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RESEARCH ARTICLE

Population history with invasive predators predicts innate immune function response to early-life glucocorticoid exposure in lizards

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ABSTRACT

Early-life stress can suppress immune function, but it is unclear whether transgenerational stress exposure modulates the immune consequences of early stress. In populations where, historically, the immune system is frequently activated, e.g. persistent stressors that cause injury, it may be maladaptive to suppress immune function after early-life stress. Thus, the relationship between early-life stress and immune function may vary with population-level historical stressor exposure. We collected gravid fence lizards (*Sceloporus undulatus*) from populations that naturally differ in long-term exposure to invasive fire ants (*Solenopsis invicta*). We manipulated early-life stress in the resulting offspring via weekly exposure to fire ants, application of the stress-relevant hormone corticosterone or control treatment from 2 to 43 weeks of age. We quantified adult immune function in these offspring with baseline and antigen-induced hemagglutination and plasma bacterial killing ability. Early-life corticosterone exposure suppressed baseline hemagglutination in offspring of lizards from populations not exposed to fire ants but enhanced hemagglutination in those from populations that were exposed to fire ants. This enhancement may prepare lizards for high rates of wounding, toxin exposure and infection associated with fire ant attack. Adult bacterial killing ability and hemagglutination were not affected by early-life exposure to fire ants, but the latter was higher in offspring of lizards from invaded sites. A population's history of persistent stress may thus alter individual long-term immunological responses to early-life stressors. Further consideration of historical stressor exposure (type and duration) may be important to better understand how early-life stressors affect adult physiology.

KEY WORDS: Corticosterone, Early-life stress, Fire ant, Invasive species, Hemagglutination, Lizard

INTRODUCTION

The immune system plays a critical role in maintaining health by protecting an organism from pathogens and parasites, repairing damage and responding to infection (Møller and Saino, 2004; Murphy, 2001). Various components of immune function are influenced by glucocorticoids, which are steroid hormones released through activation of the hypothalamic-pituitary–adrenal (HPA) axis in response to stressors (Sapolsky et al., 2000). Mild or short-duration glucocorticoid elevation can enhance immune function in preparation for wounding or subsequent infection that may occur (Deak et al., 1999; Dhabhar, 2009; Martin, 2009). Intense or long-duration glucocorticoid elevation can, by contrast, suppress immune function (Dhabhar, 2009; Martin, 2009; Sapolsky et al., 2000; Stratakis and Chrousos, 1995) as a result of energy being diverted away from immune function and toward more immediately critical stressor-relevant responses (Lochmiller and Deerenberg, 2000; McEwen and Wingfield, 2003).

Animals exposed to stressors and/or associated glucocorticoids during development or early in life (i.e. before sexual maturity) can experience sustained immunosuppressive consequences in adulthood (e.g. Michaut et al., 1981; Avitsur et al., 2006; Schmidt et al., 2015). To verify whether early-life stress can program the immune response, direct manipulation of glucocorticoids and environmentally relevant stressors in early life is necessary (Grindstaff and Merrill, 2017). The immune-suppressive effects of exposure to stressors during early life (henceforth 'early-life stress') have primarily been studied in humans (e.g. Dong et al., 2004; Danese et al., 2007; Miller et al., 2009; Fagundes and Kiecolt-glaser, 2014), rodents (e.g. Michaut et al., 1981; Avitsur et al., 2006) and birds (e.g. De Coster et al., 2011; Kriengwatana et al., 2013; Schmidt et al., 2015; Grindstaff and Merrill, 2017), with less known about lasting effects of early-life stress on immune function in ectotherms. Ectotherms are unable to internally regulate temperature and may allocate resources toward the immune system in a different manner from ectotherms (Zimmerman et al., 2010); thus, ectotherms may experience different immune consequences of early-life stress compared to endotherms. Additionally, the two branches of the immune system are differentially prioritized (i.e. innate versus adaptive) in ectotherms and therefore these animals may respond to early-life stress in a different manner from other vertebrates (Zimmerman et al., 2010).

The immune outcomes of stressor exposure can vary between populations (e.g. in response to tourism and urbanization; Martin et al., 2005; French et al., 2008, 2010). It is possible that differences in stressor outcomes reflect a population's history of stress exposure (e.g. Yehuda et al., 2000; Storm and Lima, 2010; Harris and Seckl, 2011), or how limited energy is allocated during duress (i.e. to growth, reproduction and/or immunity; Martin et al., 2004; Pörtner et al., 2005; Fischer and Thatje, 2008; Du et al., 2012). For example, in populations where the prevalent stressor (e.g. predators, aggressive competitors, pathogens) is likely to lead to injury or increase infection risk (Cox and John-Alder, 2007; Ezenwa et al., 2012), it may be maladaptive to compromise immune function in response to stressors. Such transgenerational immune changes could be induced by epigenetic processes (Bohacek and Mansuy, 2016; Fitzpatrick and Wilson, 2003; Mostoslavsky and Bergman, 1997; Teitell and Richardson, 2003), and/or maternal effects (Hasselquist et al., 2012; Ismail et al., 2015; Veru et al., 2014) on the immune

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system directly or indirectly (e.g. effects on other systems that have cascading effects on the immune system). Understanding the scope of stress effects on immune outcomes is critical to determining how species are affected by environmental challenges.

We experimentally investigated the independent and interactive effects of early-life stressor or glucocorticoid exposure and transgenerational stressor exposure on adult immune function in eastern fence lizards (*Sceloporus undulatus*). We took advantage of lizard populations that vary in exposure to an environmental stressor that can lead to wounding and venom exposure, i.e. the presence or absence of predatory invasive fire ants (Graham et al., 2012a; Langkilde, 2009a). To test the prediction that lizards from predator-exposed populations up-regulate, rather than suppress, immune function in response to predator-related stressors, we manipulated early-life stressor (i.e. fire ant) or glucocorticoid exposure and then measured adult immune function in lab-reared offspring of lizards from populations with and without fire ants. Previous research in this system has demonstrated effects of transgenerational predator exposure, but not early-life predator or glucocorticoid exposure, on adult HPA activity (McCormick et al., 2017). Here, we investigated the long-term effects of transgenerational predator exposure and early-life predator or glucocorticoid exposure on immune function in these populations.

MATERIALS AND METHODS

Study system and animal collection

Eastern fence lizards, *Sceloporus undulatus* (Bosc and Daudin 1801), and red imported fire ants, *Solenopsis invicta* Buren 1972, occupy similar habitat where their ranges overlap (Langkilde, 2009b). These invasive fire ants are predators of fence lizards (Langkilde, 2009a) and frequently bite and sting lizards in the wild (Freidenfelds et al., 2012). Envenomation from the ants can be lethal (Langkilde, 2009a). Fire ant attacks trigger the release of the stress-relevant hormone corticosterone (CORT, the primary glucocorticoid in lizards) (Graham et al., 2017), and lizard populations in fire ant-invaded areas have higher baseline CORT concentrations ('high-stress' invaded populations) than those from sites with no history of fire ant invasion ('low-stress' uninvaded populations) (Graham et al., 2012a). Fire ant attacks break the skin, leaving lizards vulnerable to infection (Elkan and Cooper, 1980; Murphy, 2001). Lizards from fire ant-invaded sites also have higher rates of tail autotomy (invaded versus uninvaded: 24.19% versus 16.57%; χ^2_1=11.71, P<0.001) and other wounding (Thawley and Langkilde, 2017), possibly as a result of behavioral responses of lizards to fire ants attracting the attention of visual predators (Freidenfelds et al., 2012).

During April and May 2012, we collected 86 gravid female eastern fence lizards from six sites across southeastern USA: (1) Blackwater River State Forest, Santa Rosa County, FL; (2) Geneva State Forest, Geneva County, AB; (3) Conecuh National Forest, Covington County, AB; (4) St Francis National Forest, Lee County, AK; (5) Edgar Evins State Park, DeKalb County, TN; and (6) Standing Stone State Park, Overton County, TN. The first three sites were invaded by fire ants 57–76 years ago ('invaded'; 31–42 generations, based on Parker, 1994), while the last three sites have no history of fire ant invasion (Callcott and Collins, 1996). All sites have the same ecoregion classification (based on nine environmental variables; Hargrove and Hoffman, 1999) and are primarily composed of temperate mixed forests, though lizards and fire ants tend to reside in open disturbed areas or along forest edges (Stiles and Jones, 1998; Trauth et al., 2004). Lizards at these sites occupy similar habitats, with similar canopy openness, perch-site diameter and ground-cover composition (Langkilde, 2009a; T.L., unpublished data).

Animal husbandry

Gravid females were transferred to the Pennsylvania State University and housed in pairs in plastic enclosures (56×40×30 cm, L×W×H) until oviposition. Each enclosure was furnished with moist sand and a water dish, with a shelter at one end for refuge and basking. Overhead lights were set to a 12 h:12 h light:dark schedule (08:00 h–20:00 h), and a 60 W incandescent light bulb placed at one end of the enclosure provided a thermal gradient for 6 h each day to allow lizards to thermoregulate. Lizards were fed crickets (*Acheta domestica*) dusted with calcium and vitamin supplements every second day, and water was available *ad libitum*.

Eggs were placed by clutch in plastic containers (11×11×7.5 cm, L×W×H) filled with moist vermiculite (−200 kPa), covered with plastic wrap and sealed with a rubber band (Langkilde and Freidenfelds, 2010). Egg containers were placed in the incubator (29±1°C) until hatching (approximately 45 days) and were rotated every other day to avoid any within-incubator effects of position. Incubators were checked twice daily for hatchlings.

Once lizards hatched, they were toe-clipped for unique identification and housed in groups of six based on age. Each enclosure contained two lizards from each of the three treatments and no more than two lizards from each clutch. Lizards from fire ant-invaded sites never shared enclosures with those from uninvaded sites. Hatchlings were housed under similar conditions to those of gravid females but without sand; the floor of each enclosure was instead lined with paper towels. Mass and snout–vent length (SVL) of all offspring were measured at 42 weeks of age. All data are from measures of these offspring and not of the parental generation.

Treatments

To determine the effects of exposure to CORT or an ecologically relevant stressor during early life, hatchlings were assigned to one of three treatments using a split-clutch design. Lizards were exposed to fire ants (FA), topical application of CORT or a handling and oil-vehicle control once a week between 2 and 43 weeks of age, at which time lizards were at or approaching sexual maturity (minimum SVL at sexual maturity at these sites: 53 mm male, 55 mm female). Each week, lizards were individually placed in a sand-lined arena (with or without fire ants) for 30 s, after which 3 µl sesame oil (with or without CORT) was applied to their backs with a pipette. This allowed us to control for effects of handling and topical application across all treatments.

Lizards in the fire ant treatment (n=10) were exposed to 15–20 fire ants, which were allowed to bite and sting the lizard as they do in nature. The trial was ended 30 s after the first ant contacted the lizard, providing a non-lethal level of fire ant exposure known to induce CORT elevation (Graham et al., 2017). They then received a topical application of sesame oil.

Lizards in the CORT treatment (n=11) were placed in an empty arena for 30 s and then received a topical dose of CORT (≥92%, Sigma C2505, Saint Louis, MO, USA) dissolved in commercial sesame oil, which was quickly absorbed as a result of the lipophilic nature of lizard skin (Belliure and Clobert, 2004). The CORT doses that lizards received each week were calculated based on the average growth of this species in the laboratory (Freidenfelds et al., 2012). This was done to avoid stress associated with weighing each lizard before dosing while ensuring that all animals received a CORT dosage within a limited range (from 0.6 to 1 µg CORT g^{-1} body

mass). A dosage within this range (0.8 µg g^{-1} body mass) resulted in circulating plasma CORT concentrations of approximately 77 ng ml^{-1} 30 min after application (Trompeter and Langkilde, 2011). This approximates plasma CORT concentrations in lizards 30 min following exposure to fire ants (61.90±7.98 ng ml^{-1}; Graham et al., 2017). To achieve the dosage range applied during the present study, the total amount of CORT an individual lizard received was adjusted regularly as determined by growth rates. During relatively rapid initial growth (weeks 3–24), adjustments in administered amounts of CORT were made every 5–6 weeks. Subsequent growth slowed (weeks 24–43) such that further adjustments were not necessary.

Lizards in the control treatment (n=11) were placed in an empty arena for 30 s and then received a topical dose of sesame oil. This sham experimental group served as a control for handling and stress effects associated with captive housing and the experimental manipulations. We selected this complex control condition rather than a simpler non-handled control condition that would only have controlled for effects of captivity.

We selected these lizards (n=32) for immune assays (described below) from a larger set of study animals to balance for age, site of mother's origin and sex (with priority given to age and site of mother's origin; Table 1) and also included these factors in analyses (described in 'Data analysis', below). The sample sizes in this study were constrained by the need to measure other parameters from a different subset of lizards; however, we were still able to detect effect sizes of relevance to these immune parameters.

Blood collection

Eight to 14 weeks after treatments ended, we collected blood from the post-orbital sinus of 32 lizards (5–6 per treatment) using 70 µl heparinized microhematocrit tubes (VWR, San Francisco, CA, USA). Blood samples were collected within 4.3 min of capture (mean±1 s.e. 116±8.9 s), and time to catch and bleed did not influence the immune parameters that we measured (see 'Data analysis', below; Table 2). We maintained blood samples on ice until blood collection was complete. Blood samples were centrifuged, and plasma was drawn off and immediately frozen (−20°C) in separate aliquots until immune assays were performed (24 h for hemagglutination assay; 4 days for bacterial killing assay; Graham et al., 2012a). Plasma can be sensitive to freezing, reducing its capacity to kill bacteria (Liebl and Martin, 2009). However, a pilot study using this species revealed an increase in bacterial killing ability of plasma from 1 day to 4 days of freezing (mean±1 s.e.m. percentage killing, 1 day 19±12%, 4 days 56±10%; paired t-test: t_8= −4.757, P=0.001). A similar lack of reduction in bacterial killing ability was observed in plasma frozen for 10 days (but not 20 days) in sparrows (*Passer domesticus*; Liebl and Martin, 2009) and up to 8 weeks in hellebenders (*Cryptobranchus alleganiensis alleganiensis*; Hopkins et al., 2016).

Table 1. Sample sizes of lizards that were used in immune assays conducted in adulthood (male, female)

	Uninvaded	Invaded	Total
Control	4,1	4,2	8,3
Fire ants	4+,1	3,2	7+,3
CORT	4,2*	1,4	5,6*
Total	12+,4*	8,8	20+,12*

Data show lizard sex distribution (M,F). Test animals were lab-reared offspring of field-caught lizards from fire ant-invaded or -uninvaded sites, and were exposed weekly during early life to a control condition, to fire ant attacks or to exogenous corticosterone (CORT). Asterisks indicate that one female was omitted from the bacterial killing analysis and crosses indicate that one male was omitted from hemagglutination analyses (described in Materials and Methods, 'Data Analysis').

Table 2. Parameters included in the full models for analysis of three parameters of immune function in adulthood: baseline hemagglutination of sheep red blood cells (SRBCs), post-inoculation hemagglutination of SRBCs and bacterial killing ability (BKA)

	Baseline SRBCs	Post-inoculation SRBCs	BKA
Maternal ID (random)	✓	✓	✓
Treatment	✓	✓	✓
Invasion status	✓	✓	✓
Treatment×invasion	✓		
Site (invasion)			
SVL			
Age			
Sex			
Bleed time		x	
Bleed order		x	
Baseline SRBCs	x	✓	x

Parameters not included in the original models are indicated with a cross, and those retained in the final models are indicated with a tick.

Hemagglutination assay

We measured baseline and acquired hemagglutination of lizard plasma to assess innate and adaptive humoral immune function, respectively (Matson et al., 2005). This assay measures the ability of lizard plasma to hold sheep red blood cells (SRBCs) in suspension *in vitro*. We first measured baseline hemagglutination, which is primarily driven by natural antibodies. SRBCs (Innovative Research, Novi, MI, USA) were washed with phosphate-buffered saline (PBS) and brought to a 2% solution with PBS. Plasma (25 µl) was plated on a 96-well plate randomly with respect to treatment, diluted 1:1 with PBS, and then serially diluted to 1:64 in a 96-well plate using a multichannel pipette. Control wells contained PBS only (25 µl). Then, 25 µl of 2% SRBC solution was added to each well, and the plate contents were mixed by gentle tapping. After incubation at room temperature for 1 h, plates were scored for agglutination. Scores were calculated as the negative $\log_2$ of the last dilution at which agglutination was attained – higher scores are associated with a stronger immune response (Matson et al., 2005). Half-scores were recorded when SRBCs precipitated partially but not to the extent of control wells. Lysis of erythrocytes was not scored as this process has been shown to be subjective (Graham et al., 2012b).

To measure acquired SRBC-specific antibody responses, after blood was collected for baseline assays, lizards were injected intraperitoneally with 50 µl of 25% SRBC solution using an insulin syringe and a 26 gauge needle. Lizards were then returned to their home enclosures for 15 days to ensure time for an appropriate antibody response (Graham et al., 2012a). We re-sampled lizards and calculated hemagglutination scores as described above.

Bacterial killing assay

We measured the ability of plasma to lyse *Escherichia coli* bacteria (American Type Culture Collection 8739; Epower Microorganisms, catalog no. 0483E7, MicroBiologics, St Cloud, MN, USA) as a coarse measure of innate immunity. Thawed plasma (14 µl) was mixed with *E. coli* (10 µl of 200 CFU bacteria dilution), and this solution was allowed to react for 1 h. This solution was then combined with a growth medium dilution [126 µl CO_2 L-glutamine, containing 400 µl L-glutamine (Thermo Scientific/Gibco catalog no. 25030081) and 19.6 ml CO_2 medium (Thermo Scientific/Gibco

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catalog no. 18045088)]. Each sample (50 µl) was spread on agar plates in duplicate and incubated at 37°C for 15 h. Control plates contained no plasma and an additional 14 µl CO_2 L-glutamine to create an equal volume (140 µl CO_2 L-glutamine medium+10 µl *E. coli*). Colonies on each plate were then counted, averaged (across duplicates) and compared with mean colony counts of four replicated control plates. Percentage bacterial killing was calculated as 100−(mean plasma treatment colony count/mean control colony count)×100. For detailed methods, see Graham et al. (2012a,b). Plates that ranged from 0 to −10% killing (n=4) were corrected to 0%. Plates with less than −10% killing (n=1) were discarded. Using the uncorrected points did not qualitatively change the outcome of the final model.

Data analysis

Percentage killing data were subjected to angular transformation prior to analysis to meet assumptions of parametric tests. One data point was omitted from analysis of both baseline and post-inoculation hemagglutination because it was >2 s.d. from the sample mean and one data point was omitted from the analysis of bacterial killing because it was >2 s.d. from the sample mean.

We analyzed hemagglutination of plasma and percentage killing separately using ANCOVA with treatment, fire ant invasion status, source population (nested within invasion status) and sex as factors, maternal ID as a random effect, and SVL and age as covariates (Table 2). Time to bleed and bleed order were included as a covariate for baseline hemagglutination and bacterial killing but were not available for post-inoculation hemagglutination scores. For SRBC-inoculated lizards, baseline hemagglutination score was included as covariates to account for initial variation in humoral activity. SVL, source population, sex, age, time to bleed and bleed order did not significantly explain variation (P>0.062) and were omitted from the final models (Table 2). In cases where interactions were non-significant, they were removed from the final model to preserve statistical power (Table 2). Cohen's f^2 (standardized local effect size) was calculated for predictors in the final models (Selya et al., 2012). By statistical convention, f^2≥0.02, f^2≥0.15 and f^2≥0.35 are interpreted as small, medium and large effect sizes, respectively (Cohen, 1988). Statistical analyses were performed using JMP (version 12.1, SAS Institute Inc., Cary, NC, USA) and SAS (version 9.4, SAS Institute Inc.) with α=0.05.

All methods used in this study adhere to the Guidelines for the Use of Animals in Research and the Institutional Guidelines of Penn State University (IACUC #35780), and animal collection was permitted by the respective states.

RESULTS

Hemagglutination

The effect of early-life treatment on adult baseline hemagglutination differed with the fire ant invasion status of the source population (Fig. 1; treatment×invasion $F_{2,18}$=5.576, P=0.013, f^2=0.366; treatment $F_{2,18}$=0.074, P=0.929; invasion $F_{1,10}$=0.675, P=0.430). Tukey's *post hoc* tests revealed that, among offspring of lizards from low-stress uninvaded populations, early-life CORT exposure suppressed baseline hemagglutination scores in adulthood relative to controls, but in offspring from invaded populations, early CORT exposure enhanced baseline hemagglutination scores. *Post hoc* analyses revealed no significant interaction of early-life fire ant exposure with population-level historical fire ant exposure.

Adult post-inoculation hemagglutination scores were greater in offspring of lizards from invaded versus uninvaded sites ($F_{1,8}$=9.007, P=0.016, f^2=0.358), but were not related to early-life

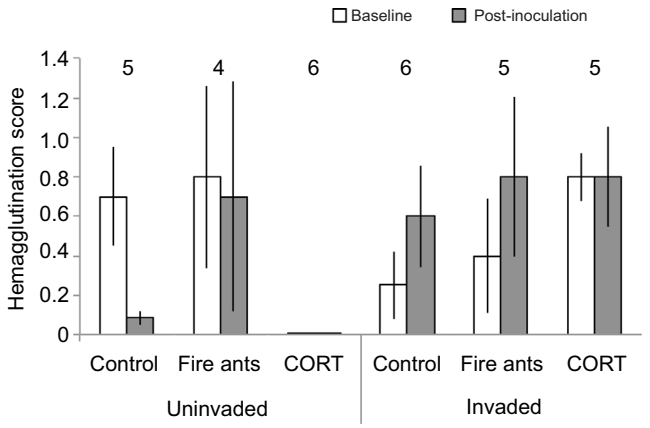

Fig. 1. Early-life and transgenerational history of stress exposure interact to affect adult baseline, but not post-inoculation, hemagglutination scores. In offspring of lizards from fire ant-uninvaded populations, corticosterone (CORT) exposure during early life suppressed adult baseline plasma hemagglutination compared with controls. The opposite effect was seen in offspring of lizards from fire ant-invaded populations: early-life CORT exposure enhanced adult baseline hemagglutination compared with controls (ANCOVA: early-life treatment×invasion status $F_{2,18}$=5.576, P=0.013, f^2=0.366). Post-inoculation hemagglutination scores were greater in offspring of lizards from invaded sites (ANCOVA: $F_{1,8}$=9.007, P=0.016, f^2=0.358) but did not differ across early-life stress treatments ($F_{2,14}$=0.256, P=0.777, f^2=−0.071; baseline hemagglutination covariate $F_{1,22}$=3.888, P=0.061, f^2=0.167). Bars represent means±s.e.m. and sample size for each group is shown above each set of bars.

treatment (Fig. 1; $F_{2,14}$=0.256, P=0.777, f^2=−0.071; baseline hemagglutination covariate $F_{1,22}$=3.888, P=0.061, f^2=0.167) or an interaction of these two factors (treatment×invasion $F_{2,12}$=0.074, P=0.929, f^2=−0.392; omitted from final model).

Bacterial killing assay

Adult percentage killing of *E. coli* by plasma was not related to early-life treatment (Fig. 2; $F_{2,21}$=0.018, P=0.983, f^2=−0.082), invasion status of the source population ($F_{1,15}$=1.788, P=0.201, f^2=0.035), or an interaction of the two ($F_{2,13}$=2.738, P=0.102, f^2=−0.088; omitted from final model).

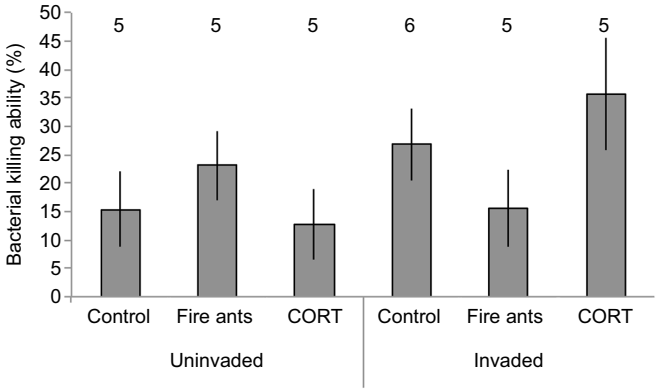

Fig. 2. Bacterial killing by plasma of adult lizards is not related to early-life or transgenerational history of stress. Offspring of lizards from fire ant-invaded and -uninvaded populations exposed weekly to fire ants, CORT or control treatment from hatching until maturity had a similar percentage bacterial killing ability of plasma in adulthood (ANCOVA: early-life treatment $F_{2,21}$=0.018, P=0.983, f^2=−0.082; invasion status $F_{1,15}$=1.788, P=0.201, f^2=0.035). Bars represent means±s.e.m. and sample size for each group is shown above each bar.

DISCUSSION

In offspring of lizards from fire ant-uninvaded populations, early-life exposure to CORT (but not to fire ants) suppressed baseline plasma hemagglutination in adulthood compared with scores in lizards in the control treatment. This finding is consistent with the suppressive effects of long-term and early-life stressor or glucocorticoid exposure on innate immune function in rodents, birds and humans (Michaut et al., 1981; Avitsur et al., 2006; De Coster et al., 2011; Kriengwatana et al., 2013; Schmidt et al., 2015; but see Grindstaff and Merrill, 2017). Development of the immune system is energetically costly (Klasing, 2004; Lochmiller and Deerenberg, 2000; Norris and Evans, 2000; Sheldon and Verhulst, 1996) and may not be prioritized if energy is limited as a result of early-life stress. In contrast, offspring from fire ant-invaded populations experienced enhanced baseline hemagglutination in adulthood following early-life CORT exposure compared with levels in controls. Offspring of lizards from invaded sites also had greater post-inoculation hemagglutination scores regardless of treatment. Enhancing immune function in response to early-life or transgenerational stress may be beneficial for lizards from these populations. Fire ants are a persistent predatory stressor (Graham et al., 2012a; Langkilde, 2009a) that cause wounding in these populations. Wounding happens directly, by fire ants breaking the skin when they bite and sting lizards (Tschinkel, 2006), and likely also indirectly, as lizards break crypsis in response to contact with fire ants (Langkilde, 2009a), making them vulnerable to attack by visual predators, as indicated by increased wounding (Thawley and Langkilde, 2017). The enhanced immune function we observed in these lizards may thus be adaptive, particularly innate immune function (e.g. baseline hemagglutination), which is essential for a rapid response to frequent wounding and infection (Murphy, 2001). Other environmental conditions at invaded versus uninvaded sites may also play a role in the observed immune investment differences [e.g. ectoparasite load (Graham et al., 2012a), disease prevalence] and should be explored. It is important to note that our sample sizes were low, and so results should be interpreted with caution. However, the effect sizes of the immune differences were considered large, even with this limitation.

The mechanisms behind the documented interaction between early-life CORT exposure and transgenerational stress exposure are unclear. Epigenetic processes (Fitzpatrick and Wilson, 2003; Mostoslavsky and Bergman, 1997; Teitell and Richardson, 2003) or the transfer of maternal immunological memory (Grindstaff et al., 2006; Hasselquist et al., 2012; Ismail et al., 2015) may play a role. Alternatively, maternal effects that influence other aspects of offspring phenotype (e.g. size, growth rate, dispersal ability, HPA activity; Meylan et al., 2002; Hayward and Wingfield, 2004; Vercken et al., 2007; Emack et al., 2008; Veru et al., 2014) may have indirect effects on how resources are allocated to immune performance. Finally, population-based differences in immune response to early-life CORT exposure may be driven by documented population-based differences in HPA responsiveness to early-life stress (McCormick et al., 2017). However, we have no evidence that hemagglutination scores are related to CORT responsiveness in individuals in this study (ANCOVA: $F_{1,22}$=0.141, P=0.711).

In contrast to the effects of CORT exposure in early life, exposure to fire ants in early life did not affect baseline hemagglutination scores. This could be because lizards habituated to the repeat exposure to fire ants (Cyr and Romero, 2009; Romero, 2004; Romero et al., 2009) or the fire ant colony became less venomous in captivity (Tschinkel, 2006; Xian-Fu et al., 2015). However, fire ant exposure did significantly elevate CORT levels 30 min after exposure in these lizards at the end of this study (McCormick et al., 2017), suggesting that neither of these was the case. Alternatively, it may be that immune-enhancing effects of CORT elevation after fire ant attack are counteracted by immune-suppressive effects of venom (Tankersley, 2008; Yi et al., 2003) or frequent wounding (Plaistow et al., 2003). It is also possible that the stress of captivity and/or handling masked potential effects of early-life stress associated with fire ant exposure.

Patterns of immune function in the field mirror the lack of immune modulation in response to early-life fire ant exposure documented in this laboratory-based study. In the field, baseline hemagglutination scores are similar for lizards at fire ant-invaded and -uninvaded sites (Graham et al., 2012a) despite the presence of both early-life and transgenerational exposure to fire ants at invaded sites. However, the results of this field study do not support the up-regulation of baseline hemagglutination of laboratory-raised lizards from invaded populations exposed to early-life CORT, or their greater post-inoculation scores, in the present study. Several possibilities could explain these seemingly conflicting findings. (1) CORT doses in the lab may have been greater than those elicited by fire ants in the field, even though we selected doses based on field lizard CORT responses to fire ants (Graham et al., 2017; Trompeter and Langkilde, 2011). (2) Younger lizards may up-regulate immune function in response to early-life CORT, as seen in this study, whereas older lizards may prioritize other traits, such as reproduction (reviewed in Forslund and Pärt, 1995). Adult lizards used in this study were smaller and likely younger than those surveyed in the field (mean±1 s.e. SVL: field-caught 63.4±0.46 mm, this study 52.6±7.04 mm; Graham et al., 2012a). (3) It may be that immune function in fire ant-invaded lizard populations would be up-regulated in response to stress but suppressed by extrinsic environmental factors in the field (e.g. frequent wounding, ectoparasite prevalence, temperature; Rios and Zimmerman, 2001), resulting in baseline CORT levels that are similar to those of lizards at uninvaded sites (e.g. Martin et al., 2011; Du et al., 2012). (4) Energy limitation in the field may prevent lizards at high-stress fire ant-invaded sites from up-regulating immune function. For example, in birds, early-life stress effects on immune function are only observed under energetically favorable conditions and not when energy demands are high (De Coster et al., 2011). (5) Experimental CORT application does not mimic the full physiological response to stressors (Seaward, 2006) and other aspects of stress physiology (e.g. noradrenaline response) that may offset effects of CORT exposure (Roozendaal, 2004). Further research into how these factors affect the interaction of within- and across-generation stress exposure is needed.

We observed modulation of innate (baseline hemagglutination) but not adaptive (post-inoculation) immunity following exposure to early-life CORT. This mirrors work in birds, in which early-life nutritional stress did not affect post-inoculation hemagglutination scores (Kriengwatana et al., 2013). However, hemagglutination scores in this study did not increase in response to SRBC injection, as would be expected if an adaptive response had occurred (Graham et al., 2012a; Ochsenbein and Zinkernagel, 2000; Zimmerman et al., 2010). The 15 day incubation should have been sufficient to allow for a significant antibody response in this species (as seen in field-caught lizards; Graham et al., 2012a). The adaptive immune system tends to be slower acting in reptiles than in mammals and birds; it can take 6–8 weeks to develop in reptiles (Zimmerman et al., 2010), and it may take longer to respond in younger animals (Hopkins and Durant, 2011; Palacios et al., 2009). Our younger lab-reared lizards may thus have taken longer to respond than our older field-caught lizards.

We did find that lizards from invaded populations had greater post-inoculation hemagglutination scores compared with those from uninvaded sites, suggesting a greater adaptive immune response in lizards that evolved in habitats with invasive wounding stressors.

In contrast to the effects of early-life CORT exposure on baseline hemagglutination, early-life exposure to CORT or fire ants did not affect bacterial killing ability of adult lizard plasma. This is supported by previous work on this species that revealed similar short-term effects of CORT exposure on baseline hemagglutination but not bacterial killing of *E. coli* (McCormick and Langkilde, 2014; McCormick et al., 2015). However, mixed results have been observed in birds (De Coster et al., 2011; Grindstaff and Merrill, 2017; Kriengwatana et al., 2013; Schmidt et al., 2015). This varied sensitivity to early-life stress may be a result of differential resource allocation to specific immune responses (e.g. complement versus cell-mediated versus antibody responses) in ectotherms compared with endotherms (Zimmerman et al., 2010), or due to species-specific effects of early-life stress on different immune components (von Hoersten et al., 1993; De Coster et al., 2011; Kriengwatana et al., 2013; Schmidt et al., 2015).

Conclusions

The results of this study demonstrate that exposure to stress-relevant hormones within a lifetime and across generations can interact to affect adult innate immune function; specifically, when the environmental stressor challenges the immune function. This is in contrast to our prior findings in this species where transgenerational stressor exposure, but not early-life stressor (fire ants) or CORT exposure, was shown to affect adult HPA activity (McCormick et al., 2017), morphology and survival (Owen et al., 2018). Together, these results suggest that transgenerational and early-life stressors affect different traits in different ways, and also serve as a caution that transgenerational history of stressor exposure likely plays an important role in determining adult phenotype. Future studies should consider the interactive effects of early-life and transgenerational stressors on adult phenotypes.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: G.L.M., T.R.R., S.A.C., T.L.; Methodology: G.L.M., T.R.R., S.A.C., T.L.; Validation: T.R.R., T.L.; Formal analysis: G.L.M., T.L.; Investigation: G.L.M., T.R.R.; Resources: T.L.; Data curation: G.L.M.; Writing - original draft: G.L.M., T.L.; Writing - review & editing: G.L.M., T.R.R., S.A.C., T.L.; Visualization: G.L.M.; Supervision: T.L.; Project administration: G.L.M., T.R.R., T.L.; Funding acquisition: S.A.C., T.L.

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Data availability

Data are available through The Pennsylvania State University's data repository, Scholarsphere: https://scholarsphere.psu.edu/concern/generic_works/bnz805z74m.

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