

DR MARIA GABRIELA PALACIOS (Orcid ID : 0000-0003-3206-5581)

DR ANNE BRONIKOWSKI (Orcid ID : 0000-0001-6432-298X)

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Genetic background and thermal environment differentially influence the ontogeny of immune components during early life in an ectothermic vertebrate

Maria G. Palacios ^{1,2*}, Eric J. Gangloff ^{1,3}, Dawn M. Reding ^{1,4}, and Anne M. Bronikowski ¹

¹. Dept. of Ecology, Evolution, and Organismal Biology, Iowa State University, Ames, IA, 50010, USA.

* Corresponding author:

Maria G. Palacios (gpalacios@cenpat-conicet.gob.ar)

Current addresses:

². Centro para el Estudio de Sistemas Marinos, CCT CONICET-CENPAT, Blvd. Brown 2915, Puerto Madryn, Chubut, 9120, Argentina.

³. Department of Zoology, Ohio Wesleyan University, Delaware, OH, USA.

⁴. Department of Biology, Luther College, Decorah, IA, USA.

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Abstract

1. An understudied aspect of vertebrate ecoimmunology has been the relative contributions of environmental factors (E), genetic background (G), and their interaction ($G \times E$) in shaping immune development and function. Environmental temperature is known to affect many aspects of immune function and alterations in temperature regimes have been implicated in emergent disease outbreaks, making it a critical environmental factor to study in the context of immune phenotype determinants of wild animals.
2. We assessed the relative influences of environmental temperature, genetic background, and their interaction on first-year development of innate and adaptive immune defenses of captive-born garter snakes (*Thamnophis elegans*) using a reciprocal-transplant laboratory experiment. We used a full-factorial design with snakes from two divergent life-history ecotypes, which are known to differ in immune function in their native habitats, raised under conditions mimicking the natural thermal regime —i.e., warmer and cooler— of each habitat.
3. Genetic background (ecotype) and thermal regime influenced innate and adaptive immune parameters of snakes, but in an immune-component specific manner. We found some evidence of $G \times E$ interactions but no indication of adaptive plasticity with respect to thermal environment. At the individual level, the effects of thermal environment on resource allocation decisions varied between the fast- and the slow-paced life-history ecotypes. Under warmer conditions, which increased food consumption of individuals in both ecotypes, the former invested mostly in growth, whereas the latter invested more evenly between growth and immune development.
4. Overall, immune parameters were highly flexible, but results suggest that other environmental factors are likely more important than temperature *per se* in driving the ecotype differences in immunity previously documented in the snakes under field conditions. Our results also add to the understanding of investment in immune development and growth during early postnatal life under different thermal environments. Our finding of immune-component specific patterns strongly cautions against oversimplification of the highly complex immune system in ecoimmunological studies. In conjunction, these results deepen our understanding of the degree of immunological flexibility wild animals present, information that is ever more vital in the context of rapid global environmental change.

Keywords: adaptive immunity, ecoimmunology, genetic background, innate immunity, life-history, phenotypic plasticity, temperature, *Thamnophis*

Introduction

An understudied aspect of vertebrate ecoimmunology is the relative contribution of genetic (G) versus environmental (E) influences on the vast variation in immune function documented in the wild. As immune function is vital to fitness, such knowledge is critical in the context of our rapidly changing world, particularly given the increased emergence of infectious diseases that threaten biodiversity (Martin, Hopkins, Mydlarz & Rohr, 2010; Altizer, Ostfeld, Johnson, Kutz, & Harvell, 2013). Some groups of ectothermic vertebrates have shown massive declines or extinctions associated with infectious disease emergence (Gibbons, Scott, Ryan, Buhlmann, Tuberville et al., 2000; Vredenburg, Knapp, Tunstall & Briggs, 2010), with alterations of environmental temperature regimes having been implicated in the dynamics of several disease outbreaks in amphibians and reptiles (e.g., Raffel, Rohr, Kiesecker & Hudson, 2006; Lorch, Knowles, Lankton, Michell, Edwards et al., 2016; Greenspan, Bower, Webb, Berger, Rudd et al., 2017). Many effector cells and molecules of the immune system are highly temperature-dependent in vertebrates (Zimmerman, Vogel & Bowden, 2010; Butler, Stahlschmidt, Ardia, Davies, Davis et al., 2013). At the same time, immune defenses have a genetic basis (Warner, Meeker & Rothschild, 1987) and genetic differentiation in vertebrate immunity has been documented in the wild (e.g., Bonneaud, Pérez-Tris, Federici, Chastel & Sorci, 2006; Hawley & Fleischer, 2012; Whiting, Magalhaes, Singkam, Robertson, D'Agostino et al., 2018). Furthermore, phenotypic plasticity (i.e., $G \times E$ interaction) is likely to be important for the complex immune phenotype in adjusting to environmental challenges. Interactions between genetic background and environmental temperature underlying disease resistance have been widely documented in invertebrates (Lazzaro & Little, 2009), but studies in vertebrates remain scant.

Our present study addressed the relative influences of environmental temperature, genetic background and their interaction on the development of immune defenses in an ectothermic vertebrate. The vertebrate immune system is inherently highly complex, with multiple interacting effector molecules, cells, and pathways that have evolved to defend hosts against pathogens and parasites (Roitt, Brostoff, & Male, 1998). Different aspects of immunity can be expected to display different sensitivities to environmental factors and/or degrees of genetic influence. As such, it is important to use diverse

measures that include aspects of both innate and adaptive immunity (Table 1) to gain a broad understanding of the contribution of G, E, and $G \times E$ on this complex phenotype. We chose as our model the well-studied system of divergent life-history ecotypes of the western terrestrial garter snake *Thamnophis elegans* (Bronikowski & Arnold, 1999; Addis, Gangloff, Palacios, Carr & Bronikowski, 2017). Populations of western terrestrial garter snakes in the vicinity of Eagle Lake (Lassen County, California, USA) inhabit two different habitat types and show distinct fast and slow life-history strategies (Table 2). Lakeshore populations (hereafter L-fast ecotype) display fast growth, large adult body size, early sexual maturation with large reproductive effort, and low annual survival; in contrast, populations in mountain meadows around the lake (hereafter M-slow ecotype) exhibit opposing traits on the ‘slow’ end of the pace-of-life continuum.

Our previous ecoimmunological studies show that M-slow snakes generally display lower innate and adaptive immunity compared to L-fast snakes when sampled in the field (Table 2). These ecotype differences could stem from genetic differentiation in immunity, as recently suggested for fish (Whiting et al., 2018). In fact, significant genetic divergence between the two garter snake ecotypes has been documented in several traits including growth rate (Bronikowski 2000, Addis et al., 2017), coloration (Manier, Seyler & Arnold 2007), and mitochondrial function (Schwartz, Areedsee & Bronikowski, 2015). Ecotype differences in immunity could also reflect plastic responses to current environmental variables experienced by individuals in their natural habitats (Palacios, Sparkman & Bronikowski, 2011). In particular, meadows inhabited by M-slow snakes have lower ambient temperatures, lower and less predictable food availability, lower predation pressure, and higher prevalence of a trematode parasite than lakeshore habitats (Table 2). All these environmental conditions can influence immune functions (Ujvari & Madsen, 2006; Zimmerman et al., 2010; Palacios, Cunnick & Bronikowski, 2013) and could thus contribute to the lower immune levels documented in wild M-slow compared to L-fast snakes. The combination of field and laboratory studies seems therefore essential to advance the understanding of drivers of immune variation in this system, as well as in most ecological and evolutionary contexts.

To parse out the effects of environmental temperature on immunity in our system, we conducted a reciprocal-transplant experiment with snakes of both ecotypes raised under laboratory conditions mimicking the different availability of optimal temperatures in the warmer lakeshore and cooler meadow habitats (Gangloff, Vleck & Bronikowski, 2015; Reding, Addis, Palacios, Schwartz & Bronikowski, 2016). Snakes were allowed to regulate their own temperature behaviorally within these differing regimes (see methods for details). Immune measurements were made at the neonate (age 3 months) and juvenile (age 12 months) stages to quantify immune development across this early-life period. The laboratory setting provided a predator- and pathogen/parasite-free environment, allowing us to test for temperature effects on immunity in the absence of these potentially confounding factors. Given temperature influences on feeding and energetics in ectotherms, we recorded food consumption and assessed body condition of individuals in order to control for their effects on immune parameters in our analyses. We evaluated the following predictions regarding the potential causes underlying the ecotype differences in immunity observed in the field (i.e., M-slow lower innate and adaptive immunity than L-fast): (1) If genetic background is the major driver, then this pattern will be maintained in the laboratory irrespective of thermal treatment and other environmental conditions. (2) If temperature is the major driver, under the ‘warmer-is-better’ hypothesis (Angilletta, 2009) snakes raised under warm thermal conditions will show higher innate and adaptive immunity than those in the cool treatment irrespective of their ecotype. (3) If ecotypes show adaptive phenotypic plasticity in immunity in relation to environmental temperature, then their immune function will be higher under the thermal conditions mimicking their natural habitats.

Materials and methods

Study animals and thermal treatments

Gravid females of each of the two ecotypes of this viviparous species were collected from populations around Eagle Lake (40.65° N, 120.75° W) during June 2010. These females were transported back to the laboratory colony at Iowa State University (L-fast: 22 individuals from 4 populations; M-slow: 22 individuals from 4 populations). Animal husbandry conditions for gravid females are described in detail by Gangloff et al. (2015). Parturition occurred between 12 August and 19 September 2010.

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Offspring ($n = 257$) were sexed by hemipene eversion and measured for mass (g) and snout-vent length (SVL in mm) within 24 h of birth. A subset of these offspring ($n = 186$, from 21 L-fast litters and 19 M-slow litters, representing 4 and 3 populations per ecotype, respectively) were included in the present study (Fig. 1), with no initial size difference between thermal treatment groups (t -test for mass: $p = 0.96$; SVL: $p = 0.62$). Males and females from each litter were split randomly between the two thermal treatment groups for a full factorial experimental design of ecotype \times thermal treatment \times sex. Offspring were housed individually in plastic boxes with paper substrate and a water dish. Ambient room temperature was 20°C and supplemental heating was supplied by heat tapes placed under one end of the box, which generated a thermal gradient (22–32°C) that allowed snakes to behaviorally thermoregulate. Optimal and preferred field-active temperatures for this species are ~28–30°C (Stevenson, Peterson & Tsuji, 1985; Peterson 1987). Snakes in the ‘Warm’ treatment received 16 h of this supplemental heating per day, while snakes in the ‘Cool’ treatment received supplemental heating for only 8 h per day. These conditions mimic the differing availability of optimum temperatures in the respective habitats, where L-fast habitats are 5–10°C warmer than M-slow (Bronikowski & Arnold, 1999). Offspring were kept on a 12:12 light:dark schedule and offered frozen/thawed pinky mice once a week. Individuals that repeatedly consumed all food were offered a greater amount in subsequent feedings. Amount of food consumed by each individual (in grams) was recorded at every feeding (Gangloff et al., 2015). Animals were maintained in these environments for the duration of the experiment, except for a period of hibernation in the dark at 4°C from January through May 2011.

Sample collection and processing

Offspring were sampled as neonates on 19–23 November 2010 and again as juveniles on 14–17 September 2011. Not all neonates survived to the juvenile stage, thus sample sizes are lower for the latter sampling period (Fig. 1). Blood samples (60–120 μ l depending on body size) were obtained from the caudal vein via heparin-rinsed syringes using sterile techniques. One drop of blood was immediately used to prepare a thin blood smear for differential leukocyte counts. Next, 30 μ l of whole blood was transferred to a sterile tube and stored on ice for use in the lymphocyte proliferation assay on the same day of collection. The remaining blood was kept on ice until plasma was separated and

stored at -80°C for use in the hemagglutination-hemolysis assay. Snakes were weighed and SVL-measured after each blood-sampling session.

Immune function assays and leukocyte profiles

All protocols included in this study have been previously adapted for use in garter snakes (Table 1). We assessed the ability of lymphocytes to proliferate *in vitro* using a whole-blood mitogenic stimulation assay (Palacios et al., 2013, Palacios & Bronikowski, 2017). We measured proliferation of B-lymphocytes using the standard B-cell mitogen lipopolysaccharide (LPS from *Escherichia coli*) and proliferation of T-lymphocytes using the standard T-cell mitogen concanavalin A (ConA). Lymphocyte responses were expressed as stimulation indices (SI). We assessed the levels of natural antibodies and complement-mediated lysis using a hemagglutination-hemolysis assay (Sparkman & Palacios, 2009). Titers were estimated as the negative log₂ of the highest dilution of plasma that showed hemagglutination/hemolysis. Fixed blood smears were stained with Wright-Giemsa and we quantified the percentages of lymphocytes, monocytes, heterophils, and basophils by classifying the first 100 leukocytes encountered under 1000× magnification (Sparkman, Bronikowski, Williams, Parsai, Manhart et al., 2014). The total leukocyte count (cells per µl of blood) was estimated by the indirect Phloxin B method using a hemocytometer (Palacios et al., 2013).

Statistical analyses

We used linear mixed models with PROC MIXED in SAS 9.4 (SAS Institute, Cary, NC, USA) to test for the effects of thermal treatment (cool/warm thermal environment), genetic background (L-fast/M-slow ecotypes), age (neonate/juvenile stages) and interactions thereof on immune measures. Models also included the fixed effect of sex and the covariate body condition, to control for these factors that can also affect immune parameters. Body condition was calculated independently for each ecotype and age group as the residuals of the log₁₀-mass on log₁₀-SVL regression, a method widely used as a measure of size-corrected mass when body composition cannot be determined directly (Weatherhead & Brown, 1996; Ujvari & Madsen, 2006). We also included population nested within ecotype as a fixed effect, litter nested within population and ecotype as a random effect, and individual identity as a repeated-measures random effect, modeled with a compound symmetric covariance structure.

Analyses of lymphocyte proliferation responses also included total lymphocyte counts as a covariate, as these responses are dependent on the number of lymphocytes initially present in the cell cultures. Except for natural antibodies and complement-mediated lysis, all dependent variables were \log_{10} -transformed to meet the assumption of normally distributed residuals. Simple correlations and principal component analyses showed that some of the immune parameters were intercorrelated when all data were analyzed together (i.e., ecotypes, ages, thermal treatments, and sexes; see Table S1 and S2, respectively). Nevertheless, because the relative influences of G, E, and $G \times E$ can be expected to vary among disparate immune measures (e.g., Versteegh, Helm, Kleynhans, Gwinner & Tieleman, 2014) and we have documented different sources of variation for some of these measures in the field (Palacios et al. 2013), we used separate mixed model analyses on individual immune parameters. We present in figures boxplots of the raw data, as these graphs better reflect the actual distribution and range of immune parameters measured.

In addition to the mixed models, we used path analyses to gain insights into how the thermal treatments influenced food consumption and allocation decisions to growth in body length (SVL) and immune development at the individual level within each ecotype. Path analyses allow the quantification of the relative influence and the significance of relationships among variables through the use of models that specify *a priori* the directionality of potentially causal relationships (Olobatuyi, 2006). Our *a priori* specified models included: 1) the influence of thermal treatment on the amount of food consumed, 2) the influences of thermal treatment and food consumed on growth, and 3) the effects of thermal treatment, amount of food consumed, and growth on the development of the different immune defenses. Growth and development of immune defenses were calculated as the within-individual change in the corresponding parameter across our two measurement times. For lymphocyte proliferation variables we used the residuals from the regression of stimulation indices on the corresponding total lymphocyte count. Path analyses were performed using the *lavaan* package (Rosseel, 2012) in R (R Core Team 2014).

Results

Relative contributions of genetic background, thermal environment, and their interaction on immune phenotype development and function

Ecotype and thermal environment differentially affected aspects of immune function and leukocyte profiles of the snakes at the neonate and juvenile stages. Three of the four parameters of immune function increased with age (Fig. 2, Table 3), with this development being independent of thermal treatment for T-cell proliferation (Fig. 2B) but positively influenced by warm rearing conditions for natural antibodies (Fig. 2C) and complement-mediated lysis (Fig. 2D). In addition, the increase in natural antibodies with age tended to be more pronounced in L-fast than in M-slow snakes (Table 3, Fig. 2C). B-cell proliferation, which did not show overall change with age or with thermal environment, exhibited ecotype dependence, with M-slow snakes showing higher levels than L-fast (Table 3, Fig. 2A).

Total leukocyte counts increased with age, an effect that was more pronounced in M-slow than in L-fast snakes (Table 4, Fig. 3A). In addition, total leukocytes were lower in the warm rearing conditions, this effect tending to be more pronounced in L-fast compared to M-slow snakes (Table 4, Fig. 3A). Among leukocyte percentages, % lymphocytes showed a three-way interaction between ecotype, thermal treatment, and age: greater increments with age were shown by L-fast snakes in the cool treatment and by M-slow snakes in the warm treatment (Table 4, Fig. 3B). In contrast to % lymphocytes, % basophils decreased with age (Table 4, Fig. 3E), whereas % heterophils and % monocytes did not show consistent changes during the developmental period (Table 4, Fig. 3C and D, respectively). Heterophil percentages were influenced by thermal environment, with slightly higher levels in the cool treatment (Table 4, Fig. 3C).

Snake body condition and sex also differentially influenced the various immune function measures (Table 3) and leukocyte profiles (Table 4). Higher body condition indices were weakly but positively associated with higher levels of T-cell proliferation, natural antibodies, complement-mediated lysis, and total leukocyte counts; whereas B-cell proliferation and the various leukocyte percentages did not show significant condition-dependence (Fig. S1 and S2). Sex had an effect on most immune parameters, with the exception of natural antibodies and complement-mediated lysis, which did not

differ between males and females. Females showed higher B- and T-cell proliferation and greater % lymphocytes than males, whereas males exhibited higher total leukocyte counts and greater % heterophils and % basophils than females (Fig. S3 and S4).

Thermal treatment effects at the individual-level within each ecotype

L-fast and M-slow snakes showed differential responses to the thermal treatments in terms of consumption of food, growth in body length, and the development of immune defenses during the period between the neonate and juvenile stages (Fig. 4, Table S3). For L-fast snakes (Fig. 4A), warm thermal treatment showed a relatively strong positive influence on the amount of food consumed, which in turn strongly determined larger growth of individuals. Thermal treatment did not have a detectable direct effect on individual growth and showed only marginally significant effects on immune development —positive for % basophils and negative for % lymphocytes. Individual growth of L-fast snakes showed only marginally significant associations with immune development, with positive trends for lysis and % lymphocytes and a negative trend for % monocytes. For M-slow snakes (Fig. 4B), warm thermal treatment also increased the amount of food consumed by individuals (although not as strongly as for L-fast), which in turn strongly increased individual growth, as with L-fast snakes. However, unlike the case for L-fast snakes, warm thermal treatment showed a direct positive influence on growth of M-slow snakes as well as more and stronger influences on immune development —positive effects for B-cell proliferation and natural antibodies, a positive trend for % lymphocytes, and a negative trend for total leukocyte counts. Lastly, M-slow snakes that grew more during this time period showed a trend towards reduced development of T-cell proliferation.

Discussion

This study contributes to the advancement of vertebrate ecoimmunology in understanding the relative contribution of genetic background, environmental factors and their interaction in determining immune phenotypes of wild animals, a topic that remains understudied in vertebrates. The intricacy of our results is likely a reflection of the inherently complex immune system of vertebrates, which generally defies description of simple patterns and explanations when studied in

the realm of real world ecological and evolutionary contexts (Matson, Cohen, Klasing, Ricklefs & Scheuerlein, 2006; French, Moore & Demas, 2009; Lazzaro & Little, 2009). Furthermore, our findings indicate that the inherent complexity of the vertebrate immune system is patent even under controlled experimental laboratory conditions designed to simplify the environment to facilitate addressing specific ecoimmunological questions. Thus, although interpretation of results becomes more challenging, appreciation and consideration of immunological complexity (i.e., inclusion of various and diverse measures of immune function), is paramount to avoid oversimplification and guarantee the continuous advancement of the field of ecological immunology (Sheldon & Verhulst, 1996; Matson et al., 2006; Palacios, Cunnick, Winkler & Vleck, 2012).

Our reciprocal transplant experiment showed that both genetic background (ecotype) and environmental temperature (mimicking the thermal environment of the cool meadow and warm lakeshore habitats) —either directly or through effects on food intake— influenced innate and adaptive immune parameters in developing snakes. We found some evidence of $G \times E$ interactions, but no suggestion of adaptive plasticity in immunity with respect to the thermal environment. Remarkably, the different immune parameters measured showed idiosyncratic patterns regarding the relative contributions of genetic background and/or environmental factors to variation in the immune phenotype (Fig. 2 and 3, Tables 3 and 4). The effects of body condition and sex also depended on the immune parameter considered. As a consequence, the main factors explaining variation in immune phenotypes of the snakes were immune-component specific, with no clear patterns in terms of their innate vs. adaptive nature. For instance, in accordance with findings in songbirds (Palacios et al., 2009), we found no evidence that adaptive immune parameters were more condition-dependent than innate ones during early life, an expectation derived from the proposed higher costs —in terms of energy and nutrients— of developing a diverse lymphocyte pool versus developing innate immune cells (Lee 2006). Similarly, we did not find adaptive immune components to be more temperature-dependent than innate ones, in contrast to reports for teleost fish (Le Morvan et al., 1998).

Only a handful of studies to date have performed common-garden experiments in the context of immune function variation across vertebrate populations (in birds: Martin, Pless, Svoboda &

Wikelski, 2004; Versteegh, Helm, Kleynhans, Gwinner & Tieleman, 2014; in reptiles: Palacios et al., 2011; Refsnider, Palacios, Reding & Bronikowski, 2015; Korfel, Chamberlain & Gifford, 2015; in fish: Whiting et al., 2018); although not all were designed for and focused on understanding the relative influences of genes versus environment on documented immune phenotype variation in the wild. Interestingly, those studies that included more than one immune measure—in most cases of innate immune components—have also reported different patterns depending on the measure being considered. For instance, a study of western painted turtle *Chrysemys picta bellii* populations found that natural antibody levels were lower in the southernmost population when individuals were sampled in their native habitats, but this difference disappeared when the same individuals were measured under common-garden conditions (Refsnider et al., 2015). At the same time, bactericidal ability of turtle plasma was relatively rigid, not evidencing across-population variation under either condition. Similarly, a common-garden study comparing five parameters of innate humoral immunity across stonechat *Saxicola torquata* subspecies concluded that while some immune measures were more flexible, others seemed relatively rigid (Versteegh, Helm, Kleynhans, Gwinner & Tieleman, 2014).

Innate and adaptive immune parameters of the two garter snake ecotypes seem to be under a relatively greater environmental than genetic influence when compared to the stonechat subspecies mentioned above. Only one of the nine immune parameters was solely influenced by genetic background in our experiment—B-lymphocyte proliferation was higher in M-slow than L-fast snakes irrespective of age, thermal treatment, sex, and body condition. Surprisingly, this pattern in B-cell function is opposite to that previously documented in the field for juvenile and adult snakes (Table 2). This suggests that the ecotype effect on B-lymphocyte proliferation might become completely masked (and even reversed) under other environmental conditions that snakes experience in their natural habitats (e.g., variation in pathogen pressure and/or food availability; Table 2) and that were controlled in our present experiment designed to evaluate the effects of thermal variation. Together with our findings of thermal environment and body condition influences on other immune parameters, this points to a high degree of immune flexibility in the snakes, which is in line with the idea that ectotherms might show

greater flexibility in immune function than endotherms (Graham, Fielman & Mendonca, 2017), as with physiology generally.

In a common-garden experiment with another garter snake cohort, innate humoral immune parameter of neonates —natural antibody levels, complement-mediated lysis, and bactericidal capacity of plasma— reflected the ecotype differences originally documented in the field (Palacios, Sparkman & Bronikowski, 2011). Interestingly, those ecotype differences were not maintained through the first year of life under common-environment conditions, suggesting developmental plasticity. We had then proposed that the initial ecotype differences could stem from early genetic effects that then become overridden by the favorable environmental conditions in the laboratory and/or from prenatal maternal effects on early offspring immunity (Palacios et al., 2011). The lack of ecotype differences in innate humoral immunity in neonates from the present study is more in line with the latter explanation.

Although neonates in both studies were born in captivity, their mothers were already pregnant when captured in the field. Ecotype differences in aspects of innate humoral immunity are only manifest in some, but not all years in adult free-ranging snakes (Palacios et al., 2013). Thus, it is possible that neonates may only display ecotype differences in years when their mothers do so in the field, as maternally-derived immune profiles can play an important role in preparing offspring immunity for their developmental environments (Grindstaff, Brodie & Ketterson, 2003; Hasselquist, Tobler & Nilsson, 2012). Gravid females that gave birth to offspring involved in the present study did not show ecotype variation in humoral innate immunity when sampled during pregnancy in the field (Palacios et al. 2017). Nevertheless, different aspects of humoral innate immunity were assessed in mothers (bactericidal capacity of plasma) and offspring (natural antibody levels and complement-mediated lysis), thus further evaluation of the role of maternal effects are warranted in this system.

Our results provided no evidence that snakes from the different ecotypes have increased immunity when raised under the thermal regime mimicking conditions of their native habitat (i.e., L-fast in warm while M-slow in cool temperature treatment). Rather, only one of the nine immune parameters showed a significant interaction between ecotype and temperature treatment. The pattern, however, was opposite to that expected if ecotypes showed adaptive phenotypic plasticity —% lymphocytes

showed greater development for M-slow snakes under warmer conditions and for L-fast snakes under cooler ones. Overall, snakes in our experimental setup showed only moderate temperature effects on immune function and its ontogeny —generally in line with the warmer is better hypothesis— which could in part stem from their opportunity to thermoregulate behaviorally during the time periods when the heat gradient was on (i.e., 8 vs. 16 h/day for the cool and warm treatment, respectively).

Behavioral thermoregulation in ectotherms has the potential to attenuate the effects of temperature challenges such as those involved in climate change (Kearney, Shine & Porter, 2009). Although we did not monitor individual behavior in our study, it is possible that snakes in the cool treatment were able to partially compensate for the reduced time that optimal temperatures were available by selecting warmer temperatures during their shorter daily cycle.

In line with the lack of evidence for adaptive phenotypic plasticity in immune function with respect to the thermal regimes in the two ecotypes, results at the individual level (i.e., from path analyses) suggested that physiology of M-slow snakes, which inhabit the cooler meadow environments, seems to be more temperature-dependent than that of L-fast snakes (Fig. 4). For instance, direct effects of temperature on immune development and growth in body size were only significant in M-slow snakes. In addition, the effects of thermal environment on resource allocation varied between the two ecotypes. Under warmer conditions that increased food consumption of individuals in both ecotypes, L-fast snakes allocated strongly into growth, whereas M-slow snakes also allocated some resources into immune development. This finding supports the genetically-influenced prioritization of early-life growth in L-fast compared to M-slow snakes (Bronikowski, 2000, Addis et al., 2017). As growth in early life has relatively greater importance for L-fast snakes, they might be privileging growth under the favorable warmer conditions. On the other hand, M-slow snakes for which self-maintenance (e.g., immune function) for a longer lifespan has relatively greater importance might be dividing resources more evenly into growth and immune development. Nevertheless, variation in immune function in this system seems highly plastic and does not seem to be tightly linked to the disparate life-history strategies of the two ecotypes, in agreement with conclusions from a recent review highlighting the importance of environmental context on the relationship between immunity and pace-of-life among avian taxa (Tieleman, 2018).

Overall, our experimental study provides insights on the relative influences of thermal environment, genetic background, and their interaction on the development of different immune defense components in an ectothermic vertebrate. Findings indicate that immune defenses can be highly flexible and suggest that in our system, other environmental factors such as food availability and/or the pathogen environment are likely more important than temperature *per se* in driving the ecotype differences in immunity observed for the snakes in the field. Our finding of immune-component specific patterns underscores in a striking manner the limitations faced by ecoimmunological studies that use single immune parameters to make broad generalizations about immune function as a whole, strongly cautioning against oversimplification of this highly complex system. Our results also add to the understanding of how ectotherms balance their investment in immune development and growth during early postnatal life under different thermal environments and whether allocation decisions relate to life-history strategies along the pace-of-life continuum. In conjunction, these results deepen our understanding of the degree of immunological flexibility wild animals present, information that is ever more vital in the face of increasing threats from emergent infectious wildlife diseases in the context of rapid global environmental change.

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Author contributions

M.G.P. and A.M.B. conceived and designed the study.

M.G.P., D.M.R. and A.M.B. collected data.

E.J.G. and M.G.P. performed statistical analyses with input from A.M.B.

M.G.P. and E.J.G. wrote the manuscript with the input from A.M.B. and D.M.R.

Data Accessibility

Data available from Mendeley: doi.org/10.17632/mmc3whj2dz.1 (Palacios et al 2020).

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Table 1. Immune defense components, their main functions, techniques used to assess them, and references for protocols previously used and adapted for garter snakes.

Immune Component	Functions	Technique	References
Innate			
- Granulocytes and monocytes	Ingest, destroy pathogens	Leukocyte profile	Sparkman et al., 2014
- Natural antibodies	Neutralize, opsonize pathogens, activate complement cascade	Hemagglutination	Sparkman & Palacios, 2009
- Complement, antimicrobial proteins	Neutralize, opsonize, destroy pathogens	Hemolysis	Sparkman & Palacios, 2009
Adaptive			
- Lymphocytes	Immunological memory of pathogens	Leukocyte profile	Sparkman et al., 2014
B-cells	Neutralize or opsonize extracellular pathogens	<i>In vitro</i> B-lymphocyte proliferation	Palacios et al., 2013; Palacios et al., 2017
T-cells	Kill infected host cells (intracellular pathogens)	<i>In vitro</i> T-lymphocyte proliferation	Palacios et al., 2013; Palacios et al., 2017

Table 2. Selected trait differences between the fast-living lakeshore (L-fast) and slow-living meadow (M-slow) ecotypes of the garter snake *Thamnophis elegans* around Eagle Lake, California (adapted from Palacios et al. 2011 and Schwartz et al. 2015). Please see cited references for more details.

References: ¹ Bronikowski & Arnold (1999), ² Miller et al. (2011), ³ Sparkman & Palacios (2009), ⁴ Palacios et

	Characteristic	L-fast ecotype	M-slow ecotype
Habitat	Substrate	Rocky lakeshore	Grassy meadow
	Elevation	1555 m	1630-2055 m
	Summer mean daytime temperature ¹	20-34°C	15-30°C
	Food/water availability ^{1,2}	Continuous	Variable across years
	Tail trematode infection ^{3,4}	Absent	Present
	Avian predation pressure ⁵	Higher	Lower
Life-history	Mean adult body size ¹	660 mm	538 mm
	Size and Age at maturity ¹	425 mm/3 years	400 mm/5-7 years
	Reproductive rate ⁶	Biennial	Resource-dependent
	Mean Litter size ⁶	8 neonates	5 neonates
	Median/maximum life span ^{1,2}	5/18 years	9/18 years
Immune Function †	Natural antibodies ³	Higher	Lower
	Complement-mediated lysis ³	Higher	Lower
	Bactericidal competence ^{3,4,*}	Higher or same	Lower or same
	T-lymphocyte proliferation ^{4,**}	Higher	Lower
	B-lymphocyte proliferation ⁴	Higher	Lower

al. (2013), ⁵ Sparkman et al. (2013), ⁶ Sparkman et al. (2007).

Notes: † Samples were obtained under field conditions from juvenile and adult snakes (gravid females excluded), * result dependent on year of sampling, ** difference is non-significant when trematode infected M-slow individuals are removed from analysis.

Table 3. Linear mixed model analyses of adaptive and innate immune function in garter snakes, *Thamnophis elegans*, measured as neonates and juveniles. Significant factors ($P < 0.05$) shown in bold with directionality of effect. For significant interactions see corresponding figure.

Source of Variation		B-cell proliferation (LPS SI)	T-cell proliferation (ConA SI)	Natural antibodies (titer)	Lysis (titer)
Ecotype		M-slow > L-fast			
	$F (df_m, df_d)$	34.23 (1, 32.6)	0.40 (1, 27.8)	0.01 (1, 32)	0.03 (1, 31.1)
	Pr > F	< 0.0001	0.5340	0.9434	0.8600
Thermal Treatment					
	$F (df_m, df_d)$	1.81 (1, 145)	1.04 (1, 144)	1.33 (1, 150)	0.72 (1, 149)
	Pr > F	0.1811	0.3096	0.2508	0.3973
Age			Juvenile > Neonate	Juvenile > Neonate	Juvenile > Neonate
	$F (df_m, df_d)$	0.00 (1, 168)	32.99 (1, 168)	457.89 (1, 160)	474.19 (1, 160)
	Pr > F	0.9749	< 0.0001	< 0.0001	< 0.0001
Ecotype × Treatment					
	$F (df_m, df_d)$	0.04 (1, 143)	1.94 (1, 142)	0.11 (1, 150)	0.09 (1, 148)
	Pr > F	0.8454	0.1664	0.7432	0.7599
Ecotype × Age					
	$F (df_m, df_d)$	0.00 (1, 160)	2.71 (1, 160)	3.89 (1, 158)	1.90 (1, 159)
	Pr > F	0.9949	0.1019	0.0503	0.1699
Treatment × Age				see Fig.3C	see Fig.3D
	$F (df_m, df_d)$	2.11 (1, 160)	0.56 (1, 160)	7.93 (1, 158)	5.77 (1, 159)
	Pr > F	0.1481	0.4534	0.0055	0.0174
Ecotype × Treatment × Age					
	$F (df_m, df_d)$	2.29 (1, 160)	0.22 (1, 159)	0.71 (1, 158)	0.76 (1, 159)
	Pr > F	0.1318	0.6360	0.4009	0.3847
Sex		Female > Male	Female > Male		
	$F (df_m, df_d)$	6.25 (1, 160)	9.23 (1, 155)	0.01 (1, 162)	0.02 (1, 161)
	Pr > F	0.0134	0.0028	0.9074	0.8839
Body Condition			Positive	Positive	Positive
	$F (df_m, df_d)$	1.86 (1, 291)	15.21 (1, 305)	27.82 (1, 314)	24.15 (1, 314)
	Pr > F	0.1735	0.0001	< 0.0001	< 0.0001
Population(Ecotype)					

	<i>F</i> (<i>df_m</i> , <i>df_d</i>)	0.15 (5, 29.7)	1.80 (5, 27)	2.14 (5, 30.6)	2.61 (5, 29.6)
	Pr > F	0.9799	0.1458	0.0866	0.0452
Total Lymphocytes	Positive				
	<i>F</i> (<i>df_m</i> , <i>df_d</i>)	5.30 (1, 304)	2.93 (1, 289)	--	--
	Pr > F	0.0220	0.0880	--	--

Table 4. Linear mixed model analyses of leukocyte profiles in garter snakes, *Thamnophis elegans*, measured as neonates and juveniles. Significant factors ($P < 0.05$) shown in bold with directionality of effect. For significant interactions see corresponding figure.

Source of Variation	Total leukocyte count (cells/ μ l)	Lymphocytes (%)	Heterophils (%)	Monocytes (%)	Basophils (%)
Ecotype					
$F (df_n, df_d)$	0.01 (1, 31.8)	0.02 (1, 28.3)	2.32 (1, 26.9)	0.00 (1, 31.2)	2.13 (1, 31)
Pr > F	0.9234	0.8958	0.1397	0.9536	0.1543
Thermal Treatment	Cool > Warm		Cool > Warm		
$F (df_n, df_d)$	6.40 (1, 159)	1.15 (1, 136)	4.84 (1, 152)	0.04 (1, 155)	0.45 (1, 144)
Pr > F	0.0124	0.2854	0.0293	0.8325	0.5016
Age	Juvenile > Neonate	Juvenile > Neonate			Neonate > Juvenile
$F (df_n, df_d)$	23.87 (1, 175)	15.84 (1, 163)	0.93 (1, 175)	0.81 (1, 175)	17.98 (1, 167)
Pr > F	< 0.0001	0.0001	0.3375	0.3680	< 0.0001
Ecotype \times Treatment					
$F (df_n, df_d)$	3.71 (1, 158)	0.03 (1, 135)	1.23 (1, 151)	0.18 (1, 154)	0.08 (1, 143)
Pr > F	0.0560	0.8608	0.2691	0.6677	0.7817
Ecotype \times Age	see Fig. 4A				
$F (df_n, df_d)$	5.48 (1, 173)	0.21 (1, 162)	1.59 (1, 173)	0.04 (1, 173)	3.57 (1, 166)
Pr > F	0.0203	0.6464	0.2092	0.8424	0.0605
Treatment \times Age					
$F (df_n, df_d)$	2.37 (1, 173)	0.69 (1, 161)	0.42 (1, 173)	0.45 (1, 173)	0.40 (1, 166)
Pr > F	0.1259	0.4087	0.5163	0.5051	0.5291
Ecotype \times Treatment \times Age		see Fig. 4B			

	$F(df_n, df_d)$	0.01 (1, 174)	5.71 (1, 162)	0.85 (1, 174)	1.54 (1, 174)	2.23 (1, 167)
	Pr > F	0.9341	0.0180	0.3576	0.2156	0.1376
Sex		Male > Female	Female > Male	Male > Female		Male > Female
	$F(df_n, df_d)$	11.19 (1, 171)	24.00 (1, 150)	12.99 (1, 168)	3.86 (1, 170)	9.62 (1, 158)
	Pr > F	0.0010	< 0.0001	0.0004	0.0510	0.0023
Body Condition		Positive				
	$F(df_n, df_d)$	4.32 (1, 301)	0.38 (1, 281)	0.15 (1, 277)	0.36 (1, 290)	0.36 (1, 239)
	Pr > F	0.0386	0.5368	0.7030	0.5473	0.5489
Population(Ecotype)						
	$F(df_n, df_d)$	1.59 (5, 29.3)	1.67 (5, 25.8)	2.43 (5, 24.2)	2.44 (5, 28.2)	1.59 (5, 26.5)
	Pr > F	0.1952	0.1768	0.0639	0.0589	0.1969

Figure legends

Fig. 1. Schematic of the reciprocal transplant experimental design with wild-sired, captive-born garter snakes *Thamnophis elegans* of the L-fast and M-slow ecotypes raised under Warm and Cool thermal treatments. Individuals of each life-history ecotype \times thermal treatment \times sex combination were sampled as neonates and as juveniles. Snakes from L-fast ecotype are shown in white boxes and snakes from M-slow ecotype are shown in shaded boxes. Notice that not all neonates survived to the juvenile stage.

Fig. 2. Adaptive and innate immune function of garter snakes *Thamnophis elegans* repeatedly measured as neonates (NEO, 3 month-old) and juveniles (JUV, 12 month-old). Boxplots show median, interquartile range, and range of raw data values for each age, thermal treatment (Cool and Warm), and ecotype (L-fast and M-slow) combination. Sample sizes are depicted beneath each box. Significant factors from mixed linear models in Table 3 are shown in legend (* : $P < 0.05$, ** : $P < 0.001$). Abbreviations: SI, stimulation index.

Fig. 3. Leukocyte profiles of garter snakes *Thamnophis elegans* repeatedly measured as neonates (NEO, 3 month-old) and juveniles (JUV, 12 month-old). Boxplots show median, interquartile range, and range of raw data values for each age, thermal treatment (Cool and Warm), and ecotype (L-fast and M-slow) combination. Sample sizes are depicted beneath each box. Significant factors from mixed linear models in Table 4 are shown in legend (* : $P < 0.05$, ** : $P < 0.001$).

Fig. 4. Path diagrams showing the relationships among thermal treatment, food consumed, SVL-growth, and development of immune parameters between 3 and 12 months of age in garter snakes *Thamnophis elegans*. Relationships were assessed separately for the L-fast (A) and M-slow (B) ecotypes. Factors predicting immune development are shown in shaded boxes; immune development variables are shown in white boxes. Black arrows indicate statistically significant paths ($P < 0.05$), grey arrows show marginally significant effects ($0.05 < P < 0.1$), non-significant paths are not depicted. Coefficient estimates \pm SE are shown, with path thickness proportional to the estimated

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effect. Positive relationships are shown with solid lines, negative relationships with dashed lines; thermal treatment effect is positive for warm treatment. See text for statistical details and Table S3 for full path analyses results.

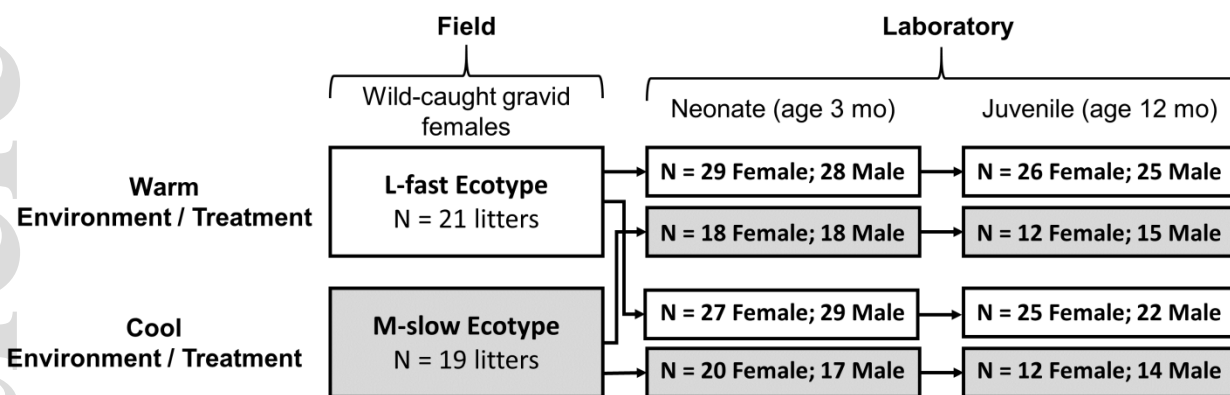
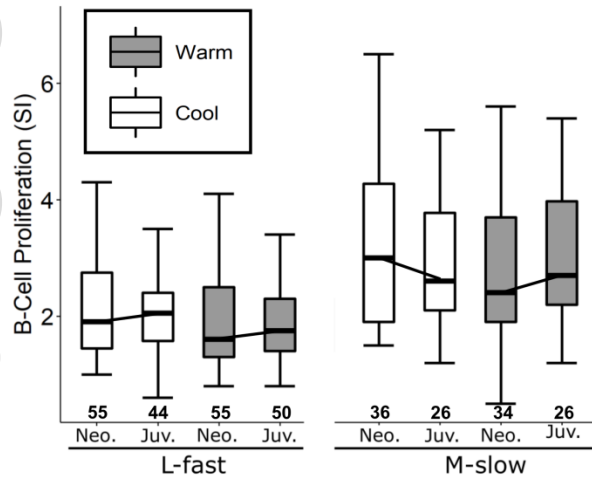
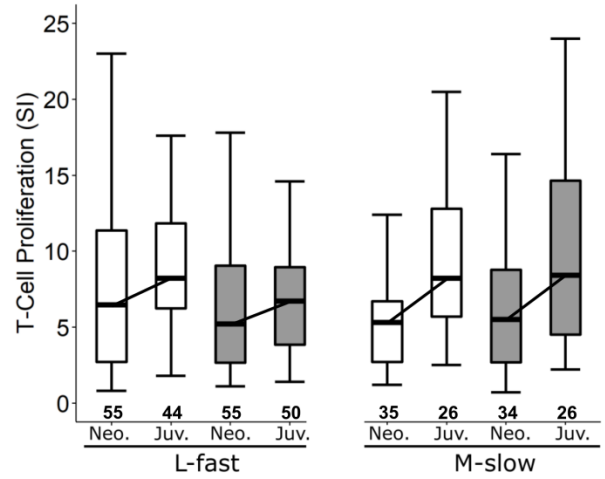


Figure 1

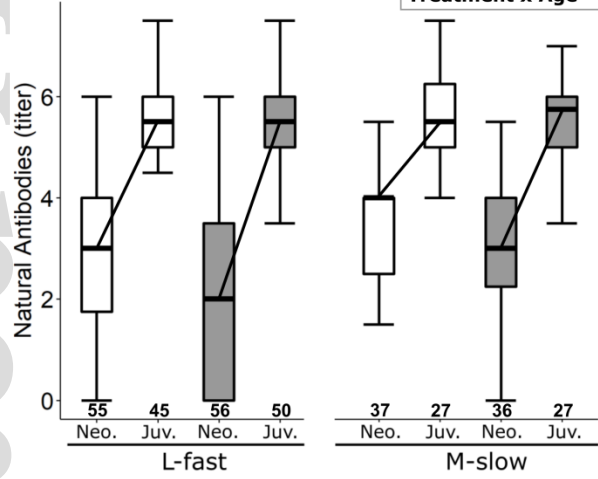
(A) B-cell Proliferation



(B) T-cell Proliferation



(C) Natural Antibodies



(D) Lysis

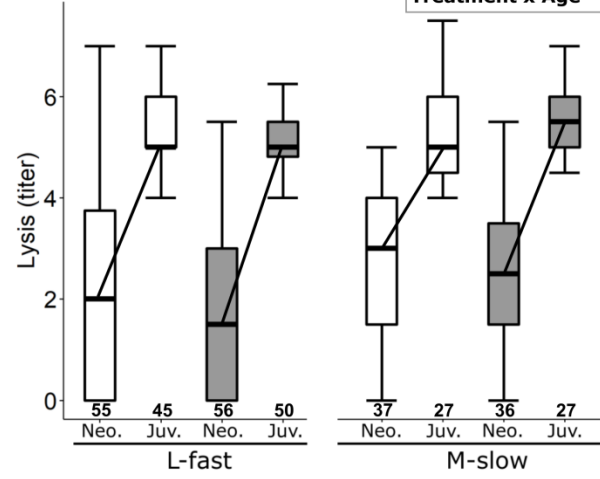


Figure 2

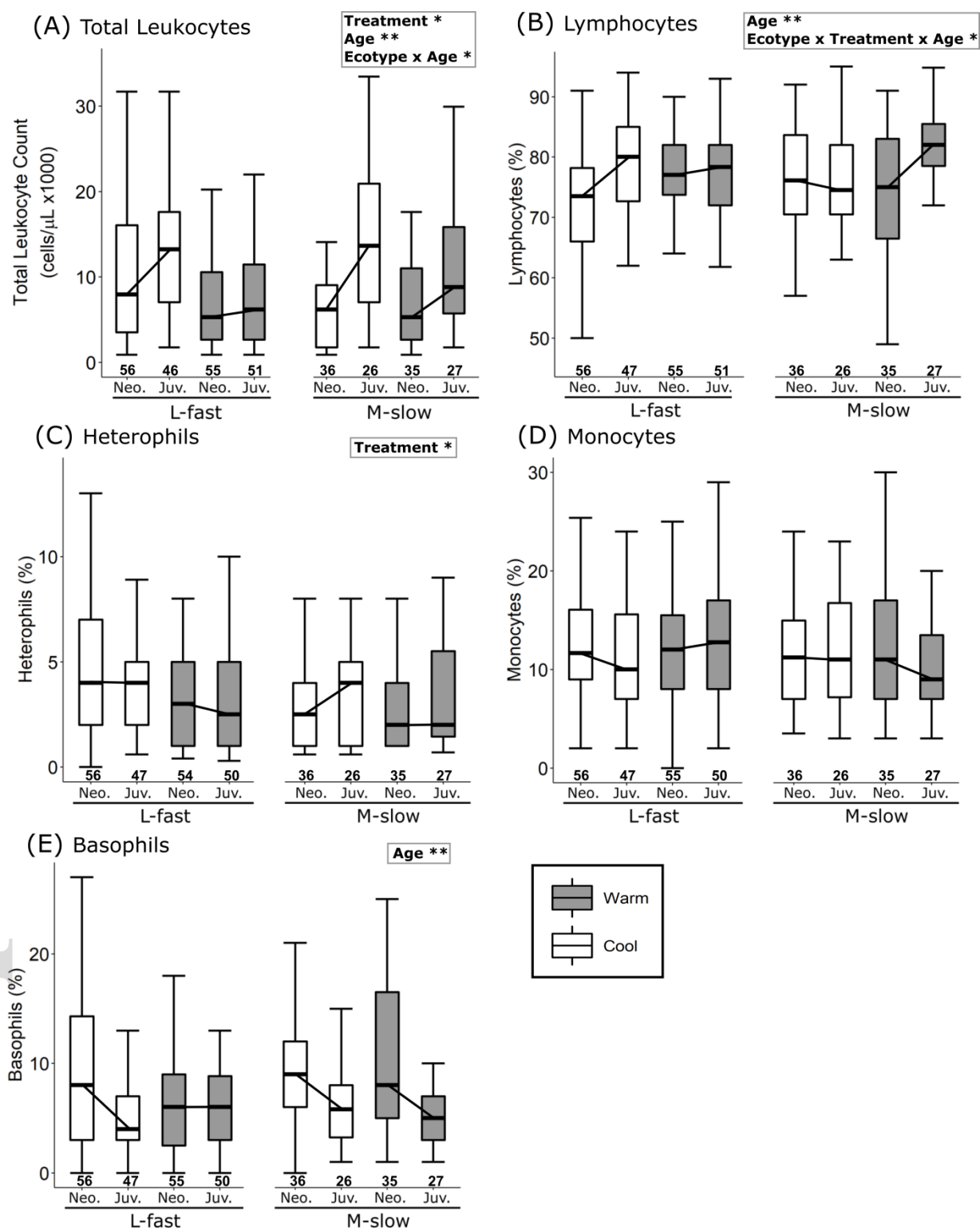
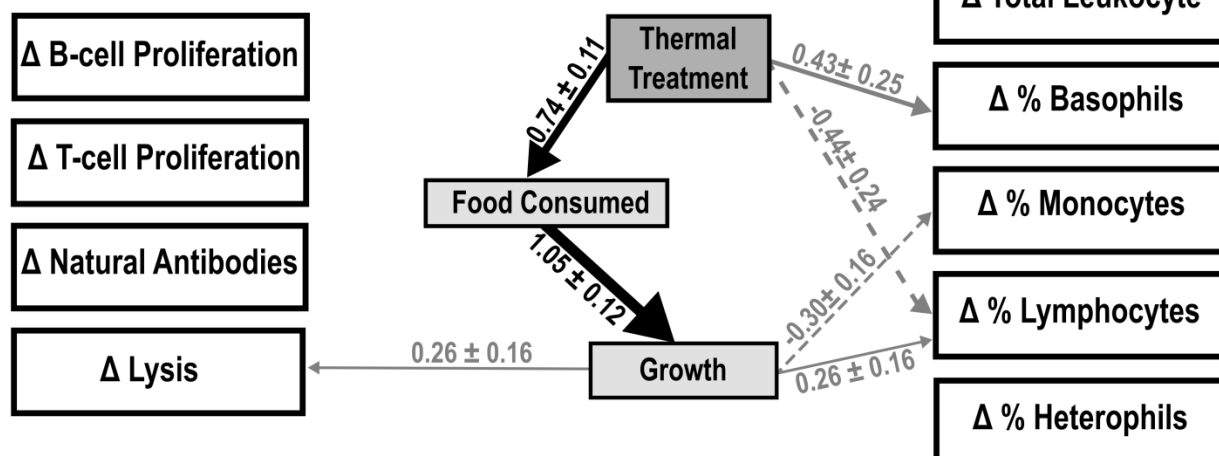


Figure 3

(A) L-fast (N = 98)



(B) M-slow (N = 53)

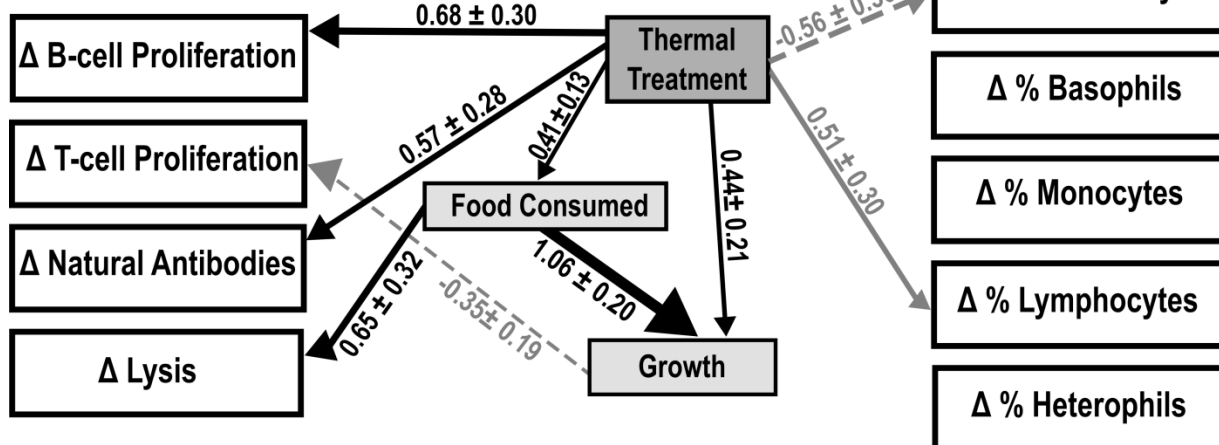


Figure 4