RESEARCH ARTICLE

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Immune variation during pregnancy suggests immune component-specific costs of reproduction in a viviparous snake with disparate life-history strategies

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Abstract

Growing evidence suggests the existence of trade-offs between immune function and reproduction in diverse taxa. Among vertebrates, however, there is still a taxonomic bias toward studies in endotherms, particularly birds. We tested the hypothesis that reproduction entails immune-related costs in the viviparous garter snake, *Thamnophis elegans*, from populations that exhibit two life-history strategies, termed ecotypes, with contrasting paces of life. Between the two ecotypes, we predicted lower immune function in gravid than non-gravid females of both strategies, but with relatively larger immunity costs in the ecotype that generally invests more in current reproduction. Across individuals, we predicted greater immune costs for females investing more in the present specific reproductive event (i.e., higher fecundity) irrespective of their ecotype. We assessed leukocyte profiles and measured bactericidal capacity of plasma (innate immunity) and T- and B-lymphocyte proliferation (adaptive immunity) in gravid and non-gravid females in their natural habitats. We also collected data on reproductive output from these same gravid females brought into captivity. Gravid females of both ecotypes showed lower T-lymphocyte proliferation responses to concanavalin A than non-gravid females, but no differential costs were observed between ecotypes. The remaining immune variables did not vary between gravid and non-gravid females. Among gravid females within each ecotype, those with larger reproductive output showed lower total leukocyte counts, suggesting a fecundity-dependent trade-off. Our study contributes to the comparative ecoimmunology of vertebrates by highlighting the immune component-specificity of trade-offs between reproduction and immune function and showing that costs can be fecundity-dependent in some, but not all cases.

1 | INTRODUCTION

At times when resources are limiting, physiological mechanisms may mediate trade-offs among costly functions such as reproduction, growth, and self-maintenance (Ricklefs & Wikelski, 2002; Zera & Harshman, 2001). Central to self-maintenance and survival is the immune system, a physiological system in charge of defense against parasites and disease. Immune defenses, however, are not cost-free (Lochmiller & Deerenberg, 2000) and growing evidence suggests a trade-off between immune function and reproduction in diverse taxa, including both vertebrates (e.g., Knowles, Nakagawa, & Sheldon, 2009; Norris & Evans, 2000; Sheldon & Verhulst, 1996) and invertebrates (e.g., Adamo, Jensen, & Younger, 2001; Schmid-Hempel, 2003). Among vertebrates, nonetheless, there is still a taxonomic bias toward studies in endotherms, and birds in particular. Squamate reptiles (lizards and snakes) have complex immune systems similar to those of birds and

mammals (Zimmerman, Vogel, & Bowden, 2010), but have been relatively less studied, making them an interesting group for expanding the knowledge on life-history trade-offs involving immunity.

Given the complexity of the immune system, a thorough assessment of the relationship between reproduction and immunity requires measurement of multiple immune parameters, which can allow detection of differential responses by diverse immune components based on their interactions and/or relative costs of use (Lee, 2006). The vertebrate immune system can be divided into innate (or non-specific) immunity and acquired (or adaptive) immunity, with each comprised of both cellular and humoral (i.e., soluble) components (Roitt, Brostoff, & Male, 1998). Innate immunity is the first line of defense against invading pathogens. Innate cellular effectors include monocytes and macrophages, granulocytes, and natural killer cells, whereas humoral effectors include natural antibodies, the complement system, and other antimicrobial proteins. Acquired immunity is the second line of

TABLE 1 Life-History Differences Between Fast-Living Lakeshore (L-Fast) and Slow-Living Meadow (M-Slow) Garter Snake Ecotypes around Eagle Lake, California

Life-History Trait	Fast-Living Ecotype	Slow-Living Ecotype
Maximum body size	700 mm	550 mm
Size at maturity	425 mm	400 mm
Age at maturity	3 years	5–7 years
Reproductive rate	Biennial	Resource dependent
Mean litter size	8 neonates	5 neonates
Median life span	5 years	9 years

Notes: Data from Bronikowski and Arnold (1999), Sparkman et al. (2007), and Miller et al. (2011).

defense and is mediated by B- and T-lymphocytes, which upon recognition of the invader proliferate mitotically to form an army of cells that can more effectively destroy it (Roitt et al., 1998). Acquired cell-mediated responses have cytotoxic T-lymphocytes as main effectors and are directed against intracellular pathogens (e.g., viruses) and tumor cells (Roitt et al., 1998). On the other hand, acquired humoral responses are mediated by B-lymphocytes, and the specific antibodies that these cells produce, and are effective mainly against extracellular pathogens (e.g., bacteria and macroparasites). These different innate and acquired components interact to defend an organism against disease.

In the present study, we investigated the link between reproduction and immune function in the western terrestrial garter snake, *Thamnophis elegans*, assessing aspects of both innate and acquired immunity. Populations of *T. elegans* inhabiting the vicinity of Eagle Lake, California, have been the focus of study for over 30 years and offer an ideal system showing two clearly divergent life-history ecotypes that differ substantially in their growth rate, adult body size, age of reproductive maturation, longevity, and investment in current reproduction (Table 1). Thus, the existence and characteristics of trade-offs between reproduction and immune function can be evaluated both within and between life-history ecotypes, providing the opportunity of a more complete understanding of reproductive and immune investment in the context of disparate pace-of-life strategies. The fast-living lakeshore (L-fast) ecotype consists of populations found along the shore of Eagle Lake, with individuals characterized by rapid growth to large body sizes, early maturation, high and frequent reproduction, and short median life span (Table 1). On the other hand, the slow-living meadow (M-slow) ecotype consists of populations that inhabit mountain meadows that surround the lake and show slow growth to smaller body sizes, late maturity, low and less frequent reproduction, and longer median life span (Table 1). Gravid (i.e., pregnant) and non-gravid females of this viviparous snake can be readily found and sampled at the same time in their natural habitats, preventing potentially confounding effects of seasonality and/or the stress of captivity on immune function.

In addition, assays for assessing aspects of both innate and acquired immunity have been developed for use in this system (Palacios, Cun-

nick, & Bronikowski, 2013; Sparkman & Palacios, 2009). The selected immune measures (Table 2) can be performed with a relatively small blood sample and do not require recapture of individuals or species-specific reagents (Demas, Zysling, Beechler, Muehlenbein, & French, 2011; Salvante, 2006), making them suitable for use in ecological studies of non-model organisms. The leukocyte profile provides insights regarding the major cellular components of the immune system (Beldomenico et al., 2008), including those involved in innate (e.g., granulocytes, monocytes) as well as acquired (i.e., lymphocytes) immunity. Bactericidal capacity (or competence) of plasma is a functional measure of the integrated bacterial killing ability of plasma proteins, including complement, lysozyme, and constitutively produced acute phase proteins (Matson, Tieleman, & Klasing, 2006). Assessment of acquired immune components in free-living squamates has been rare (Palacios et al., 2013), as most assays require recapture of individuals (Demas et al., 2011). *In vitro* lymphocyte proliferation assays, widely used by traditional immunologists and immunotoxicologists (Fairbrother, Smits, & Grasman, 2004; Froebel et al., 1999; Grasman, 2002) offer a viable alternative. They measure the functional ability of T- and B-lymphocytes to become activated and multiply in response to a challenge, which constitute critical initial steps of acquired immune responses (Roitt et al., 1998).

To test the hypothesis that reproduction entails costs in terms of immune function, we measured these immune components in reproductive (gravid) and non-reproductive (non-gravid) female garter snakes from the two life-history ecotypes in their native habitats. In addition, those gravid females sampled in the field were transported to the laboratory and data on their reproductive output were obtained to determine whether any immunological costs of reproduction are fecundity-dependent (i.e., whether individuals producing more and/or larger offspring suffer greater costs; e.g., Stahlschmidt et al., 2013). We predicted: (1) lower immune function in gravid than non-gravid females of both ecotypes, (2) relatively larger costs in terms of immunity in the L-fast ecotype given their greater investment in current reproduction, and (3) greater immune-function costs for individual females investing more in current reproduction (i.e., higher fecundity) irrespective of their ecotype. In addition, under the hypothesis that trade-offs can be immune component-specific, we predicted that different aspects of immunity could show different responses to reproduction, with some aspects being more affected than others.

2 | METHODS

2.1 | Sample collection

Populations of the western terrestrial garter snake (*T. elegans*) are distributed over a 25-km^2 study area at 1,555–2,055 m above sea level at the northern end of the Sierra Nevada Mountains in northeastern California, USA. We sampled snakes from L-fast and M-slow populations during the reproductive season (June–July) in two consecutive years (2009 and 2010). Snakes were hand captured and blood samples (70–150 μl) were obtained from the caudal vein via heparin-rinsed syringes using sterile techniques. Mean ($\pm$SD) time elapsed

TABLE 2 Immune Defense Components, Their Main Functions, and Techniques Used to Measure Them in Garter Snakes

Immune Component	Functions	Technique
Innate		
-Granulocytes and monocytes	Ingest, destroy pathogens	Cell counts
-Complement, antimicrobial proteins	Neutralize, opsonize, destroy pathogens	Bactericidal competence of plasma
-Natural antibodies[a]	Neutralize, opsonize pathogens, activate complement cascade	Hemagglutination–hemolysis
Acquired		
–Lymphocytes	Immunological memory of pathogens	Cell counts
B-cells	Neutralize or opsonize pathogens.	*In vitro* B-lymphocyte proliferation
T-cells	Kill infected host cells.	*In vitro* T-lymphocyte proliferation

[a]Not measured in the present study, but used in previous garter snake studies (e.g., Sparkman & Palacios 2009, Palacios et al., 2011).

between capture and bleeding was 9.1 ± 5.1 min (median $=8$ min). Time elapsed between capture and bleeding did not explain significant variation in any of the immune parameters measured (Spearman rho $= -0.16$ to 0.17, all $P > 0.17$); nevertheless, due to interindividual variation, time elapsed between capture and bleeding was evaluated as a covariate in statistical models (see below). Blood smears were made with a drop of fresh blood and fixed with methanol for leukocyte quantification (performed only in 2010). For the bactericidal capacity assay (performed both years), blood was kept cold and centrifuged within a few hours of collection, then plasma was separated and snap-frozen in liquid nitrogen. Upon arrival in the laboratory, plasma was stored at $-80°C$ until analyses. For the lymphocyte proliferation assay (performed only in 2010), $60\ \mu$l of whole blood were transferred to a sterile tube and diluted 1:1 in AIM-V lymphocyte medium containing heparin (15 units/ml) and supplemented with 25 mM HEPES, 2 mM L-glutamine, and 50 μg/ml gentamicin (all from Life Technologies, Rockville, MD). These samples were stored on ice and shipped overnight to Iowa State University for analysis the following morning as described in Palacios et al. (2013). After blood collection and processing, snakes were weighed (g), measured (mm, snout-to-vent length, SVL), sexed via hemipene eversion, and females were classified as gravid or non-gravid based on palpation of their abdomen for yolks/embryos. All snakes were also examined for the presence of the trematode *Alaria* sp., which causes inflammation and necrosis in the tail region. A total of 69 females (16 gravid, 53 non-gravid) were sampled in 2009 and 76 (16 gravid, 60 non-gravid) in 2010. Sample sizes vary among analyses because we could not perform all assays in some individuals, especially in 2010, due to limited blood volume (Supplementary File 1). Work with the snakes was carried out in accordance with standard animal care protocols and approved by the Iowa State University Animal Care and Use Committee (IACUC no. 3-2-5125-J). The State of California Department of Fish and Game granted collecting permits.

2.2 | Reproductive output

In both years, gravid females were transported to the laboratory in order to gather data regarding their reproductive output. Females were housed individually in 10-gallon glass aquaria containing corn-cob substrate and water dishes with hollow rims that conferred shelter. Aquaria maintained a permanent thermal gradient that ranged between 25 and 34°C at opposite ends to allow thermoregulation. Females were fed thawed frozen mice once a week until satiation and maintained on a 12:12 light–dark daily cycle. Females gave birth in the laboratory between mid-August and mid-September in both years. Each female gave birth on a single day and was weighed and measured on the day post parturition. The number and mass of neonates, stillborns, and yolks (unfertilized eggs) were also recorded within 24 hr of birth. Reproductive variables considered were: total litter size (i.e., including live and non-live components), live litter size (i.e., number of live offspring), total litter mass (i.e., sum of the mass of neonates, stillborns, and yolks), relative litter mass (residuals of the regression of total litter mass on female post-parturition mass), and reproductive failure (dead litter size/total litter size) (Sparkman, Arnold, & Bronikowski, 2007). None of the reproductive parameters of the 32 gravid females that gave birth in captivity differed between the two study years (all $P > 0.25$), thus data for 2009 and 2010 were analyzed and presented together. Analyses by year were not conducted given the limited sample sizes for gravid females of each ecotype within each study year (see Supplementary File 1).

2.3 | Leukocyte profile

Fixed blood smears were stained with Wright-Giemsa stain at the College of Veterinary Medicine at Iowa State University. Relative abundances (proportion) of lymphocytes, monocytes, heterophils, and basophils were estimated by classifying the first 100 leukocytes encountered under 1000 $\times$ magnification using a compound microscope (Sparkman et al., 2014). The total leukocyte count (TLC, number of cells per μl of blood) was estimated by the indirect Phloxin B method (Campbell and Ellis 2007) using 0.1% phloxin stain (Vetlab Supply, Palmetto Bay, FL) and a hemocytometer. Total counts of each type of leukocyte were calculated by multiplying their proportion by TLC (Palacios et al., 2013).

2.4 | Bactericidal capacity of plasma

We assessed bactericidal capacity of plasma following the protocol by Matson, Tieleman et al. (2006), which we had previously adapted for use in garter snakes (Palacios, Sparkman, & Bronikowski, 2011). Briefly, a pellet of lyophilized *Escherichia coli* (ATCC 8739, Cat# 0483E7, Microbiologics) was reconstituted using 40 ml of phosphate-buffered saline

(PBS) and a fraction was further diluted with PBS to produce a working solution containing approximately 200–300 colony-forming bacteria per 10 μl. All plasma samples were diluted 1:10 with PBS and sample reactions were prepared by adding 10 μl bacterial working solution to 100 μl of the diluted plasma samples. We incubated all sample reactions for 20 min at 28°C to provide adequate time for bacterial killing to occur. Control reactions were prepared by adding 10 μl of the bacterial working solution to 100 μl PBS and were plated before, in the middle, and after plating of the sample reactions. All sample reactions and controls were plated in duplicate using 50 μl aliquots on 4% tryptic soy agar and incubated overnight at room temperature ($\sim$25°C). The number of bacterial colonies on each plate was then counted and the percentage of bacteria killed calculated. Bactericidal capacity did not differ between the two study years for gravid and non-gravid females of either ecotype (all $P > 0.16$), thus data for 2009 and 2010 were analyzed together.

2.5 | *In vitro* lymphocyte proliferation

We assessed aspects of acquired immunity using a whole-blood mitogenic stimulation assay that we adapted and optimized for use in garter snakes (Palacios et al., 2013). Within 24 hr of collection, blood samples were further diluted to 1:20 using supplemented AIM-V lymphocyte medium and 50 μl of the dilution were dispensed into flat-bottomed 96-well microculture plates containing 50 μl of mitogen (stimulated wells) or 50 μl of medium (non-stimulated, control wells). We measured proliferation of B-lymphocytes using the standard B-cell mitogen lipopolysaccharide (LPS from *E. coli*, 20 μg/ml). Proliferation of T-lymphocytes was measured using two standard T-cell mitogens, phytohemagglutinin (PHA, 40 μg/ml) and concanavalin A (ConA, 10 μg/ml), given that these mitogens are thought to stimulate different T-cell subsets (Jones, 1973) and can show different patterns in ecoimmunology studies (e.g., Palacios, Cunnick, Vleck, & Vleck, 2009). Triplicate blood cultures were incubated in a 7% CO_2, 28°C humidified atmosphere for a total of 96 hr and pulsed during the last 24 hr of incubation with tritiated [^{3}H] thymidine (0.5 μCi/well). We then harvested cultures onto glass-fiber filters using a cell harvester (Combi Cell Harvester; Skatron Instruments, Sterling, VA) and quantified radioactive thymidine incorporation (counts per minute, cpm) using a liquid scintillation counter. Lymphocyte proliferative responses were expressed as a stimulation index (SI) calculated as the ratio between the mean cpm of replicate mitogen-stimulated wells and the mean cpm of replicate non-stimulated (control) cultures.

2.6 | Statistical analyses

We conducted general linear (and mixed) model analyses using SAS software (SAS Institute, Cary, NC). Normality of the residuals was visually inspected and further assessed using Shapiro-Wilk tests; when deviations were detected, variables were transformed to achieve normally distributed residuals. To assess whether reproductive status has an effect on immune function of female garter snakes, we compared immune parameters of gravid females with those of non-gravid ones taking ecotype into consideration. Thus, all models included the

fixed effects of reproductive status, ecotype, and their interaction. Initial models also tested for the potential effects of SVL, date of sampling, number of days elapsed between sampling and parturition, time between capture and bleeding, and trematode infection status. Non-significant terms, except for those of the main predictors mentioned above, were removed from the final models. Significance was assessed with an α-level of 0.05. Effects excluded from the final models exceeded a P value of 0.1.

Data on total and differential leukocyte counts were analyzed separately for each individual variable and also summarized using a multivariate analysis to determine whether the integrated indices reflect the same patterns as the individual variables (e.g., Buehler, Versteegh, Matson, & Tieleman, 2011; Matson, Cohen, Klasing, Ricklefs, & Scheuerlein, 2006). Thus, we ran a principal component analysis (PCA) that included the TLC and the proportion of each leukocyte type and used the first two principal components (PCs) as independent variables in the general linear models. Bactericidal competence of plasma was arcsine square-root transformed before analysis, and year was included as a random effect in the model. Lymphocyte proliferation responses were $\log_{10}$-transformed and the total lymphocyte count (lymphocytes/μl) was included as a covariate to correct for differences in the initial number of lymphocytes available for proliferation among individual snakes (Palacios et al., 2013).

To assess whether gravid females that invest more in the present reproductive output show lower immune function than those investing less, we evaluated the relationships among immune function variables and reproductive effort both within and across ecotypes. Given that measures of reproductive output were highly intercorrelated, we calculated an integrated measure of reproductive effort as the first PC from a PCA that included total liter size, live litter size, total litter mass, and relative litter mass. Due to the smaller sample sizes available for these analyses (i.e., only gravid females considered), we performed simple Spearman correlations between variables of immune function and reproductive effort. For lymphocyte proliferation parameters, stimulation indices corrected for total lymphocyte counts (i.e., residuals) were used.

3 | RESULTS

In accordance with previous data on this system, our results confirm that the two ecotypes differ widely in their reproductive output in terms of total and live litter size and total and relative litter mass (Table 3), with L-fast females investing significantly more in current reproduction than M-slow ones. On the other hand, reproductive failure, that is, the proportion of the litter that consisted of yolks and still-borns, did not differ between the two ecotypes (Table 3).

The PCA of leukocyte counts resulted in two PCs with eigenvalues greater than one (Table 4), which together explained 73% of the variance in the data. The first PC showed positive loadings for the percentage of phagocytic cells (heterophils and monocytes) together with negative loadings for the percentage of lymphocytes. The second PC showed positive loadings for the TLC and negative loadings for the percentage of basophils. Neither the integrated leukocyte profile

TABLE 3 Comparison of Body Size (SVL) and Reproductive Variables Between Gravid Females of L-Fast and M-Slow Garter Snakes in the Present Study

	Body Size[a] SVL (mm)	Total[a] Litter Size (Number)	Live[a] Litter Size (Number)	Total[a] Litter Mass (gr)	Relative[b] Litter Mass (Residuals)	Reproductive Failure (Proportion)
L-fast (n = 20)	629.5 ± 49.1 (547–692)	11.1 ± 3.4 (4–18)	9.8 ± 3.8 (2–16)	34.3 ± 10.6 (17.2–58.3)	3.1 ± 11.6 (−22.4 to 18.5)	0.12 ± 0.21 (0–0.85)
M-slow (n = 12)	483.5 ± 67.9 (415–608)	4.3 ± 1.4 (2–7)	3.9 ± 1.7 (1–7)	11.8 ± 5.9 (4.4–24.6)	−3.5 ± 3.8 (−12.3 to 0.5)	0.11 ± 0.23 (0–0.67)

Notes: Data for both years (2009 and 2010) were pooled together. Means ± standard deviations and ranges (min-max) are depicted. Wilcoxon rank tests for an ecotype effect were performed due to unequal variances. Significant differences are indicated by letters.
[a]$P < 0.01$.
[b]$P < 0.05$.

TABLE 4 Result of Principal Component Analysis of Leukocyte Counts of Female Garter Snakes

Variable	PC1	PC2
TLC (cells/μl)	−0.24	**0.63**
Lymphocytes (%)	**−0.62**	−0.09
Heterophils (%)	**0.48**	−0.07
Monocytes (%)	**0.52**	0.44
Basophils (%)	0.24	**−0.62**
Variance (%)	49.1	24.0

Notes: Only principal components (PCs) with eigenvalues over 1 are depicted. Values in boldface indicate the highest loading for each immune variable across PCs. Percentage of variance explained by each PC is shown at the bottom of the table. TLC, total leukocyte count.

(PC1 and PC2) nor bactericidal capacity of plasma differed between gravid and non-gravid females, between the L-fast and M-slow ecotypes, or their interaction (Table 5 and Figure 1). Separate analysis for each leukocyte variable provided the same results and conclusions as the leukocyte PCA approach (data not shown). Date of sampling, number of days elapsed between sampling and parturition (for gravid females), SVL, time elapsed between capture and bleeding, and trematode infection status were excluded from all the final models (all $P > 0.1$).

Lymphocyte proliferation responses increased with the initial number of lymphocytes present in the cultures (Table 5). Response of T-lymphocytes to ConA was lower in gravid than in non-gravid females, whereas the response of T-lymphocytes to PHA and of B-lymphocytes to LPS was not significantly affected by female reproductive status (Table 5 and Figure 1). There was no effect of ecotype (or its

interaction with reproductive status) on any of the lymphocyte proliferation responses. T-lymphocyte responses to ConA were also lower for females infected with tail trematodes (least-square mean ± SE: 0.19 ± 0.06) than for non-infected females (least-square mean ± SE: 0.40 ± 0.04) irrespective of their reproductive status (Table 5). Date of sampling, SVL, time elapsed between capture and bleeding, and number of days elapsed between sampling and parturition (for gravid females) had no significant effects on any of the lymphocyte proliferation variables and were thus excluded from the final models (all $P > 0.1$).

Among gravid females, PCA analysis of reproductive effort variables resulted in a first PC that explained 84% of the variance in the data and had an eigenvalue of 3.3, with positive loadings for all four reproductive output variables included. That is, females having larger total and live litter sizes and larger total and relative litter masses displayed higher scores than those with the opposite characteristics. The remaining PCs had eigenvalues lower than 1 and explained less than 12% of the variance each. A significant trade-off was found between TLCs and the reproductive effort PC within each ecotype, with females investing more in reproduction (i.e., higher PC scores) showing lower total counts of leukocytes (Figure 2). The remaining pairwise correlations between immune variables and reproductive effort were not significant (all $P > 0.2$).

4 | DISCUSSION

Based on the hypothesis of costs of reproduction on immune function we predicted that (1) gravid garter snakes of both ecotypes would show lower immune function than non-gravid females, (2) the eco-

TABLE 5 General Linear Models for Immune Function Variables of Free-Living Female Western Terrestrial Garter Snakes

Effect	Leukocytes PC1	PC2	BC	ConA SI	PHA SI	LPS SI
Reproductive status (RS)	1.11$_{1,63}$	1.23$_{1,63}$	0.18$_{1,114}$	**8.42$_{1,62}$**	0.10$_{1,63}$	2.06$_{1,63}$
Ecotype (Ec)	0.49$_{1,6}$	0.21$_{1,6}$	0.98$_{1,6}$	0.32$_{1,6}$	0.00$_{1,6}$	0.49$_{1,6}$
RS x Ec	1.93$_{1,63}$	1.35$_{1,63}$	2.85$_{1,114}$	0.24$_{1,62}$	0.04$_{1,63}$	0.36$_{1,63}$
Total lymphocytes	na	na	na	**11.3$_{1,62}$**	**21.6$_{1,63}$**	**14.6$_{1,63}$**
Trematodes				**7.86$_{1,62}$**		

Notes: Leukocyte PC1 and PC2 are the scores from principal component analysis (Table 3). Bactericidal competence (BC) was arcsine square root - transformed and lymphocyte proliferation variables (ConA SI, PHA SI, and LPS SI) were $\log_{10}$-transformed before analysis. Values denote F-statistics with df1,df2 as subscripts. Significant effects are shown in bold (all $P < 0.01$). na, not applicable.
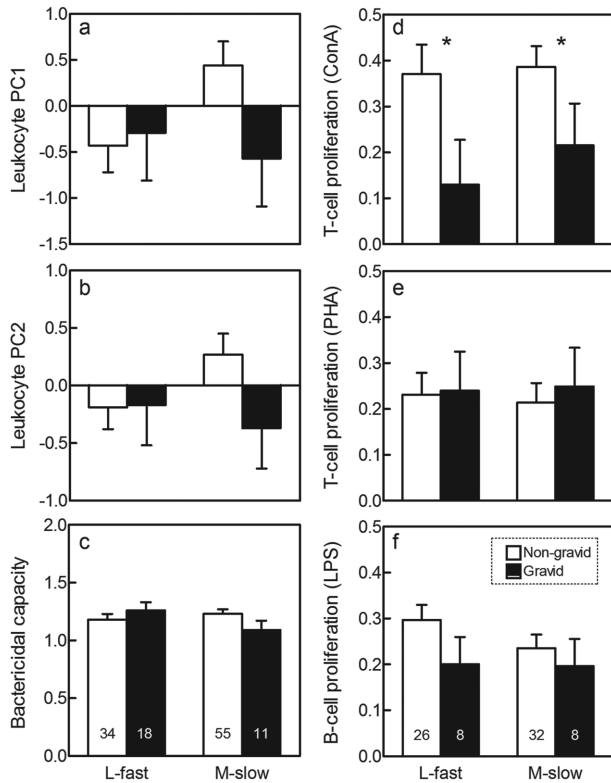
FIGURE 1 Comparison of immune function parameters between non-gravid (open bars) and gravid (black bars) female garter snakes of the L-fast and M-slow ecotypes sampled in the field. a) and b) Scores of principal components 1 and 2, respectively, from the leukocyte PCA shown in Table 3. c) Bactericidal competence of plasma (arcsine square root-transformed). d) to f) Lymphocyte proliferation responses (log10-transformed stimulation indices) to the two T-cell mitogens (ConA and PHA) and to the B-cell mitogen (LPS), respectively. Depicted values are least-square means ± SE from the statistical models in Table 4. Sample sizes for bactericial competence are shown inside each corresponding bar in panel c). Sample sizes for the remaining variables are the same as those shown in panel f). Asterisks highlight significant differences between gravid and non-gravid females.

type investing more in current reproduction (i.e., L-fast) would show greater costs in terms of immunity, and (3) costs would be greater for individual females investing more in current reproduction irrespective of their ecotype (i.e., fecundity-dependent costs). In addition, we evaluated whether different immune components would be affected differently by reproduction. Our results suggest the existence of immune component-specific costs of reproduction on immune function of free-living western terrestrial garter snakes. Gravid females displayed lower T-lymphocyte proliferation in response to stimulation by ConA than non-reproductive females, but other immune measures were not affected by reproductive status, suggesting an immune component-specific trade-off. Contrary to our prediction that the ecotype investing more in current reproduction (i.e., L-fast) would show greater costs in terms of immunity, the observed pattern was not more pronounced in L-fast snakes. Among gravid females, there was evidence of a fecundity-dependent trade-off between TLCs and reproductive effort within each ecotype. Below we discuss these findings in the context of ecoimmunological theory and previous work on the link between reproduction and immunity in squamate reptiles and other vertebrates.

Among squamates, results to date generally support the idea of a trade-off between reproduction and immune function (Table 6), including both observational and experimental studies and those involving animals in captivity or in the field. Most studies have, nevertheless, measured a single aspect of immune function (i.e., innate, acquired, or integrated immunity). Interestingly, those few studies including more than one immune measure, as the present one, suggest that the trade-off between reproduction and immunity does not necessarily involve all aspects of immune function; that is, patterns can be immune component-specific. For instance, Stahlschmidt et al. (2013) found that reproduction was associated with reduced lytic, but not agglutinating, ability of plasma (two aspects of innate humoral immunity) in captive colonies of children´s python *Antaresia children* (Table 6). Similar immune component-specificity of the trade-off with reproduction has been documented in birds (e.g., Ardia, 2005a; Hanssen, Hasselquist, Folstad, & Erikstad, 2005). In our study, gravid garter snakes showed depressed function of T-lymphocytes in response to ConA, whereas lymphocyte responses to the other two mitogens (PHA and LPS), bactericidal capacity of plasma, and the leukocyte profile were no different from those of non-reproductive females (irrespective of their ecotype).

Although ConA and PHA are mitogens known to stimulate T-lymphocytes, differential responses between them are not uncommon
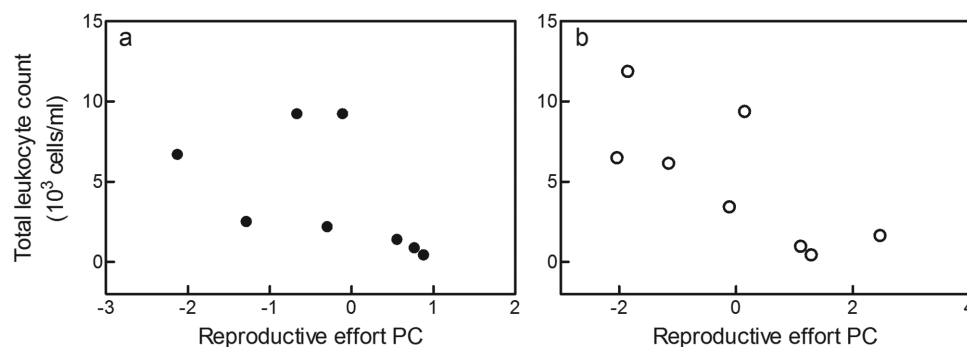

FIGURE 2 Negative correlations between reproductive effort PC scores and total leukocyte counts within each garter snake ecotype. a) L-fast snakes, Spearman rho = −0.72, P = 0.04, n = 8. b) M-slow snakes, Spearman rho = −0.71, P = 0.04, n = 8. Higher PC scores indicate larger reproductive effort.

TABLE 6 Studies on the Relationship Between Reproduction and Immunity in Squamates

Species (Reproductive Mode)	Study Design	Main Findings	Reference
Chalcides ocellatus (viviparous lizard)	Observational, G and NG females.[a]	Gravid females lower in vitro proliferation of spleen lymphocytes in response to ConA, PHA, and LPS than non-gravids.	Saad and el Deeb (1990)
Psammodromus algirus (oviparous lizard)	Experimental increase in reproductive effort by hormonal treatment in males.[b]	Experimentally elevated testosterone in males decreases leukocyte counts.	Veiga, Salvador, Merino, and Puerta (1998)
Ctenophorus fordi (oviparous lizard)	Experimental immune challenge (LPS injection) after mating in females.[a]	Immune challenged females reduced egg mass, but not clutch size or future reproduction.	Uller, Isaksson, and Olson (2006)
Urosaurus ornatus (oviparous lizard)	Experimental Exp.1: Increased reproductive effort by hormonal treatment in food restricted PV females Exp.2: Food restriction in PV and V females.[a]	Experimentally elevated vitellogenesis decreases wound healing in food limited females. Only vitellogenic females show slower wound healing during food restriction.	French et al. (2007)
Urosaurus ornatus (oviparous lizard)	Experimentally elevated investment in immunity by wounding in food restricted and control females.[a]	Wounded females show smaller follicles under food restriction, but not with *ad lib* food.	French et al. (2007)
Urosaurus ornatus (oviparous lizard)	Observational, PV, V, G*, and PR females.[a,b] *shelled-eggs retained in oviduct	Vitellogenic females show slower wound healing than other stages in field, but not in lab conditions.	French and Moore (2008)
Anolis sagrei (oviparous lizard)	Experimental manipulation of reproductive effort by ovariectomy.[a]	Ovariectomized females show higher in vivo PHA response than control females.	Cox et al. (2010)
Agkistrodon piscivorus (viviparous snake)	Observational, G and NG females.[b]	Gravid females show lower bactericidal capacity of plasma than non-gravids.	Graham et al. (2011)
Zootoca vivipara (viviparous lizard)	Experimental manipulation of reproductive effort by surgical litter reduction during gestation.[a]	Litter reduction (by surgery) has no effect on in vivo PHA response or survival.	Bleu, Massot, Haussy, and Meylan (2012)
Zootoca vivipara (viviparous lizard)	Experimental manipulation of reproductive effort by hormonal litter reduction during gestation.[a]	Litter reduction (by hormonal treatment) increased in vivo PHA response, but only in largest females.	Bleu, Massot, Haussy, and Meylan (2013)
Zootoca vivipara (viviparous lizard)	Experimental immune challenge (SRBC injection) during pregnancy.[a]	Effects of immune challenge on reproduction and in vivo PHA response dependent on interactions with SVL and body condition.	Meylan, Richard, Bauer, Haussy, and Miles (2013)
Antaresia children (oviparous snake)	Observational, G*, BR, and PR females.[a] *shelled-eggs retained in oviduct.	Gravid and brooding females show lower fRBC lysis (fecundity-independent), but not fRBC agglutination capacity of plasma, than post-reproductives.	Stahlschmidt et al. (2013)
Thamnophis elegans (viviparous snake)	Observational, G and NG females of two life-history ecotypes.[b]	Gravid females show depressed in vitro proliferation of T-lymphocytes in response to ConA, but similar lymphocyte responses to the other two mitogens (PHA and LPS), bactericidal capacity of plasma, and leukocyte profile compared to non-gravids. Trade-off between reproductive effort and TLC among gravid females.	This study

Abbreviations: BR, brooding eggs; fRBC, foreign red blood cells; G, gravid; NG, non-gravid; LPS, lipopolysaccharide; PHA, phytohemagglutinin; PR, post-reproductive; PV, pre-vitellogenic; SRBC, sheep red blood cells; SVL, snout-vent length; TLC, total leukocyte count; V, vitellogenic.
[a]Captivity.
[b]Field.

(e.g., Palacios et al., 2013; Palacios et al., 2009; Suresh, Sharma, & Belzer, 1993) and suggest that these mitogens likely stimulate different T-lymphocyte subsets (Suresh et al., 1993). Reduced lymphocyte proliferation responses during pregnancy are well documented in humans (Brunham et al., 1983) and, among squamates, have also been reported in the viviparous lizard *Chalcides ocellatus* (Saad & el Deeb, 1990, Table 6). T-lymphocytes are important effectors of cell-mediated responses to viruses, and reduced activity of T-lymphocytes during pregnancy has been linked to attenuated responses to viral disease (Pazos, Kraus, Munoz-Fontela, & Moran, 2012). It is interesting to note that reduced T-lymphocyte responses to ConA were also associated with infection by tail trematodes in garter snakes independent of their reproductive status. Whether reduced immune function leads to infection with trematodes, trematode infection depresses immune function, or both are a consequence of variation in individual quality or body condition, for instance, is not known at present and deserves further study. Nevertheless, our results suggest that in our study system, T-lymphocyte responses to ConA are more sensitive to variation in individual traits than the remaining immune components measured.

Body size and/or body condition can influence immune responses of vertebrates, including snakes (e.g., Palacios et al., 2011, 2013; Sparkman & Palacios, 2009; Ujvari & Madsen, 2006); however, this is not necessarily always the case. For instance, no relationship was found between SVL and bactericidal capacity of plasma in pregnant and non-pregnant female cottonmouth snakes (Graham, Earley, Guyer, & Mendonca, 2011). Likewise, SVL did not explain significant variation in any of the immune parameters measured in gravid and non-gravid female garter snakes (including bactericidal capacity) in the present study. Interestingly, bactericidal capacity increased and lymphocyte responses to ConA slightly decreased with SVL in non-reproductive male and female snakes from this same study system (Palacios et al., 2013), but there were no effects of SVL on immune parameters of neonate and juvenile snakes (Palacios et al., 2011). Taken together, these results highlight the diversity of relationships (or lack thereof) that can be found between body size and different immune parameters even within a species. Regarding body condition (i.e., an estimate of fat/energy reserves), we could not use in the present study the residuals of body mass on body size, a common index of body condition that we have previously used in non-reproductive garter snakes (Palacios et al., 2011, 2013; Sparkman & Palacios, 2009). This index of body condition is not adequate for use in gravid females (Graham et al., 2011), and more direct, non-lethal alternatives are not available for snakes (Gregory & Skebo, 1998). Hence, whether and how individual body condition affects the observed relationships between reproduction and immune function in our study system is not known at present and deserves further study.

Ecoimmunologists have hypothesized a link between life-history strategy and immune defense strategy (Lee, 2006; Martin, Pless, Svoboda, & Wikelski, 2004; Norris & Evans, 2000). Results consistent with differential reproductive costs in terms of reduced immunity have been reported for tree swallow populations (Ardia, 2005b): females from Alaska (faster pace of life) showed depressed immune function when forced to raise enlarged broods, whereas females from Tennessee (slower pace of life) did not show this trade-off. In the present study, we did not find the predicted differential costs between the two garter snake life-history ecotypes despite their pronounced differences in current reproductive investment and pace-of-life strategies (Table 1, Table 3). Instead, and in accordance with previous findings in the garter snake system, the absence of predicted immunological differences between the L-fast and M-slow ecotypes are consistent with the hypothesis that current environmental factors experienced by the two ecotypes in their natural habitats might have a stronger influence on immunity than their disparate life-histories (Palacios et al., 2013; Palacios et al., 2011). For instance, L-fast non-reproductive females and males, living in a habitat with higher food availability and mean ambient temperature (Bronikowski & Arnold, 1999; Miller, Clark, Arnold, & Bronikowski, 2011) showed higher lymphocyte proliferation responses to ConA and LPS than M-slow ones (Palacios et al., 2013). Perhaps, reproductive L-fast snakes might thus be able to invest more resources into current reproduction than M-slow snakes without suffering more pronounced costs in terms of immune function. This possible explanation would be in line with the idea of context-dependent trade-offs (Table 6; French & Moore, 2008; French, DeNardo, & Moore, 2007; French, Johnston, & Moore, 2007). Thus, at present, evidence for the hypothesized link between life-history strategy and immune defense strategy remains mixed (Ardia, 2005b; Martin, Hasselquist, & Wikelski, 2006; Martin, Weil, & Nelson, 2007; Palacios et al., 2013; Palacios et al., 2011; Previtali et al., 2012). Among squamates, a recent study in female *Zootoca vivipara* is along our findings in L-fast and M-slow garter snakes, showing rather weak influence of life-history strategy on various aspects of immune function (Bleu, Heulin, Haussy, Meylan, & Massot, 2012).

Finally, among reproductive females, we found evidence of fecundity-dependent costs of reproduction on TLCs within each ecotype. That is, females producing larger/heavier litters, both in absolute terms and relative to their body size, showed lower TLCs compared to females making a smaller reproductive investment. The remaining immune function variables measured were not correlated with female reproductive effort, again highlighting the immune component-specificity of patterns and the importance of assessing diverse measures of immunity in ecoimmunological studies. It is interesting to note that T-lymphocyte responses to ConA, which were significantly lower in gravid than non-gravid females, were nevertheless fecundity-independent among gravid females; whereas TLCs, which did not differ on average between gravid and non-gravid females, were nevertheless fecundity-dependent among gravid females. In addition, other aspects of immune function measured that were not related to female reproductive status or fecundity in this study (e.g., bactericidal capacity of plasma, lymphocyte responses to PHA and LPS), have been reported as being lower in gravid than non-gravid females in other squamates species (Graham et al., 2011; Saad & el Deeb, 1990; Table 6). This complexity precludes, at least with the data available at present, making generalizations about immune costs of reproduction across squamate species and/or immune function components. Rather, results suggest that detection of these costs depends on the combination of study species, ecological context (e.g., food resources available), and specific immune components measured.

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SUPPORTING INFORMATION