A Simulated Heat Wave Has Diverse Effects on Immune Function and Oxidative Physiology in the Corn Snake (*Pantherophis guttatus*)

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ABSTRACT

Animals will continue to encounter increasingly warm environments, including more frequent and intense heat waves. Yet the physiological consequences of heat waves remain equivocal, potentially because of variation in adaptive plasticity (reversible acclimation) and/or aspects of experimental design. Thus, we measured a suite of physiological variables in the corn snake (*Pantherophis guttatus*) after exposure to field-parameterized, fluctuating temperature regimes (moderate temperature and heat wave treatments) to address two hypotheses: (1) a heat wave causes physiological stress, and (2) thermal performance of immune function exhibits adaptive plasticity in response to a heat wave. We found little support for our first hypothesis because a simulated heat wave had a negative effect on body mass, but it also reduced oxidative damage and did not affect peak performance of three immune metrics. Likewise, we found only partial support for our second hypothesis. After exposure to a simulated heat wave, *P. guttatus* exhibited greater performance breadth and reduced temperature specialization (the standardized difference between peak performance and performance breadth) for only one of three immune metrics and did so in a sex-dependent manner. Further, a simulated heat wave did not elicit greater performance of any immune metric at higher temperatures. Yet a heat wave likely reduced innate immune function in *P. guttatus* because each metric of innate immune performance in this species (as in most vertebrates) was lower at elevated temperatures. Together with previous research, our study indicates that a heat wave may have complex, modest, and even positive physiological effects in some taxa.

Keywords: acclimation, antioxidant, climate change, hemagglutination-hemolysis, reptile.

Introduction

Animals will continue to encounter the increasingly warm environments (i.e., those with elevated mean temperatures) that have already resulted in shifts in species' geographic ranges, activity patterns, and abundances (IPCC 2014). These increasing environmental temperatures are also associated with increased temperature variance, and heat waves (acute, high-intensity elevations in temperature) are expected to become more frequent and intense (IPCC 2014). Such temperature variability may pose a greater risk to species and biodiversity than gradual warming (Vasseur et al. 2014). While it is clear that temperature, in general, influences a range of biological processes (e.g., enzymatic efficiency, energy use, behavior, and reproduction; reviewed in Angiletta 2009), the physiological effects of heat waves—which are short-term, fluctuating increases in temperature—are less understood. Nonetheless, the negative impacts of heat waves have been shown to span several levels of biological organization—from the molecule (Quinn et al. 2011; Madeira et al. 2016*b*) to the organism (Dittmar et al. 2014; Fischer et al. 2014; Madeira et al. 2016*a*) to the population (Parmesan 2006; Smale and Wernberg 2013) to the community (McKechnie and Wolf 2010; Sentis et al. 2013; Ma et al. 2015; Seifert et al. 2015). Thus, examining the stressful effects of acute, extreme warming events continues to enrich our understanding of thermal biology in our rapidly changing world.

Yet although many studies demonstrate detrimental effects of heat waves on animals, others offer conflicting results. For example, a greater number of extremely hot days was related to increased hatching success—but decreased fledgling body size—in passerine birds (Pipoly et al. 2013). Such discrepancies may be rooted in potentially confounding factors (e.g., temperature regime and access to food) that may be strongly associated with biological patterns. Controlled, laboratory-based studies demonstrate that food availability (which is also influenced by climatic variation; Altermatt 2010; Pearce-Higgins et al. 2010; Cahill et al. 2013) can strongly interact with the effects of warmer temperatures on animals. For example, in field crickets, simulated heat waves had a positive effect on egg production when food was available, but negative effects

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on both egg production and survival when food was limited (Adamo et al. 2012). Further, the specific dynamics of temperature manipulation may obscure important biological phenomena. To examine the effects of simulated heat waves, researchers typically measure animal traits after exposure to different but constant temperatures (Adamo et al. 2012; Dittmar et al. 2014; Fischer et al. 2014; Seifert et al. 2015). However, an animal's performance in a constant environment is not always the same as its performance in a fluctuating, more ecologically relevant environment with the same mean temperature (reviewed in Angilletta 2009; Niehaus et al. 2012; Kingsolver et al. 2015; but see Michel and Bonnet 2010). In support, constant-temperature heat waves induced strong negative effects on butterfly development (Fischer et al. 2014), while fluctuating-temperature heat waves did not (Klockmann et al. 2016). Thus, food availability and temperature variability should be carefully considered because they can influence the observed effects of heat waves on animals.

Animals mediate the effects of temperature shifts—including heat waves—through a suite of physiological responses (e.g., increasing antioxidant defenses to offset potential oxidative damage; Madeira et al. 2016*b*). Therefore, examining shifts in animals' thermal performance curves (TPCs; nonlinear relationships between temperature and given performance traits; fig. 1*a*) due to a heat wave may elucidate animals' propensity for adaptive plasticity or reversible acclimation in response to heat waves. Though traditionally explored in the context of locomotor performance (reviewed in Angilletta 2009), the TPC has also been used to examine the thermal sensitivity of other important physiological traits (e.g., Gangloff et al. 2016). Both invertebrates and vertebrates rely on innate immunity more than adaptive immunity (Sandmeier and Tracy 2014; Pap et al. 2015), and TPCs of innate immune function have been identified across every vertebrate class (Butler et al. 2013).

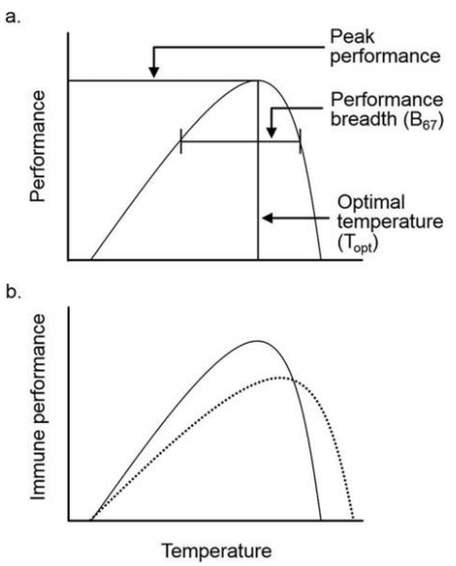

Figure 1. Thermal performance curves. *a*, General nonlinear relationship between temperature and performance (e.g., sprint speed or immune performance). Peak performance occurs at a specific optimal temperature (T_{opt}). In this example, performance breadth (B_{67}) is the range of temperatures over which performance is two-thirds (67%) of peak performance. *b*, Hypothetical thermal performance of immune function after moderate temperature treatment (solid line) and simulated heat wave treatment (dotted line), wherein a heat wave was predicted to reduce peak performance, increase T_{opt}, and increase B_{67}.

Although snakes from temperate regions exhibit variation in metrics of oxidative stress and immune function because of a range of variables (e.g., reproduction, feeding, restraint stress, and sex; Stahlschmidt et al. 2013; Neuman-Lee et al. 2015; Butler et al. 2016; Luoma et al. 2016), the lability of TPCs for these physiological metrics is unknown. Also, temperate snakes may actually experience population- and individual-level benefits from warming (Sperry et al. 2010; Weatherhead et al. 2012; Huang et al. 2013; Stahlschmidt et al. 2015*b*). Further, snakes from temperate regions typically encounter significant intra- and interannual variation in temperature, and theory predicts that animals in variable thermal environments should acclimate more readily than those in more stable thermal environments (Angilletta 2009). Thus, temperate snakes may yield particular insight into heat wave–induced physiological plasticity.

To better understand how animals respond to a heat wave, we used a repeated-measures experimental design in corn snakes (*Pantherophis guttatus*) that both controlled for food intake and used fluctuating temperature regimes to address two hypotheses. First, we hypothesized that a heat wave causes physiological stress. From this hypothesis, we predicted that a simulated heat wave would result in (1) an increased loss of body mass, (2) increased oxidative damage with no commensurate increase in antioxidant defenses, and (3) reduced immune function (fig. 1*b*). Second, we hypothesized that thermal performance of immune function exhibits adaptive plasticity in response to a heat wave (sensu the theory of optimal acclimation; reviewed in Angilletta 2009). From this hypothesis, we predicted that a simulated heat wave would result in (1) better immune performance at higher temperatures (e.g., higher optimal temperature [T_{opt}]) and (2) reduced temperature specialization (e.g., higher performance breadth [B_{67}] and/or lower peak performance; fig. 1*b*). The potential interactions among oxidative damage, antioxidant defenses, and immune performance as a function of exposure to a heat wave will elucidate how animals cope with an increasingly relevant aspect of their environment.

Material and Methods

Study Species and Husbandry

Pantherophis guttatus are nonvenomous, medium-sized colubrid snakes that are native to the southeastern United States (Gibbons and Dorcas 2005). A captive sample of 24 *P. guttatus* (aged 22–23 mo) were used to address our hypotheses. Snakes were the offspring (first to third generation) of wild-caught individuals from Beaufort County, South Carolina. Snake hus-

bandry has been described in detail previously (Stahlschmidt et al. 2015*b*). Briefly, snakes were kept individually in translucent plastic enclosures (27 cm × 41 cm × 15 cm) in a room with a 12L : 12D cycle before and between temperature treatments. Enclosures had subsurface heating elements that allowed snakes to thermoregulate along a gradient of temperatures from 24.5° to 33°C. This thermal gradient encompasses the preferred temperature range for *P. guttatus* (26°–29°C; Roark and Dorcas 2000; Stahlschmidt et al. 2015*b*). Though raised in captivity under relaxed predation pressure, study snakes retain a strong preference for shelter (e.g., they prefer shelter over nonshelter >95% of the time; Todd et al. 2016). Thus, to mimic a cool refuge similar to those found in a natural environment (e.g., a subterranean burrow), a folded newspaper was placed at the cool end of each enclosure. Snakes had ad lib. access to water throughout the study (May–July 2015). All snakes were well fed and in good body condition as they were offered food (frozen/thawed adult mice weighing 15%–20% of snake body mass) every 2 wk between temperature treatments (described below). All procedures were approved by the Institutional Animal Care and Use Committee at Georgia Southern University (protocol I14004).

Experimental Design

Because digestive state influences immune function, oxidative damage, and antioxidant defenses in *P. guttatus* (Butler et al. 2016; Luoma et al. 2016), all snakes were fed 5 d before temperature treatments to ensure that all individuals were postabsorptive (Crocker-Buta and Secor 2014). Immediately before temperature treatments, snakes were weighed and then returned to their individual enclosures. Next, they were transferred to an incubator (model I-36; Percival Scientific, Perry, IA) with a 12L : 12D cycle and one of the following temperature regimes: moderate temperature (diel cycle: 19.5°–29.1°C, which is the average daily temperature range in Beaufort during the snakes' active season [April–September]; National Weather Service) and heat wave (diel cycle: 25.6°–35°C, which was the average daily temperature range during a heat wave in July 2010 in Beaufort; National Weather Service). In the heat wave treatment, snakes were forced to experience temperatures above their preferred temperature range (26°–29°C: Roark and Dorcas 2000; Stahlschmidt et al. 2016) more than 60% of the time. Each snake experienced both treatments in random order (i.e., half of the snakes first experienced the moderate treatment), and treatments were separated by 4 wk. After 7 d of treatment, snakes were reweighed, and 0.3 mL of intracardiac blood samples were taken between 0900 and 1030 hours. Samples were placed on ice before centrifugation at 2,350 *g* for 5 min. Plasma was removed, and subsampled into aliquots of 20–60 μL that were stored at −80°C before assays. Because of variation in the volume of each initial plasma sample, there was not enough plasma to perform all of the assays on each snake's blood sample. All procedures were approved by the Institutional Animal Care and Use Committee at Georgia Southern University (protocol I14004).

Assays of Oxidative Damage and Antioxidant Defenses

The amount of hydroperoxides—which are pro-oxidant intermediates of lipid peroxidation that lead to the production of oxidative damage—was quantified with the d-ROMs test (MC002, Diacron International). This test reliably detects ecologically relevant levels of reactive oxygen metabolites (ROMs), such as hydroperoxides, using Fenton's reaction (reviewed in Costantini 2016). The directions of the kit were amended for smaller sample volumes sensu Costantini et al. (2011). In brief, 4 μL of the plasma sample was mixed with 200 μL of kit regent, which was then incubated at 37°C for 75 min. Next, the absorbance was read at 505 nm using a Tecan (Switzerland) Infinite M200Pro plate reader. The amount of ROMs relative to control blanks and the kit's calibrator was calculated, and values are reported as mM H_2O_2 equivalents, with larger numbers corresponding to a greater amount of ROMs. The nonenzymatic antioxidant capacity of the plasma was quantified using the OXY-adsorbent test (MC435, Diacron International), which measures the plasma's ability to oppose the pro-oxidizing action of hypochlorous acid. Also following Costantini et al. (2011), the kit's instructions were amended by combining 5 μL of a 1 : 100 dilution of plasma with 200 μL of the hypochlorous acid–based oxidizing solution and then incubating the plate for 10 min at 37°C. Next, 5 μL of the chromagen solution was added, and absorbance was read at 505 nm using a Tecan Infinite M200Pro plate reader. The antioxidant capacity relative to control blanks and the kit calibrator was calculated, and values are reported in mM HClO neutralized, with larger values corresponding to a more robust antioxidant barrier. A metric reflecting the balance between oxidative damage and antioxidant defenses (oxidative index; Vassalle et al. 2008) was determined as the difference between the standardized values of each individual's d-ROMs and OXY scores.

Assays of Innate Immunity

Three assays of innate immunity—which is the first line of defense against foreign microbes in the body (Matson et al. 2005)—were performed. Hemagglutination is mediated by natural antibodies (NAbs; polyreactive immunoglobulins), which can opsonize foreign microbes. Because of their structure, NAbs can also promote agglutination and initiate the complement enzyme cascade (Matson et al. 2005). Complement activation can reduce the integrity of cellular membranes resulting in lysis of foreign erythrocytes (Trouw and Daha 2011). Additionally, the killing of foreign bacteria requires an integrated action of natural antibodies, circulating leukocytes (in whole blood), antimicrobial peptides, and the complement system for final removal of bacterial pathogens (Demas et al. 2011; French and Neuman-Lee 2012).

Plasma from each snake at both temperature treatments underwent hemagglutination, hemolysis, and bacteria-killing (BK) assays at five incubation temperatures (5°, 15°, 25°, 35°, and 45°C) using previously described methods with a bacterial concentration of 1×10^4 colony forming units and a plasma

dilution of 0.27 (French and Neuman-Lee 2012; Butler et al. 2013). The interassay coefficient of variation for BK assays was 0.0594. Hemagglutination and hemolysis assays were scored blind to treatment independently by A. Ahn and Z. R. Stahlschmidt. Because scores were highly correlated (Pearson correlation: $R = 0.8$, $P < 0.001$), the mean value of the scores was used to perform statistical analyses on hemagglutination and hemolysis titers (see "Statistical Analyses").

Thermal Performance Curves (TPCs)

As in the study by Butler et al. (2013), several methods were used to characterize thermal performance of immune function and its plasticity in response to several factors (e.g., sex and temperature treatment). First, linear mixed models were used to determine the factors influencing variation in innate immune function—specifically, whether hemagglutination, hemolysis, and BK were influenced by the incubation temperatures of assays. For these models, incubation temperature, temperature treatment, sex, and a treatment × sex interaction were included as fixed effects. The dependent variables were hemagglutination titers, hemolysis titers, and BK values.

Second, an open-source graphing software (CurveExpert Basic, ver. 1.4) was used to determine the polynomial or Gaussian function that best fit each individual's data set. These best-fit functions were used to determine three metrics for hemagglutination and hemolysis from each snake for both treatments: peak performance (highest level of titer or BK capacity), optimal temperature (T_{opt}), and 67% performance breadth (B_{67}; range of temperature over which performance is ≥67% of peak performance). The data from some snakes were not amenable to nonlinear function fitting (e.g., strong linear relationship between incubation temperature and hemolysis titer). In these cases, the peak performance and T_{opt} values were determined from the raw data, with mean values taken in the event of ties (e.g., if titer peaked at both 5° and 15°C, T_{opt} would be determined at 10°C). Peak performance, T_{opt}, and B_{67} for hemagglutination, hemolysis, and BK were used in subsequent statistical analyses. To examine variation in the specialist-generalist trade-off, the difference between the standardized values of each individual's peak performance and B_{67} score was determined (specialization index sensu the oxidative index; Vassalle et al. 2008): a high specialization index represented a plasma sample that exhibited relatively high temperature specialization.

Third, principal component (PC) analyses were used to generate PC scores for each snake's TPC for hemagglutination, hemolysis, and BK after both temperature treatments. Using hemagglutination scores across all five temperatures for all individuals after both treatments, the eigenvalues were 1.8 for PC1 and 1.4 for PC2, explaining 36% and 27% of the variation, respectively. For hemolysis, the eigenvalues were 2.0 for PC1 and 1.0 for PC2, explaining 41% and 21% of the variation, respectively. For BK, the eigenvalue was 3.1 for PC1, explaining 62% of the variation. For all three immune metrics, PC1 loaded positively with the overall magnitude of the response (i.e., higher PC1 corresponded to higher immune performance across incubation temperatures). PC2 loaded positively on lower temperatures and negatively on higher temperatures for hemagglutination (i.e., higher PC2 corresponded to relatively better hemagglutination at lower temperatures), and it loaded in the opposite direction for hemolysis. Thus, PC2 for hemagglutination reflected cool-biased performance, while PC2 for hemolysis reflected warm-biased performance, with particularly high loading at the temperature associated with heat wave (35°C; fig. A1). These PCs were used in subsequent statistical analyses.

Statistical Analyses

Several linear mixed models were performed in SPSS (ver. 22; IBM, Armonk, NY), data were log transformed when necessary, and two-tailed significance was determined at $\alpha < 0.05$. Models were used to determine the factors influencing variation in metrics of d-ROMs, OXY, oxidative index, TPCs (peak performance, T_{opt}, B_{67}, specialization index, and PCs for hemagglutination, hemolysis, and BK), and posttreatment body mass. For these models, temperature treatment, sex, and a treatment × sex interaction were included as fixed effects. Initial body mass was included as a covariate in the model examining posttreatment body mass. Animal identification was included as a random effect in all mixed models.

To determine relationships between traits, Pearson correlation analyses were performed on parametric data, and Spearman's rank-order correlation analyses were performed on nonparametric (nonnormally distributed) data. Correlation analyses were performed on hemagglutination titers, hemolysis titers, and BK values to determine relationships among metrics of immune function. To determine relationships among oxidative biomarkers and metrics of TPCs of immunity, correlation analyses were used to compare d-ROMs, OXY, and oxidative index with PCs of hemagglutination, hemolysis, and BK. To assess the generalist-specialist trade-off of thermal performance, correlation analyses were also used to examine relationships between peak performance and B_{67} for hemagglutination, hemolysis, and BK.

Results

Posttreatment body mass was significantly predicted by initial (pretreatment) body mass ($F_{1, 34} = 2{,}020$, $P < 0.001$) and temperature treatment ($F_{1, 21} = 14$, $P = 0.001$), where snakes lost more body mass during heat wave treatment relative to moderate temperature treatment (3.5% ± 0.6% vs. 1.4% ± 0.5% of initial body mass, respectively). However, posttreatment body mass was not influenced by sex or a treatment × sex interaction (both $P > 0.35$).

Levels of d-ROMs were affected by temperature treatment ($F_{1, 19} = 6.7$, $P = 0.018$), where d-ROMs were higher after moderate temperature treatment relative to heat wave treatment (fig. 2). Yet there was no statistically significant effect of temperature treatment on OXY (fig 2; $F_{1, 23} = 0.41$, $P = 0.53$). Levels of d-ROMs and OXY were not affected by sex or a treatment × sex interaction (both $P > 0.47$; fig. 2). The oxida-

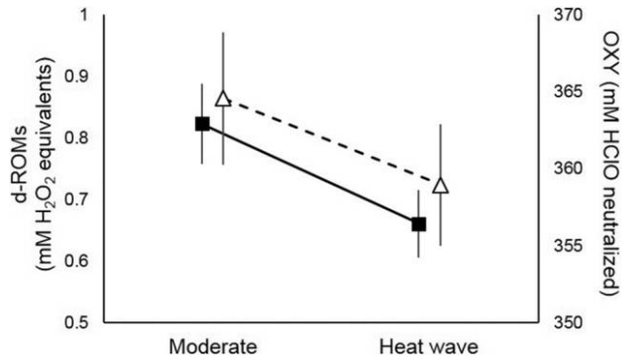

Figure 2. Effects of temperature treatment (moderate or heat wave) on plasma levels of oxidative damage (d-ROMs; squares) and antioxidant defenses (OXY; triangles) in *Pantherophis guttatus*. Temperature treatment affected levels of oxidative damage but not OXY or the oxidative index (a metric reflecting the balance between oxidative damage and antioxidant defense; not shown).

tive index was not significantly affected by temperature treatment, sex, or a treatment × sex interaction (all $P > 0.33$).

Hemagglutination titers, hemolysis titers, and BK values were all positively correlated with one another (all $R > 0.43$, $P < 0.001$; fig. 3*a*). Levels of OXY and d-ROMs were not correlated with each other or most of the PCs of hemagglutination, hemolysis, and BK (all $R < 0.32$, $P > 0.09$), but they were both positively correlated with overall hemagglutination performance (PC1; OXY: $R = 0.38$, $P = 0.011$; d-ROMs: $R = 0.36$, $P = 0.017$; fig. 3*b*). Oxidative index was not correlated with any of the PCs of hemagglutination, hemolysis, and BK ($R < 0.27$, $P > 0.14$)

Hemagglutination titers, hemolysis titers, and BK values were influenced by the temperature at which assays were performed (fig. 4; hemagglutination: $F_{4,194} = 23$, $P < 0.001$; hemolysis: $F_{4,194} = 45$, $P < 0.001$; BK: $F_{4,135} = 13$, $P < 0.001$) but not by temperature treatment, sex, or a treatment × sex interaction (all $F < 2.3$, $P > 0.06$). Peak performance and B_{67} were negatively correlated for hemagglutination ($R = -0.38$, $P = 0.039$) and BK ($R = -0.42$, $P = 0.035$) but not for hemolysis ($R = 0.29$, $P = 0.17$).

Peak performance for hemagglutination was influenced by a treatment × sex interaction ($F_{1,22} = 5.4$, $P = 0.029$), where moderate temperature females exhibited higher peak performance than moderate temperature males, but heat wave males exhibited higher peak performance than heat wave females. Males also exhibited greater B_{67} for hemagglutination than females ($F_{1,26} = 6.6$, $P = 0.017$). The T_{opt} for BK was influenced by temperature treatment ($F_{1,16} = 7.8$, $P = 0.013$), where T_{opt} was lower after snakes experienced the heat wave treatment. Performance breadth (B_{67}) for BK was affected by treatment ($F_{1,13} = 13$, $P = 0.014$), where immune performance was less sensitive to assay temperature after the heat wave treatment. Temperature specialization (specialization index) for hemagglutination was influenced by sex ($F_{1,14} = 9.5$, $P = 0.008$), where females exhibited greater temperature specialization than males. The specialization index for BK was influenced by treatment ($F_{1,10} = 6.8$, $P = 0.026$) and a treatment × sex interaction ($F_{1,10} = 5.2$, $P = 0.046$), where temperature specialization was lower after the heat wave treatment, particularly for males. Temperature-biased hemolysis performance (PC2) was influenced by treatment ($F_{1,22} = 4.8$, $P = 0.040$), sex ($F_{1,22} = 4.4$, $P = 0.047$), and a treatment × sex interaction ($F_{1,22} = 6.2$, $P = 0.021$), where results were driven by males exhibiting a bias in hemolysis performance toward cooler and non–heat wave temperatures after the heat wave treatment. All other TPC metrics for hemagglutination, hemolysis, and BK were not significantly affected by temperature treatment, sex, or a treatment × sex interaction (all $P > 0.08$).

Discussion

This study demonstrated that a simulated heat wave had diverse, seemingly opposing physiological effects, resulting in

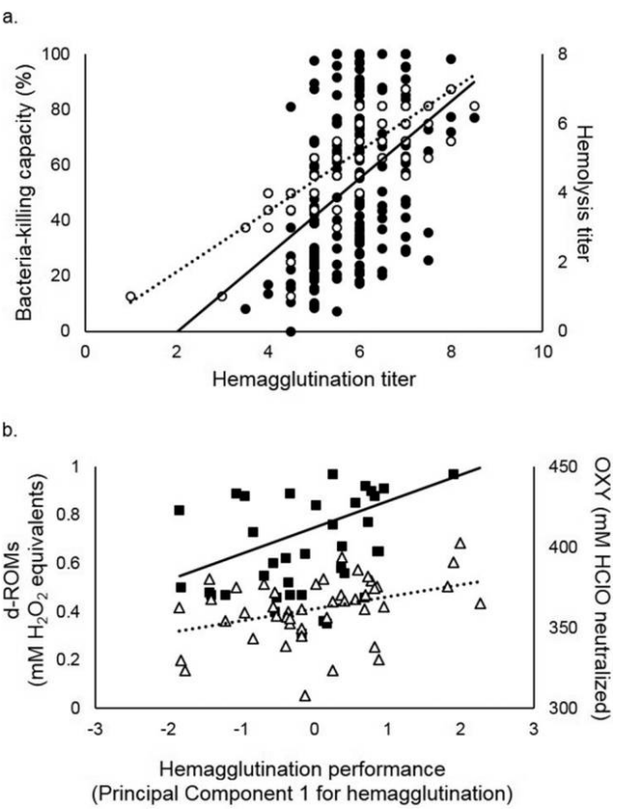

Figure 3. Relationships among metrics of immune function and oxidative status in *Pantherophis guttatus*. *a*, Hemagglutination titers were positively correlated with bacteria-killing capacity (filled symbols, solid line) and hemolysis titers (open symbols, dotted line). All three metrics of immune function were positively correlated. *b*, Overall hemagglutination performance (principal component 1 for hemagglutination) was positively correlated with levels of oxidative damage (d-ROMs; squares, solid line) and antioxidant defenses (OXY; triangles, dotted line).

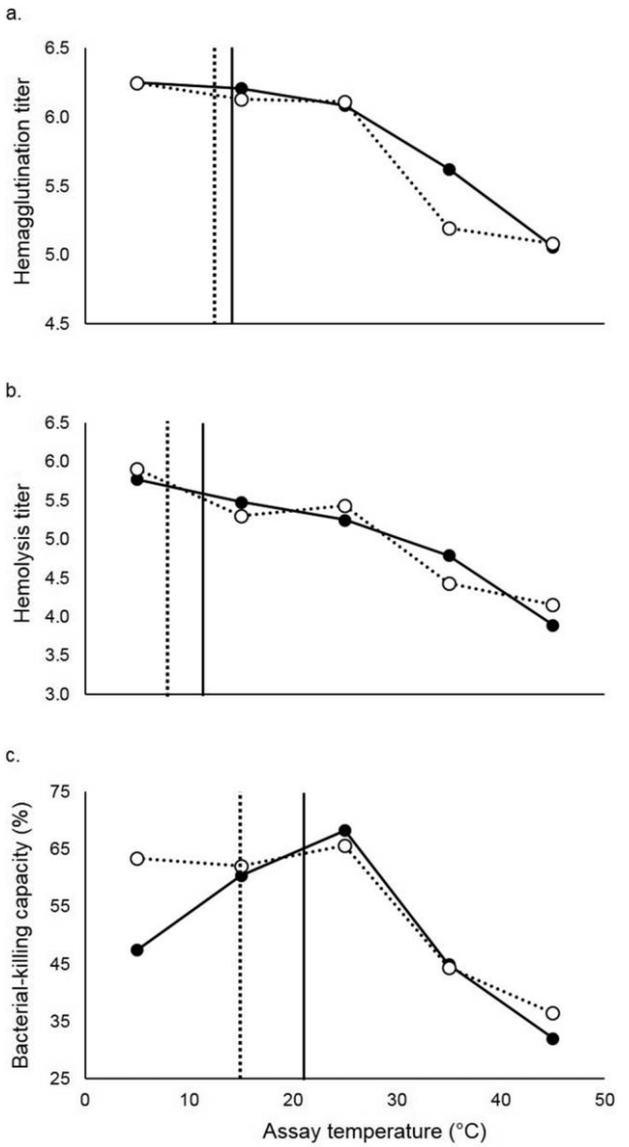

Figure 4. Effects of assay temperature on hemagglutination (*a*), hemolysis (*b*), and bacteria-killing (BK) capacity (*c*) in *Pantherophis guttatus* after moderate temperature treatment (filled symbols, solid lines) and a simulated heat wave treatment (open symbols, dotted lines). Vertical lines denote the temperatures at which performance was optimized (T_{opt}) for each immune metric. For BK capacity, T_{opt} was affected by temperature treatment. For clarity, error bars (which largely overlapped) were omitted, and data from both sexes were pooled. Sex-based differences in individual-level thermal performance are described in "Results."

a negative effect on body mass, a positive effect on oxidative damage (i.e., reduced d-ROMs: fig. 2), no effect on peak performance of three immune metrics (fig. 4), and a cool-biased shift in immune function in *Pantherophis guttatus*. Thus, our first hypothesis (a heat wave causes physiological stress) was largely unsupported, whereby heat waves resulted in a greater loss in body mass, but neither immune function nor oxidative stress was negatively impacted. Likewise, we found only partial support for our second hypothesis (thermal performance of immune function exhibits adaptive plasticity in response to a heat wave). After exposure to a simulated heat wave, *P. guttatus* did exhibit greater performance breadth and reduced temperature specialization for BK but not for hemagglutination or hemolysis capacity. Further, a heat wave did not elicit greater performance of any immune metric at higher temperatures—in fact, hemolysis and BK were greater at lower temperatures even after heat wave treatment (fig. 4).

Some results from our study contribute to the growing list of physiological costs associated with heat waves recently demonstrated by others (e.g., Quinn et al. 2011; Dittmar et al. 2014; Fischer et al. 2014; Madeira et al. 2016*a*, 2016*b*). After a simulated heat wave, nonabsorptive *P. guttatus* in our study lost more body mass after heat wave temperature treatment. Thus, heat wave treatment putatively resulted in increased energy expenditure. However, the relative amount of body mass lost as a result of simulated heat wave (3.5%) was modest and could easily be offset by feeding; *P. guttatus* can eat meals between 5% and 45% of their body mass (Crocker-Buta and Secor 2014). Like most vertebrate taxa, *P. guttatus* exhibited T_{opt} for innate immunity well below its preferred or actual body temperature (fig. 4; Butler et al. 2013; Dittmar et al. 2014). Thus, as temperatures increase as a result of heat waves and climate change, in general, innate immune function is expected to become less effective, particularly given that thermal performance of immune function was minimally plastic. Further, other physiological variables not measured in our study may have been affected by simulated heat waves. For example, environmental temperatures typically influence levels of stress hormones (glucocorticoids) across vertebrate taxa (Jessop et al. 2016), and suboptimal temperatures can elicit elevations in levels of glucocorticoids in some reptiles (Dupoué et al. 2013; Telemeco and Addis 2014; but see Sykes and Klukowski 2009; Li et al. 2011). Further, because of the specificity of the OXY and d-ROMs assays, it is possible that other aspects of oxidative physiology (e.g., enzymatic antioxidant expression or markers of protein oxidation) could have varied in ways we were unable to detect, because many aspects of oxidative physiology are uncorrelated with each other (Christensen et al. 2015).

Yet many of our results indicate that an ecologically relevant simulation of a heat wave did not entail physiological costs in *P. guttatus*. For example, antioxidant defenses were unaffected by simulated heat wave treatment, although high interindividual variation in this metric may obscure a biological pattern (fig. 2; power $[1 - \beta] = 0.23$), and oxidative damage was actually lower after simulated heat wave treatment (fig. 2). Prior work with reptiles has demonstrated that hotter temperatures do not inherently result in oxidative damage (Treidel et al. 2016), and prolonged exposure to warmer temperatures in fish can actually decrease oxidative damage (Enzor and Place 2014). Recent evaluation of the dynamics at the mitochondrial membrane indicates that increased metabolic rate (e.g., due to increased temperature) may actually reduce—rather than increase—the production of reactive oxygen species associated with oxidative damage (Speakman and Garratt 2014; Salin et al. 2015). Thus, thermal conditions that are not acutely stressful (e.g., the

gradual warming expected under climate change scenarios) may benefit ectotherms' oxidative states because of small increases in metabolic rate. Such putatively beneficial consequences of small-scale stressors are receiving renewed attention using a hormetic conceptual framework. Indeed, multiple lines of evidence support hormesis—which is the nonlinear relationship between stressors and fitness—with regard to oxidative stress (Costantini 2014). Frequently, hormetic patterns emerge in response to repeated exposure to stressors (including heat stress), and mild heat stress during development has been linked to decreased oxidative stress later in life (sensu developmental plasticity; Costantini et al. 2012). However, even the first exposure to a mild stressor may provide benefits under a hormetic framework (Costantini et al. 2010). While further work using multiple magnitudes of heat waves would be necessary to evaluate whether there is a nonlinear relationship between temperature treatment and oxidative physiology, the results presented here suggest that such a pattern is possible.

In terms of effects on immune function, our results indicate no effect of heat wave on overall immune performance (e.g., peak performance or PC1 for hemolysis, hemagglutination, or BK), which is in contrast to work in the three-spined stickleback (*Gasterosteus aculeatus*; Dittmar et al. 2014). This discrepancy may be due to taxonomy or to variation in temperature treatments. In addition to distant phylogenetic relatedness, *G. aculeatus* can be found across microenvironments with a narrower temperature range than *P. guttatus* (16°C vs. ≥23°C, respectively: Allen and Wootton 1982; Z. R. Stahlschmidt, unpublished data). Thus, *P. guttatus* may be more adapted to and less physiologically sensitive to temperature shifts than *G. aculeatus*. Further, the simulated heat wave in the Dittmar et al. (2014) study (which did not characterize TPCs) was nonfluctuating, longer (2 wk vs. 1 wk), and more intense (magnitude of mean temperature shift: 10°C vs. 6°C) and occurred in a medium characterized by a much higher conduction constant than our study (water vs. air, respectively). Clearly, the physiological responses to heat waves can be subtle and complex. Therefore, future heat wave studies should be carefully designed to incorporate ecologically relevant temperature regimes and to measure a suite of important variables (e.g., Madeira et al. [2016*a*] measured the effect of heat waves on mortality, eight stress biomarkers, and tissue histopathology).

Consistent with many immunological studies, we found sex differences in immunity as well as sex-dependent effects of temperature treatment on the immune system (Klein and Nelson 1997, 1998; Rolff 2002; Zuk et al. 2004). Females display greater immunocompetence in many taxa (Klein and Nelson 1997; Zuk et al. 2004), and we likewise found that moderate temperature females in this study exhibited a greater peak agglutination performance than males. Yet the opposite relationship was true for *P. guttatus* exposed to heat waves, with male snakes showing a greater peak agglutination performance than females, thus suggesting that females are more susceptible than males to temperature shifts. Related, we also found that females displayed a greater temperature specialization for agglutination than males. While the mechanisms in this study are not clear, past work has attributed apparent sex differences in immunity and pathogen resistance to the suppressive effects of high androgen levels in males, but these effects are also likely mediated via the expression of resistance genes (Klein 2000) or sex-dependent differences in hormone-mediated gene expression (Waxman and Holloway 2009). Furthermore, the idea of sex-dependent effects on thermal biology is not without precedent; in reptiles, the sexes are known to vary in their preferred body temperature, thermoregulation, and locomotion (reviewed in: Lailvaux 2007).

For decades, scientists have demonstrated that the thermal environment in which organisms develop influences several aspects of thermal performance (reviewed in Angilletta 2009). However, the degree to which animals exhibit reversible thermal acclimation—as opposed to developmental plasticity (irreversible acclimation)—is less straightforward. When an animal faces a fluctuating environment over the course of its life, the theory of optimal acclimation predicts that reversible acclimation should be favored (Gabriel 1999; Angilletta 2009). Yet, like many other empirical tests of this theory (reviewed in Angilletta 2009), the vast majority of our results did not support the notion that the thermal performance of immune function was plastic or capable of reversible acclimation in *P. guttatus* (fig. 4). Given the complex, interactive role of temperature in gene networks (e.g., Sikkink et al. 2015), recent work is only beginning to demonstrate the genetic capacity for developmental plasticity related to thermal performance (van Heerwaarden and Sgro 2013; Cooper et al. 2014; but see Kristensen et al. 2015); the genetic constraints on reversible thermal acclimation are even less understood. Developmental plasticity and reversible acclimation related to temperature may be causally linked (Beaman et al. 2016), but future work is required to better understand the mechanisms underlying this link.

We measured how different temperature regimes influenced variables related to immune function and oxidative physiology because these variables can covary. For example, the immune response is associated with oxidative damage in many animal taxa (e.g., insects: Sadd and Siva-Jothy 2006; reptiles: Tobler et al. 2015; birds: Costantini and Møller 2009; mammals: Graham et al. 2010; but see Cram et al. 2015), which has led to growing interest in how animals balance immune resistance (ability to destroy pathogens) with infection tolerance (e.g., ability to mitigate self-damage associated with immune resistance; Schneider and Ayres 2008; Ayres and Schneider 2012; Stahlschmidt et al. 2015*a*). Potentially because antioxidant defenses mitigate oxidative damage associated with immune resistance (Ayres and Schneider 2012; Stahlschmidt et al. 2015*a*), we found positive correlations between metrics of oxidative state (levels of d-ROMs and OXY) and immune resistance (hemagglutination; fig. 3*b*). Similar results have been demonstrated in insects: immune challenge leads to oxidative damage (Sadd and Siva-Jothy 2006), and immune resistance is positively correlated with antioxidant defenses (Stahlschmidt et al. 2015*a*). Thus, high levels of immune resistance may obligate oxidative damage, despite increased antioxidant defenses.

As expected, immune metrics were also correlated with one another. This interrelatedness is likely due to the fact that the killing and removal of foreign *Escherichia coli* bacteria (assessed via BK) is partially dependent on both opsonization via NAbs (assessed by hemagglutination) and hemolysis and removal via the complement system (assessed by hemolysis). Further, hemagglutination and hemolysis can be linked because the complement system is activated by NAbs via the classical pathway (Matson et al. 2005). Despite these statistical correlations, there were still notable differences among immune metrics: (1) levels of OXY and d-ROMs were correlated with hemagglutination performance (PC1) but not with hemolysis or BK performance; (2) there was a peak-breadth trade-off for hemagglutination but not for hemolysis; (3) temperature-biased hemolysis performance (PC2) was influenced by heat wave treatment and a treatment × sex interaction, but PC2 for hemagglutination was not; and (4) T_{opt} and B_{67} for BK were influenced by heat wave treatment but were not for hemagglutination or hemolysis. Previous research has demonstrated that hemagglutination and hemolysis respond differently to several factors, including assay temperature, reproductive status, sex, and digestive state (Butler et al. 2013; Stahlschmidt et al. 2013; Luoma et al. 2016). These differences between hemolysis and hemagglutination may be due to hemolysis activation that is independent of NAbs (reviewed in Luoma et al. 2016). Clearly, studies should continue to measure a suite of immune metrics (Demas et al. 2011; Zylberberg 2015; Vermeulen et al. 2016) and further strive to understand mechanistic links among these metrics. Though it was not possible for our study (e.g., because of limited sample size), incorporating the concept of integration (i.e., the degree to which within-individual biological variables are correlated and interdependent: Pavlicev et al. 2009; Haber 2011; Costantini et al. 2013) into experiments may yield new insight into the dynamics by which heat waves shift (or dysregulate) whole-organism physiology.

By using field-parameterized, fluctuating temperature regimes and measuring a suite of physiological variables, our study clarifies the dynamics by which animals respond to a heat wave. While a simulated heat wave resulted in reduced oxidative damage (fig. 2), it also shifted the body temperature of *P. guttatus* away from its peak immune function, given its relative lack of reversible acclimation in the thermal performance of immune function (fig. 4). Together, our results indicate that heat waves have mixed physiological effects in some taxa. Because certain warming scenarios may actually benefit snakes (Sperry et al. 2010; Weatherhead et al. 2012; Huang et al. 2013; Stahlschmidt et al. 2015*b*; this study), future work should determine the physiological effects of heat waves in other taxa. When examining animals' responses to heat waves, we also encourage the continued examination of other important independent variables (e.g., food availability or reproductive state) and dependent variables (e.g., reproductive output, growth, or survival; Adamo et al. 2012; Madeira et al. 2016*a*, 2016*b*). Finally, field-based efforts should focus on characterizing behavioral responses (e.g., shifts in refuge use or temporal patterns in daily activity patterns) due to a natural heat wave.

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APPENDIX

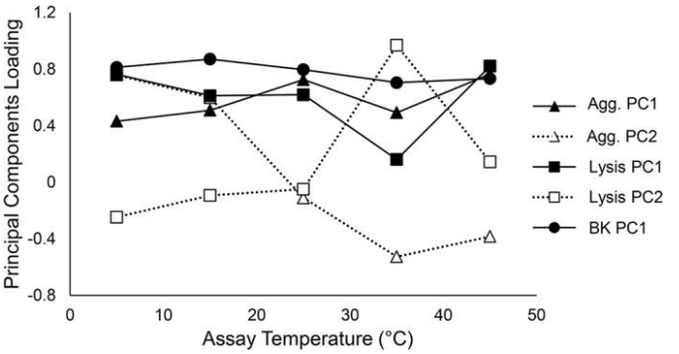

Figure A1. Principal component (PC) loadings for hemagglutination (triangles), hemolysis (squares), and bacteria-killing (BK; circles) capacity. Higher PC1 values (filled symbols, solid lines) for all immune metrics correspond to greater immune responses, regardless of temperature. Higher PC2 scores for hemagglutination (open triangles, dotted line) are associated with individuals that have greater responses at cooler temperatures, while higher PC2 scores for hemolysis (open squares, dotted line) are associated with greater responses at warmer temperatures. For BK, only one PC generated had an eigenvalue >1; thus, only PC1 for BK is displayed.

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