

History of predator exposure affects cell-mediated immunity in female eastern fence lizards, *Sceloporus undulatus* (Squamata: Phrynosomatidae)

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On exposure to stressors, energy is diverted from non-urgent functions towards those important for immediate survival. The degree and nature of reallocation may be affected by the evolutionary history of the animal. The eastern fence lizard (*Sceloporus undulatus*) coexists in parts of its range with invasive fire ants (*Solenopsis invicta*), which attack and wound lizards and elevate stress-relevant hormones (corticosterone), whereas other populations have never been exposed to fire ants. We examined how a history of fire ant invasion affected the immune response in female lizards after exposure to exogenous corticosterone (mimicking exposure to a stressor) during gestation (dosing regimens differed among corticosterone-exposed lizards owing to the constraints of the original studies, but we found no evidence that this affected the outcome of the present study). A history of exposure to predatory stressors (fire ants) and corticosterone treatment affected cell-mediated immunity. Lizards from fire ant-invaded sites had a reduced immune response compared with those from uninvaded sites. Corticosterone treatment had no effect on the immune response of lizards from invaded sites but reduced the immune response of lizards from uninvaded sites. This suggests that an evolutionary history of exposure to wounding alters the immune response to corticosterone. Future work on how the immune system responds to environmental threats will be informative for the prediction and management of these threats.

ADDITIONAL KEYWORDS: corticosterone – evolutionary history – fence lizards – fire ants – glucocorticoids – immunity – invasive species – phytohaemagglutinin – stress

INTRODUCTION

An animal's response to environmental, social and physical stressors often includes physiological alterations that involve energy reallocation (Sapolsky *et al.*, 2000), via changes to reproductive cycles (Wingfield & Sapolsky, 2003), digestion (Bhatia &

Tandon, 2005) and immunity (Franchimont, 2004; Tuckermann *et al.*, 2005). Important mediators of these modifications are glucocorticoid hormones, which are released during activation of the hypothalamic–pituitary–adrenal (HPA) axis to facilitate response to a stressor (Bateman *et al.*, 1989; Buchanan, 2000; Bonier *et al.*, 2009). Glucocorticoid-mediated energy reallocation can result in immunosuppression, especially of lymphocytic immunity (Tuckermann

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et al., 2005), which can have both immediate and long-term consequences for survival (Romero & Wikelski, 2001; MacLeod *et al.*, 2018). For example, although immunosuppression might be energetically favourable for immediate survival of a stressor, chronic (long-term) immunosuppression is disadvantageous in some environments (Lochmiller & Deerenberg, 2000), such as those that carry a high risk of wounding from encounters with predators (McCormick *et al.*, 2019).

An increasingly prevalent stressor is the presence of invasive species, which can act as novel predators in many systems (Langkilde *et al.*, 2017). Eastern fence lizards (*Sceloporus undulatus* Bosc & Daudin in Sonnini & Latreille, 1801) are one of many native species adversely affected by the introduction of a predator, the red imported fire ant (*Solenopsis invicta* Buren, 1972) (Langkilde, 2009), which was accidentally introduced into the USA in the 1930s in the southern portion of the range of the fence lizard (Callcott & Collins, 1996). Fire ants pose a predatory threat to eastern fence lizards; their stings are a frequent cause of skin trauma (Freidenfelds *et al.*, 2012) and can be fatal (Langkilde, 2009). Encounters are frequent where their ranges overlap (Freidenfelds *et al.*, 2012). Eastern fence lizards captured from sites invaded by fire ants display higher baseline concentrations of corticosterone (CORT), the primary glucocorticoid hormone in this species, and higher CORT concentrations when restrained, compared with those from uninvaded sites (Graham *et al.*, 2012). Even when raised in captivity, lizards originating from fire ant-invaded sites have elevated CORT responsiveness (McCormick *et al.*, 2017).

The classic understanding of CORT-induced effects on immunity would predict that fence lizards from fire ant-invaded sites are immunosuppressed, given the history of exposure to fire ants and associated increase in CORT concentrations (Coutinho & Chapman, 2011; Graham *et al.*, 2012, 2017). Alternatively, selection might have moderated the immune response to higher CORT concentrations, given the negative impacts of frequent immunosuppression on longevity (Lochmiller & Deerenberg, 2000; Møller & Saino, 2004; Martin *et al.*, 2005). There is evidence that this might be the case, because eastern fence lizard hatchlings from sites invaded by fire ants had increased natural antibody function when treated with CORT, whereas those from uninvaded sites exhibited the classic signs of CORT-induced immunosuppression (McCormick *et al.*, 2019). This would be consistent with an evolutionary perspective, because lizards from fire ant-invaded sites would suffer costs if immunity was generally reduced in response to CORT; lizards in these populations are frequently bitten and stung by fire ants, increasing CORT concentrations and also breaking their skin and leaving them at risk of infection (Graham *et al.*,

2012; McCormick *et al.*, 2019). Thus, it is possible that certain parts of the immune system might be maintained, or even upregulated, in response to CORT to reduce associated costs.

We examined the effects of an evolutionary history of exposure to a prevalent predatory stressor that causes frequent wounding (i.e. fire ants) and short-term CORT treatment on cell-mediated immunity in eastern fence lizards. Our goals were to determine: (1) the effects of evolutionary exposure to a wounding stressor on the cell-mediated immune response; (2) the effects of short-term CORT treatment on the cell-mediated immune response; and (3) how an evolutionary history of exposure to a wounding stressor might alter the effects of short-term CORT treatment on the cell-mediated immune response. We predicted that: (1) fence lizards from sites invaded a long time ago by fire ants would have a lower cell-mediated immune response than those from uninvaded sites, owing to wounding and associated CORT increases and suppression of lymphocyte function (Morici *et al.*, 1997; Tuckermann *et al.*, 2005); (2) lizards from uninvaded sites treated with CORT would have a lower cell-mediated immune response compared with the control group, resulting from the immunosuppressive effects of CORT; and (3) lizards from fire ant-invaded sites would show an unchanged or even upregulated immune response when treated with CORT, in comparison to the control group. Understanding how the evolutionary history of stressor exposure of an individual interacts with their immunological response to CORT will provide important insights into the health consequences of exposure to stressors and resulting CORT production.

MATERIAL AND METHODS

The animals used here were drawn from two separate studies run concurrently, but independently (MacLeod KJ, unpubl. data; Owen *et al.*, 2018). As a result, there were differences in the housing and handling of lizards from invaded vs. uninvaded sites. These differences, and how we accounted for them, are detailed in the relevant sections below.

FIELD CAPTURE

Gravid female eastern fence lizards ($N = 29$ from the invaded sites and $N = 29$ from the uninvaded sites) were captured by noosing or hand from six sites in the south-eastern USA. Four sites were in areas with long histories (55–70 years) of fire ant invasion ('invaded'): Geneva State Forest and surrounding areas ($31^{\circ}10'N$, $86^{\circ}20'W$, AL; $N = 6$), Conecuh National Forest ($31^{\circ}20'N$, $86^{\circ}60'W$, AL; $N = 20$), Gulf State Park ($30^{\circ}26'N$, $87^{\circ}64'W$, AL; $N = 1$) and Blakely

State Park (30°74'N, 87°91'W, AL; $N = 2$). Two sites were in locations that have not yet been invaded by fire ants ('uninvaded'): Standing Stone State Park (36°28'N, 85°25'W, TN; $N = 7$) and Land Between the Lakes National Recreation Area (36°50'N, 88°5'W, KY; $N = 22$).

ANIMAL HUSBANDRY

Snout–vent length (to the nearest 0.5 mm) was measured, and the lizards were transported to the Pennsylvania State University, where they were housed in plastic tubs (56 cm × 40 cm × 30 cm length × width × height). Each tub was furnished with a plastic water bowl, a shelter that served as a refuge and basking perch, and damp sand as a substrate for egg-laying when females reached late-stage gestation (determined by palpation; [Graham et al., 2012](#)). Females from fire ant-invaded sites were housed individually, whereas females from uninvaded sites were housed in pairs, according to the designs of their original respective studies (MacLeod KJ, unpubl. data; [Owen et al., 2018](#)). Lamps with 60 W bulbs were hung above one end of each tub to provide a thermal gradient for thermoregulation for 8 h each day. Overhead (room) lights were set on a 12:12 h light:dark schedule. Lizards were fed to satiation with vitamin-dusted (Zoo Med Reptivite with D₃ and Zoo Med Repti Calcium without D₃) crickets (*Acheta domestica*) on 3 days each week, and water was available *ad libitum*.

EXPERIMENTAL DESIGN

All female lizards were randomly assigned to either CORT or control treatment (invaded: CORT $N = 15$ and control $N = 14$; uninvaded: CORT $N = 13$ and control $N = 16$). CORT-treated females were dosed topically with a 4 mg/mL solution of CORT (Sigma C2505, St Louis, MO, USA) in sesame seed oil at a dosage of 0.8 µg CORT/g lizard body mass ([Trompeter & Langkilde, 2011](#)). This solution is rapidly absorbed through the skin into the bloodstream ([Belluore et al., 2004](#)) and results in an increase in plasma CORT concentrations to approximately twice that of baseline values 30 min after administration; levels return to baseline within 90 min ([MacLeod et al., 2018](#)). This dosage and application method effectively increases fence lizard plasma CORT equivalent to the increase in CORT seen with fire ant encounters ([Graham et al., 2017](#)). Females in the control group received the sesame seed oil vehicle control only. CORT and control solutions were applied to the middle of back of each lizard between 19:00 and 21:00 h, after lizards had become inactive for the day, to avoid the need to handle the animals. Females from invaded sites were dosed with CORT or vehicle control every other day,

and females from uninvaded sites were dosed every day owing to logistic constraints associated with the original studies (MacLeod KJ, unpubl. data; [Owen et al., 2018](#)). Treatment ceased when eggs were laid, with females from invaded sites being dosed for an average of 35.5 ± 16.7 days and females from uninvaded sites being dosed for an average of 23.13 ± 12.92 days. Maternal mass and the mass of resulting clutches were measured (to the nearest 0.01 g) immediately after laying.

TESTING IMMUNE FUNCTION

The phytohaemagglutinin (PHA) skin test was used to measure the cell-mediated immune response of these lizards 24 h after laying. This test is a good measure of cell-mediated immunity in the skin, which is likely to be particularly important to organisms exposed to frequent skin wounding, such as from fire ant attacks. We used PHA-L, which preferentially stimulates a T-lymphocyte response, the magnitude of which is determined by measuring the swelling of the injected area ([Tylan & Langkilde, 2017](#)). We randomly selected either the left or the right hindfoot, measured the thickness of this foot in triplicate with a pressure-sensitive micrometer (Mitutoyo 7301 dial thickness gauge; to the nearest $0.01 \text{ mm} \pm 15 \text{ µm}$), then injected the foot pad subcutaneously with 10 µL of 2 mg/mL PHA-L (L2769; Sigma-Aldrich). Twenty-four hours after injection, the thickness of the injected foot was measured again in triplicate.

STATISTICAL ANALYSIS

Triplicate measures of foot thickness were averaged, and the difference between pre- and post-injection foot thickness was calculated to determine the magnitude of the swelling response. Swelling responses were Johnson SI transformed to meet assumptions of normality. Body condition was calculated as the residuals of a linear regression of the logarithm of snout–vent length and the logarithm of post-partum mass.

To determine the effects of the invasion status of the site of capture of a lizard and of CORT treatment on cell-mediated immunity, we used an analysis of covariance (ANCOVA), with the magnitude of the PHA swelling response as the dependent variable and fire ant invasion status (invaded vs. uninvaded), treatment (CORT vs. control) and an interaction between invasion status and treatment as factors. We included as covariates in initial models body condition, clutch mass, date of PHA skin test, number of doses of CORT or vehicle control the lizard had received, number of days since the last dose and the site of capture nested within invasion status. Body condition and clutch mass were included as measures of body

resources and reproductive allocation, which can alter the immunological effects of CORT treatment (French *et al.*, 2007) and the date of the PHA test was included to account for possible effects of season (Huyghe *et al.*, 2010). The number of CORT or vehicle control doses and the number of days since the last dose were included to account for the effects of overall CORT dosage and recency of treatment, respectively, on the immune response. Only the number of days since the last CORT or vehicle control dose contributed significantly to explaining the variation in our data; the other covariates were omitted from final models to preserve degrees of freedom. Our final model thus included only the site of capture nested within invasion status, and the number of days since the last CORT or vehicle control dose, in addition to the factors of fire ant invasion status, treatment and their interaction.

We used LS Means Contrast, a post-hoc test which compares specific factors of interest while avoiding the loss of statistical power associated with including uninformative comparisons. In order to determine the effect of invasion status on the PHA swelling response, we set LS Means Contrasts to compare the swelling of invaded and uninvaded lizards within each treatment (i.e. effect of invasion status within control lizards and within CORT-dosed lizards separately, rather than comparing invaded control lizards with uninvaded CORT-dosed lizards, for example). To determine the effect of treatment on the PHA swelling response, we set LS Means Contrasts to compare the swelling

of CORT-treated and control lizards within each invasion status (rather than comparing control lizards from invaded sites and CORT-treated lizards from uninvaded sites, for example). Statistical analyses were performed using JMP Pro (v.12.0.0; SAS Institute Inc., Cary, NC, USA), with $\alpha = 0.05$.

RESULTS

Female lizards from fire ant-invaded sites had a lower swelling response to PHA injection than lizards from uninvaded sites (ANCOVA, invasion status: $F_{1,49} = 7.51$, $P < 0.01$; LS Means Contrast, invasion status: $F_{2,49} = 4.95$, $P = 0.02$; Fig. 1). Corticosterone treatment reduced the swelling response to PHA (ANCOVA, treatment: $F_{1,49} = 5.92$, $P = 0.02$). There was no interaction between CORT treatment and invasion status in the overall model that included all comparisons, including those which were uninformative (see 'Statistical analysis' section; ANCOVA, treatment \times invasion status: $F_{1,49} = 0.72$, $P = 0.40$). However, when assessed using post-hoc LS Means Contrasts, the effect of CORT on PHA-induced swelling differed between lizards from fire ant-invaded and uninvaded sites. Within invaded sites, there was no difference in the swelling response between treatment groups (LS Means Contrast, treatment within invaded sites: $t = 1.17$, $P = 0.27$), whereas in uninvaded sites, CORT-treated lizards had a lower swelling response than those treated with vehicle control (LS Means

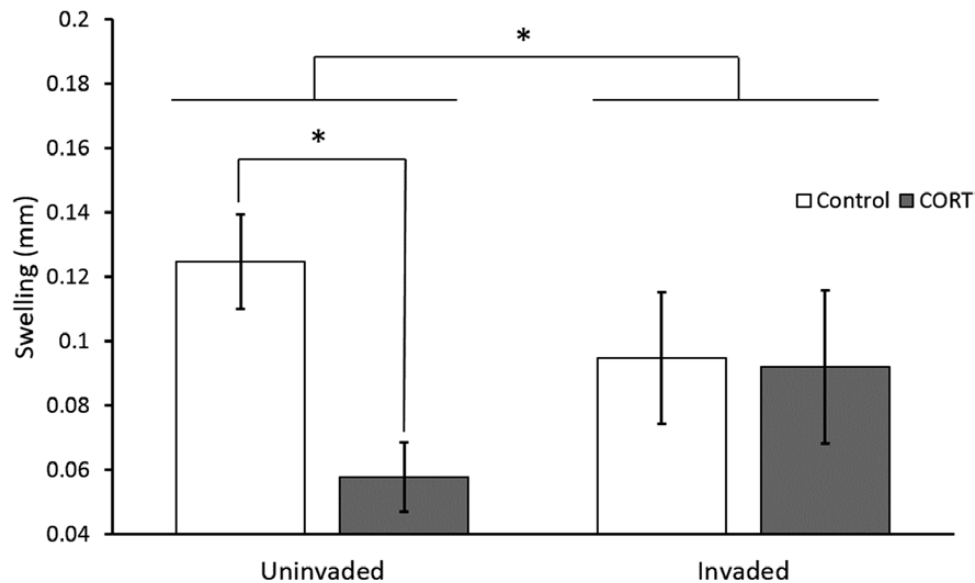


Figure 1. The swelling response to injection of phytohaemagglutinin in lizards from sites invaded by fire ants and uninvaded sites that were treated with corticosterone (grey bars) or a vehicle control (white bars). Swelling was determined as the difference in foot thickness measured before and 24 h after injection with phytohaemagglutinin. Bars represent means ± 1 SE. *Indicates a statistically significant difference, $P \leq 0.05$.

Contrast, treatment within uninvaded sites: $t = 2.31$, $P = 0.03$; Fig. 1).

Phytohaemagglutinin-induced swelling did not vary among capture sites with the same invasion status (ANCOVA, site nested within invasion status: $F_{4,49} = 1.20$, $P = 0.32$), but was lower in lizards that had been dosed more recently with CORT or vehicle control (ANCOVA, number of days since last dose: $F_{1,49} = 7.80$, $P < 0.01$).

DISCUSSION

The effects of CORT on the immune response of the lizards differed according to their history of exposure to fire ants. Lizards from sites with long histories of fire ant invasion and associated wounding and elevated CORT concentrations (Graham *et al.*, 2012; McCormick *et al.*, 2017, 2019) had lower swelling responses to PHA than did lizards from sites not yet invaded by fire ants. Corticosterone-treated lizards from uninvaded sites had a lower swelling response to PHA than did control-treated lizards from the same sites, which is consistent with the current understanding of the immunosuppressive effects of CORT (Tuckermann *et al.*, 2005). However, among lizards from fire ant-invaded sites, CORT-treatment and control-treatment groups did not differ in the swelling response to the PHA skin test.

The CORT treatment schedule in this study differed between lizards from fire ant-invaded and uninvaded sites; lizards from invaded sites were dosed with CORT every other day, whereas lizards from uninvaded sites were dosed every day. Thus, in lizards from invaded sites the lower-frequency dosing regimen might have resulted in reduced immunological effects of the CORT treatment. However, this does not appear to confound our results for the following reasons. Whether females were dosed every day or every other day, the total number of CORT doses administered did not affect the PHA swelling response. Additionally, alternate-day and every-day dosing of gravid lizards from invaded sites had similar effects on hatching success and hatchling morphology (Ensminger *et al.*, 2018; MacLeod *et al.*, 2018; MacLeod KJ, unpubl. data). Within lizards from fire ant-invaded sites (dosed every other day), those dosed more recently exhibited a decreased PHA-induced swelling response (ANCOVA, number of days since last dose: $F_{1,23} = 5.26$, $P = 0.03$); however, this was also true for lizards that received vehicle control only, indicating that apparent differences in response owing to recency of dosing are not attributable to differences in exposure to CORT, but instead some other effect associated with the oil or its application. Taken together, this suggests that the differences we saw in the PHA swelling response between lizards from fire ant-invaded and

uninvaded sites were likely to be attributable to factors other than the dosing regimen.

Housing also differed between lizards from fire ant-invaded and uninvaded sites, with females from invaded sites being housed individually and females from uninvaded sites in pairs (MacLeod KJ, unpubl. data; Owen *et al.*, 2018). Although it is possible for this difference to have affected our results, it is unlikely, because other differences in husbandry and enrichment have no effect on CORT concentrations and other measures of wellness in this species (Rosier & Langkilde, 2011). Additionally, females do not compete for thermal resources when housed in these conditions (Assis BA, unpubl. data; MacLeod KJ, unpubl. data), and sufficient food was provided to account for any (mild) competition for prey.

The reduction in PHA swelling response in lizards from fire ant-invaded sites can be attributed to immunosuppression from chronic stress and associated CORT (Tuckermann *et al.*, 2005; Dhabhar, 2006). Downregulation of the immune response after chronic increases in CORT (in this case, associated with frequent encounters with fire ants; Graham *et al.*, 2012, 2017) is also seen in other species, both within a lifetime and across generations. For example, in tree lizards, chronic handling stress impairs wound healing (French *et al.*, 2006), and the offspring of snowshoe hares exposed to increased predation risk have decreased eosinophil counts (Sheriff *et al.*, 2010). These results support potential immune consequences of CORT exposure across contexts and time scales, suggesting strong selective pressure for and the adaptive potential of the 'emergency life history' stress response (Wingfield *et al.*, 1998; Boonstra, 2013).

In addition to reductions in the immune function of lizards from fire ant-invaded vs. uninvaded sites, we found a difference in CORT responsiveness of the immune system of lizards from sites with different fire ant histories. Lizards from uninvaded sites showed the expected immunosuppression in response to CORT administration (Lochmiller & Deerenberg, 2000; Norris & Evans, 2000; Martin *et al.*, 2008), whereas CORT administration did not suppress the immune response of lizards from fire ant-invaded sites. This might be explained by some minimal level of immune function being necessary for survival (Martin *et al.*, 2008; Zimmerman *et al.*, 2010). Pathogen invasion is particularly likely when an animal is wounded, such as from fire ant bites and stings. Wounding is thus likely to be a potent selective pressure for the maintenance of immune function and might explain why lizards from invaded sites experienced no immunosuppression in response to CORT application. Within sites invaded by fire ants, the history of chronic CORT increase might have reprogrammed the immune response of the lizards to increases in CORT, preventing further

immunosuppression. This might be a response that lizards have developed within their lifetime, after repeated encounters with fire ants, or a product of multigenerational exposure to fire ants (Langkilde, 2009; Trompeter & Langkilde, 2011; Schrey *et al.*, 2016; McCormick *et al.*, 2017), and future research should focus on determining the mechanism underlying this pattern.

Alterations to immune function in response to CORT may vary between different aspects of the immune system, and possibly between life stages. The reduction of the immune response to CORT in lizards from fire ant-invaded sites was seen here in response to injection with PHA, which induces a cell-mediated immune response in skin (Tylan & Langkilde, 2017) and might be particularly relevant to skin wounding that results from fire ant attacks. In a previous study, eastern fence lizard hatchlings from uninvaded sites reduced natural antibody function when treated with CORT, whereas hatchlings from fire ant-invaded sites increased natural antibody function (McCormick *et al.*, 2019). In another study, natural antibody function was upregulated in CORT-treated adults from both invaded and uninvaded sites (McCormick & Langkilde, 2014). Natural antibodies might be useful for resisting the effects of fire ant envenomation, and thus the early adoption of a CORT-mediated increase might be adaptive in hatchlings from fire ant-invaded sites and is complemented by a maintained cell-mediated immune response in the face of CORT treatment. However, it is possible that measuring other tissues or immune functions that are less useful in responding to fire ant attacks might reveal the classic response of immunosuppression to treatment with CORT. Such immunosuppression might even be increased in severity to account for the preserved or enhanced energy allocated to fire ant-relevant immune functions.

Our results show that a history of exposure to stressful encounters with invasive fire ants and associated elevations in CORT is correlated with reduced cell-mediated immunity in eastern fence lizards. Additionally, within-lifetime exposure to CORT reduces cell-mediated immunity in this species, although this might differ according to the history of exposure to fire ants; lizards from sites invaded by fire ants exhibited a lower swelling response than did lizards from uninvaded sites, but did not show further immunosuppression when treated with CORT, unlike lizards from uninvaded sites. This suggests that a history of exposure to a stressor can alter immune function and energy reallocation by CORT. Energy reallocation in response to CORT and the effects of a history of exposure to wounding and associated stress on this reallocation can be complicated and may vary across different branches of the immune system. By preserving investment in cell-mediated immunity and

other immune functions crucial for survival in their particular environment, animals may increase their survival in the face of frequent wounding. Future examination of different immune functions, such as using wound healing or phagocytic response assays, would be important to enhance our understanding of this complex response.

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