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SYMPOSIUM

Contrasting Responses of Lizards to Divergent Ecological Stressors Across Biological Levels of Organization

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Synopsis It is frequently hypothesized that animals employ a generalized "stress response," largely mediated by glucocorticoid (GC) hormones, such as corticosterone, to combat challenging environmental conditions. Under this hypothesis, diverse stressors are predicted to have concordant effects across biological levels of an organism. We tested the generalized stress response hypothesis in two complementary experiments with juvenile and adult male Eastern fence lizards (*Sceloporus undulatus*). In both experiments, animals were exposed to diverse, ecologically-relevant, acute stressors (high temperature or red imported fire ants, *Solenopsis invicta*) and we examined their responses at three biological levels: behavioral; physiological (endocrine [plasma corticosterone and blood glucose concentrations] and innate immunity [complement and natural antibodies]); and cellular responses (gene expression of a panel of five heat-shock proteins in blood and liver) at 30 or 90 min post stress initiation. In both experiments, we observed large differences in the cellular response to the two stressors, which contrasts the similar behavioral and endocrine responses. In the adult experiment for which we had innate immune data, the stressors affected immune function independently, and they were correlated with CORT in opposing directions. Taken together, these results challenge the concept of a generalized stress response. Rather, the stress response was context specific, especially at the cellular level. Such context-specificity might explain why attempts to link GC hormones with life history and fitness have proved difficult. Our results emphasize the need for indicators at multiple biological levels and whole-organism examinations of stress.

Introduction

The concept of stress is foundational to our understanding of how organisms cope with environmental variation. Generally, conditions that cause stress are assumed to reduce fitness whereas those that minimize stress are assumed optimal (Broom and Johnson 1993; Bradshaw 2003; Bonier et al. 2009; Busch and Hayward 2009). Researchers endeavor to quantify stress to indicate how organisms, both in natural and laboratory settings, perceive environments (Levine 2005; Busch and Hayward 2009; Koolhaas et al. 2011; Wingfield and Boonstra 2013). "Stress" is difficult to define and measure, as previously discussed at length (Selye 1976; Broom and Johnson 1993; Bradshaw 2003; McEwen and Wingfield 2003; Levine 2005; Romero et al. 2009; Koolhaas et al. 2011; Del Giudice et al. 2018). Recent definitions cast stress as a multidimensional concept involving an atypical and challenging stimulus (stressor), the biological processing of that stimulus (stress response), and the resulting organismal condition (stressed individual, Broom and Johnson 1993; Bradshaw 2003;

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Romero 2004; Levine 2005; Koolhaas et al. 2011). The majority of stress definitions suggest the presence of a generalized "stress response" (either implicitly or explicitly) that allows organisms to cope with diverse stressors, largely mediated through the hypothalamic-pituitary-adrenal axis and the action of GC hormones (also known as "stress hormones"; Selye 1976; Sapolsky et al. 2000; Romero 2004; Koolhaas et al. 2011; Wingfield and Boonstra 2013). GCs broadly affect intermediary metabolism, behavior, and immunity (Sapolsky et al. 2000; Bradshaw 2003; Romero 2004; Norris 2007) and are frequently quantified or manipulated as the sole indicator of stress, despite numerous authors advocating against this practice (Bradshaw 2003; Romero 2004; Busch and Hayward 2009; Cockrem 2013; Doom and Gunnar 2013). Even so, the pervasive assumption that GC quantity and stress magnitude are functionally linked results in "stress" being defined, effectively, as the physiological condition of elevated GCs in many studies (Broom and Johnson 1993; Busch and Hayward 2009).

Use of GCs as primary indicators of stress (Broom and Johnson 1993; Bonier et al. 2009) and conceptual models such as the general adaptation syndrome, allostatic load, reactive homeostasis, and the emergency life-history stage that treat stress as a univariate phenotype (Selye 1976; Wingfield et al. 1998; McEwen and Wingfield 2003; Romero et al. 2009; Wingfield and Boonstra 2013) implicitly propose that diverse stressors induce a similar physiological response with comparable consequences for cellular processes, energetics, behavior, and fitness. However, the consequences of GC elevation for these traits are diverse and difficult to predict (Broom and Johnson 1993; Breuner et al. 2008; Bonier et al. 2009; Busch and Hayward 2009; Crespi et al. 2013). For example, stressed and baseline GC concentrations can be positively, negatively, or non-correlated with survival and fecundity, often in context-specific ways (reviewed in Breuner et al. 2008; Bonier et al. 2009; Crespi et al. 2013). Thus, cellular and organismal consequences of "stress" can vary markedly even when GCs are elevated and GCs might have stressor-specific action. Gaining a better understanding of which components of the "stress response" are general and which are context-specific is imperative for predicting the survival and fitness of organisms, and ultimately populations responding to challenging environments with multiple types of stressor.

We examined the response of Eastern fence lizards (*Sceloporus undulatus*) to diverse, yet ecologically relevant stressors—high-temperature exposure and attack by red imported fire ants (*Solenopsis invicta*, an invasive predator of *S. undulatus*; Langkilde 2009). Both stressors are frequent threats experienced by *S. undulatus* populations in the southeastern United States (Angilletta et al. 2009; Langkilde 2009; Buckley et al. 2015; Graham et al. 2017). Intriguingly, both heat challenge and fire ant stressors induce similar up-regulation of GCs in reptiles, although in *S. undulatus* GC response has only been examined for fire ants, not high temperature (Dupoué et al. 2013; Schwartz and Bronikowski 2013; Telemeco and Addis 2014; Gangloff et al. 2016; Jessop et al. 2016; Graham et al. 2017; but see Sykes and Klukowski 2009 for a high-temperature exception). However, these stressors challenge homeostasis in very different ways: high temperatures compromise protein and cell membrane function (Hochachka and Somero 2002; Gangloff et al. 2016) and possibly induce cellular hypoxia (Pörtner 2002; Smith et al. 2015), whereas fire ant venom lyses cells, inhibits ATPase activity, and disrupts the neuromuscular system (Schmidt 1986; Allen et al. 2004; Langkilde and Freidenfelds 2010). The divergent proximate action of these potent natural stressors of *S. undulatus* makes this system a unique opportunity to explore the generality of the vertebrate "stress response" in an ecologically relevant context.

We examined the responses of *S. undulatus* exposed to heat and fire ants at three biological levels: organismal (behavior), physiological (endocrinological via plasma corticosterone [CORT hereafter] and blood glucose, and innate immune function via complement and natural antibodies [NAb]), and cellular (gene expression of a panel of five heat-shock proteins [HSPs] in blood and liver). We focus on expression of HSPs as our indicator of cellular response because they are molecular chaperones that stabilize protein folding and prevent dangerous aggregations of denatured proteins against multiple types of stressors (e.g., heat, desiccation, caloric restriction, disease, hypoxia, and toxin exposure), and have been used as stress indicators similar to GCs (Lewis et al. 1999; Kregel 2002; Sørensen et al. 2003; Hill et al. 2013; Tedeschi et al. 2015). Moreover, HSPs play a crucial role in maintaining the conformation of receptors and enzymes, including the GC-receptor that binds CORT (Csermely et al. 1998; Norris 2007).

We use two complimentary experiments, one with juveniles and the other with adult male lizards to test the generalized stress response hypothesis. This hypothesis predicts that acute heat stress and fire ant attack (FA) will have similar effects at each biological level examined. At the behavioral level, we predict that animals will display similar distress and

avoidance behaviors in response to both stressors (Broom and Johnson 1993). At the endocrine level, we predict that both stressors will elevate CORT as previously observed and consistent with CORT being a key player in the stress response (Sapolsky et al. 2000; Bonier et al. 2009; Gangloff et al. 2016; Graham et al. 2017). Similarly, we predict that blood glucose concentrations will elevate because increased glucose availability is a primary action of CORT and other hormones (e.g., catecholamines) commonly associated with the stress response (Wingfield et al. 1998; McEwen and Wingfield 2003; Romero 2004; Norris 2007; Gangloff et al. 2016). Moreover, elevated glucose should be a functionally appropriate response to both heat and FA because it provides fuel for additional responses (Wingfield et al. 1998; Mateo 2007; Rhoads et al. 2013). At the innate immunity level, we predict our immune measures will be reduced by both stressors, and negatively correlated with CORT, although due to the acute nature of our stress exposures there is the potential for immunoenhancement (Tuckermann et al. 2005; Dhabhar 2009; Dhabhar et al. 2012). Finally, at the cellular level, we predict that HSP-gene expression will be similar across both stressors and tissues, and positively correlated with behavior, CORT, and glucose. Alternatively, if the generalized stress response hypothesis is not supported, we expect the response to these two stressors to diverge at one or more of these biological levels.

Materials and methods

Stressor experiments

We utilized young-of-the-year lizards ("juvenile experiment" hereafter) and adult-male lizards ("adult-male experiment" hereafter). Although experimental protocols were similar, we used results from the juvenile experiment to inform modifications to the adult-male experiment. We measured the same dependent variables for both experiments except we examined HSP-gene expression in the liver and blood of juveniles, but only the liver in adult-males. We only examined liver HSP-gene expression in adult males because both tissues responded similarly to stressor treatments in juveniles (no tissue by treatment interaction, see results for details). Additionally, we measured innate immunity only in the adult experiment because we could not collect sufficient plasma volume from juveniles to measure both CORT and innate immunity. Differences between these two experiments are illustrated in Fig. 1 and described below. Full methodological details are provided as Supplementary Material.

Juvenile experiment

We collected *S. undulatus* young-of-the-year and acclimated them to a standard environment for 7–13 days prior to experiments (details in Supplementary Table S1). Animals were randomly assigned to one of four treatments: Control ($N = 7$), FA ($N = 7$), Heat ($N = 7$), and Heat-30 ($N = 8$; Supplementary Table S1; Fig. 1).

For the FA treatment, we introduced ~15–20 freshly collected fire ants and allowed lizards to receive ~12 stings (either directly or via ingestion; Boronow and Langkilde 2010; Graham et al. 2017), which required <5 min. Fire ants were then removed and the lizard remained undisturbed for 30 min prior to processing. In a previous study, this treatment induced a significant increase in plasma circulating CORT between 30 min and 1 h post treatment (Graham et al. 2017).

For both the Heat and Heat-30 treatments, we warmed animals until they opened their mouths to gape (T_{GAPE}). Gaping and associated panting behaviors are useful indicators of heat stress in lizards (Dawson and Templeton 1966; Heatwole et al. 1973; Tattersall et al. 2006). The Heat group was then allowed to recover at room temperature (~22°C) for 30 min, whereas the Heat-30 group remained in the thermal challenge for 30 min until processing. To warm the animals, we suspended two 75 W ceramic infrared heaters (Repticare; Zoo Med Laboratories Inc., San Luis Obispo, CA) overhead (31 cm above the floor of the center of the enclosures). Surface temperatures within enclosures reached a maximum of 43°C, which approximates the critical thermal maximum temperature (CT_{MAX}) of nearby populations (Buckley et al. 2015).

We recorded behavioral observations throughout the trials, including whether or not lizards gaped, length of time lizards were exposed to the stressor prior to gaping, location of the lizard in the experimental bin (i.e., middle or center), its posture (up and alert, or laying down), and the presence/absence of escape behaviors and what those behaviors were (i.e., running, jumping, and thrashing). Immediately following the trials, we tested righting ability, measured body temperature, collected blood, measured blood glucose with a glucometer (Contour®), euthanized animals, and snap-froze tissues for gene expression. We centrifuged blood in a mini-fuge (VWR) for 30 s, separated plasma and stored at −20°C for CORT assays, and froze the red blood cells in liquid nitrogen prior to storage at −80°C

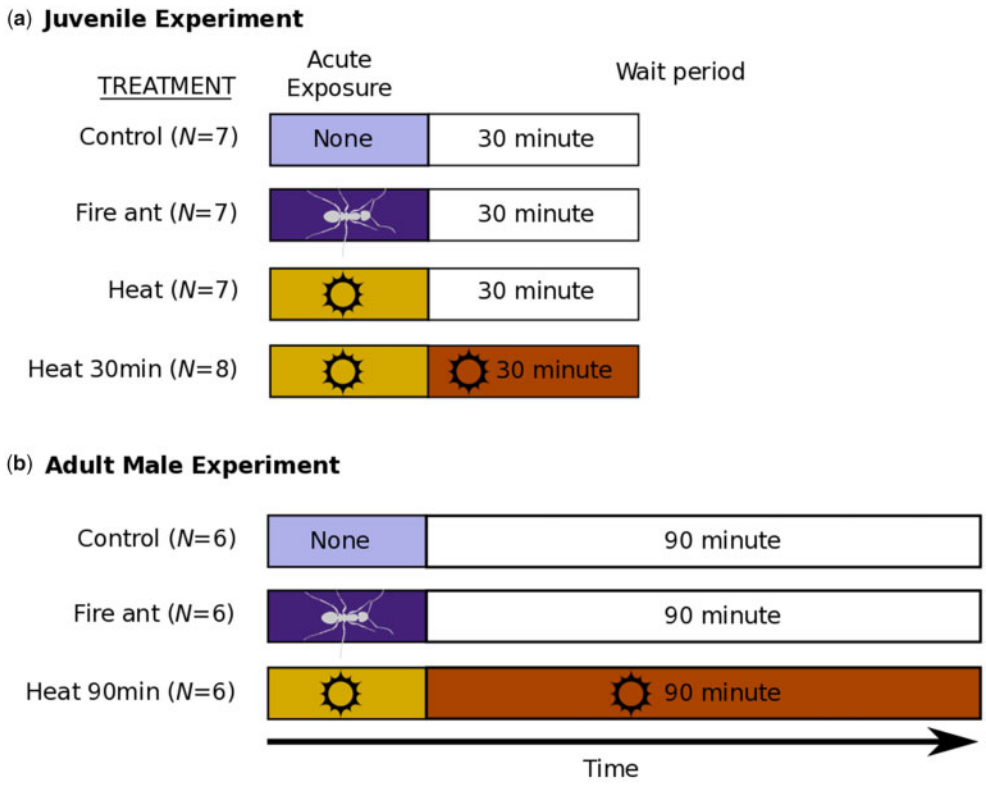

Fig. 1 Schematic of experimental design. *Sceloporus undulatus* lizards were exposed to one of three stressors or control and then allowed a wait period prior to tissue collection. (**A**) Juvenile lizards were exposed to all stressors with a 30 min wait period. (**B**) Adult male lizards were exposed to stressors with a 90 min wait period and the acute "Heat" treatment was removed. For both the "Heat 30 min" and "Heat 90 min" treatments, lizards were held at gaping temperature for the entire waiting period. For all other treatments, this wait period took place in benign conditions. Colors (online version) match points in Figs. 2 and 3 and Supplementary Fig. S2.

for gene expression. We validated our glucometer for repeatability, and for a correlation between plasma and whole-blood glucose ($F_{1,\ 19} = 132.5$, $P < 0.0001$, $R^2 = 0.87$), but not with glucose standards; therefore, absolute glucose values should be interpreted with caution. We quantified plasma CORT in duplicate using enzyme immunoassay (EIA) (Corticosterone High Sensitivity EIA Kit, Immunodiagnostic Systems, Fountain Hills, AZ) following (Graham et al. 2017; mean CV = 2.3%).

We used two-step reverse transcription-quantitative polymerase chain reaction (RT-qPCR) to quantify gene expression of five genes encoding for HSPs in red blood cells and liver tissue that span known HSP function: *HSPA1A*, *HSPA9*, *HSP90AB1*, *HSP90B1*, and *HSPD1*. HSP genes were chosen based on their known function in non-reptiles and expression patterns in reptiles (Ulmasov et al. 1992; Lewis et al. 1999; Kregel 2002; Kampinga et al. 2009; McMillan et al. 2011; Hill et al. 2013; Schwartz and Bronikowski 2013; Gao et al. 2014; Tedeschi et al. 2015; Simoniello et al. 2016; Tedeschi et al. 2016). Further details on these genes are provided in the Supplementary Material and Supplementary Table S3. Additionally, we examined expression of two control genes, *GAPDH* and *POLR2A*. In brief, we extracted total ribonucleic acid (RNA) from blood and liver using the Invitrogen RiboPure^{TM}-Blood Kit (ThermoFisher Scientific) and RNeasy Minikit (Qiagen), respectively, with DNase I digestion. We synthesized complementary DNA (cDNA) from 200 ng total RNA using random hexamer primers with the Invitrogen SuperScript^{TM} IV First-Strand Synthesis System. We created standard curves for each gene and plate using serial dilutions of a common pool of cDNA for each experiment. We performed relative qPCR in duplicate using ABsolute qPCR SYBR Green Fluorescein Mix (AB-1220/A, ThermoFisher Scientific) on the BioRad CFX platform. Further details are provided in the Supplementary Material, Supplementary Table S4, and Supplementary Fig. S2.

Adult male experiment

For the adult-male experiment, we followed the protocols for the juvenile experiment with the following modifications. First, we removed the "Heat" treatment, and extended the wait times post stressor

from 30 min to 90 min before collecting tissues. This left three treatments: Control ($N = 6$), FA ($N = 6$), and 90-min heat challenge (Heat-90, $N = 6$, Fig. 1B; Supplementary Table S2). We exposed adult males to the heat stressor in a temperature-controlled chamber (136NL, Percival Scientific Inc., Perry, IA) set to 42.5°C with internal lights switched on, rather than using overhead heat lamps. Because animals were enclosed in opaque chambers during these trials, it was not possible to make behavioral observations. Lizards were in the chamber for a total of 150 min, allowing 60 min for body temperature to rise to gaping temperature (validated via an earlier pilot study) followed by 90 min of continuous stressor exposure (Heat-90 treatment). Because animals were forced to reach an equilibrium temperature with the chambers, we did not record cloacal body temperature of adult males at processing. Control and FA animals were housed in plastic bins at room temperature during trials, and were not placed in a thermal chamber. We also modified HSP qPCR by (1) only analyzing the liver, (2) the assay was performed in triplicate, and (3) we were only able to amplify the control gene *GAPDH* in adult males despite numerous attempts to further optimize our protocols for *POLR2A*. Further details are provided in the Supplementary Material. The CV for the adult CORT assay was 5.4%. Adult plasma was also used to measure complement and NAb as proxies of innate immune function. We modified methods from French et al. (2010) to measure plasma complement ability to lyse heterologous red blood cells in an antibody-independent manner, reported as CH50, the reciprocal of the dilution of plasma required to lyse 50% of the red blood cells (Mayer 1961). We modified methods from Matson et al. (2005) to measure the ability of NAb to agglutinate heterologous red blood cells, recorded as the negative $\log_2$ of the lowest plasma dilution that exhibited agglutination. Further details are provided in the Supplementary Material.

Statistical analyses

We performed all analyses using R software (version 3.3.2; R Core Team 2016). For each experiment separately, we examined the effects of our treatments on body temperature, blood glucose, and natural-log transformed plasma CORT concentration using Analysis of Variance (ANOVA). Simple models without interactions were preferred based on backward selection and small-sample corrected Akaike's Information Criterion (AICc). For the adult-male experiment, we also examined the effects of stressor treatment, natural-log transformed plasma CORT concentration, and blood glucose on our measures of innate immunity using Multivariate Analysis of Covariance (MANCOVA), and then individually using Analysis of Covariance (ANCOVA). Again, simple models without interactions were preferred. For MANCOVA, CH50 and NAb were scaled to a mean of zero and unit variance.

We then tested whether HSP-gene expression responded differently to our stress treatments in the two experiments. For juveniles only, we also assessed whether this response differed between blood and liver. We used ANOVA to examine the effects of tissue, treatment, and their interaction (juvenile experiment) or treatment (adult-male experiment) on log-transformed relative starting quantities for each gene individually, and Euclidean-distance based permutational MANOVA (pMANOVA) to analyze all *HSP* together. We analyzed all treatments and tissues in the same models for the juvenile experiment to allow us to simultaneously examine their effects. Because control-gene expression varied among tissues for juveniles (see section 'Results' for details), we could not normalize the data by control-gene expression. Instead, we relied on our experimental protocol (i.e., cDNA synthesized from 200 ng total RNA for all samples, multiple biological replicates) to control for technical variation. To keep results commensurate, we also analyzed non-normalized data for the adult-male experiment. We further explored the effects of treatment (both experiments) and tissue (juvenile experiment only) on HSP-gene expression with principal components analyses (PCA). Next, we tested for correlations between HSP-gene expression and plasma CORT using multivariate regressions. To visualize the relationships between CORT and HSP-gene expression, we plotted plasma CORT against the top two principal components from the PCAs described above. Behavioral observations could only be compared among groups qualitatively. Additional details for all analyses are available as Supplementary Material.

Ethics

Animals were collected with permission from Auburn University Solon-Dixon Forestry Education Center, Andalusia, AL under permit from the Alabama Department of Conservation and Natural Resources (permits: 2016104343468680 and 2017026240868680). All research was approved by the Pennsylvania State University IACUC (44595) or Auburn University IACUC (2017-3066). Data and R-scripts uploaded as Online Supplementary Material.

Results

Juvenile experiment

Behavioral response

Animals exposed to either heat or fire ant stressors displayed similar stereotyped behaviors (Langkilde 2009) that differed from Control animals, which generally remained motionless throughout trials. Lizards first attempted to escape the stressor, but eventually moved to a corner of their enclosure and remained motionless with their eyes closed. Lizards in the heat treatments began to gape ~13.9 min into Heat and Heat-30 trials (SD = 6.4 min) when adjacent surface temperatures averaged 41.3°C (SD = 1.6°C). Toward the end of the Heat-30 treatment, some lizards closed their mouths, possibly to avoid over-desiccation. All animals successfully righted themselves following treatments, indicating that the stressors, while acute, where not debilitating.

Endocrine response

The endocrine response to both stressors was the same: neither mean plasma CORT concentration ($F_{3,\ 24} = 1.64$, $P = 0.2063$, Fig. 2A) nor blood glucose concentration ($F_{3,\ 25} = 1.37$, $P = 0.2755$, Fig. 2B) was affected by either treatment. Plasma CORT and blood glucose were negatively correlated ($F_{1,\ 23} = 7.41$, $P = 0.0121$, $R^2 = 0.37$, Fig. 2C). Post-hoc tests suggest that the correlation was driven by the Heat-30 treatment (Control: $F_{1,\ 5} = 1.07$, $P = 0.3486$; FA: $F_{1,\ 5} = 0.01$, $P = 0.9199$; Heat: $F_{1,\ 5} = 3.35$, $P = 0.1269$; Heat-30: $F_{1,\ 5} = 5.68$, $P = 0.0630$, Fig. 2C), although the interaction between blood glucose and treatment was not significant ($F_{3,\ 20} = 1.41$, $P = 0.27$, ΔAICc = 6.64).

Cellular response

FA had the opposite effect on HSP-gene expression compared to Heat or Heat-30 treatment in juvenile lizards. Generally, FA treatment reduced *HSP* expression, whereas Heat and Heat-30 treatment elevated *HSP* expression (Tables 1 and 2; Supplementary Fig. S1). Principal Component 1 (PC1) and PC2 explained 82% of *HSP* expression variation (Table 4 gives all variable loadings), and allow qualitative separation of the tissues, and treatments within the tissues. Analyzed as a panel of HSP genes, both tissue and treatment had significant effects (Table 1, Figure 4A). Although the effect of treatment did not vary between juvenile blood and liver, the quantity of transcripts differed between tissues for three of the *HSP* genes and the control genes (Table 1; Supplementary Fig. S1).

Multivariate regression revealed that plasma CORT correlated with *HSP* expression in juvenile liver (Pillai's trace = 0.45, df = 1, $P = 0.0239$), but not blood (Pillai's trace = 0.14, df = 1, $P = 0.6469$). Although the correlation was significant in juvenile liver, plasma CORT concentration only explained 2% of *HSP*-expression variation (R^2 = -0.02). Moreover, post-hoc univariate regressions revealed that plasma CORT did not correlate with the expression of any individual *HSP* in either tissue ($P > 0.10$ for all).

Adult-male experiment

Behavioral response

Adults in FA treatment performed the same avoidance behaviors as described for juveniles. Since the adult male Heat-90 experiment was conducted in an enclosed incubator, we could not observe behaviors during this treatment. All animals successfully righted themselves following treatments indicating that the stressors, while acute, where not debilitating.

Endocrine response

Both the FA and Heat-90 treatments similarly elevated CORT concentration relative to Controls, with no difference between the treatments (Control–FA: $t_{14} = -5.85$, $P = 0.0001$; Control–Heat-90: $t_{14} = -4.77$, $P = 0.0008$; FA–Heat-90: $t_{14} = 0.81$, $P = 0.7018$, Fig. 2D). However, stressor treatments differed in their effect on plasma glucose with Heat-90 lizards displaying lower concentrations than FA lizards ($t_{12} = 2.85$, $P = 0.0361$), but neither differed from Control lizards (FA: $t_{12} = -1.39$, $P = 0.3789$; Heat-90: $t_{12} = 1.47$, $P = 0.3401$, Fig. 2E). Plasma CORT and blood glucose were not correlated in adults ($F_{1,\ 11} = 0.02$, $P = 0.888$, Fig. 2F).

Innate immune response

Both stressor treatments similarly affected the innate immune response (Fig. 3A, Pillai's trace = 0.984, df = 2, $P = 0.012$) when plasma CORT and blood glucose were included as covariates in MANCOVA (Pillai's trace = 0.512 and 0.539, $P = 0.057$ and 0.045, respectively, df = 1 for both covariates). When analyzed individually, CH50 was affected by treatment (Fig. 3B, $F2_{,\ 9} = 6.96$, $P = 0.015$), whereas we could only detect a trend for treatment affecting NAb (Fig. 3C, $F2_{,\ 9} = 3.30$, $P = 0.084$). Pairwise tests revealed that CH50 was reduced after high temperature exposure ($t_9 = 3.48$, $P = 0.017$) but unaffected by FA ($t_9 = 2.12$, $P = 0.140$). CORT correlated with both immune measures, but in opposite directions: CH50 was positively correlated with CORT ($F_{1,\ 9} = 5.21$, $P = 0.048$), whereas NAb was negatively

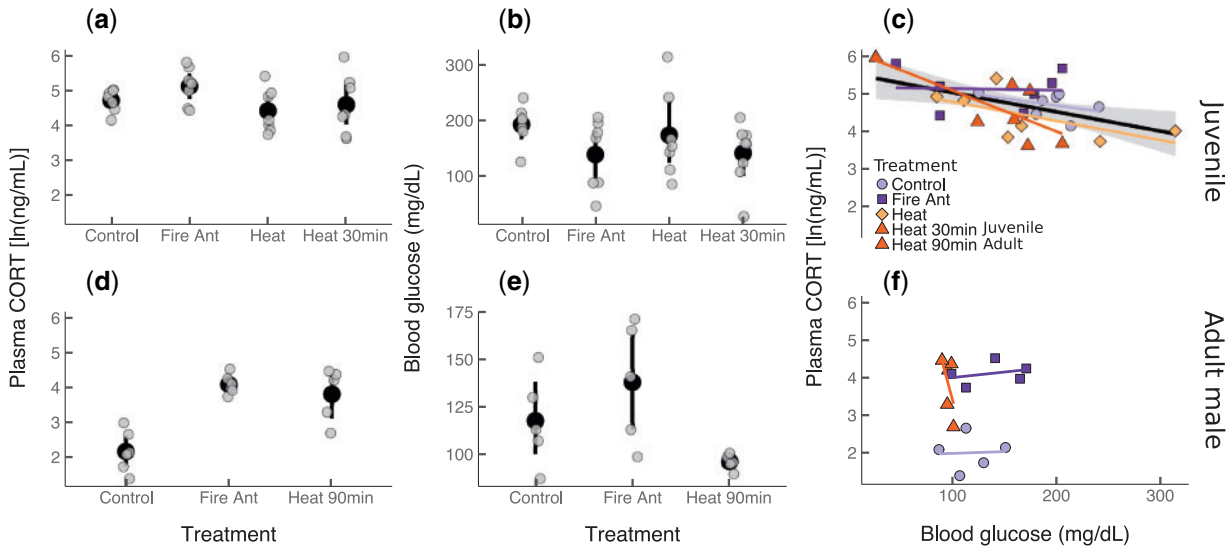

Fig. 2 Endocrine responses to Heat Challenge and Fire Ant attack. Neither mean corticosterone (CORT, $P = 0.21$) nor glucose ($P = 0.28$) were elevated 30 min after stressor exposure in juvenile *Sceloporus undulatus*. By contrast CORT was similarly elevated by both fire ant and high temperature exposure in adult males ($P < 0.001$), and Glucose was reduced by exposure to high temperature ($P = 0.01$). Additionally, CORT and glucose were negatively correlated ($P = 0.01$) in juveniles, but not adults ($P = 0.88$). Panels (**A–B**, **D–E**) display individual data points in grey (jittered along the x-axis) and means with 95% CI in black for plasma CORT and whole-blood glucose concentrations, respectively. Both were analyzed with ANOVA. Panels (**C, F**) displays the relationship between CORT and glucose analyzed with linear regression. The black line and shaded region depict the mean and 95% CI for the entire dataset whereas the colored lines represent the relationship (or lack thereof) for each treatment. For juvenile experiment analyses, CORT $N = 7,7,7,7$ and Glucose $N = 7,7,7,8$ for Control, Fire Ant, Heat, and Heat-30 treatments, whereas for adult-male experiment analyses, CORT $N = 6, 6, 5$ and Glucose $N = 5, 5, 5$ for Control, Fire Ant, and Heat-90 treatments, respectively.

Table 1 Results from (pM)ANOVAs testing effects of tissue (blood and liver) and treatment (Control, Fire Ant, Heat, and Heat-30) on gene expression in juvenile experiment, and treatment (Control, Fire Ant, and Heat-90) on gene expression in adult male experiment. Each gene was analyzed with ANOVA and the HSPs were analyzed together with pMANOVA. Significant *P*-values (<0.05) are indicated in bold type. Results for tissue and treatment are from the preferred, reduced models, whereas interaction terms are from full models

	Juvenile experiment									Adult male experiment		
	Tissue			Treatment			Interaction			Treatment		
Variable	*F*	d.f.	*P*	*F*	d.f.	*P*	*F*	d.f.	*P*	*F*	d.f.	*P*
GAPDH	720.37	1, 48	**<0.0001**	0.62	3, 48	0.6073	0.88	3, 45	0.4651	2.41	2, 14	0.1259
POLR2A	82.83	1, 48	**<0.0001**	1.94	3, 48	0.1355	0.94	3, 45	0.4279			
HSPA1A	2.48	1, 49	0.1218	3.47	3, 49	**0.0229**	1.66	3, 46	0.1883	11.95	2, 13	**0.0011**
HSPA9	11.66	1, 48	**0.0013**	0.94	3, 48	0.4285	1.66	3, 46	0.1883	2.87	2, 14	0.0903
HSP90AB1	0.01	1, 48	0.9282	3.26	3, 48	**0.0294**	1.09	3, 45	0.3617	1.92	2, 14	0.1840
HSP90B1	159.86	1, 48	**<0.0001**	3.16	3, 48	**0.0331**	0.03	3, 45	0.9918	1.28	2, 14	0.3082
HSPD1	17.27	1, 48	**0.0001**	3.60	3, 48	**0.0201**	0.19	3, 45	0.8999	1.09	2, 10	0.3717
All *HSP*	50.00	1, 48	**0.0001**	6.75	3, 48	**0.0001**	0.58	3, 45	0.7788	3.46	2, 9	**0.0113**

correlated with CORT ($F_{1,\ 9} = 7.31$, $P = 0.024$). Blood glucose did not correlate significantly with either immune measure ($P \approx 0.1$ for both).

Cellular response

The effect of FA on HSP-gene expression differed from that of the high-temperature treatment in adult male lizards (Tables 1 and 3). Together, PC1 and PC2 explained 70% of the variation in *HSP* expression and allowed qualitative separation of the treatments (Table 4 and Fig. 4B). Treatment effects on *HSP*-gene expression were largely driven by Heat-90 exposure increasing (cDNA) *HSPA1A* expression, with FA having little effect on any HSP gene expression (Supplementary Fig. S1). Multivariate regression revealed that plasma CORT did not correlate with *HSP* expression in adult-male liver (Pillai's trace = 0.65, df = 1, $P = 0.2646$). Moreover, post-hoc

Table 2 Pairwise comparisons testing effects of stressor treatments on expression of five HSP genes (across both tissues) in juvenile *S. undulatus*. Values above diagonals are *P*-values. For multivariate contrasts, *F*-ratio statistics are below the diagonal, whereas *t*-ratio statisitcs are below the diagonal for univariate contrasts. Positive *t*-ratios indicate that the column treatment had relatively higher gene expression (univariate contrasts only). True, uncorrected *P*-values are presented, but bold- and italic-font indicate significant ($P < 0.05$) and nearly-significant ($0.05 \leq P < 0.10$) effects, respectively, after Tukey correction

Treatment	Control	FA	Heat	Heat 30
All *HSP*				
Control		**0.0168**	0.2062	**0.0097**
FA	3.93		**0.0002**	**0.0001**
Heat	1.53	10.78		0.1934
Heat 30	4.81	16.96	1.63	
HSPA1A				
Control		0.1027	0.2593	0.3826
FA	1.663		**0.0053**	*0.0109*
Heat	−1.141	−2.919		0.7877
Heat 30	−0.881	−2.648	0.271	
HSPA9				
Control		0.4729	0.4793	0.5469
FA	0.723		0.1414	0.1773
Heat	−0.713	−1.495		0.9228
Heat 30	−0.607	−1.369	0.097	
HSP90AB1				
Control		0.0842	0.8588	0.2295
FA	1.763		0.0488	**0.0036**
Heat	−0.179	−2.021		0.2845
Heat 30	−1.217	−3.065	−1.082	
HSP90B1				
Control		0.9991	0.3087	*0.0122*
FA	0.001		0.2891	**0.0093**
Heat	−1.029	−1.072		0.1038
Heat 30	−2.606	−2.71	−1.658	
HSPD1				
Control		*0.021*	0.8342	0.5576
FA	2.387		0.028	**0.0037**
Heat	0.21	−2.266		0.4114
Heat 30	−0.591	−3.051	−0.829	

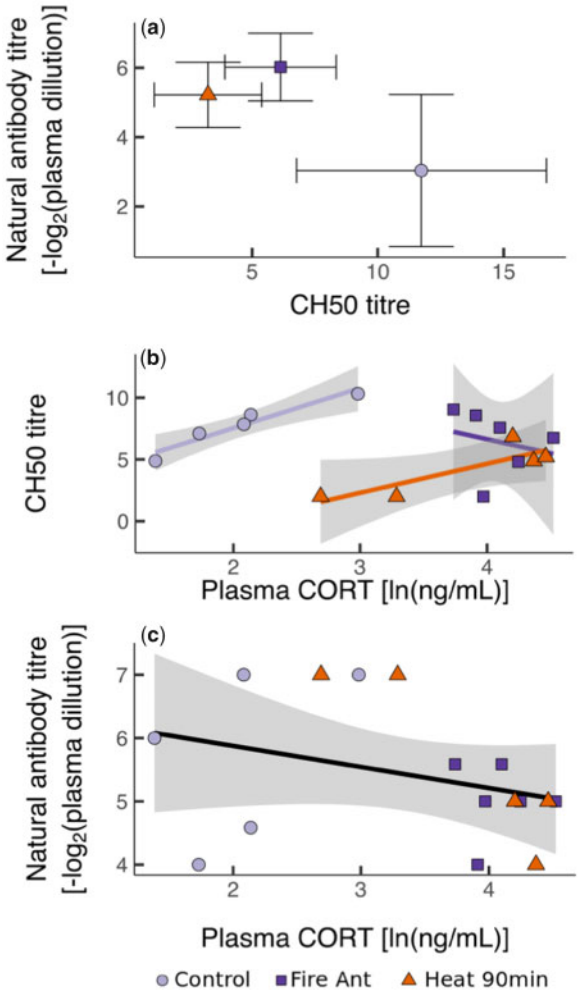

Fig. 3 Innate immune response to heat challenge, FA, and Corticosterone. (**A**) Stressor treatments similarly affected the innate immune response of adult male lizards, which changed relative to Control ($P = 0.012$). Points are lsmeans and 95% CI. (**B**) When examined in isolation, complement lysing ability (CH50) was reduced after exposure to high temperature for 90 min ($P = 0.017$), whereas FA had no effect relative to Control ($P = 0.19$). Moreover, CH50 was marginally positively correlated with plasma corticosterone concentration (CORT, $P = 0.048$). (**C**) By contrast, there were positive trends for the effects of treatment on natural antibody titre (NAb, $P = 0.084$), and NAb was negatively correlated with CORT ($P = 0.024$). For (B) and (C), points are individual observations, whereas lines and shaded regions are means and 95% CI from linear regression. Data were analyzed via MANCOVA (A) or ANCOVA (B,C) and are only available for adult male lizards. For both dependent variables, $N = 6, 6, 5$ for Control, Fire Ant, and Heat 90 min treatments, respectively.

univariate regressions revealed that plasma CORT did not correlate with the expression of any individual *HSP* ($P > 0.10$ for all).

Discussion

The generalized stress response hypothesis, implicitly assumed in much stress research, suggests that stressor exposure induces GC production, which initiates a cascade of cellular and immunological responses, and that these responses are similar across different stressors. However, our results add to a growing literature challenging the concept of a single, generalized stress response. Across two

Table 3 Pairwise comparisons testing effects of stressor treatments on expression of five HSP genes in adult male *S. undulatus*. Values above diagonals are *P*-values. For multiivariate contrasts, *F*-ratio statistics are below the diagonal, whereas *t*-ratio statisitcs are below the diagonal for univariate contrasts. Positive *t*-ratios indicate that the column treatment had relatively higher gene expression (univariate contrasts only). True, uncorrected *P*-values are presented, but bold- and italic-font indicate significant ($P < 0.05$) and nearly-significant ($0.05 \leq P \leq 0.10$) effects, respectively, after Tukey correction

Treatment	Control	FA	Heat 90
All *HSP*			
Control		0.7224	**0.0089**
FA	0.64		0.0857
Heat 90	5.80	4.88	
HSPA1A			
Control		0.1488	**0.0004**
FA	−1.53		**0.0076**
Heat 90	−4.76	−3.16	
HSPA9			
Control		0.1475	0.1241
FA	−2.005		1.0
Heat 90	−2.108	−0.01	
HSP90AB1			
Control		0.1212	0.1090
FA	−1.65		0.9862
Heat 90	−1.71	0.018	
HSP90B1			
Control		0.1620	0.2322
FA	−1.48		0.7794
Heat 90	−1.25	0.286	
HSPD1			
Control		0.6364	0.1701
FA	−0.49		0.4433
Heat 90	−1.48	−0.80	

independent experiments, *S. undulatus* lizards responded differentially to high temperature and fire ant exposure, both of which are ecologically-relevant acute stressors. The degree of difference in stress responses varied across biological levels: stressors induced very different responses at the cellular level (*HSP* expression), mixed responses at the physiological level (moderately different innate immunity responses, contrasting relatively more similar endocrine [GC] responses), but relatively similar behavioral responses. Because our sample sizes were modest ($N \approx 6$/treatment/experiment), it is possible that we were unable to detect smaller disparities in responses to the two stressors. Even so, our observations highlight the importance of broadly assessing the response of organisms to diverse stressors across multiple organismal levels.

Divergent responses to high-temperature and fire ant exposure were most pronounced at the cellular level with HSP-gene expression, but also apparent for plasma glucose concentration. High temperatures increased HSP-gene expression in both life stages, whereas fire ant exposure had the opposite effect and decreased *HSP* expression in juveniles and had no effect on *HSP* expression in adult males. High-temperature exposure reduced plasma glucose concentration in adult males, but no other treatments had an effect on plasma glucose in either life stage. Moreover, we generally did not observe correlations between plasma CORT and either *HSP* expression or plasma glucose, although such correlations are expected if GCs mediate a generalized stress response. Neither trait correlated with CORT in the adult male experiment, and correlations were either extremely weak (CORT explained 2% of *HSP* expression in liver) or in the opposite direction of predictions (plasma glucose) in the juvenile experiment. For example, we predicted that plasma glucose would positively correlate with plasma CORT because CORT acts to elevate circulating glucose via gluconeogenesis, glycogenolysis, and reduced glucose uptake by peripheral tissues (Stevenson et al. 1957; McEwen and Wingfield 2003; Norris 2007). However, plasma glucose was negatively correlated with CORT in juvenile lizards. We suspect that this negative correlation resulted from increased metabolic demand from high temperature and escape attempts depleting glucose reserves more rapidly than glucose could be released via CORT action, but more research is needed to test this hypothesis. Regardless, the differences in *HSP* expression and plasma glucose response to stressors, and the lack of correlations between these responses and CORT strongly suggest that these stressors induce specialized, context-specific responses.

Plasma CORT concentration and innate immunity measures were similarly affected by high temperature and fire ant exposure, contrasting the *HSP* expression and plasma glucose responses. We expected plasma CORT concentration to increase in response to both stressors in all lizards because both stressors were previously observed to elevate CORT in reptiles within 0.5–1 h of exposure (Schwartz and Bronikowski 2013; Telemeco and Addis 2014; Gangloff et al. 2016; Jessop et al. 2016; Graham et al. 2017), and because stress elevating CORT is general dogma. However, we only observed the expected pattern in adult male lizards; plasma CORT concentration was unaffected by either

Table 4 Summary of variable loadings from PCA of cDNA representing the expression of five *HSP* genes in *S. undulatus* juveniles (blood and liver) and adult males (liver only). Cummulative variance explained by the principal components is given in parentheses. Scores from these analyses are depicted in Fig. 4.

Juvenile experiment					
Gene	**PC1 (59%)**	**PC2 (82%)**	**PC3 (93%)**	**PC4 (98%)**	**PC5 (100%)**
HSPA1A	0.3	−0.69	0.54	−0.06	−0.37
HSPA9	0.52	0.12	−0.26	−0.8	−0.04
HSP90AB1	0.42	−0.37	−0.69	0.45	−0.06
HSP90B1	0.41	0.6	0.19	0.33	−0.57
HSPD1	0.54	0.1	0.35	0.21	0.73
Adult experiment					
Gene	**PC1 (44%)**	**PC2 (70%)**	**PC3 (86%)**	**PC4 (95%)**	**PC5 (100%)**
HSPA1A	0.44	−0.51	−0.37	−0.37	0.53
HSPA9	0.44	0.48	0.29	−0.68	−0.18
HSP90AB1	−0.05	−0.62	0.77	−0.11	−0.1
HSP90B1	0.53	0.25	0.37	0.56	0.45
HSPD1	0.57	−0.26	−0.22	0.27	−0.69

stressor in juveniles. Even so, the behavioral and *HSP*-expression responses that we observed confirm that the juveniles were challenged and "stressed." These 1- to 2-month-old offspring may have been in a developmental period during which they do not mount a CORT response to stressors (Sapolsky and Meaney 1986), but instead exist in a chronic high-CORT endocrine state. As evidence of this, Control juveniles displayed "stress-like" plasma CORT concentrations more similar to stressor-exposed individuals (adults and juveniles) than to Control adults (compare Fig. 2A,B). Recent work with the same source population of our juvenile *S. undulatus* also observed high baseline CORT concentrations from 1-week old hatchlings, a limited CORT response following Adrenocorticotropic hormone (ACTH) challenge (Ensminger et al. 2018), and failure to elevate CORT following exposure to a natural stressor (D. Ensminger, personal communication). Such ontogenetic variation in CORT physiology demonstrates an additional limitation to using GCs to indicate stress.

When variation associated with plasma CORT and blood glucose was accounted for, we observed similar effects of both stressor treatments on innate immunity. However, these effects disappeared when the covariates were not included. Innate immunity appears to be affected both directly by stressor exposure and indirectly by CORT (or an unmeasured correlate), and these effects are in opposite directions. Stressor exposure generally induced reductions in the lysing ability of complement (CH50), while CORT was positively associated with CH50; the stressors induced increases in the agglutinating ability of NAb, while CORT was negatively correlated with NAb. Reduced immune function is frequently associated with increased CORT concentrations (Cain and Cidlowski 2017), which fits with our correlation between CORT and NAb. However, the positive association seen with complement runs counter to this model, although a similar trend has been observed in the complement of box turtles (West and Klukowski 2018). Our complement measure is an antibody-independent function of the innate immune system that may be more likely to be spared or even enhanced by GCs, which may explain this result (Schleimer 2004; Zhang et al. 2007). Additionally, the opposing effects of treatment and CORT on immune function likely indicate that other responses to the experimental stressors acted counter to the effects of CORT on immunity; for example, some HSPs have been found to activate complement (Prohaszka et al. 1999, 2002). These observations further suggest that the stress response can be highly context dependent, with numerous cascades and feedbacks acting until an equilibrium is reached.

Numerous studies demonstrate that up-regulation of GC hormones has extremely varied effects on organisms, particularly on complex fitness-related traits such as reproduction and survival (Breuner et al. 2008; Bonier et al. 2009; Busch and Hayward

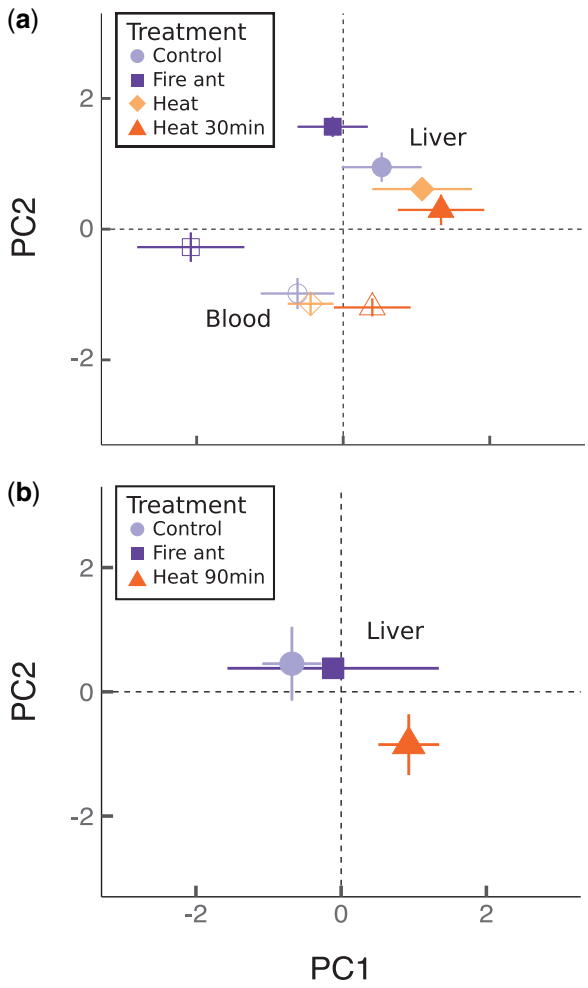

Fig. 4 PCA of cellular response. Fire ant and heat exposure induced opposite patterns of HSP expression after stressor exposure in both blood and liver from juvenile *S. undulatus* (**A**, pMANOVA, $P < 0.01$); only heat affected expression in adult males (**B**, pMANOVA, $P < 0.01$). Data are means and SE for gene expression from five *HSPs* projected onto the primary components from a principle component analysis. Individual expression of each HSP is presented in Supplementary Fig. S1 and PC loadings are presented in Table 4.

2009; Crespi et al. 2013). The stressor- and stage-specific differences that we observed between the behavioral, endocrine, immune, and cellular responses can explain some of this variation. For example, if animals employ opposing strategies at the cellular level, as we observed, it follows that resource and energy allocation decisions differ, which will have broad downstream consequences. Some of the observed variation in fitness-related traits in diverse taxa might therefore result from molecular stress pathways acting independently, but simultaneously, with GCs. Additionally, the direct action of GCs might be context- and ontogenetic-stage specific. GCs act by binding to GC receptors (GRs), which enables the GR to move into the nucleus and mitochondria to bind the GC response elements (GREs) on the DNA as a transcription factor, or bind and repress other transcription factors (Heck et al. 1994; Reichardt et al. 2001; Hunter et al. 2016). It is likely that a myriad of mechanisms modulates the transcription factor action of the GR-complex, such as cofactors that may be stressor specific, the accessibility of DNA regions via epigenetic regulation, or the ability of CORT to bind GR. HSP90, for example, maintains the GR complex for CORT binding (Csermely et al. 1998; Norris 2007). Thus, stressor-induced variation in HSP90 could affect the number of bound GR-CORT complexes in the cell and all downstream components of the CORT-mediated response to that stressor.

The "stress response" appears too varied and complex for a simple indicator, such as up-regulation of GCs or individual HSP proteins, to allow adequate description or fitness-related predictions of responses to challenges. Further research examining the context- and age-specific effects of GC hormones and the interactions between the GC-induced cascade and other cellular responses to diverse stressors are needed for a mechanistic understanding of the stress response. Such a mechanistic understanding will be especially important for understanding the consequences of exposure to multiple stressors, which will likely be non-additive. Our results add to a growing consensus among stress researchers that stressors induce diverse responses, which are poorly represented by simple models (Romero 2004; Breuner et al. 2008; Bonier et al. 2009; Busch and Hayward 2009). Still, the idea of a generalized stress response is pervasive, both among those attempting to utilize GCs to estimate stress for conservation or treatment validation, and implicitly in many conceptual models proposed to explain "stress" as a univariate phenomenon (e.g., allostatic load, reactive homeostasis, and emergency life-history stage; Wingfield et al. 1998; McEwen and Wingfield 2003; Romero et al. 2009; Wingfield and Boonstra 2013). Our prior work is no exception. Broadening the stress indicators used by ecophysiologists, such as gene expression and behavior, can provide important insight into the specialized functions and adaptive significance of diverse stress responses. We hope that additional research on the context- and tissue-specific effects of stressors will both increase our understanding of stress responses, and increase the visibility of a "many-stressors-to-many-responses" model of stress among the scientific community.

Authors' contributions

RST, TL, and TSS conceptualized the experiments. All authors collected data, contributed critically to the drafts, and gave final approval for publication. RST analyzed the data and drafted the manuscript.

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Supplementary data

Supplementary data are available at *ICB* online.

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