Complex Interplay of Body Condition, Life History, and Prevailing Environment Shapes Immune Defenses of Garter Snakes in the Wild

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ABSTRACT

The immunocompetence "pace-of-life" hypothesis proposes that fast-living organisms should invest more in innate immune defenses and less in adaptive defenses compared to slow-living ones. We found some support for this hypothesis in two life-history ecotypes of the snake *Thamnophis elegans*; fast-living individuals show higher levels of innate immunity compared to slow-living ones. Here, we optimized a lymphocyte proliferation assay to assess the complementary prediction that slow-living snakes should in turn show stronger adaptive defenses. We also assessed the "environmental" hypothesis that predicts that slow-living snakes should show lower levels of immune defenses (both innate and adaptive) given the harsher environment they live in. Proliferation of B- and T-lymphocytes of free-living individuals was on average higher in fast-living than slow-living snakes, opposing the pace-of-life hypothesis and supporting the environmental hypothesis. Bactericidal capacity of plasma, an index of innate immunity, did not differ between fast-living and slow-living snakes in this study, contrasting the previously documented pattern and highlighting the importance of annual environmental conditions as determinants of immune profiles of free-living animals. Our results do not negate a link between life history and immunity, as indicated by ecotype-specific relationships between lymphocyte proliferation and body condition, but suggest more subtle nuances than those currently proposed.

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Introduction

Ecological immunology, or ecoimmunology, seeks to understand the causes and consequences of the broad variation in immune function observed within, as well as among, species (Sheldon and Verhulst 1996; Norris and Evans 2000). An important influence on immune function is thought to be the life-history strategy of the organism (Sheldon and Verhulst 1996; Norris and Evans 2000; Zuk and Stoehr 2002). Specifically, populations/species with disparate life-history strategies have been hypothesized to invest preferentially in different immune components, that is, have different immune defense strategies, based on the costs and benefits associated with the different types of defense (Klasing and Leshchinsky 1999; Klasing 2004; Lee 2006; Martin et al. 2007). Vertebrate immune defenses are generally classified into two arms, innate and acquired, that interact broadly to fight disease but are characterized by different effectors and modes of action against pathogens. Innate (nonspecific) immune defenses constitute the first line of defense by responding faster and more generally to a broad range of pathogens than acquired defenses. In addition, innate defenses are thought to develop earlier during ontogeny and at a lower developmental cost, energetically and nutritionally, than acquired defenses. On the other hand, acquired (specific or adaptive) defenses constitute a second line of defense, are highly specific and more effective against recurring infections, and are thought to be less costly to deploy than some aspects of innate immunity such as inflammation. Given these characteristics, it has been hypothesized that organisms with fast pace-of-life strategies—characterized by rapid growth, early maturation, and high reproductive rate but a short life span—should rely more on innate immune defenses, whereas slow-living organisms—characterized by a long life span but slow growth, delayed maturation, and a lower reproductive rate—should rely more on adaptive immune defenses (Lee 2006). Despite the intuitive appeal of this hypothesis, few studies to date have tested its predictions, and support remains mixed (Lee et al. 2008; Sparkman and Palacios 2009; Cutrera et al. 2010; Previtali et al. 2012).

An excellent system for testing the predictions linking immune defense strategy to pace of life exists in evolutionary-divergent populations of two life-history ecotypes of the western terrestrial garter snake *Thamnophis elegans* (table 1). Replicate populations of the fast-living ecotype inhabit the shoreline of Eagle Lake in the northeastern Sierra Nevada range of California and display rapid growth to large body sizes, early maturation, high reproductive rate, and short median life span (hereafter referred to as the lakeshore L-fast ecotype). Surrounding the lake, replicate populations of the slow-living eco-

Table 1: Life-history and habitat differences between replicate populations of L-fast and M-slow garter snake ecotypes around Eagle Lake, California

Trait	L-fast ecotype	M-slow ecotype
Life history:[a,b]		
Mean body size (range; mm)	660 (425–876)	538 (370–598)
Size at maturity (mm)	450	400
Age at maturity (yr)	3	5–7
Reproductive rate	Annual	Resource dependent
Mean litter size (neonates)	8	5
Median life span (yr)	4	8
Habitat:[a]	Rocky lakeshore	Grassy meadow
Elevation (m)	1,555	1,630–2,055
Summer daytime temperature (°C)	20–34	15–30
Food/water availability	Continuous	Variable across years
Major prey types	Fish, leech	Anurans, leech
Tail trematode parasite	Absent	Present

[a]Bronikowski and Arnold 1999.
[b]Sparkman et al. 2007.

type inhabit mountain meadows and display the opposite set of life-history characteristics (hereafter referred to as the meadow M-slow ecotype). These ecotypes have been the focus of study for more than 30 yr, and in addition to their dramatic variation in pace of life, they also differ in various physiological parameters (summarized in Bronikowski and Vleck 2010) and show heritable differences in morphology (Manier et al. 2007). Furthermore, a common-environment experiment has revealed a genetic component to differences in growth rate between ecotypes (Bronikowski 2000), suggesting that several of the ecotypic differences are evolved rather than a plastic response to disparate habitats.

Our previous ecoimmunological research in this system has provided some support for the hypothesized association between immune and life-history strategies. Consistent with the hypothesis, free-ranging snakes from L-fast populations had higher levels of natural antibodies, complement-mediated lysis, and bactericidal competence of plasma—all innate immune components—than snakes from M-slow populations (Sparkman and Palacios 2009), and these ecotypic differences were also observed in 4-mo-old neonate snakes born and raised in a common environment (Palacios et al. 2011). On the other hand, some findings suggest that current environmental conditions might be as important as or even more important than life-history strategy in determining the immune defense profiles of these snakes. In particular, the ecotypic differences in innate immunity observed in 4-mo-old neonate snakes born and raised under common conditions were no longer detectable when individuals were sampled at 19 mo of age (Palacios et al. 2011). Meadow habitats have lower and less predictable food availability, lower ambient temperatures, and presence of a trematode parasite compared to lakeshore habitats (table 1). These environmental factors can have important impacts on immune function in vertebrates, including reptiles (Cooper et al. 1985; Chandra and Chandra 1986; Ujvari and Madsen 2006; Madsen et al. 2007), and could thus explain the lower innate immune levels in M-slow snakes compared to L-fast snakes in their natural environments (Sparkman and Palacios 2009).

Our previous work in this system, however, has been based solely on indexes of innate immunity, and assessment of aspects of acquired immunity is warranted to address the complementary prediction of the pace-of-life hypothesis regarding differential investment in acquired immune defenses. A bias toward measures of innate immune function in studies of free-living organisms is not uncommon due to their ease of sampling under field conditions, especially for animals for which recapture in the wild is difficult (e.g., Tieleman et al. 2005; Graham et al. 2011; Hegemann et al. 2012). Unlike many innate immune measures, most measures of acquired immunity routinely used by ecoimmunologists often necessitate two captures (e.g., in vivo antibody response to a challenge and delayed-type hypersensitivity response). Thus, in cases where recapture of individuals in the field is difficult, acquired immune measures have been obtained mainly from animals brought into captivity (e.g., Martin et al. 2006; Cutrera et al. 2010; Previtali et al. 2012). An alternative approach is to use measures of acquired immunity that do not require repeated captures in the field. Here, we optimized an in vitro lymphocyte proliferation assay that requires a single small blood sample to assess aspects of acquired immune function of free-ranging garter snakes. The in vitro lymphocyte proliferation assay measures the ability of T- and B-lymphocytes—the main effector cells of the acquired immune system—to become activated and proliferate on stimulation. Activation and proliferation of lymphocytes are critical initial steps of acquired immune responses—both cellular, mediated by cytotoxic T-lymphocytes, and humoral, mediated by B-lymphocytes and helper T-lymphocytes—leading to the formation of clones of cells that can more effectively fight the invader (Roitt et al. 1998). In vitro lymphocyte proliferation assays are widely employed by immunologists and immunotoxicologists (Froebel et al. 1999; Grasman 2002; Fairbrother et al. 2004) and can provide val-

uable insights into the immune function of free-living animals (Palacios et al. 2007, 2009).

Following the pace-of-life hypothesis, we predicted that M-slow snakes would display stronger B- and T-lymphocyte proliferative responses than L-fast snakes, opposing the pattern already documented for innate immune defenses for these ecotypes in their natural environments. Alternatively, we predicted that if prevailing environmental conditions have a stronger influence than life-history strategy in determining the immune profiles of the snakes (hereafter referred to as environmental hypothesis), then M-slow snakes would display weaker B- and T-lymphocyte proliferative responses than L-fast snakes, given the harsher environmental conditions they face and paralleling the already documented differences in innate immune defenses. These hypotheses are not mutually exclusive as both life history and current environment likely interact to affect immune functions of wild animals; however, the ecotypic patterns predicted by the hypotheses are expected to differ based on the relative importance of these factors in shaping the immune profiles of the snakes. Thus, in this study we tested these predictions while controlling for the potentially confounding effects of sex, size, body condition, and levels of the stress hormone corticosterone. In addition, we measured bactericidal capacity of plasma as an index of innate immune function to compare to the patterns previously documented in our study system (Sparkman and Palacios 2009; Palacios et al. 2011).

Material and Methods

Study Populations

More than 35 populations of the western terrestrial garter snake *Thamnophis elegans* are arrayed over a 100-km^2 study area at 1,555–2,055 m at the northern end of the Sierra Nevada in northeastern California. Lakeshore (L-fast) populations are found at widely spaced intervals along the shore of Eagle Lake, a large (15 km × 3 km) natural lake, whereas meadow (M-slow) populations inhabit mountain meadows that surround the lake at distances of several kilometers (Bronikowski and Arnold 1999). In this project we focused on four replicate lakeshore and four replicate meadow populations in our study system that have been the focus of study for more than 30 yr. The snakes in our study system emerge from hibernacula and mate in the spring, actively forage during the summer, give birth to live young in late August–September, and then hibernate for an extended period (October–April).

Field Work

We sampled snakes from four replicate L-fast ($n = 60$ individuals) and four replicate M-slow ($n = 70$ individuals) populations between June 11 and June 22, 2010. This sampling time ensured the inclusion of all ages of snakes, including young of the previous year that had survived their first winter hibernation while excluding young of the current year because birthing begins in late August. Snakes ranging from 200 to 700 mm snout-vent length (SVL)—that is, the full range of potential body sizes—were hand-captured from under rocks, in grasses, or while out basking or actively foraging. Blood samples (70–150 μL, depending on snake body size) were obtained from the caudal vein via heparin-rinsed syringes using sterile techniques. Mean ($\pm$SE) time elapsed between capture and bleeding was 16.6 $\pm$ 2.21 min (median, 8 min). Time elapsed between capture and bleeding did not explain significant variation in corticosterone levels measured in these samples (linear regression of $\log_{10}$-transformed variables: $r^2 = 0.01$, $P = 0.33$, $n = 97$). One drop of blood was immediately used to prepare a thin blood smear for differential leukocyte counts and detection of blood parasites. Next, 60 μL of whole blood was 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 for use in the lymphocyte proliferation assay the following morning. The remainder of the blood was kept on ice until plasma was separated by centrifugation and snap-frozen in liquid nitrogen in the field. Upon arrival in the laboratory, plasma was stored at −80°C until used in the bactericidal capacity and corticosterone assays. After blood collection and processing, snakes were weighed, measured (SVL), and sexed via hemipene eversion, and females were palpated to determine gravidity (i.e., the presence or absence of embryos). Because gravidity can influence immune function of females (e.g., Graham et al. 2011) and its effects are the main focus of another study in this system (M. G. Palacios and A. M. Bronikowski, unpublished data), only data for nongravid females (M-slow: $n = 33$; L-fast: $n = 27$) and males (M-slow: $n = 28$; L-fast: $n = 24$) are included in this work. Before release at their site of capture, snakes were examined for the presence of the trematode *Alaria* spp., which causes inflammation and necrosis in the tail region. In addition, once in the laboratory, we checked for the presence of extracellular and intracellular blood parasites by scanning the blood smears under a compound microscope as described elsewhere (Palacios and Martin 2006).

In Vitro Lymphocyte Proliferation

Previous studies in reptiles that have assessed in vitro lymphocyte proliferation have used terminal sampling (i.e., requiring euthanasia) for obtaining the necessary lymphocytes (e.g., Eldeeb and Saad 1987; Saad and Shoukrey 1988; Saad 1989; Muñoz and De la Fuente 2001*a*, 2001*b*). Here we used a whole-blood mitogenic stimulation assay that does not require terminal sampling (Palacios et al. 2007, 2009) and that we adapted for use in free-living garter snakes. Parameters reported here provided optimal proliferation responses for garter snake blood in preliminary trial analyses (refer to the appendix in the online edition of *Physiological and Biochemical Zoology* for information on assay optimization). Because prolonged storage of blood can affect lymphocyte proliferation assays in some species (e.g., Raj et al. 1997), we tested the effect of storage on proliferation responses of garter snake blood cells diluted

1 : 1 in supplemented AIM-V lymphocyte medium and kept on ice for 24 h. Proliferation responses of snake blood stored in the aforementioned conditions did not differ from those of fresh cells (paired samples *t*-test: $t = 0.669$, $P = 0.519$, $n = 11$). Within 24 h of collection, blood samples were further diluted to 1 : 20 using supplemented AIM-V lymphocyte medium, and 50 μL of the dilution was dispensed into flat-bottomed 96-well microculture plates containing 50 μL of mitogen (stimulated wells) or 50 μL of medium (nonstimulated control wells). We tested for proliferation of lymphocytes using two standard T-cell mitogens, phytohaemagglutinin (PHA; 40 μg/mL) and concanavalin A (ConA; 10 μg/mL), and the B-cell mitogen lipopolysaccharide (LPS from *E. coli*; 20 μg/mL), all from Sigma (St. Louis, MO) and prepared using supplemented AIM-V lymphocyte medium. Triplicate blood cultures were incubated in a 7% CO_2, 28°C humidified atmosphere for a total of 96 h and pulsed during the last 24 h 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 the mean cpm of replicate mitogen–stimulated cultures or as a stimulation index (SI), calculated as the ratio between the mean cpm of replicate mitogen–stimulated wells and the mean cpm of replicate nonstimulated (control) cultures (Farag and Ridi 1986; Palacios et al. 2009).

Whole-blood assays of lymphocyte proliferation, like those used in this study, are considered more representative of in vivo conditions than assays that use isolated lymphocytes (Lee 1978). However, because they use a constant volume of blood, not number of lymphocytes, the proliferation response is likely a combination of both the initial number of lymphocytes present and the activation and proliferative ability of these lymphocytes. To control for the potential effect of different initial lymphocyte numbers, we estimated the total lymphocyte count (no. lymphocytes/μL blood) for inclusion as a covariate in the statistical analyses. Total lymphocyte counts were estimated by multiplying the total leukocyte count by the proportion of lymphocytes in the sample. The total leukocyte count 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, whereas the proportion of lymphocytes was derived from a differential leukocyte count on blood smears stained with Wright's stain and scanned under × 1,000 oil immersion using a compound microscope as described in Palacios et al. (2009).

Bactericidal Competence of Plasma

We assessed bactericidal competence of plasma following the method of Matson et al. (2006), with a few modifications for use in garter snakes (Sparkman and Palacios 2009; Palacios et al. 2011). A pellet of lyophilized *E. coli* (ATCC8739; Microbiologics, St. Cloud, MN) was reconstituted using 40 mL × 1 phosphate-buffered saline (PBS), and a fraction was further diluted 1 : 64 with PBS to produce a working solution containing approximately 150 colony-forming bacteria per 10 μL. All plasma samples were diluted 1 : 10 with PBS, and sample reactions were prepared by adding 10 μL of the 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 (three duplicates) were prepared by adding 10 μL of the bacterial working solution to 100 μL PBS, and duplicates 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 (~25°C). The number of bacterial colonies on each plate was then counted, and the percentage of colonies on each plate per the mean number of colonies in the control plates was calculated. This percentage was subtracted from 100 to obtain the percentage of bacteria killed.

Corticosterone Radioimmunoassay

The concentration of plasma corticosterone in the samples was determined using a double-antibody radioimmunoassay kit (catalog no. 07-102103; MP Biomedical, Orangeburg, NY) that had already been validated for use in our study system (Robert et al. 2009; Palacios et al. 2012). All samples were run in duplicate and assayed within a 24–48-h time period, with samples from L-fast and M-slow snakes included in each assay.

Statistical Analyses

We used general linear models to test the effects of ecotype (L-fast vs. M-slow) on immune responses while controlling for several factors that can confound the relationships of interest. Body condition and age are known to influence immune responses of vertebrates, including snakes (e.g., Ujvari and Madsen 2006, 2011; Sparkman and Palacios 2009; Palacios et al. 2011). We estimated body condition as the size-corrected weight of the snakes, calculated as the residuals of the regression of log body weight on log SVL (Weatherhead and Brown 1996), and used SVL (i.e., body size) as a proxy of age of the snakes (e.g., Ujvari and Madsen 2006; Sparkman and Palacios 2009). In addition, immune responses can vary with the sex of individuals (e.g., Saad and Shoukrey 1988; Saad 1989) and with the levels of stress (reviewed in Martin 2009). In fact, concentrations of the stress hormone corticosterone can differ between the two ecotypes of garter snakes, with M-slow snakes having higher levels than L-fast snakes (Robert et al. 2009; Palacios et al. 2012). Thus, these four factors—condition, SVL, sex, and corticosterone—were tested as covariables in the models.

General linear models for the four immune variables (i.e., lymphocyte proliferative responses to ConA, PHA, and LPS and bactericidal competence) thus included the fixed effects of ecotype, population nested within ecotype, and sex, while body condition, SVL, and corticosterone concentration were in-

Table 2: General linear models for mitogen-induced lymphocyte proliferation responses and bactericidal capacity of plasma of free-living garter snakes of the fast- and slow-living ecotype

	ConA cpm		PHA cpm		LPS cpm		BC	
Effect	*F*	df	*F*	df	*F*	df	*F*	df
Ecotype	4.82*	1, 95	.04	1, 95	4.08*	1, 95	.36	1, 156
Pop(ecotype)	2.41*	6, 95	1.47	6, 95	1.50	6, 95	2.45*	6, 156
Sex	3.07	1, 95	4.61*	1, 95	3.93*	1, 95	.04	1, 156
Control cpm	15.70**	1, 95	12.01**	1, 95	41.94**	1, 95	...	...
Total lymphocytes	8.85**	1, 95	16.03**	1, 95	10.41**	1, 95	...	...
SVL	6.24*	1, 95	2.61	1, 95	1.36	1, 95	18.96**	1, 156
Condition	.01	1, 95	1.52	1, 95	.19	1, 95	.02	1, 156
Ecotype × condition	12.52**	1, 95	2.90	1, 95	20.33**	1, 95	.03	1, 156

Note. Lymphocyte proliferation variables (ConA cpm, PHA cpm, and LPS cpm) were $\log_{10}$ transformed, while bactericidal competence (BC) was arcsine square root transformed before analyses. ConA, concanavalin A; cpm, counts per minute; LPS, lipopolysaccharide; PHA, phytohaemagglutinin; SVL, snout-vent length.

*$P < 0.05$.

**$P < 0.01$.

cluded as covariates. We also assessed all two-way interactions with ecotype (i.e., ecotype × sex, ecotype × body condition, ecotype × SVL, and ecotype × corticosterone). Population nested within ecotype represents the effect of the different replicate populations within each ecotype (L-fast and M-slow; $n = 4$ populations per ecotype), treated as a fixed effect to reflect complex microhabitat variation among populations even within ecotypes. In addition to these effects and interactions, the models for lymphocyte proliferative responses also included as covariates the total lymphocyte count (both for mean cpm of mitogen-stimulated cultures and SI) and mean cpm of control cultures (only for mean cpm of mitogen-stimulated cultures). For bactericidal competence, in addition to the samples collected in 2010 (described above), we also had samples similarly collected the previous year (July 2009; $n = 95$). We therefore tested year (2009 vs. 2010) as a fixed effect and interactions with year in the model for bactericidal competence in addition to the effects and interactions described above. In all cases nonsignificant interactions were removed from the final models if they exceeded an α level of 0.1. Corticosterone concentration differed between the two ecotypes (Wilcoxon $Z = -2.85$, $P = 0.004$; M-slow median, 82 pg/mL; L-fast median, 48 pg/mL) but did not explain significant variation in any of the four immune variables when included in the analyses (all $P > 0.16$) and was therefore excluded from all final models.

Lymphocyte proliferative responses and total lymphocyte counts were $\log_{10}$ transformed, while bactericidal competence was arcsine square root transformed to obtain normally distributed residuals in the models. Results and conclusions for lymphocyte proliferative responses (i.e., to ConA, PHA, and LPS) obtained using the SI as a dependent variable did not differ from those obtained using the mean cpm of mitogen-stimulated cultures as dependent variables; therefore, only the latter are presented and discussed. Sample sizes differ among analyses because not all variables could be measured in some individuals because of limited blood volume. Figures depict the least square means from the general linear models, whereas descriptive summary statistics for the raw immune function data are available in the appendix. All statistical analyses were performed using SAS 9.2 (SAS Institute, Cary, NC).

Results

Parasite Loads

The prevalence of trematode infection was 55% in M-slow snakes and 0% in L-fast snakes, which was evident in both the 2010 and 2009 samples. Thus, as previously documented in this system, tail trematodes are present only in snakes inhabiting meadow habitats (Sparkman and Palacios 2009). Also as previously reported in this system (Sparkman and Palacios 2009), no blood parasites were detected in the blood smears of any snake of either ecotype in this study.

Acquired Immunity: In Vitro T- and B-Lymphocyte Proliferation

All three lymphocyte proliferative responses (i.e., mean cpm of mitogen-stimulated cultures) increased with an increase in the corresponding control values (i.e., mean cpm of control unstimulated cultures) and with an increase in the total number of lymphocytes initially present in the cultures (table 2). Even after controlling for these effects, the three lymphocyte proliferative responses were positively correlated with each other, such that individuals with relatively strong responses to one mitogen also had relatively strong responses to the other two mitogens (Pearson correlations, ConA-PHA: $r = 0.48$; ConA-LPS: $r = 0.60$; PHA-LPS: $r = 0.37$; $P < 0.0001$ and $n = 110$ in all cases). Despite this intercorrelation, analyses indicated somewhat different sources of variation for these proliferative responses; thus, we present a separate model for each proliferative variable.

Proliferation by T-lymphocytes in response to ConA showed a significant effect of ecotype, population nested within ecotype, ecotype × condition interaction, and SVL (table 2). L-fast

snakes had overall stronger responses than M-slow snakes (fig. 1), but as indicated by the interaction term between ecotype and body condition, these proliferative responses decreased with body condition in L-fast snakes while they increased in M-slow snakes (fig. 2*a*). Lymphocytes of longer/older snakes showed weaker proliferative responses to ConA than those of shorter/younger snakes, irrespective of ecotype, as indicated by the negative effect of SVL (fig. 3*a*). In relation to sex, males tended to have higher proliferative responses to ConA than females, also irrespective of ecotype, but this effect was only marginally significant ($P = 0.08$; fig. 4*a*). Given the high proportion of meadow snakes infected by trematodes, we tested whether trematode infection affected their proliferative response to ConA and found that infected meadow snakes displayed reduced responses compared to noninfected meadow snakes ($F_{1,56} = 4.43$, $P = 0.039$, $n = 60$). To determine whether the effects described in the main model (table 2) were influenced by the presence of trematode-infected meadow snakes, we repeated the analysis after removal of the 32 infected meadow individuals. This removal resulted in the effects on ecotype, population nested within ecotype, and sex becoming nonsignificant (all $P > 0.13$) and the effect of SVL becoming marginally significant ($P = 0.075$), while the ecotype × condition interaction remained unchanged ($P = 0.002$).

Proliferation by T-lymphocytes in response to PHA showed only a significant effect of sex (table 2), with males displaying stronger responses than females irrespective of ecotype (fig. 4*b*). Although nonsignificant ($P = 0.09$), an ecotype × condition interaction patterned similarly to that for ConA was observed for the proliferative response to PHA (fig. 2*b*; table 2). In contrast to the T-lymphocyte response to ConA, however, when we restricted our analysis to just meadow snakes, their response to PHA was not affected by their trematode infection status ($F_{1,56} = 1.10$, $P = 0.298$, $n = 60$).

Finally, proliferation by B-lymphocytes in response to LPS showed a significant effect of ecotype, ecotype × condition interaction, and sex (table 2). L-fast snakes had overall stronger responses than M-slow snakes (fig. 1), and the interaction pattern was the same as described for the other two mitogens, with responses of L-fast snakes decreasing and those of M-slow snakes increasing with body condition (fig. 2*c*). Once again, males showed stronger proliferative responses than females (fig. 4*c*). Similar to the T-cell response to PHA, B-cell response to LPS by meadow snakes was not affected by the presence or absence of trematode infection ($F_{1,56} = 1.12$, $P = 0.294$, $n = 60$).

Innate Immunity: Bactericidal Capacity of Plasma

Bactericidal capacity, assessed as the percentage of *E. coli* killed by plasma, was not correlated with any of the three lymphocyte proliferative responses (Pearson correlations: all $r < 0.13$, $P > 0.263$, $n = 75$ in all cases) but showed significant effects of population nested within ecotype and body size/age (SVL; table 2). Year (2009 vs. 2010) and interactions between year and other effects were not significant (all $P > 0.1$) and were therefore removed from the model. Contrary to the pattern observed for T-lymphocyte proliferative responses to ConA, longer/older snakes displayed stronger bactericidal capacity, irrespective of ecotype, than shorter/younger ones (fig. 3*b*). Trematode infection status in meadow snakes had a marginally significant effect on this immune response ($F_{1,100} = 3.82$, $P = 0.054$, $n = 102$), with infected individuals tending to have lower bactericidal capacity than uninfected ones. Removal of the 54 infected meadow individuals from the data set resulted in the effect of population nested within ecotype becoming nonsignificant ($P > 0.1$), while the effect of SVL remained unchanged ($P = 0.0002$).

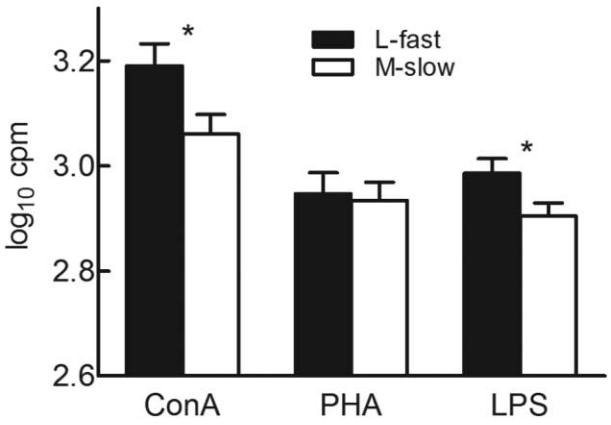

Figure 1. In vitro lymphocyte proliferative responses of garter snakes from L-fast and M-slow life-history ecotypes (trematode-infected individuals included). Bars represent least square means ± SE from the models in table 2. Asterisk = $P < 0.05$.

Discussion

The ecoimmunological pace-of-life hypothesis proposes a link between immune and life-history strategies, such that fast-living organisms should rely relatively more on innate immune defenses and less so on adaptive defenses compared to slow-living ones (Lee 2006). We had previously found some support for this hypothesis in two life-history ecotypes of the western terrestrial garter snake sampled in their natural environments; that is, fast-living (L-fast) snakes showed higher levels of three measures of constitutive innate immunity than slow-living (M-slow) snakes (Sparkman and Palacios 2009). In this study we optimized an in vitro assay of acquired immune function to test the complementary prediction that M-slow snakes should in turn show stronger adaptive immune defenses than L-fast snakes. Based on this assay we did not find support for the prediction. In fact, in vitro lymphocyte proliferation responses to the T-cell mitogen ConA and to the B-cell mitogen LPS were on average higher in L-fast snakes than in M-slow ones, opposing the pattern predicted by the ecoimmunological pace-of-life hypothesis.

Instead, the documented pattern is more in line with the environmental hypothesis, which states that the differences in

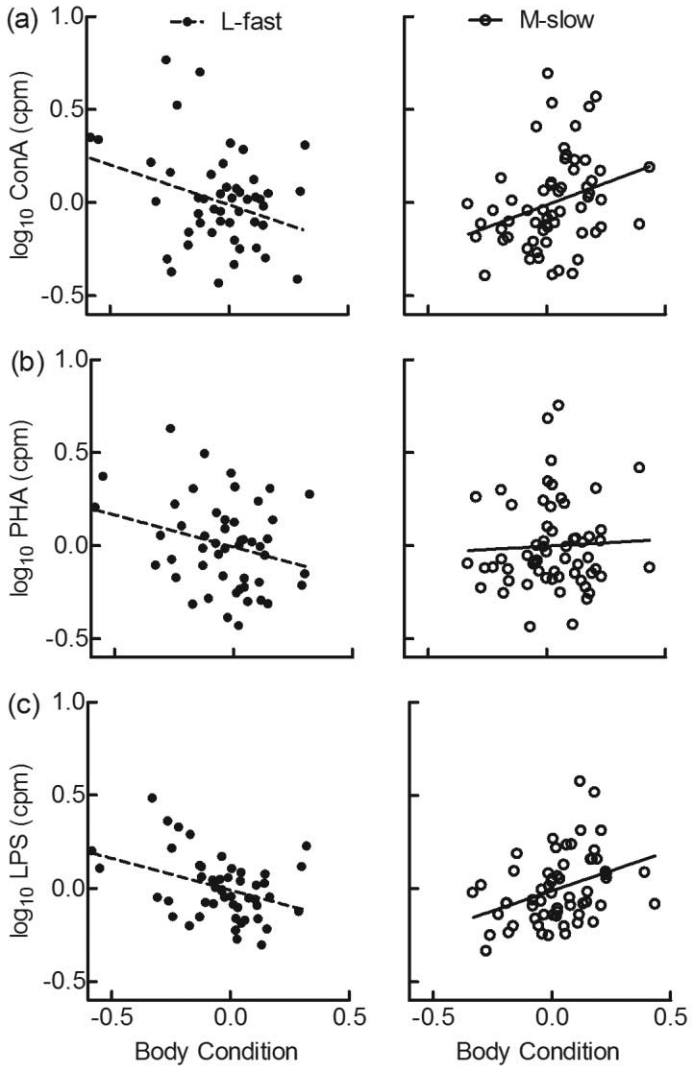

Figure 2. In vitro lymphocyte proliferative responses as a function of body condition in garter snakes from L-fast and M-slow life-history ecotypes (trematode-infected individuals included). *a*, T-lymphocyte response to ConA; *b*, T-lymphocyte response to PHA; *c*, B-lymphocyte response to LPS. Points represent residuals from the models in table 2 with body condition removed from the model. These residuals are plotted against body condition to reveal the relationship of each dependent variable with body condition while all other effects in the model are held constant.

immune defense observed between the two snake ecotypes are caused mainly by differences in environmental conditions currently experienced by snakes in the meadow and lakeshore habitats. Consistent with this hypothesis, snakes inhabiting the lakeshore (L-fast snakes) show overall higher immunity—assessed through various indexes of innate and adaptive immune function—than snakes that live in the meadows surrounding the lake (M-slow snakes) likely due to the more favorable environmental conditions in the former. Several environmental factors known to influence immune responses differ between the lakeshore and meadow habitats (table 1) and could individually or in conjunction explain the patterns of immune function observed.

First, lakeshore habitats show higher and more predictable food resources compared to meadow ones. While L-fast snakes feed primarily on fish that are always available in the lake, M-slow snakes feed primarily on anurans that depend on standing water in the meadows for breeding and are thus abundant only in years with adequate levels of precipitation (Bronikowski and Arnold 1999). Good nutrition is paramount for strong immune defenses (Chandra and Chandra 1986); thus, L-fast snakes might show higher innate and adaptive immunity thanks to the more plentiful and less variable food resources available in lakeshore habitats. Second, lakeshore habitats are at lower altitude than the mountain meadows surrounding the lake and therefore have higher (by about 4°C) ambient temperatures. Reptile physiology is highly dependent on environmental temperature, and immune function is no exception. In general,

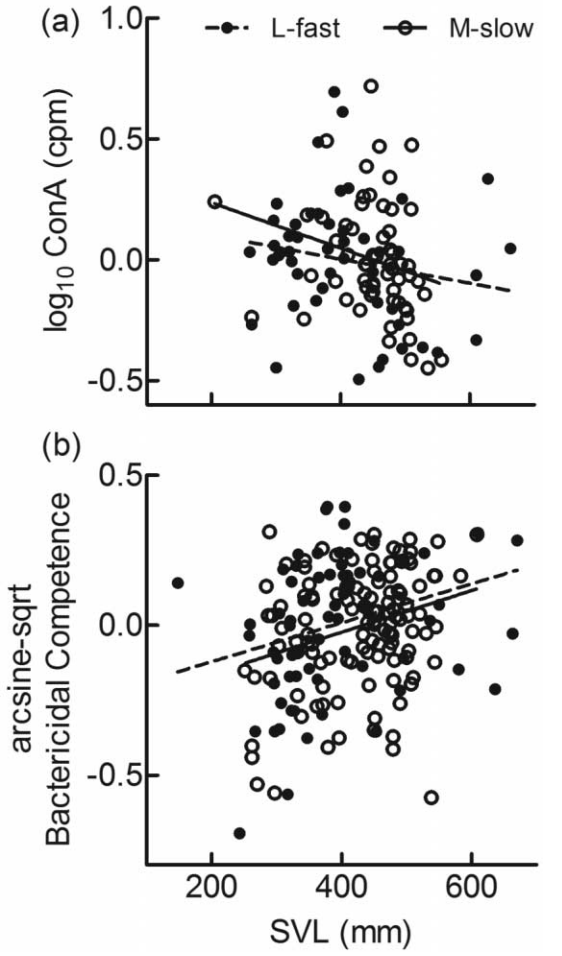

Figure 3. Immune responses as a function of snout-vent length (SVL) in garter snakes from L-fast and M-slow life-history ecotypes (trematode-infected individuals included). *a*, T-lymphocyte response to ConA; *b*, bactericidal competence of plasma. Points represent residuals from the models in table 2 with SVL removed from the model. These residuals are plotted against SVL to reveal the relationship of each dependent variable with SVL while all other effects in the model are held constant.

higher temperatures (but below the upper temperature limits) favor stronger immune responses in reptiles (Cooper et al. 1985); thus, the stronger responses of L-fast snakes could be a result of the higher ambient temperatures they are exposed to in the lakeshore. Third, although no data exist on the overall parasite pressure that L-fast and M-slow snakes face in their environments, M-slow snakes show high prevalence (more than 50%) of infections by a trematode that causes necrosis of the tail, while L-fast snakes do not show such infections. Infection can have a negative impact on immune function of the host. For instance, antibody responses of water pythons *Liasis fuscus* decreased as blood parasite load increased (Ujvari and Madsen 2006). Although we found no blood parasites in either ecotype, we found that M-slow snakes showing trematode infections displayed reduced T-cell proliferative responses to ConA (and a trend toward reduced bacterial killing capacity) compared to uninfected M-slow snakes. Thus, the weaker responses of M-slow snakes compared to L-fast ones could be due to the presence of trematode-infected individuals among the former but not among the latter. In agreement with this scenario, removal of infected M-slow snakes from the analysis resulted in the vanishing of the overall ecotypic difference in the lymphocyte proliferative response to ConA.

Further evidence in support of the environmental hypothesis in this snake system comes from our finding that the bactericidal capacity of plasma of free-living individuals differs between the two life-history ecotypes in some years (2008; Sparkman and Palacios 2009) but not in others (2009 and 2010; this

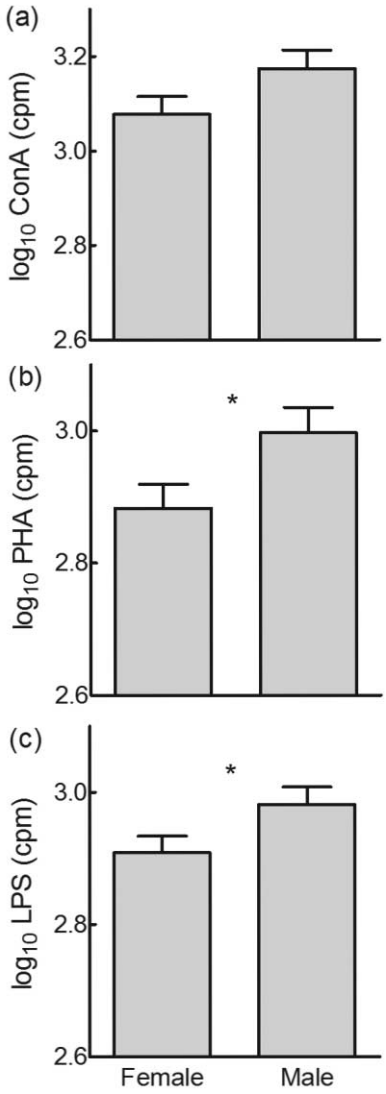

Figure 4. In vitro lymphocyte proliferative responses as a function of sex in garter snakes (both ecotypes combined). *a*, T-lymphocyte response to ConA; *b*, T-lymphocyte response to PHA; *c*, B-lymphocyte response to LPS. Bars are least square means ± SE from the models in table 2. Asterisk = $P < 0.05$.

study). This finding, together with the fact that bactericidal capacity of plasma does not show an ecotypic difference when snakes of the two ecotypes are born and raised in a common environment (Palacios et al. 2011), highlights the importance of prevailing environmental conditions rather than life-history strategy per se in shaping the immune profiles of these snakes. Interannual variation in ecotypic patterns seems the rule rather than the exception in this system, as we have documented for other physiological functions of the L-fast and M-slow snakes (i.e., insulin-like growth factor 1 levels [Sparkman et al. 2009], baseline corticosterone levels [Palacios et al. 2012]). The need for further studies that span more than a single year when testing hypotheses about physiological patterns in free-living animals has recently been emphasized by Hegemann et al. (2012), and our work supports this notion.

An interesting finding of our study that points to a complex interplay between prevailing environmental factors and life history is the interaction between ecotype and body condition on our measures of adaptive immunity. While proliferation of B- and T-lymphocytes increased with increasing body condition in M-slow snakes, the opposite relationship was found in L-fast snakes. Most ecoimmunological studies show either a positive relationship or a lack of relationship between immune function and body condition (e.g., Ujvari and Madsen 2006; Palacios et al. 2009); thus, the negative relationship in L-fast snakes is intriguing. We hypothesize that the divergent patterns observed here might be a result of different resource allocation strategies based on both current energy reserves available for competing demands (i.e., body condition) and life-history strategy (i.e., L-fast or M-slow). Under this scenario, M-slow snakes in better body condition would be expected to allocate relatively more resources to immune defense (self-maintenance) than those in worse condition in order to further increase their chances of survival and future reproduction. On the other hand, L-fast snakes in better body condition might favor an even larger investment in current reproduction at the expense of immune function compared to those in worse body condition, which could potentially underlie the negative relationship between body condition and lymphocyte proliferative responses observed in the L-fast ecotype. A possible caveat to this interpretation is that the indirect proxy for body condition used here (i.e., mass-length residuals) may not have the same meaning for both ecotypes given their different growth trajectories (Bronikowski and Arnold 1999). Thus, further research incorporating alternative measures of body condition is warranted to understand the effects of this variable on immune functions of the snakes.

Finally, we found that male garter snakes showed higher lymphocyte proliferation responses than females irrespective of ecotype, whereas the sexes did not differ in bactericidal capacity of plasma. Our results contrast with those of previous studies that have measured in vitro lymphocyte proliferation responses in reptiles. While studies in another snake species found higher immune responses in females (Saad and Shoukrey 1988; Saad 1989), two studies in turtles reported no sex differences (Muñoz and De la Fuente 2001*b*; Keller et al. 2006). On the other hand, the lack of sex differences in innate immunity parallels findings in water pythons (Ujvari and Madsen 2011) and marine iguanas (French et al. 2010). Differential investment in immune function by the sexes is widespread in animals, although not universal, with females generally showing higher immune responses than males (Rolff 2002; Nunn et al. 2009). Thus, the finding of higher T- and B-lymphocyte proliferation responses by male garter snakes is interesting and deserves further study. We hypothesize that sex-specific reproductive history may impact the ability of the snakes to invest in immune defense. In any given growth season, adult females—of both ecotypes—are either building up energetic resources for their next reproductive event or actively pregnant. On the other hand, males would not be expected to have a signature of previous years' reproduction on current immune function due to the expectation of annual fertility based on sperm production. An alternative and not mutually exclusive hypothesis for higher immune function in males irrespective of ecotype might be the prevalence of multiple paternity and intrasexual competition among males in these populations (M. Manes and A. M. Bronikowski, unpublished data) to the extent that immune function is a target of selection for successful paternity.

Assessment of acquired immunity of free-living animals continues to be a challenge for ecoimmunologists as most techniques require recapture of the individuals (Salvante 2006; Ardia and Schat 2008; Demas et al. 2011). This can be facilitated by bringing the animals into captivity. Artificial conditions and stress in captivity, however, can cause uncharacteristic immune responses (Ardia and Schat 2008; Kuhlman and Martin 2010) that might be difficult or even impossible to relate to immune defenses of free-living animals in their natural habitats. In this respect, the in vitro lymphocyte proliferation assay used here is a good option for assessment of adaptive immunity because it can be performed with a single small blood sample collected in the field and measures important aspects of acquired immune responses, that is, the ability of lymphocytes to become activated and proliferate upon challenge. Using different mitogens (e.g., PHA, ConA, LPS), different lymphocyte subsets are targeted, providing information on more than one cell type (Fairbrother et al. 2004). Although performance of this assay requires a specialized laboratory setting, it can be accomplished through fruitful collaborations among ecoimmunologists and more traditional immunologists, as is generally the case for other measures of immune function (e.g., Matson et al. 2005, 2006; Millet et al. 2007). The lymphocyte proliferation assay—as do most assays of immune function—has some limitations in addition to its strengths (Fairbrother et al. 2004; Demas et al. 2011). In particular, this assay assesses only early steps of acquired immune responses; thus, for a more complete assessment of acquired immunity of animals that cannot be easily recaptured in the wild, additional in vitro assays that measure effector functions such as B-cell antibody production or T-cell cytotoxicity and that also involve a single blood sample could be performed (Fairbrother et al. 2004).

In conclusion, we found no support for the hypothesis linking pace of life with a more innate versus more adaptive im-

mune defense strategy in this garter snake system, using in vitro lymphocyte proliferation responses as indexes of acquired immunity of free-living individuals. Instead, our results point to a complex interplay of life history, individual characteristics, and prevailing environmental factors in shaping the immune profiles of these wild reptiles. Such complex interactions are probably widespread in natural populations and should be taken into account when testing ecoimmunological theory, as exemplified here by the pace-of-life hypothesis. In particular, few ecoimmunological studies test for pattern variation across years; thus, conclusions from such studies might provide only a partial look at the bigger picture that could arise if additional years were included and could also help explain the pervasiveness of mixed support for some hypotheses. In addition, our study highlights that elucidation of the main environmental contributors to interannual variation in physiological patterns is crucial if we are to understand the diversity in immune profiles displayed by free-living animals. In this respect, and as highlighted by a recent study by Horrocks et al. (2012), understanding the link between disease environment and immune function in natural populations is paramount.

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