INTERACTIONS BETWEEN INNATE IMMUNITY, STEROID HORMONES, AND BODY CONDITION IN FEMALE FENCE LIZARDS

(SCELOPORUS UNDULATUS)

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ABSTRACT

This study tests for potential interactions between innate immunity, the steroid hormones corticosterone (CORT) and progesterone, and body condition in female Eastern fence lizards (*Sceloporus undulatus*). Seasonal changes were investigated during the active season and we tested for an affect of one-hour confinement on innate immunity. Innate immunity was measured by an individual's plasma bacterial killing capacity, total circulating leukocyte counts, and differential circulating leukocyte percentages. Baseline circulating blood or plasma CORT concentrations positively correlated with percent monocytes but not the other immune measures. CORT increased with confinement but failed to elicit a change in immune function. We observed a seasonal change in percent lymphocytes, which peaked in the spring, percent heterophils, which peaked in the summer, and ectoparasitic mites, which peaked in late spring. Overall, our predictions that baseline steroid hormones would be immune suppressing and that one-hour confinement would be immune enhancing were not strongly supported.

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CHAPTER I

INTRODUCTION

Purpose of study

The immune system of an animal and their adrenal stress response are essential components of maintaining homeostasis and survival. The immune system can respond to a diversity of stimuli from environmental, behavioral, or physiological events (Coe 2002), and an organism's ability to respond to these stimuli and sustain the energetically costly functions of the immune system can also be influenced by numerous factors (Demas *et al.* 2012) such as reproductive state (French and Moore 2008), body condition (Berger *et al.* 2005), steroid hormone concentration, and seasonal changes (Saad and El Ridi 1988). A survey of the current literature shows a need for context dependent and species-specific evaluations of interactions between immunity, steroids, and measures of body condition (Berger *et al.* 2005; French *et al.* 2006; Roberts *et al.* 2007; Butler *et al* 2009). The purpose of this study is to test for interactions among innate immunity, steroid hormones, stress, and body condition in female *Sceloporus undulatus* during the active season (Fig. 1).



Figure 1. Interactions among stress, reproduction, body condition, and immunity.

Stress and immunity

Animals regularly face stressors and perturbations throughout their lifetime that can threaten fitness and survival. Common stressors include predators, conspecific interaction, and changing environmental or physiological conditions. An organism's response to the disruption of homeostasis is essential to survival (Sapolsky 2002). Glucocorticoid release from the adrenal gland is part of the physiological response to stress and can act by altering behavior (DeNardo and Sinervo 1994; Thaker et al. 2009), mobilizing energy stores, and suppressing functions, such as growth and reproduction, that are not necessary for immediate survival (Moore and Jessop 2003). The acute, or short term, glucocorticoid stress response is a means of promoting survival through maintaining homeostasis (Nelson and Demas 2004; Angelier and Wingfield 2013). The function of glucocorticoid release can have preparative actions, which prepare an organism for anticipated stressors, or modulating actions, which mediate the response once a stressor is presented (Sapolsky et al. 2000). The function of the acute stress response differs in comparison to that of chronic stress. Chronic stress can be difficult to identify (Dickens and Romero 2013) but is generally characterized by the prolonged exposure to elevated circulating glucocorticoids, which can have detrimental effects on an organism (Sapolsky et al. 2000; Nelson and Demas 2004).

Corticosterone (CORT) is the primary glucocorticoid in reptiles secreted in response to stress (Wilson and Wingfield 1994). Seasonal changes in CORT circulation correlate with changes in immunity, often with the most robust immune activity found when concentrations of CORT are the lowest (Saad and El Ridi 1988). Elevated CORT levels have been associated with a decrease in body condition and several measures of immune function (Berger *et al.* 2005), including decreased circulating leukocytes (Morici *et al.*1997), slower wound healing rate (French *et al.* 2006), and a suppression of bacterial killing capacity (French *et al.* 2010). Glucocorticoids can have a pronounced affect on leukocyte profiles, often increasing heterophils and decreasing lymphocytes, and a high heterophil to lymphocyte ratio (H:L) often occurs in correlation with high glucocorticoid levels in several animal taxa (Davis *et al.* 2008).

Glucocorticoids, however, are not exclusively immunosuppressive. Some studies report immunoenhancing effects (Roberts *et al.* 2007), or no effect (Butler *et al.* 2009) of increased CORT circulation, suggesting the need for a context-dependent analysis of how glucocorticoids interact with the immune system. Short-term stressors, and the increases in plasma CORT concentrations that follow, are often immune enhancing (Coe 2002; Seddon and Klukowski 2012), while long-term stressors and CORT exposure tend to be immune suppressing (Coe 2002; Martin 2009). In Martin's (2009) review of stress and immune function, the energy savings of down regulating the immune system are discussed as being beneficial only over long periods of time. Immune suppression can be a slow process requiring apoptosis, which is itself energetically expensive (Martin 2009).

Reproduction, body condition, and immunity

Many reptilian species, including *Sceloporus undulatus* (Phillips and Klukowski 2008), have a peak in baseline glucocorticoid levels during the breeding season (Wilson and Wingfield 1994; Woodley and Moore 1999; Romero 2002; Moore and Jessop 2003; John-Alder *et al.* 2009). Reproductive state has been observed to further influence baseline CORT concentrations in female fence lizards with the highest levels found in

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gravid individuals (M Klukowski, pers comm.). The mass of reproductive tissues has similarly been positively correlated with increased CORT (Wilson and Wingfield 1992). By shifting energy allocation and mobilizing glucose, CORT can facilitate certain aspects of reproductive behavior and physiology (Moore and Jessop 2013). Elevated CORT during reproduction could represent chronic stress or a facilitating activity in which elevated CORT helps mobilize glucose to meet the intense energetic demands of reproduction. Moderate elevations of plasma CORT can facilitate reproduction while more extreme elevations of CORT are likely inhibitory (Moore and Jessop 2003).

When under chronic stress, decisions about energy allocation must be confronted (Nelson and Demas 2004), and an organism is faced with potential trade-offs between maintenance, growth, reproduction, and immunity (Demas et al. 2012). With a finite amount of resources to be utilized, reproduction, growth, and immunity can encounter difficulties in functioning optimally when stress is increased (Nelson and Demas 2004). Reproduction, the stress response, and the immune system are all crucial to an organism's fitness and survival, which presents a challenge in understanding how resources are allocated to each of these energetically costly and essential processes. The presence of trade-offs has been identified between reproduction and immunity (French and Moore 2008; French et al. 2007a; French et al. 2007b), growth and immunity (Uller and Olsen 2003), and activity level and growth (John-Alder et al. 2009) in reptiles. Studies have also demonstrated that these compromises are made primarily during the most energyintensive times (French and Moore 2008; Ruiz et al. 2011) or when food sources are limited (French et al. 2007b), suggesting that the interactions among steroids, stress, and immunity are not always limited to this model of energy allocation as a trade-off. The

decision that animals make about resource allocation and expenditure is often dependent upon their ecological context, including current energy stores and seasonal patterns of energy fluctuation (Demas *et al.* 2012). One example includes reproduction of seasonally breeding animals, which occurs in synchrony with an increased availability of resources and optimal environmental conditions (Nelson and Demas 2004). In contrast, when resources become limited during winter months, energy is often expended on enhancing the immune system and diverted away from reproduction (Nelson and Demas 2004).

Measures of body condition, such as length-adjusted mass, plasma triglycerides, and maximum girth, can provide valuable insights into an animal's physiological condition. When times of high energetic demands are faced, glucocorticoids help meet the demands by mobilizing glucose, potentially lowering measures of body condition and reducing body fat, which can in turn impair immunity (Demas *et al.* 2003). Body mass residuals, a measure of body condition, have been negatively correlated with both baseline plasma CORT concentrations (Berger *et al.* 2005) and experimentally elevated plasma CORT concentrations (Hews and Baniki 2013). Poor body condition has also been associated with an increased acute CORT response (Berger *et al.* 2005). Elevated plasma triglyceride levels may reflect either the mobilization of stored energy that is needed to support vitellogenesis, or recent feeding activity.

Seasonal changes in steroids and immunity

The immune system responds to many stimuli, including hormones, that are not stress related (Coe 2002). Among seasonally breeding animals, seasonal changes in sex hormone concentrations are widespread (Wingfield *et al.* 1987; John-Alder *et al.* 2009).

Changes in the immune system occur seasonally (Nelson and Demas 2004) and have been associated with the annual fluctuation of sex steroid hormones (Casto *et al.* 2001). In reproductive males, correlations have been observed between seasonally and experimentally increased testosterone and immune suppression, possibly through the glucocorticoid increase that follows testosterone increase (Casto *et al.* 2001; Klukowski 2011). Experimentally increased testosterone has also been found to suppress immune function in both non-reproductive male and female starlings (Duffy *et al.* 2000). In female reptiles, a correlation has been observed between increased progesterone and decreased innate immunity (Graham *et al.* 2011). The immune system is often suppressed during reproduction (Saad and El Deeb 1990; Graham *et al.* 2011) and enhanced during the nonbreeding season (Coe 2002; Nelson and Demas 2004). Changes in immunity can also be specific to certain reproductive stages that require the most amount of energy, as has been demonstrated by a decrease in wound healing ability during vitellogenesis for female tree lizards (*Urosaurus ornatus*) (French *et al.* 2008).

Adrenal hormones and sex hormones can dually influence the immune system and seasonal changes occur in both steroid hormone concentrations and immunity (Coe 2002). One cause of seasonal change is photoperiod, or day length, which has significant effects on multiple parameters of the immune system (Nelson and Demas 1996; Garcia *et al.* 2010). Just as steroids can influence the immune system, the immune response can influence steroid hormone secretion by activating the hypothalamic-pituitary-adrenal (HPA) axis (Ashley and Wingfield 2012), possibly to mediate the magnitude of immune response and initiate sickness behavior (Coe 2002).

Study objectives

Two studies were conducted during the active season of female fence lizards, Sceloporus undulatus. In 2011, the study objectives were to determine the potential interactions between innate immunity, steroid hormones, and body condition and to investigate the seasonal changes among these variables. Due to the energetic demands of reproduction in female lizards, I predict a down regulation of the immune system via decreased circulating leukocytes and bacterial killing capacity that is associated with increased steroid hormone concentrations and decreased measures of body condition. Alternatively, immunosupression might only be evident in gravid females as indicated by higher baseline CORT levels, lower progesterone, greater length-adjusted mass, greater maximum girth, and decreased triglyceride levels. Additionally, an influence of season on immunity and body condition would be expected due to seasonal changes in the availability of resources as well as reproductive physiology and behavior. In addition to the above objectives, the purpose of the 2012 study was to determine the effects of restriction stress on innate immunity and the role steroid hormones play in modulating innate immunity. Seasonal changes were not investigated in the 2012 study. Short-term stressors have been reported to have immune enhancing effects (Coe 2002; Seddon and Klukowski 2012) and I predict a similar change in innate immune function, observed as an increase in circulating leukocytes levels and an increase in bacterial killing capacity.

CHAPTER II

MATERIALS AND METHODS

Design and overview of project

To test for potential interactions between innate immunity, steroids, stress, and body condition, adult female fence lizards, *Sceloporus undulatus*, were captured and bled over the course of two field studies in 2011 and 2012. The first year focused on testing for possible associations between innate immunity, plasma steroid hormone concentrations, and measures of body condition. In the second year, females were additionally subjected to an acute restriction (confinement) stress to test for effects on innate immunity and plasma steroid concentration.

Animals and study site

Eastern fence lizards, *Sceloporus undulatus*, are widely distributed across southern and eastern North America (Conant and Collins 1998). *Sceloporus undulatus* are phrynosomatid lizards with a maximum snout-vent length (SVL) of 86 mm (Jones and Droge 1980; Conant and Collins 1998). Adult male fence lizards are territorial and engage in courtship with females and aggressive encounters with other males (Haenel *et al.* 2003). Female eastern fence lizards reproduce continuously during the late spring and summer months with average clutch sizes of 7-12 (Ferguson and Brockman 1980; Parker 1994). Fence lizards prey on a diversity of arthropods with larger individuals ingesting larger prey (Jameson *et al.* 1980; Parker 1994). Adult female fence lizards were collected at three sites in Middle Tennessee: Flat Rock Nature Preserve (35°51'N, 86°23'W), Cedars of Lebanon State Forest (36°12'N, 86°19'W), and Barfield Crescent Park (35°77'N, 86°41'W). Each site had characteristics consistent with a cedar glade environment: exposed limestone surrounded by dense herbaceous growths and patches of cedar trees. Lizards were collected in close proximity to the main hiking trails by noosing.

2011 Associations between steroids, condition, and immunity

In 2011, forty-one adult female lizards were captured and bled as quickly as possible to test for potential associations between steroids, measures of body condition, and innate immunity. All females were bled within 3 min of capture (Mean: 137 sec; Range: 60-180 sec). A blood smear was made in the field, and females were measured (see below) and then released at the capture site. The plasma concentration of corticosterone and progesterone was determined via enzyme-linked immunosorbant assay (ELISA). A bacterial killing assay, differential leukocyte counts, and total leukocyte counts were conducted to measure innate immunity of the individual lizard. Body condition measures included the plasma triglyceride levels, maximum girth, and length-corrected mass of an individual. To test for seasonal effects on body condition, immunity, steroids, and ectoparasites, individuals were divided in four groups based on collection date: early spring (May 25 - June 8; n = 14), late spring (June 13 - 24; n = 16), summer (July 12 - 13; n = 6), and fall (Sept.10 - 24; n = 5).

2012 Effect of restriction stress

In 2012, twenty-four adult female lizards were divided into two treatment groups, baseline or confined. Confined females were placed in a cloth bag for 1 hour prior to bleeding. Baseline females were bled within 3 min of capture (Mean: 149 sec; Range: 60-180 sec). Treatment group was assigned by alternating baseline and confined. Gravid lizards

(n = 4) were equally divided between treatment groups to account for potential effects of reproductive state on immunity and steroid hormone levels. Gravidity was determined by palpation of eggs and visual inspection.

General lizard processing

All blood samples were collected between the hours of 9:30 and 13:30 to control for potential circadian rhythms in hormone levels, triglyceride levels, and innate immunity. All lizards that were bled weighed a minimum of 7.5 g or had a snout vent length (SVL) of at least 64 mm. Blood was collected from the postorbital sinus in heparinized microcapillary tubes. Immediately after collecting a blood sample a blood smear was made for each individual. Once collected, blood samples were kept on ice in a cooler until they were centrifuged in the lab to extract plasma which was stored at -80°C.

Each lizard was measured for weight (nearest 0.1 g), snout-vent length (SVL) (nearest 0.5 mm), tail length, maximum girth (see below) and ectoparasite load (ticks and chiggers). Ectoparasites were visually counted in the field by use of a magnifying glass. Mites were found primarily behind the ears on the side of the head and between the legs and body of the lizard. Ticks were less frequently found in the same locations.

Measures of innate immunity

Bacterial killing assay

The bacterial killing assay measures the ability of a host to eliminate a pathogen, providing an assessment of immune function (Liebl and Martin 2009, Demas *et al.* 2011). Following the protocol of S. French (pers comm) and Leibl and Martin (2009), *Escherichia coli* was used as the pathogen (Microbiologics, ATCC #8739) to measure the bacterial killing capacity of lizard plasma. In brief, 30 µl of lizard plasma (previously diluted to 8.85 x in PBS) was mixed with 5 µl of *E. coli* (1 x 10^{5} /ml) on a 96-well plate and incubated for 30 min at 37° C. After this killing step, tryptic soy broth was added and the initial absorbance read at 340 nm. After 12 hr of incubation at 37° C the final absorbance was recorded. Bacterial killing capacity was calculated as: [1 - (mean abs. change of sample / mean abs. change of positive control)] x 100Negative controls (PBS and tryptic soy broth but no *E. coli*) were run on each plate.

Leukocyte counts

Leukocyte counts were done on individual blood smears that had been prepared in the field immediately after blood collection. Blood smears were air dried before storage in the field, fixed in methanol the day collected, and stored until Giemsa staining (J.T. Baker Co.) for 75 min at the end of each field season.

Differential leukocyte counts were done manually with a microscope at 600x and 1000x with oil immersion. A total of 150 leukocytes were counted and scored as heterophils, basophils, eosinophils, lymphocytes, and monocytes (Campbell and Ellis 2007). Monocytes were further identified as monoblasts, mature monocytes, or

developing monocytes. Heterophils were large, round cells, identified by the presence of a dark staining, eccentrically located, multi-lobed nucleus, and granulated cytoplasm. Although infrequently observed, basophils were identified by a dark staining, highly clustered mass of granules in the cytoplasm. Eosinophils were similar in structure to heterophils but differed in their staining of cytoplasmic granules to an orange-red color. Monocytes were easily identified by their size in comparison to surrounding cells. The shape was consistently round with a large, round, offset nucleus. The nucleus of developing monocytes resembled the lobes of heterophils but lacked the granulated cytoplasm. Monoblasts were scored as the largest and most round of the monocytes. The distinction between monocytes, monoblasts, and developing monocytes was not used in the statistical analysis. Lymphocytes were identified by their relatively small size, dark blue staining cytoplasm, and a large, purple staining nucleus. Lymphocytes were the only leukocytes smaller than surrounding erythrocytes.

Total leukocytes were counted as the number of leukocytes per 1×10^4 red blood cells (RBC). Total leukocyte counts were completed at 400x using ImageJ (http://rsb.info.nih.gov/ij/) to count cells. The threshold adjustment feature highlighted the nucleus of each red blood cell and counted the number of red blood cells per image. The threshold was adjusted low enough to exclude leukocyte nuclei. Leukocytes were then manually counted for each image to calculate total leukocytes per 1×10^4 RBC.

Plasma hormone assays

Baseline levels of two steroids known to influence immunity, corticosterone and progesterone, were measured via commercially available enzyme-linked immunosorbant assay (ELISA) kits (Corticosterone EIA kit ADI-900-097 and Progesterone ELISA kit ADI-900-011, Enzo Life Sciences). For the progesterone and corticosterone ELISA, lizard plasma was thawed at room temperature for 20 minutes and vortexed before diluting with a stock steroid displacement reagent (SDR) and assay buffer. Plasma was diluted 1:2 with SDR and 40 μ l was added to 560 μ l of the assay buffer for a total dilution of 1:30. All plasma samples were assayed in duplicate for corticosterone and progesterone. A polyclonal antibody was used to competitively bind to the hormone in the standard (provided in kit) and the sample. After a 2 hr incubation at room temperature, excess reagents were washed off and the substrate solution was added. Color development was quantified on a microplate reader at 405 nm. Hormone concentrations (ng/mL) were determined by comparing the absorbance values of lizard samples to known concentrations on a standard curve. The intraassay coefficient of variation was 7.5% for CORT and 12.5% for progesterone. The interassay coefficient of variation was 14% for CORT and 18% for progesterone.

Estimators of body condition

Triglycerides assay

A commercially available enzyme kit was used to measure plasma triglycerides (reagent no. T7532, Point Scientific, Inc.). Plasma triglycerides were quantitatively determined through a series of enzymatic reactions involving lipase, glycerol kinase, and glycerol phosphate oxidase to produce a measurable production of red-colored quinoneimine dye. The intensity of the color development measured by absorbance in a spectrophotometer (Spectronic Genesys 5, Fisher Scientific) at 500 nm is directly proportional to the triglyceride concentration in the sample. Triglyceride concentrations (mg/dL) were then calculated by comparison to a triglyceride standard. The intrassay coefficient of variation was 6.4%.

Maximum girth and length-adjusted mass

A lizard's maximum girth (nearest 0.1 mm) was measured by laying each female lizard on a flat surface, pressing lightly on the spine, and sliding calipers (SPI model no. 314153) over the widest part of the mid-section. Girth measurements were taken when no visual inflation of the body was observed. Length-corrected body mass was calculated as the residuals from a linear regression of body mass on snout-vent length (SVL).

Statistical analyses

The Spearman's rank correlation test was used in determining associations between immune parameters, steroid concentrations, and measures of body condition in each year of the study. The Kruskal-Wallis one-way ANOVA on ranks was used in determining the effect of season on immune parameters, steroid concentrations, measures of body condition, and ectoparasitic mite loads on female fence lizards in 2011. The student's t-test was used to compare mass, SVL, tail, girth, and length-adjusted mass between control and confined females in 2012. The nonparametric Mann-Whitney Rank Sum test was used to compare bacterial killing capacity, leukocyte counts, corticosterone, progesterone, and triglyceride concentrations in confined versus control females since these variables did not fulfill the assumptions of the parametric test.

CHAPTER III

RESULTS

Associations between steroid hormones and immune measures (2011)

Baseline plasma CORT concentrations were positively correlated with the percent monocytes (Rs = 0.49, P = 0.0022, n = 38, Fig. 2). There were no significant correlations between baseline plasma CORT and the bacterial killing capacity (%) of the plasma, or total leukocyte counts per 10,000 RBC, or heterophils (%), or lymphocytes (%), or the heterophil to lymphocyte ratio (P > 0.05 for each, Table 1). Plasma progesterone concentrations were not significantly correlated with any of the immune measures (P > 0.05 for each, Table 1).



Figure 2. Relationship between baseline plasma corticosterone concentration and circulating monocytes (%) in female *Sceloporus undulatus* (2011).

Table 1. Correlations between baseline steroid hormones and innate immune measures in female Sceloporus undulatus (2011). The percent basophils and eosinophils are not shown since these cells were so rare. The last column shows the heterophil to lymphocyte ratio (H:L). The sample size is n = 39 for bacterial killing and n = 38 for the other variables.

T:H	ns Rs = -0.16 $P = 0.33$	ns $Rs = -0.033$ $P = 0.84$
Lymphocyte (%)	ns $Rs = 0.13$ $P = 0.44$	ns $Rs = -0.00044$ $P = 1.0$
Heterophil (%)	ns Rs = -0.21 $P = 0.20$	ns Rs = -0.029 P = 0.86
Monocyte (%)	(+) correlation Rs = 0.49 P = 0.0022	ns Rs = 0.04 P = 0.81
Total leukocyte count	ns Rs = -0.032 $P = 0.85$	ns $Rs = 0.13$ $P = 0.43$
Bacterial killing (%)	ns $Rs = -0.054$ $P = 0.74$	ns Rs = 0.072 P = 0.66
	Corticosterone (ng/mL)	Progesterone (ng/mL)

Associations between measures of body condition and innate immune measures (2011)

Plasma triglyceride levels were positively correlated with the percent lymphocytes (Rs = 0.39, P = 0.013, n = 39). Triglycerides were negatively correlated with the heterophil: lymphocyte ratio (Rs = -0.35, P = 0.031, n = 39; Table 2). Plasma triglycerides levels were not correlated with the other immune parameters, though the negative correlation with the percent monocytes approached significance (Table 2). Maximum girth was positively correlated with the percent monocytes (Rs = 0.36, P =0.027, n = 39; Table 2) but was not correlated with any other immune measures (P > 0.05for each, Table 2). Length-adjusted mass was not correlated with any immune measure (P > 0.05 for each, Table 2). Table 2. Correlations between measures of body condition and immune measures in female Sceloporus undulatus (2011). The percent basophils and eosinophils are not shown since these cells were so rare. The last column shows the heterophil to lymphocyte ratio (H:L). The sample size is n = 39 for triglycerides and max girth and n = 40 for length-adjusted mass except where noted.

	Bacterial killing (%)	Total leukocyte counts	Monocyte (%)	Heterophil (%)	Lymphocyte (%)	H:L
Triglycerides (mg/dL)	ns $Rs = -0.23$ $P = 0.17$ $n = 40$	ns Rs = -0.16 $P = 0.34$	ns $Rs = -0.30$ $P = 0.064$	ns Rs = -0.27 $P = 0.096$	(+) correlation Rs = 0.39 P = 0.013	(-) correlation $R_S = -0.35$ P = 0.031
Max girth (mm)	ns $Rs = 0.26$ $P = 0.12$ $n = 40$	ns Rs = -0.29 $P = 0.080$	(+) correlation Rs = 0.36 P = 0.027	ns Rs = -0.17 $P = 0.30$	ns Rs = 0.14 $P = 0.41$	ns Rs = -0.15 $P = 0.37$
Length-adjusted mass (g)	ns $Rs = 0.15$ $P = 0.34$ $n = 41$	ns Rs = -0.07 P = 0.67	ns Rs = 0.16 P = 0.32	ns Rs = -0.16 $P = 0.33$	ns $Rs = 0.19$ $P = 0.24$	ns $Rs = -0.16$ $P = 0.32$

Associations between measures of body condition and steroid hormones (2011)

Plasma triglycerides levels were negatively correlated with plasma corticosterone concentrations (Rs = -0.38, P = 0.018, n = 39; Table 3) and positively correlated with plasma progesterone concentrations (Rs = 0.32, P = 0.049, n = 39; Table 3). The other potential associations between the steroids and measures of body condition were not significant though progesterone had a nearly significant negative correlation with maximum girth (Rs = -0.31, P = 0.055, n = 39; Table 3).

Effect of season (2011)

Plasma corticosterone levels (H = 0.86, df = 3, P = 0.83) and progesterone levels (H = 1.86, df = 3, P = 0.60) did not differ significantly between seasons. There were no significant effects of season on plasma triglycerides (H = 4.91, df = 3, P = 0.18), length-adjusted mass (H = 5.49, df = 3, P = 0.14), or girth (H = 0.20, df = 3, P = 0.98). Lymphocyte percentages were significantly affected by season with a peak in median values observed in early spring (H = 19.09, df = 3, P < 0.001; Fig. 3). Significant seasonal changes in heterophil percentages and H:L ratios were observed with a peak in median values in the summer (H = 20.97, df = 3, P = 0.001; Fig. 4; H:L ratio: H = 19.89, df = 3, P < 0.001). Other immune measures did not differ significantly between seasons (monocytes: H = 2.94, df = 3, P = 0.401; bacterial killing capacity: H = 3.32, df = 3, P = 0.34; total leukocytes: H = 3.91, df = 3, P = 0.27;). The number of ectoparasitic mites was significantly affected by season with a peak observed in late spring (H = 10.22, df = 3, P = 0.017; Fig. 5). Chiggers forming thick clusters behind the ears accounted for the majority of ectoparasite counts.

	Length-adjusted mass (g)	Triglycerides (mg/dL)	Max girth (mm)
Corticosterone (ng/mL)	ns Rs = 0.23 P = 0.170 n = 38	(-) correlation Rs = -0.38 P = 0.018 n = 39	nsRs = 0.19P = 0.25n = 38
Progesterone (ng/mL)	ns Rs = -0.073 P = 0.66 n = 38	(+) correlation Rs = 0.32 P = 0.049 n = 39	Nearly significant Rs = -0.31 P = 0.055 n = 39

Table 3. Correlations between measures of body condition and baseline steroid hormones in female *Sceloporus undulatus* (2011).



Figure 3. Seasonal changes of circulating lymphocytes (%) in female *Sceloporus undulatus* (2011). The box plots indicate the median, upper and lower quartiles, and the range of values observed. Sample sizes shown above bars.



Figure 4. Seasonal changes of circulating heterophils (%) in female *Sceloporus undulatus* (2011). The box plots indicate the median, upper and lower quartiles, and the range of values observed. Sample sizes shown above bars.



Figure 5. Seasonal changes of ectoparasitic mite loads on female *Sceloporus undulatus* (2011). The box plots indicate the median, upper and lower quartiles, and the range of values observed. Sample sizes shown above bars.

Effects of confinement stress on steroid hormones (2012)

Plasma CORT concentrations were significantly greater in the females confined for 1 hr than in the females bled immediately upon capture (Mean CORT: confined 19.68 \pm 3.76 (n = 12), baseline 8.09 \pm 4.02 (n = 12); Mann-Whitney Rank Sum Test, P = 0.004; Fig. 6.). Plasma progesterone concentrations did not differ significantly between the confined and baseline treatment groups (Mean progesterone: confined 0.39 \pm 0.10 (n = 10), baseline 0.73 \pm 0.26 (n = 5); Mann-Whitney P = 0.43; Fig. 7).



Figure 6. Mean (\pm SEM) plasma corticosterone in female *Sceloporus undulatus* either bled immediately upon capture (baseline, n = 12) or after confinement for 1 hr (n = 12) in the 2012 study.



Figure 7. Mean (\pm SEM) plasma progesterone in female *Sceloporus undulatus* either bled immediately upon capture (baseline, n = 5) or after confinement for 1 hr (n = 10) in the 2012 study.

Effects of confinement stress on innate immunity (2012)

The bacterial killing capacity of female fence lizard plasma was not affected by confinement stress (Mean % killed: confined 45.56 ± 13.64 (n = 12), baseline 52.28 ± 12.7 (n = 12); Mann-Whitney, P = 0.30). Total leukocyte counts were similarly unaffected by confinement (Mean TLC: confined 34.52 ± 10.09 (n = 12), baseline 43.12 ± 9.14 (n = 12); Mann-Whitney P = 0.34). The percent of circulating heterophils did not significantly differ between confined and baseline treatment groups (Mean % heterophils: confined 10.6 ± 3.05 (n = 12), baseline 16.65 ± 2.3 (n = 12); Mann Whitney P = 0.089; Fig. 8) nor did the H:L ratio (Mean H:L: confined 0.273 ± 0.14 (n = 12), baseline 0.256 ± 0.044 (n = 12); Mann-Whitney P = 0.089). Lymphocyte percentages did not differ significantly between groups (Mean % 1ymphocytes: confined 73.67 ± 6.3 (n = 12), baseline 70.9 ± 3.38 (n = 12); Mann-Whitney P = 0.23). Similarly, monocytes percentages did not differ significantly between the treatment groups (Mean % monocytes: confined 12.89 ± 2.58 (n = 12), baseline 12.28 ± 1.85 (n = 12); Mann-Whitney P = 0.71).



Figure 8. Mean (\pm SEM) circulating heterophils (%) in female *Sceloporus undulatus* either bled immediately upon capture (baseline, n = 12) or after confinement for 1 hr (n = 12) in the 2012 study.

Associations between steroid hormones and innate immunity (2012)

Neither plasma corticosterone nor progesterone were correlated with any immune

measures (Table 4).

Table 4. Correlations between steroid hormones for the combined treatment groups (confined, baseline) and innate immune measures in female *Sceloporus undulatus* (2012). The sample size is n = 24 for corticosterone and n = 15 for progesterone.

e (%) H:L	$\begin{array}{c} ns \\ Rs = -0.094 \\ P = 0.658 \end{array}$	$\begin{array}{c c} \mathbf{ns} \\ \mathbf{ns} \\ Rs = 0.48 \\ P = 0.07 \end{array}$
Lymphocyt	ns Rs = -0.0 $P = 0.9$	Rs = -0.7 $P = 0.1$
Heterophil (%)	ns $Rs = -0.19$ $P = 0.38$	ns Rs = 0.44 $P = 0.10$
Monocyte (%)	ns Rs = 0.11 $P = 0.60$	ns Rs = 0.27 $P = 0.33$
Total leukocyte count	ns Rs = 0.104 $P = 0.62$	ns Rs = 0.043 $P = 0.87$
Bacterial killing (%)	ns $Rs = -0.29$ $P = 0.17$	ns $Rs = -0.0018$ $P = 0.99$
	Corticosterone (ng/mL)	Progesterone (ng/mL)

Associations between body condition and innate immunity (2012)

Length-adjusted mass was negatively correlated with bacterial killing capacity (R_s = -0.51, P = 0.013, n = 23; Fig. 9). Neither plasma triglycerides nor maximum girth were associated with any immune measures (Table 5).

Associations between body condition and steroid hormones (2012)

Maximum girth was positively correlated with plasma corticosterone concentrations (Rs = 0.46, P = 0.035, n = 21). Neither plasma triglycerides nor lengthadjusted mass were correlated with plasma corticosterone or progesterone concentrations (P > 0.05; Table 6).



Figure 9. Correlation between length-adjusted mass and plasma bacterial killing capacity in female *Sceloporus undulatus* (2012). The sample size is n = 23 for the combined treatment groups (confined, baseline).

adjusted mass, n = 23) and immune measures in female *Sceloporus undulatus* (2012). The confined and baseline treatment Table 5. Correlations between measures of body condition (triglycerides, n = 19; maximum girth, n = 21; and lengthgroups have been combined.

T:H (%)	$\begin{array}{c} ns \\ Rs = -0.14 \\ P = 0.56 \end{array}$	5 $R_{s} = -0.009$, $P = 0.96$	$7 \qquad ns \\ Rs = 0.014 \\ P = 0.94$
Lymphocyte	ns Rs = 0.06 $P = 0.79$	ns Rs = 0.04; P = 0.85	ns Rs = -0.17 $P = 0.44$
Heterophil (%)	ns Rs = -0.064 P = 0.79	ns Rs = -0.088 $P = 0.70$	ns $Rs = -0.024$ $P = 0.91$
Monocyte (%)	ns Rs = -0.16 $P = 0.50$	ns Rs = -0.105 $P = 0.65$	ns Rs = 0.34 $P = 0.12$
Total leukocyte counts	ns $Rs = -0.047$ $P = 0.84$	ns Rs = 0.16 $P = 0.49$	ns $Rs = 0.015$ $P = 0.94$
Bacterial killing (%)	ns Rs = -0.061 $P = 0.804$	ns Rs = -0.204 $P = 0.37$	(-) correlation Rs = -0.51 P = 0.013
	Triglycerides (mg/dL)	Max girth (mm)	Length-adjusted mass (g)

been combined.			
	Length-adjusted mass (g)	Triglycerides (mg/dL)	Max girth (mm)
Corticosterone (ng/mL)	ns Rs = 0.312 P = 0.145 n = 23	ns Rs = -0.34 P = 0.15 n = 19	(+) correlation <i>Rs</i> = 0.46 <i>P</i> = 0.034 <i>n</i> = 21
Progesterone (ng/mL)	ns Rs = -0.13 P = 0.62 n = 15	ns Rs = -0.29 P = 0.29 n = 15	ns Rs = 0.25 P = 0.39 n = 13

Table 6. Correlations between measures of body condition and steroid hormones in female *Sceloporus undulatus* (2012). The confined and baseline treatment groups have been combined.

CHAPTER IV DISCUSSION

The evidence of CORT's ability to suppress the immune system varies between studies (reviewed in Martin 2009) as some have found CORT to suppress the immune system (Morici et al 1997; Berger et al. 2005; French et al. 2006; French et al. 2010), enhance the immune system (Roberts et al. 2007), or have shown CORT to have no effect on the immune system (Butler et al 2009). However, several studies of CORT's interactions with immune function have positively correlated either baseline or experimentally increased CORT concentration with a decrease in immunity (Berger et al. 2005; French et al. 2006; French et al. 2010). A similar result was expected for this study, but contrary to our initial hypothesis, the data shows little evidence that either baseline CORT or stress-induced CORT were immunosuppressive. In 2012, CORT concentrations were significantly increased during confinement but confinement failed to elicit a change in bacterial killing capacity, differential leukocyte counts, total circulating leukocyte counts, or H:L ratios. In 2011, female lizards were not subjected to confinement but baseline concentrations of CORT were tested for associations with immune measures. The baseline CORT concentrations were positively correlated with monocytes percentages but CORT was not correlated with any of the other immune measures. On the whole, our results indicate little effect of an acute stressor or elevated CORT on innate immunity in female fence lizards.

The stress of confinement has previously been found to increase plasma CORT concentrations and subsequently decrease some measures of immunity. In a study on

restriction stress and cutaneous wound healing, restrained male tree lizards (Urosaurus ornatus) had higher CORT levels and a slower wound healing rate compared to lizards that were not restricted (French et al. 2006). Unlike our study of acute stress, their 1 hr confinement was repeated daily for 21 days and thus constituted a chronic stress, which could explain why we did not observe a similar decrease in immunity. Confinement in adult male and female Galapagos marine iguanas resulted in increased plasma CORT and decreased bacterial killing capacity during the non-breeding season (French et al. 2010). In male Galapagos marine iguanas, other types of stressors such as conspecific interactions, changes in social status, and injection of CORT have been observed to elicit an increase in plasma CORT concentration and a concurrent suppression of innate immunity measured by a phytohemagglutinin (PHA) skin test (Berger et al. 2005). In contrast to these studies, bacterial killing capacity positively correlated with the length of confinement in the amphibian, Cryptobranchus alleganiensis (Hopkins and Durant 2011) and another study on wall lizards (Podarcis melisellensis) found no relationship between CORT and immunity (Huyghe et al. 2009). In contrast to our results, a study of restriction stress on the leukocyte profiles of five-lined skinks (Plestiodon inexpectatus) found a positive correlation between CORT and the H:L ratio after a 1 hr confinement (Seddon and Klukowski 2012). The differing environment, season, location, sex, body condition, and species studied could all contribute to the differing results and explain the insignificant associations between CORT and immune measures we observed since other studies have found pronounced differences in immune function associated with the variables mentioned above (reviewed in Klein 2000). In our studies of female fence lizards, exposure to natural stressors, either acute or chronic, prior to capture could also

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affect both baseline CORT and the CORT response making interpretations of results difficult. For example, compared to uninfected individuals, western fence lizards (*Sceloporus occidentalis*) chronically stressed by malarial infection were found to release higher levels of CORT in response to confinement (Dunlap and Schall 1995). Another potential confounding variable in our 2012 confinement study is that the body mass of females confined for 1 hr was significantly higher than the baseline group (Mean confined mass = 12.2 g, mean baseline mass = 9.3 g), however, the overall magnitude of the difference (2.9 g) was relatively small and is likely of little biological relevance.

Food availability has been observed to influence an individual's innate immunity, especially as the energetic demands of reproduction increase (Ruiz *et al.* 2011). The bacterial killing capacity of female lizards (*Sceloporus graciosus*) has been observed to decrease during times of high reproductive investment but showed no decrease when females were supplemented with food (Ruiz *et al.* 2011). Another study found that female lizards (*Urosaurus ornatus*) were capable of maintaining both reproduction and innate immune function, measured via wound healing, when they had unlimited access to food but not when they were on a restricted diet (French *et al.* 2007b). It is possible that food availability could have influenced our studies findings and we may not have seen an effect if 2012 was a year in which food resources were especially plentiful. Populations can exhibit different levels of investment in immune function as environmental factors change from year to year (Nelson and Demas 1996). If our study was repeated in a year of food scarcity, we may have observed different results.

The positive correlation between baseline CORT and the percent monocytes we observed (Fig. 2) could be an example of the body reacting to acute stress by

redistributing leukocytes or possibly indicative of chronic inflammation. Acute stressors can be immune enhancing by mobilizing phagocytic cells to make them more likely to encounter invading pathogens. As circulating monocytes have not been found to exhibit significant seasonal changes in reptiles, they typically indicate phagocytic activity and inflammation (Campbell and Ellis 2007). The 2012 study did not show any effect of confinement on monocyte percentages despite the significant increase in CORT. This may suggest that the increase in monocytes percentages observed in 2011 is a result of long-term exposure to CORT or chronic inflammation. One study on juvenile alligators (*Alligator mississippiensis*) found experimentally elevated CORT to be correlated with a decrease in the percentages of circulating lymphocytes, eosinophils, and basophils, but not monocytes (Morici *et al.* 1997). Further studies on of the effects of acute versus chronic stressors on the percent monocytes are needed.

Based on the literature, we hypothesized that a negative correlation between progesterone concentration and immunity would be observed (Saad and El Deeb 1990; Cartledge *et al.* 2005; Graham *et al.* 2011). As with CORT, we saw little evidence to support a role for progesterone in down-regulating immunity. In both the 2011 and 2012 studies, progesterone was not correlated with bacterial killing capacity, differential leukocyte counts, total circulating leukocyte counts, or H:L ratios. Pregnancy in lizards have been correlate to a suppressed immune response of splenic cells stimulated in vitro to multiple mitogens (*Chalcides ocellatus*, Saad and El Deeb 1990) and to mediate a decrease in capacity for bacterial lysis compared to non-pregnant females (*Agkistrodon piscivorus*, Graham *et al.* 2011). Similarly, vitellogenesis has been found to significantly decrease wound healing ability in *Urosaurus ornatus* (French *et al.* 2008). Gestation has also been associated with an increase in circulating lymphocytes and a decrease in heterophils in female skinks (*Egernia whitii*) (Cartledge *et al.* 2005). In my study there was no evidence for the ability of progesterone to interact with the immune system in female fence lizards during their active season. Increasing the sample size and measuring additional variables such as reproductive status, plasma estradiol concentrations, humoral immunity, and cutaneous wound healing would benefit this and future studies in better understanding the role progesterone and reproduction play in immune system regulation.

Plasma CORT and progesterone were not correlated in female fence lizards in either year of the study. This appears to be common despite the role each can play in modulating the immune system. Other studies have found no correlation between progesterone and CORT in female lizards (*Cnemidophorus sexlineatus*, Grassman and Hess 1992; *Sceloporus undulatus*, Phillips and Klukowski 2008) and no difference in CORT concentrations between pregnant and non-pregnant cottonmouth snakes (*Agkistrodon piscivorus*, Graham *et al.* 2011).

In general, body condition is believed to have an influence on both baseline (Berger *et al* 2005) and stress-induced plasma CORT levels (Hews and Banikik 2013). A few associations between steroid hormone concentrations and measures of body condition were observed in this study. In female fence lizards, baseline CORT was negatively correlated with levels of plasma triglycerides (2011) and post-confinement CORT levels were positively correlated with max girth (2012). Other studies have specifically associated gravidity in female lizards (*Urosaurus ornatus;* Woodley and Moore 2002), and more broadly reproduction (Wilson and Wingfield 1994; Woodley and Moore 1999; Romero 2002; Moore and Jessop 2003; Phillips and Klukowski 2008; John-

Alder et al. 2009), with elevated baseline CORT concentrations. In vitellogenic female lizards, plasma triglycerides levels are elevated as lipids are transported to and incorporated into the developing eggs. Since this lipid transport is completed in gravid females, and since gravid females may feed less, gravid females have lower triglyceride levels, thus, the negative correlation we observed between baseline plasma CORT concentrations and plasma triglyceride concentrations is likely driven by gravid females. I would predict females with the lowest triglycerides levels to be gravid and thus exhibit increased plasma CORT concentrations. High plasma triglyceride levels could also indicate a surplus of energy stores, in which case there would be no need for elevated CORT to mobilize stored energy. I found the relationship between plasma progesterone concentrations and triglycerides to be opposite to that of baseline CORT concentrations and triglycerides. Progesterone positively correlated with triglycerides (2011) and had a nearly significant negative correlation with max girth (2011)(Table 3). One study has observed female lizards (Urosaurus ornatus) in earlier stages of reproduction, such as vitellogenesis, to have elevated progesterone concentrations and decreased CORT concentrations compared to gravid females, which have elevated levels of both CORT and progesterone (Woodley and Moore 2002). However, earlier work on female fence lizards in our lab also suggests that gravid females should have elevated baseline CORT, low progesterone, and elevated plasma triglyceride concentrations (M Klukowski, pers comm.).The increased plasma progesterone concentrations we observed could be associated with vitellogenesis and subsequently elevated triglycerides as suggested above.

Females in relatively poor body condition would be expected to be immunosuppressed, but I found that BKA was negatively correlated with length-adjusted mass in 2012. In other words, females that weighed the most for their SVL, tended to have plasma with relatively weak bacterial killing capacity. This may again indicate that gravid females, which would tend to have the larger length-adjusted mass, are immunosuppressed. Plasma triglyceride concentrations were positively correlated with the percent lymphocytes (2011) and negatively correlated with the H:L ratio in the 2011 study. One possible explanation for this would be that females with large energy stores are able to invest more heavily in humoral immunity, as indicated by the elevated lymphocyte percentages. Also gravid females, expected to have low triglyceride levels, may be immunosupressed. Max girth was positively correlated with monocytes percentages (2011) and to the extent that max girth may indicate gravidity, monocytes may play some role in preparing for oviposition. This may also explain the positive association found between baseline CORT and the percent monocytes (Fig. 2), if the females with the higher baseline CORT tended to be gravid.

An influence of season on immunity and body condition was anticipated as reproductive status, behavior, and the availability of resources and energetic investments change during an animal's active season (Coe 2002; Nelson and Demas 2004). An effect of season was observed for lymphocyte percentages, which peaked in the spring (May 25-June 8, Fig 3), and heterophil percentages, which peaked in July (Fig. 4). The innate immune response is energetically expensive and a change in energy investment into this component of the immune system could represent a change in the availability of resources and energy stores available to female lizards, such as environmental or

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reproductive status changes. Adrenal hormones and sex hormones concentrations, which fluctuate with season, can also influence the function of the immune system causing a similar change in immune response with season (Coe 2002). Though in the current study I failed to detect significant seasonal changes in either baseline CORT or progesterone. Studies have found pronounced differences in the immune function of vertebrates across the year, with daily photoperiod having a strong influence on the strength of the immune system by acting as a seasonal cue (reviewed in Nelson and Demas 1996; Garcia et al. 2010). A general trend that begins to emerge among studies of seasonally breeding animals is that, compared to long photo period days, short photo period days tend to cue an enhancement of immune function to compensate for an increased amount of pathogens or stressors that are anticipated to be encountered (Nelson and Demas 1996). Many of these studies investigate annual changes, however, my study looked more specifically within the parameters of the active season of fence lizards, which could provide evidence for the changes in resource allocation associated with reproductive status. The seasonal shift we observed in percent lymphocytes and percent heterophils could be associated with the specific functions of these leukocytes. Heterophils are one of the primary phagocytic cells in reptiles which act by rapidly mobilizing to the site of infection to engulf and kill pathogens, playing a strong role in inflammation and the hosts nonspecific defense (Campbell and Ellis 2007; Genovese et al. 2013). Unlike heterophils, lymphocytes can provide a longer and more focused response to specific antigens (Parham 2009), although they have been reported in some cases to have phagocytic activity in some reptiles (Trachemys scripta, Zimmerman et al. 2010) suggesting a heavier reliance on innate immunity over adaptive immunity. The seasonal shift in

leukocytes I observed could be due to a change in the type or abundance of pathogens and stressors this population is encountering. An increase in percent lymphocytes might indicate that the innate immune cells have been overwhelmed, as is often the case in an active infection (Parham 2009), and as the daily photoperiod increases with season the peak in heterophils may represent a decreased dependence on the expensive humoral immune response possibly to facilitate reproduction. Over the course of the active season no significant seasonal changes were observed between the other measures of immunity, steroid hormone concentrations, or measures of body condition. Although seasonal influences on innate immune function were observed, identifying the proximate mechanisms will require further investigation.

Ectoparasitic mites were found to have a peak in abundance during late spring (Fig. 5). Similar seasonal changes in the abundance of ectoparasites, with a peak during June-August, have previously been observed in both male and female *Sceloporus undulatus* (Klukowski 2004). Ectoparasitism tends to increase as testosterone, activity, and home-range area increase in *Sceloporus undulatus* (John-Alder *et al.* 2009). The changes in ectoparasitic mite abundance observed on female fence lizards could indicate a time of increased activity and movement among reproductive female lizards or simply that mites are most abundant in the environment during late June. A study on male wall lizards (*Podarcis melisellensis*) found a seasonal peak in ectoparasite mites during September, the end of their active season, and also observed a positive relationship between an increased parasite load and swelling in response to phytohaemagglutinin injection (Huyghe *et al.* 2010). While I did not find any associations between ectoparasite mite loads and either BKA or leukocytes, investigating potential correlations between

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ectoparasite mite loads and other aspects of immunity could provide valuable insights for future studies.

Overall, my prediction that baseline steroid hormones would be correlated with a decrease in immunity was not strongly supported. Similarly, our prediction that an acute stressor, such as 1 hr of confinement, would cause an enhancement of innate immune function was also not strongly supported. The effect of season on the measurements of immune function was somewhat supported by the change in leukocyte profiles but ideally the remaining measures of immunity would have also shown distinct differences throughout the season. Several factors could have affected my results including the small sample size, measuring only a few parameters of innate immunity, yearly variation in food resources, and an uneven distribution of female body weight between baseline and confined treatment groups in 2012. Looking to the future, this study could be expanded to include additional measures of both innate and humoral immunity, a more robust sample size, identification of female reproductive status, and quantification of plasma estradiol concentrations in female lizards to better understand the interactions between steroids, reproduction, and immunity.

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