation (n=6 per trial for each of a given snake's four trials) was the dependent variable (0: under shelter, 1: not under shelter). Observation (but not trial) order significantly influenced shelter preference; thus, I included observation order (1-6) as a random effect. To examine whether animals had a preference for warmer or cooler shelter in the partial shelter trials,  $\chi^2$  goodness of fit tests were performed for each immune status. For preferred  $T_s$ , a linear mixed model was used where shelter status and immune status were included as fixed effects, and each temperature observation (n=6 per trial for each of a given snake's four trials) was the dependent variable. Trial (but not observation) order significantly influenced  $T_s$ ; thus, I included trial order (1-4) as a random effect. To account for repeated sampling, animal ID was included as a random effect in all mixed models.

## RESULTS

Shelter preference was influenced by the interaction between shelter status and immune status ( $\chi^2=12$ , d.f.=1,  $P=0.001$ ), but it was not affected by shelter status ( $\chi^2=0.47$ , d.f.=1  $P=0.49$ ) or immune status independently ( $\chi^2=2.8$ , d.f.=1  $P=0.094$ ) (Fig. 4). When immune-challenged and given only partial shelter, snakes exhibited reduced shelter preference relative to other treatment combinations (83% vs. >96% of observations under shelter) (Fig. 4). When forced to choose between shelters that were cooler- and warmer-than-preferred (i.e., during partial shelter trials), snakes strongly preferred cooler shelters when LPS-challenged (89% of observations) ( $\chi^2=26$ , d.f.=1  $P<0.001$ ) and when non-challenged (99% of observations) ( $\chi^2=46$ , d.f.=1  $P<0.001$ ). Preferred  $T_s$  was independently influenced by shelter status ( $F_{1,517}=8.6$ ,  $P=0.003$ ) and immune status ( $F_{1,517}=11$ ,  $P=0.001$ ), but these effects were largely driven by a shelter $\times$ immune interaction ( $F_{1,517}=33$ ,  $P<0.001$ ) (Fig. 5). When non-challenged and given only partial shelter, snakes exhibited reduced  $T_s$  relative other treatment combinations (24°C vs. 27°C) (Fig. 5).

## DISCUSSION

Although I did not detect a strong independent effect of immune activation on thermoregulatory or shelter use behavior (Figs. 4 and 5; which does not support my first hypothesis), I did find support for my second hypothesis that immune activation influences the tradeoff between thermoregulation and shelter use. The snakes utilized shelter at least 96% of the time when shelter was readily available (Fig. 4). However, when forced to choose between thermoregulation and shelter use, immune-challenged snakes were more likely to maintain their  $T_s$  at the expense of shelter use—specifically, they spent 9-fold as much time exposed relative to control snakes (Fig. 4).

Across taxa, the effects of immune activation can have varying effects on temperature preference. Although fever is demonstrated by many animals (e.g., reviewed in Kluger 1986; Stahlschmidt & Adamo 2013b), some species exhibit a hypothermic response to an immune activation or even no response at all, including several reptile species (Laburn et al. 1981; Zurovsky et al. 1987; Don et al. 1994; Burns et al. 1996). Fever is a complex response, and several factors (e.g., the type and dosage amount of pyrogen, and the timing of measurement: Stahlschmidt & Adamo 2013b) must be considered when determining its presence or absence. Although immune activation can also influence predator avoidance behavior and shelter preference (Lefcort & Eiger 1993; Joop & Rolff 2004), experiments examining fever typically do not include shelter (Zurovsky et al. 1987; Ortega et al. 1991; Don et al. 1994; Burns et al. 1996; Cichoń et al. 2002; Stahlschmidt & Adamo 2013a). Shelter is important for a plethora of behavioral processes, such as competition (Fero & Moore 2014; Kintzing & Butler 2014) and oviposition site selection (Stahlschmidt & Adamo 2013a)—thus, it may be similarly important



for behavioral fever. Future studies examining fever should provide varying degrees of shelter to determine the importance of shelter in thermoregulatory responses to immune activation.

Given my results (Figs. 4 and 5) and those of others (Webb et al. 2009; Bonnet et al. 2013; Stahlschmidt et al. 2013b; Villén-Pérez et al. 2013), the prioritization of shelter use over thermoregulation by animals may be widespread. When forced to choose between thermoregulation and shelter use, the control snakes in my study did not alter their shelter utilization (Fig. 4) and this came at the expense of their  $T_s$  (Fig. 5)—that is, they prioritized shelter use over thermoregulation. Both shelter use and thermoregulation are important (i.e., an animal lacking both aspects will likely exhibit higher risk of predation and hindered physiological performance) (Colishaw 1997; Regal 1966). However, a lack of shelter typically outweighs the negative effects of undesirable ambient temperature in insects (Stahlschmidt & Adamo 2013b), reptiles (Webb et al. 2009; Bonnet et al. 2013), and birds (Villén-Pérez et al. 2013). When lacking shelter, snakes exhibit reduced energy intake (high regurgitation rate) and chronically elevated levels of corticosterone (CORT, a hormone secreted in response to stress via the hypothalamic-pituitary-adrenal [HPA] axis) (Moore & Mason 2001; Bonnet et al. 2013). However, some snakes do not experience an increase in baseline plasma CORT levels when occupying thermally suboptimal environments (Sykes & Klukowski 2009; but see Dupoue et al. 2013). Further, although little data on thermal performance exists for *P. guttatus*, the swimming and striking performances of other colubrid snakes are not highly sensitive to shifts in  $T_b$  (e.g., the range of  $T_b$ s over which performance is 80% of maximal is 15-18°C: Blouin-Demers et al. 2003). Therefore, shelter use may be ultimately prioritized over thermoregulation due to its direct effect on fitness (i.e., avoiding predators increases survival), and this prioritization may be mediated at a proximate level by the HPA axis (Smith & Vale 2006).

My second hypothesis (immune activation influences the tradeoff between thermoregulation and shelter use) was supported because shelter use became deprioritized over thermoregulation due to immune activation (Figs. 4 and 5). Although this hypothesis had never been explicitly tested, others had similar results in different taxa. For example, immune activation in blue tits (*Cyanistes caeruleus*) does not cause a change in  $T_b$ , but it may cause birds to sacrifice perceived safety (i.e., remain roosting in nest boxes where previous captures occurred) to maintain preferred  $T_b$  in the context of energy conservation (Nord et al. 2014). Similarly, immune activation in field crickets (*Gryllus campestris*) increases the amount of time spent exposed, and this may occur to facilitate shifts in thermoregulation (e.g., increased basking: Otti et al. 2011). Thus, the benefits of such behavioral shifts to the immune system (i.e., creating an undesirable environment for a pathogen) may begin to outweigh the costs (e.g., sub-optimal temperature for other physiological processes, reduced food consumption, and increased predation risk). Given the potentially widespread nature of thermoregulation-shelter use tradeoffs, I encourage others to test its plasticity in response to immune activation using other taxa.

When forced to choose between shelters that were either cooler or warmer than preferred, snakes in my study preferred to utilize cooler shelters (Fig. 5). I offer several possible reasons for this result. First, some benefits of the warmer shelter were minimized in my study. For example, snakes were post-absorptive and, thus, did not reap the benefits of digestion related to warmer temperatures (Michel and Bonnet 2010). Second, there are physiological costs to occupying and conforming to warmer microhabitats. Energy expenditure and water loss increase with increasing  $T_b$ , and thermal performance declines more rapidly at temperatures above the thermal optimum relative to temperatures below the thermal optimum (reviewed in Angilletta

2009; Michel and Bonnet 2010; Bonnet 2011). Third, the thermal optimum for at least one widespread metric of performance (innate immune function: Butler et al. 2013) is lower than  $T_s$  in my study snakes (Stahlschmidt et al., unpublished). Future research should continue to examine the relationship between thermal optima for other performance traits (e.g., locomotion) and temperature-based decision-making in snake taxa and beyond.

Fever responses are complex and influenced by several factors (e.g., reviewed in Stahlschmidt & Adamo 2013b). Thus, I suggest using the following standard protocol when studying behavioral responses to immune activation in vertebrates. (1) Use the genus *Aeromonas* as the pyrogen because this group can elicit a febrile response in ectothermic vertebrates (e.g., fish: Tsai & Hoh 1995; amphibians: Casterlin & Reynolds 1977; reptiles: Merchant et al. 2007) and is associated with infections of birds and mammals (Janda and Abbott 1998; Tomás 2012). (2) Use a comprehensive range of sampling time points because febrile responses can range from hours (Haukenes and Barton 2004) to days (Don et al. 1994; Burns et al. 1996), to weeks (Moore & Freehling 2002) depending on taxon. (3) Control for shelter availability (see rationale above and below). Adopting a more standardized protocol should allow us to better understand the prevalence of fever and the role of fever in behaviors associated with shelter use.

Results from my study suggest we should re-evaluate how immune activation influences decisions related to behavioral thermoregulation. For example, I demonstrate that immune-challenged animals not exhibiting fever may still exhibit important shifts in the prioritization of thermoregulation. My results also demonstrate that immune activation can affect the potentially widespread tradeoff between thermoregulation and shelter use. In the future, researchers should continue to investigate the plasticity of this tradeoff in response to other common factors, such as reproductive status, digestive state, age, or motivation for predator avoidance or foraging (e.g.,

by scenting areas of the arena with predators or prey, respectively, *sensu* Downes and Shine 1998). We should also continue to examine this pattern across other taxa and in other contexts (e.g., the role of immune activation in tradeoffs between thermoregulation and foraging behavior).

Table 1: Abundance of species found across seasons at five forested sites in southern Georgia.

Genera with more than one species observed are denoted with an \* (*Ambystoma: opacum*, *talpoideum*, *Bufo: quercicus, terrestris*, *Eumeces: fasciatus, inexpectatus, laticeps*).

Class	Genera	Season			
		Spring	Summer	Fall	Winter
Amphibia	<i>Ambystoma*</i>	8	1	7	7
	<i>Bufo*</i>	3	5	0	3
	<i>Gastrophryne carolinensis</i>	4	0	0	0
	<i>Lithobates sphenoccephalus</i>	3	1	7	10
Reptilia	<i>Anolis carolinensis</i>	8	2	52	62
	<i>Aspidoscelis sexlineatus</i>	9	0	0	1
	<i>Coluber constrictor</i>	7	11	1	1
	<i>Diadophis punctatus</i>	3	3	1	4
	<i>Eumeces*</i>	69	37	0	0
	<i>Lampropeltis getula</i>	1	1	0	1
	<i>Masticophis flagellum</i>	2	1	2	1
	<i>Nerodia fasciata</i>	2	0	0	1
	<i>Ophisaurus ventralis</i>	2	0	1	0
<i>Scincella lateralis</i>	0	3	28	19	
Mammalia	<i>Blarina carolinensis</i>	1	0	0	2
	<i>Peromyscus gossypinus</i>	0	0	26	34

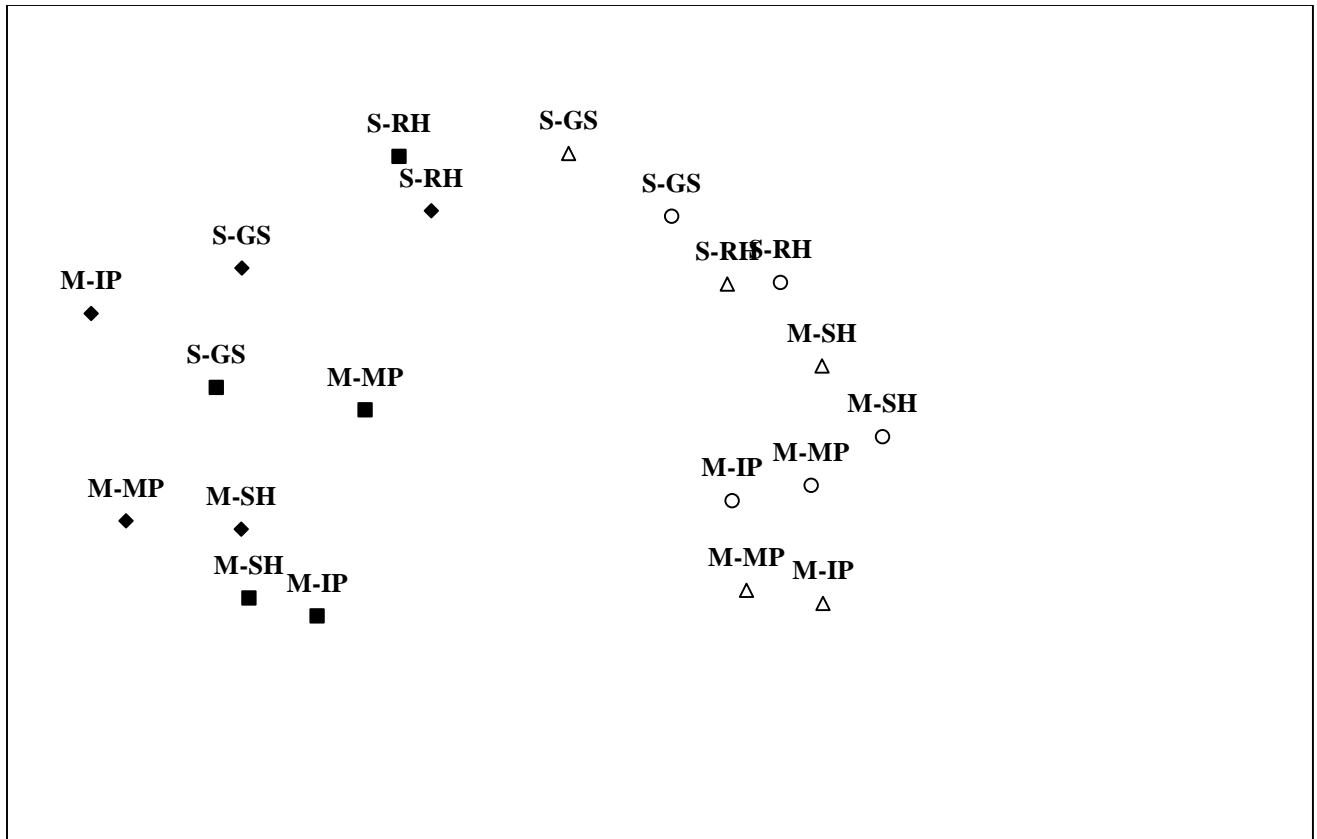


Figure 1: Nonmetric multidimensional scaling ordination plot based on the relative abundance of vertebrates observed across five forested sites and all seasons. Distances between sample points represent differences based on Bray-Curtis dissimilarity (e.g., symbols that cluster close to one another exhibited similar levels of vertebrate abundance; Bray and Curtis 1957). Triangles represent fall, circles: winter, squares: spring, diamonds: summer. Black symbols represent spring and summer seasons, and white symbols represent fall and winter seasons. Sites are as follows: M-SH, M-MP, and M-IP (for sand hill, mature pine, and immature pine sites near Metter, GA), and S-GS and S-RH (for Georgia Southern University and residential housing sites in Statesboro, GA).

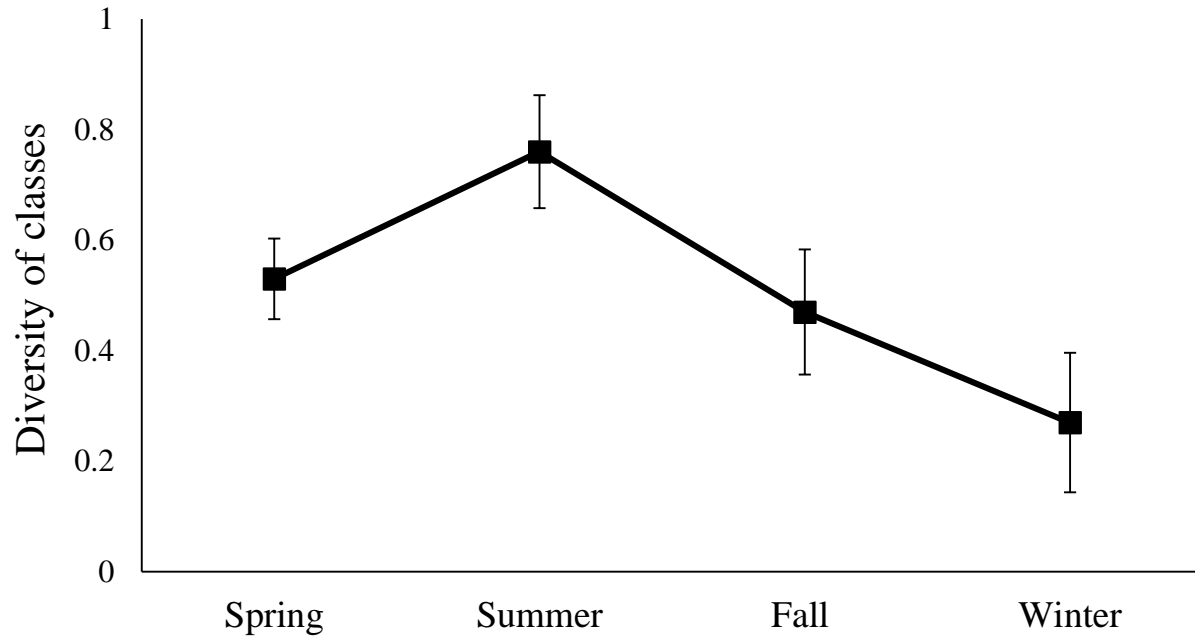


Figure 2: Effect of season on Shannon's index of diversity (H) of classes in forests in southeastern Georgia, U.S. where H of classes was significantly greater in the summer than in the fall or winter. Shared letters above points represent non-significant ( $p > 0.05$ ) differences between seasons. Symbols represent mean  $\pm$  1 SE.

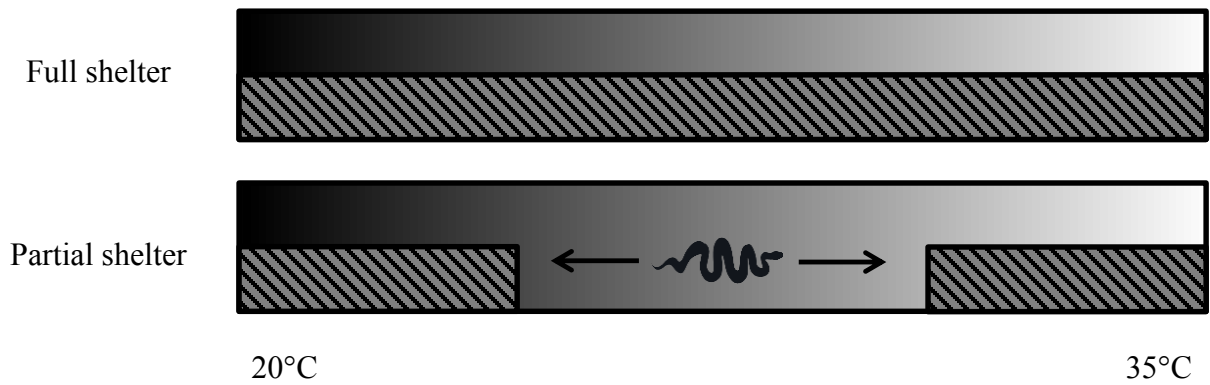


Figure 3: Diagram of shelter treatments in thermal gradient. Open area represents exposed space and diagonally patterned area represents sheltered space. Each snake underwent trials with both shelter types (full and partial) at two immune states (challenged and control). See text for details. A sample snake is shown to demonstrate how each snake had the option to move freely in any direction based on its temperature or shelter preference.



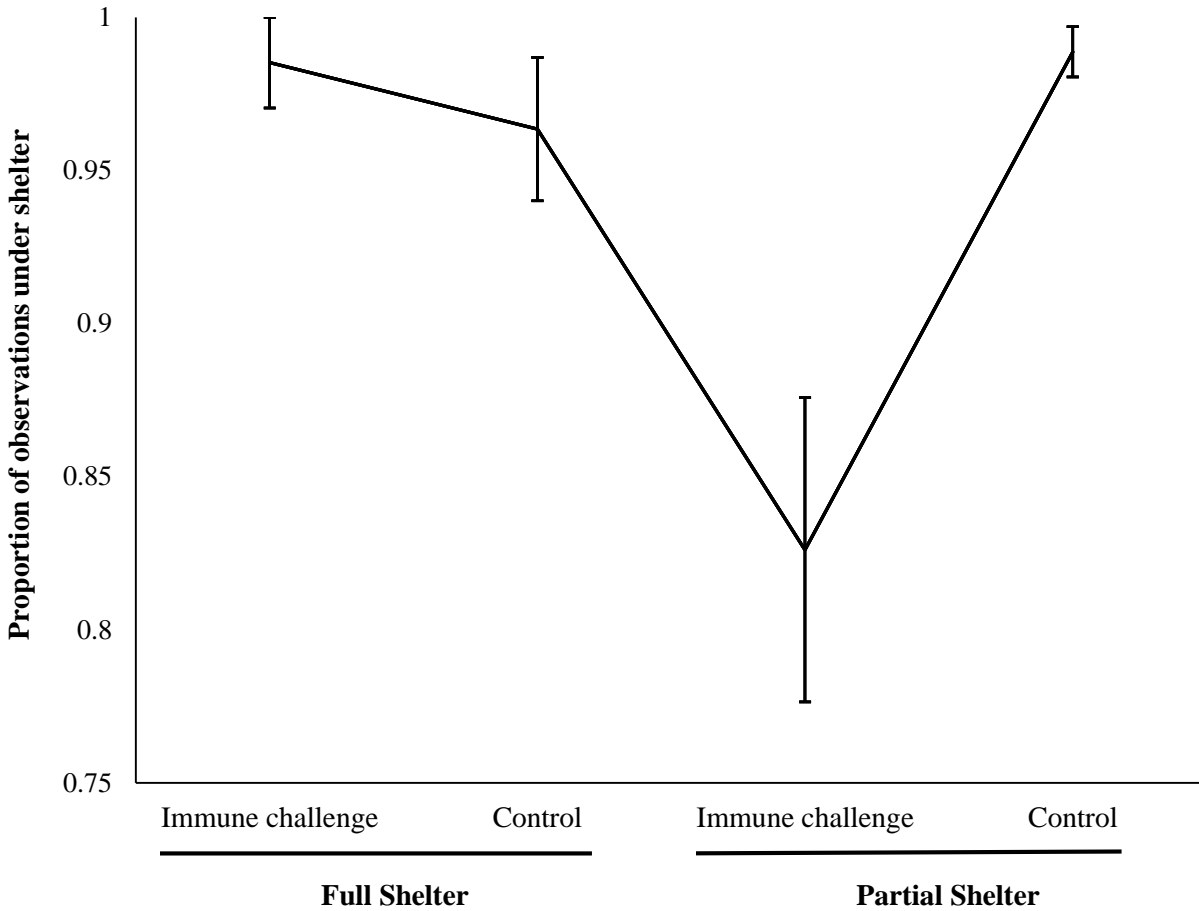


Figure 4: Effects of shelter treatment (full or partial) and immune status (challenged or control) on the proportion of observations of *P. guttatus* ( $n=23$ ) under refuge. Each snake underwent all combinations of trials in randomized order. See text for details about shelter and immune status treatments. Symbols represent  $\text{mean} \pm 1$  SE.

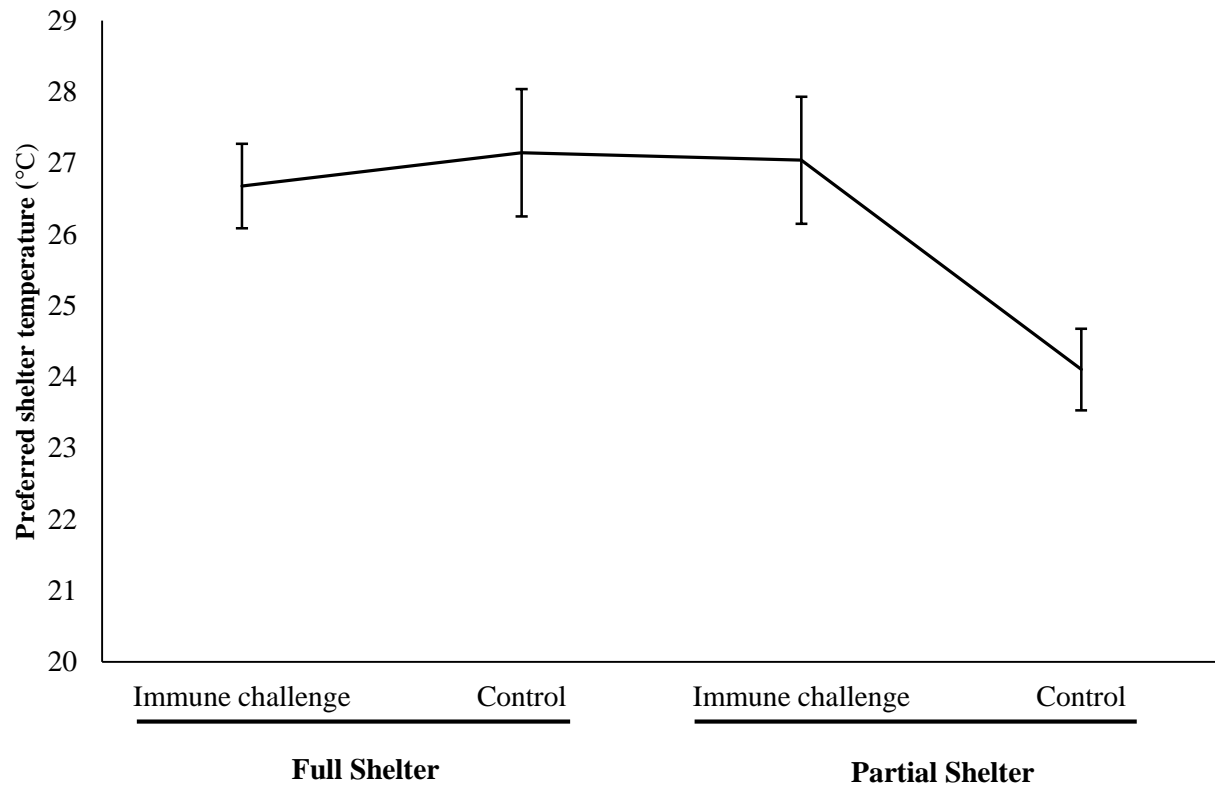


Figure 5: Effects of shelter treatment (full or partial) and immune status (challenged or control) on mean temperature selected by *P. guttatus* ( $n=23$ ). Each snake underwent all combinations of trials in randomized order. See text for details about shelter and immune state treatments.

Symbols represent mean $\pm$ 1 SE.

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