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Drivers of Immune Cost and Implications for Host Protection from Parasites

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Drivers of Immune Cost and Implications for Host Protection from Parasites

by

Amber J. Brace

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy Department of Integrative Biology with a concentration in Ecology and Evolutionary Biology College of Arts and Sciences University of South Florida

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Keywords: immune costs, life history, exposure-dependence, ectotherm, malaria, *Anolis sagrei*

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DEDICATION

I dedicate this dissertation to my husband for his unwavering support and my sister, because a little bit of healthy competition never hurt anyone. I also dedicate this work to my parents for always telling me I could be whatever I wanted to be, and to May Howell, my grandmother, whose strength and poise in all matters of academics, family, and life in general I hope to someday emulate. Lastly to Thorin, who has my whole heart and for whom I do all things.
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ABSTRACT

Among species, populations, and individuals, there exists a tremendous amount of variation in how hosts respond to, and are thus protected from parasites. Such variation inevitably affects host-parasite dynamics and ultimately how parasites will move through and evolve in communities. A likely factor in the diversity of immune responses seen in nature are the costs associated with activation of the immune system upon exposure to parasites. Costs can manifest in many ways, including changes in resource usage or metabolism, self-damage from inflammatory reactions, lost opportunities (e.g., foraging reproduction), and often as tradeoffs with other physiological processes. However, we do not yet fully understand the factors that influence costs of immune activation across ecological scales, nor the relationship between immune costs and protection from parasites, despite the common assumption that greater costs equates to better protection. For my dissertation, I have investigated large-scale drivers of immune costs, specifically whether life history and/or body mass influence costs of immune activation (Chapter 1), whether magnitude of parasite exposure affects immune activation costs at the population level (Chapter 2), and the relationship between costs of immune activation and benefit in terms of parasite protection (Chapter 3).

Across taxa, immune costs are likely to be affected by host life history traits such as longevity and reproductive scheduling such that long-lived and slow to mature species (i.e., slow-paced) should experience lower costs than animals that die comparatively early (i.e., fast-paced). By reducing immune costs, slow-paced species could reduce the accumulation of damage associated with repeated activations of the immune response that could reduce successful reproduction over
a long life. Likewise, body mass should also be an important determinant of immune costs as physical size should affect the extent to which hosts are exposed to parasites as well as their ability to combat infection. For the first chapter, I used meta-analysis to determine whether life history traits (lifespan and time to maturity) and/or body mass affected functional costs of immune activation (e.g., changes in performance, food intake, growth, mass, reproductive effort/success, survival) across taxa. The results of this study showed that, in general, animals incur costs of immune activation and that costs are influenced by life history and body mass such that species that are relatively long-lived experience greater costs of immune activation than species that are relatively short-lived. We also found that small species experienced relatively greater costs than large species. Such patterns may arise because long-lived species may have been selected to endure high costs of immune activation to develop more robust adaptive responses in order to extend lifespan. In addition, small animals may experience greater costs than large animals because of a higher cell turnover rate potentially resulting in greater self-damage (via oxidative damage).

While costs of immune activation can be broadly predicted by lifespan and body mass, smaller-scale factors, such as magnitude of exposure to parasites are also likely important drivers of immune costs within populations. Indeed, if costs increase linearly with magnitude of exposure, then at a certain level of parasite burden, costs may become too great for the host and selection may favor hosts that tolerate, rather than eliminate infection. In the second chapter, I investigated how exposure to multiple concentrations of Salmonella lipopolysaccharide (LPS), a immunogenic component of Gram negative bacteria cell walls, affected immune costs in brown anole lizards (Anolis sagrei). To quantify costs, I examined allocation of leucine, a critical amino acid to immune tissue (liver) and reproductive tissue (gonads). I predicted that immune
costs would increase with exposure, however, because females often suffer decrements in immune function during reproduction, I expected their immune costs to be less pronounced than males. I also hypothesized that leucine allocation to reproductive tissue would decrease with increasing magnitude of LPS exposure because immune activation often results as tradeoffs as competing physiological systems vie for limited resources. I found that costs of immune activation increased with magnitude of LPS exposure, and that costs differed between the sexes as males allocated more leucine to the liver at high LPS doses, but did not shift allocation from gonads to livers. Females, however, tended to bias resources to livers and away from gonads with increasing LPS doses. Interestingly, I also found that cost of immune activation increased linearly with magnitude of exposure, indicating that this species may tolerate high levels of infection with *Salmonella* bacteria as the cost of resisting infection is likely to become unmanageable. Such a finding suggests that at the population level, brown anoles may be particularly important contributors to the spread of *Salmonella* within populations, or even communities.

Although costs of immune activation may drive a host’s response to infection by limiting the magnitude of its response when costs of a strong response are too great, we cannot predict how immune costs may be driving parasite prevalence and selection on hosts unless we understand the relationship between costs of immune activation and protection. While it is commonly assumed that high costs of immune activation are indicative of a stronger immune response and ultimately better protection for the host, the relationship between immune costs and benefits remains unstudied. In the third chapter, I examined host- and parasite-mediated costs of parasite exposure and the relationship between costs and benefits of immunity by exposing brown anole lizards to live or killed malaria parasites and following the course of infection over 7 weeks. I
measured costs by quantifying glucose oxidation during the 12-36 hours following exposure to
determine whether and how cost of initial exposure related to protection from malaria parasites.
As glucose is the primary fuel source for malaria parasites, we predicted that hosts that oxidized
little glucose would also experience lower parasite burdens. We found that lizards infected with
killed parasites oxidized less glucose than control-infected animals, however lizards infected
with live parasites did not differ from either the control or killed parasite group, indicating the
possibility of a parasite-mediated mechanism to slow host-driven glucose sequestration.
Importantly, we also found that the cost of exposure appeared to come with a benefit: lizards
infected with live parasites that experienced the lowest glucose oxidation also had lower parasite
burdens over the 7 weeks following exposure. The results from this study show that brown
anoles that experience greater costs of malaria exposure also experience greater protection,
contributing to our understanding of how individual-level processes drive disease dynamics
within communities.
Altogether, my dissertation has identified that broad characteristics such as life history and body
mass can be helpful in predicting costs of immune activation and has additionally demonstrated
the importance of smaller-scale processes such as the relationship between immune costs and
magnitude of exposure at the population level and the relationship between immune costs and
benefits at the individual level. These studies have expanded our understanding of the factors
that drive variation in immunity at multiple levels and give the field of ecoimmunology a more
holistic picture of the species, population and individual-level processes that affect host-parasite
interactions within communities.
CHAPTER 1: LIFE HISTORY AND BODY MASS DRIVE COSTS OF IMMUNE RESPONSE

Authors

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Abstract

A central assumption in the field of ecological immunology is that immune responses to infection exact costs on hosts, which can manifest directly (e.g., increases in metabolic rate, increased amino acid usage) or as tradeoffs with other life processes (e.g., reduced growth and reproductive success). Across taxa, how hosts schedule reproductive life should affect immune investment; species that spread breeding effort over a long life should experience lower immune costs than those that die comparatively early. Likewise, body mass should also be an important determinant of immune costs as physical size should affect the extent to which hosts are exposed to parasites and their ability to combat infections. Here, we used meta-analysis to synthesize ecological immunology studies to demonstrate that in general, animals incur costs of immune activation, but long-lived species have relatively greater immune costs than short-lived ones and small animals pay relatively greater costs than large animals. These patterns might arise because i) long-lived species may have been selected to endure high costs of immune activation to extend lifespan and ii) small animals experience greater oxidative damage and higher metabolic turnover of immune cells involved in an initial immune response than larger animals. Together,
these findings demonstrate the fundamental links between selection on lifespan, body size and immune function.

Introduction

Protection against infection often comes at a cost to the host (Lochmiller & Deerenberg 2000). Once exposed to a pathogen, costs of immune activation can occur in ways that are directly measurable, such as increases in metabolic rate or amino acid use (Lochmiller & Deerenberg 2000; Brace, Sheikali & Martin 2015), or they can manifest as tradeoffs with life history traits such as growth and reproduction (Lochmiller & Deerenberg 2000; Bonneaud et al. 2003). Although it is possible for costs to be mitigated by increased resource intake (Ruiz et al. 2010), in natural environments resources are typically limited and must be distributed among multiple competing physiological processes. A number of studies have demonstrated that immune costs are present, marked, and variable among populations (Ricklefs & Wikelski 2002; Bonneaud et al. 2003; Lee 2006; Cox & Calsbeek 2010). However, the large-scale drivers of these observed costs remain under debate. Exposing broad patterns might refine and clarify the ecological and evolutionary processes driving variation in immune costs across taxa.

Potential drivers of immune system variation, which in turn underlies immune costs are likely to include life history traits such as lifespan and time to maturity. Longer-lived and slower-to-mature organisms likely experience different trajectories of immune ontogeny and overall greater lifetime exposure to parasites than fast-developing, prolifically breeding species. For instance, long-lived species tend to have specific, adaptive immune defenses (Lee 2006) rather than non-specific responses. The reasons for this are thought to be two-fold; longer-lived organisms are more likely to encounter the same parasites multiple times during their lives, making it beneficial to control infections via specific defenses, which can be costly to develop (Schmid-Hempel
2011). However, by developing robust adaptive defenses with the capacity for precision and
rapidity, long-lived organisms might avoid the collateral damage associated with less specific
defenses (Ricklefs & Wikelski 2002), ultimately resulting in a greater initial investment followed
by decreased subsequent costs of immune activation when exposed to the same pathogen more
than once.

Additionally, because body size influences nearly all aspects of physiology (West, Brown &
Enquist 1997; Ricklefs & Wikelski 2002; Gillooly & Allen 2007), it also likely influences how
the immune system is structured (e.g., reliance on general vs. specific responses) as a result of
costs incurred in response to immune activation. For example, the surface area of digestive and
respiratory tracts and skin, which are the most common sites of parasite invasion and infection,
scale with size (West, Brown & Enquist 1997; Brown et al. 2004; Wiegel & Perelson 2004),
consequently increasing the volume of tissue requiring surveillance and protection. As
metabolic rate (scaling coefficient of $\frac{3}{4}$) and number of cells (scaling coefficient of 1) also
increase with body mass, (Lindstedt & Calder 1981; Savage et al. 2004; Savage et al. 2007), the
energy required to activate more cells, specifically leukocytes, should be greater in large than
small organisms.

Here, we investigated whether and how activation of the immune system imparts functional
costs, including by not limited to changes in mass, performance, food intake, growth rate, egg
production, egg size, gonad size, breeding effort, individual survival, recruitment rate, and
dispersal behavior across diverse animal hosts (Klasing & Korver 1997; Laugero & Moberg
2000a; Bonneaud et al. 2003; Bonneaud et al. 2004; Cox & Calsbeek 2010). Using phylogenetic
meta-analysis, we tested whether interspecific diversity in lifespan, body size and/or time to
maturity predict costs of immune activation. We focused on studies that experimentally
quantified the costs of exposure to non-replicating immune challenges; we avoided studies using live pathogens because of difficulties in disentangling the effects of hosts and parasites on costs. In total, we estimated 236 immune costs among 39 invertebrate and vertebrate species with body sizes spanning 9 orders of magnitude (0.71mg to 200kg) and lifespans and times to maturity spanning 3 orders of magnitude (21 days to 40 years and 8 days to 5 years, respectively). As explained above, we expected that long-lived and/or long developing species would exhibit comparatively low costs of immune activation and we predicted that larger animals would exhibit larger immune costs than small animals (Scheuerlein & Ricklefs 2002; Wiegel & Perelson 2004).

**Methods**

*Search methods and inclusion/exclusion criteria*

Candidate studies were identified using Web of Science and Google Scholar based on the search terms: cost* and immun*, trade* and immun*, and fitness and immun*. Between November 2011 and January 2012, approximately 179 candidate studies published between 1991 and 2010 identified with these search terms were selected by screening titles and abstracts. We vetted the full text of these candidates for inclusion in our meta-analysis using the following criteria: experimental manipulations must have used a treatment (immune stimulant) that did not include (live) infections with parasites or pathogens, treated animals must have been compared to a control group that was either unmanipulated or procedural (e.g., sham, saline), and costs were required to involve fitness, performance, or other functional responses (e.g., activity, food intake, growth rate, mass change, egg production, egg size, gonad size, breeding effort, individual survival, recruitment rate, dispersal behavior). Studies that only reported statistical model outcomes or marginal means were excluded, along with study outcomes based on physiological
traits (e.g., cortisol), immune traits (e.g., antibody levels), and medical research on humans or other medically-related model organisms (i.e., non-human primates). In total, 46 research articles met these inclusion criteria (Table 1).

Predictor extractions

From each research article, the following methodological predictors of outcome heterogeneity were extracted: the study species and its taxonomic affiliation; the type of control (i.e. unmanipulated and procedural controls, including sham injected and saline injected individuals); and the stimulant used to activate the immune system (e.g., LPS, vaccines, nylon thread). These stimulants were grouped among broader elicitor types (e.g., antibody, simulated parasite, deactivated pathogen), overall reactive immune class (i.e. innate or adaptive), or grouped as ‘mixed’ if they were difficult to assign to a single class (e.g., single vaccine treatments with dual or multiple antigens and/or adjuvants). When possible, we also extracted average body mass, lifespan and time to maturity for each study species from the primary papers; but if these species characteristics were not reported, data were supplemented using other studies, books and online databases (Table 2).

Outcome extractions and experimental-design modeling

Study outcomes were quantified as the standardized mean difference between a treatment (T) and control (C) group using effect sizes based on Hedges’ $d$ (Hedges 1981). We interpret negative values for Hedges’ $d$ as evidence for a ‘cost’ to immune activation by a stimulant manipulation. This effect size metric requires the extraction of means ($\bar{X}$), standard deviations ($SD$), and sample sizes ($N$) of each group, and combines these study parameters into an effect size ($d$) using:
\[
d = \frac{X_T - X_C}{\sqrt{\frac{(N_T - 1)SD_T^2 + (N_C - 1)SD_C^2}{N_T + N_C - 2}} \left[ 1 - \frac{3}{4(N_T + N_C - 9)} \right]},
\]

which has a variance:

\[
\text{var}(d) = \frac{N_T + N_C}{N_T N_C} + \frac{d^2}{2(N_T + N_C)}.
\]

When possible, these study parameters (\(X\), \(SD\), and \(N\)) were extracted from tables, text, and supplemental materials, or extrapolated from plots manually. When standard errors (\(SE\)) were reported these were converted to \(SD = SE\sqrt{N}\). Marginal or least square means that were adjusted with covariates were not included in our study; however, very few studies statistically adjusted outcomes this way. Several studies reported relevant outcomes based on counts or proportions (e.g., individuals surviving with or without immune activation). The outcomes of these studies were first quantified with an odds ratio effect size and then converted to Hedges’ \(d\) using the equation reported in Lajeunesse (Lajeunesse et al. 2013); less than 6% of effect sizes were derived from these conversions.

Several outcomes were often extracted from a single study; in total, 236 effect sizes were extracted from 46 studies. This approach can be a problem for meta-analysis since multiple extractions derived from the same experimental design will share study parameters that are statistically dependent; these dependencies can increase type II error of hypothesis tests (Lajeunesse 2011). In our meta-analysis, we modeled several forms of experimental dependencies to improve our variance estimates and hypothesis tests. For example, many studies used a design that compared several types of controls to a single treatment group. Typically, one control group consisted of unmanipulated (U) individuals and a second with individuals that underwent a procedural treatment (P) intended to quantify administration effects of the stimulant (e.g., to control for injury effects a sham/injury or saline injection was used when a stimulant
was administered with a syringe). However, effect sizes derived from this design share a common treatment group (i.e., $\bar{X}_T - \bar{X}_{C-U}$ and $\bar{X}_T - \bar{X}_{C-P}$) and therefore do not represent independent study outcomes. Here we modeled the covariance between these two effects, using a modified version of Gleser and Olkin’s (Gleser & Olkin 2009) covariance (cov) equation for Hedges’ $d$ effect sizes with a common control $\text{cov}(d_{C-U}, d_{C-P}) = (1 + 0.5d_{C-U}d_{C-P})/(N_T + N_{C-U} + N_{C-P})$ where both $d_{C-U}$ and $d_{C-P}$ use the pooled standard deviation:

$$\sqrt{\frac{(N_T-1)SD_T^2+(N_{C-U}-1)SD_{C-U}^2+(N_{C-P}-1)SD_{C-P}^2}{N_T+N_{C-U}+N_{C-P}-3}}.$$  

When studies reported multiple outcomes as well as the correlation between these outcomes (e.g., outcomes based on two vaccines that are correlated), we modeled their dependencies using the covariance equation between multivariate $d$ for unequal sample sizes reported in Robinson et al. (Robinson, Lajeunesse & Forbes 2012). Finally, when outcomes were reported as a time series (either pre- and post-effects designs, or through multiple repeated measurements through time), we modeled dependencies by first estimating phi (an optimized autocorrelation value for the white noise in a weighted regression model using the nlme R package; (Pinheiro et al. 2007)) using a simple ARMA ($p$, $q$) model assuming a first-order autoregressive structure ($p = 1$) and no moving average correlations ($q = 1$). When phi could not be estimated, we assumed phi had a small autoregressive structure between time series of 0.25. Phi’s were then converted into a variance/covariance matrix following Trikalinos and Olkin (Trikalinos & Olkin 2012).

**Traditional and phylogenetic analyses**

We analyzed our effect size data using multi-factor mixed-model meta-analyses (for categorical predictors) and meta-regressions (for continuous predictors). All regression models assumed a maximum-likelihood (ML) based between-study variance estimate ($\tau^2$) required for random-effects meta-analysis, and included a block-diagonal sampling variance-covariance matrix that
provides the weights for each effect size used in the weighted regressions with the variances of $d$
on main diagonal and the covariances used to model dependencies arising from various experimental designs (i.e. multiple control groups). The regression equations used to perform meta-analyses require that each variance-covariance matrix used to model dependencies be symmetric and positive definite; therefore when necessary, this was achieved using Higham’s (Higham 2002) method.

Phylogenetic analyses included two additional random-effects: a random factor designating the multiple effect sizes derived from single species, and a second unstructured random-effect matrix modeling the shared evolutionary history (phylogenetic correlations) of these species (Lajeunesse 2009). Our phylogenetic correlation matrix was derived from a composite phylogeny assuming a Brownian motion model of phenotypic evolution (Rohlf 2001) using the vcv() function of the ape package in R (Paradis, Claude & Strimmer 2004). The taxonomic composition of this ultrametric tree was broad and included the following: 39 species from three invertebrate (Insecta, Gastropoda, and Bivalvia) and three vertebrate classes (Aves, Mammalia, Reptilia). The deep divergence times and topology were based on Hedges et al. (Hedges, Dudley & Kumar 2006) for classes, Meredith et al. (Meredith et al. 2011) for mammals, Jetz et al. (Jetz et al. 2012) for birds, and Trautwein et al. (Trautwein et al. 2012) for insects. In cases when divergence times were unavailable, we arbitrarily scaled branch-lengths distances using Grafen’s method using $\rho$ to the power of 1.0 to model divergence times emerging from a Brownian motion model of evolution. Finally, all analyses were performed using the rma.mv() function from the metafor package in R (Viechtbauer 2010) assuming the nlminb optimizer. Pseudo-$R^2$ (proportional reduction in the total variance explained) for each model was estimated as follows:

$$R^2 = (\sum \tau^2_{base} - \sum \tau^2_{predictors})/ \sum \tau^2_{base},$$

where $\tau^2_{base}$ are all the estimated random-effect
variances from a model without predictors (base model) and $\tau^2_{predictors}$ are the variances of a model including predictors. Between-group $Q$-tests were used as omnibus tests for comparing differences among predictor categories (Hedges & Olkin 2014) and continuous predictors (e.g., mass) were log transformed prior to analyses.

**Results**

Overall, we found a significant functional cost of immune activation across taxa ($k = 236$ effect sizes; conventional grand mean $d = -0.34$, 95% CI: -0.47 to -0.21; phylogenetic grand mean $d = -0.33$, 95% CI: -0.57 to -0.09; Figure 1.1); variation in these costs was substantially beyond that which is predicted by sampling error (fixed-effect $Q_{within} = 1253.5$, d.f. = 235, p<0.001).

Although some variation in immune costs was explained by the type of immune challenges used to activate immune systems as well as the type of costs measured, these sources of variation were negligible when the phylogenetic history of taxa was modeled in our meta-analysis.

Contrary to predictions, lifespan, time to maturity and body mass did not predict costs of immune activation when modeled alone in meta-regressions (lifespan: conventional $\beta = -0.02$, SE = 0.05, 95% CI: -0.13 to 0.08, p = 0.64; phylogenetic $\beta = -0.03$, SE = 0.07, 95% CI: -0.18 to 0.11, p = 0.64); time to maturity: conventional $\beta = 0.05$, SE = 0.05, 95% CI: -0.04 to 0.16, p = 0.29; phylogenetic $\beta = 0.03$, SE = 0.08, 95% CI: -0.11 to 0.19, p = 0.65; body mass: conventional $\beta = 0.01$, SE = 0.02, 95% CI: -0.03 to 0.05, p = 0.58; phylogenetic $\beta = 0.03$, SE = 0.04, 95% CI: -0.04 to 0.11, p = 0.40). However, when lifespan and body mass were modelled simultaneously, longer-lived animals had larger costs than short-lived ones (Figure 1.2) in conventional ($\beta = -0.23$, SE = 0.11, 95% CI: -0.42 to -0.03, p = 0.02) but not phylogenetic models ($\beta = -0.23$, SE = 0.13, 95% CI: -0.49 to 0.03, p = 0.08 and body mass was a significant predictor of immune costs in both conventional ($\beta = 0.1$, SE = 0.04, 95% CI: 0.02 to 0.18, p =
such that smaller animals appear to experience greater immune costs than larger ones (Figure 1.3). In a separate meta-regression model, there was no significant interaction between lifespan and body mass in either conventional ($\beta = -0.004, SE = 0.01, 95\% CI: -0.03 to 0.02, p = 0.70$) or phylogenetic analyses ($\beta = -0.004, SE = 0.02, 95\% CI: -0.04 to 0.03, p = 0.79$), as such, the interaction term was removed.

When mass was included in the meta-regression model testing for whether time to maturity predicted costs of immune activation, neither time to maturity (conventional $\beta = 0.03, SE = 0.08, 95\% CI: -0.13 to 0.19, p = 0.74$; phylogenetic $\beta = -0.04, SE = 0.13, 95\% CI: -0.29 to 0.22, p = 0.77$) nor body mass (conventional $\beta = 0.02, SE = 0.03, 95\% CI: -0.05 to 0.08, p = 0.62$; phylogenetic $\beta = 0.05, SE = 0.06, 95\% CI: -0.05 to 0.16, p = 0.35$) significantly predicted costs of immune activation; there was also no significant interaction between time to maturity and body mass in a separate model (conventional $\beta = 0.01, SE = 0.01, 95\% CI: -0.01 to 0.03, p = 0.59$; phylogenetic $\beta = 0.003, SE = 0.02, 95\% CI: -0.03 to 0.04, p = 0.84$).

Among all the major taxonomic groups, the conventional meta-analysis model compared to the phylogenetic model was the better fit (among all animals: $LR \chi^2 = 10.78, p < 0.001$; among taxonomic groups of vertebrates and invertebrates: $LR \chi^2 = 7.99, p < 0.001$; and among taxonomic classes: $LR \chi^2 = 4.96, p < 0.001$).

**Discussion**

Our phylogenetic meta-analysis supports a central tenet of ecological immunology that functional costs of immune activation exist (Figure 1.1). Second, and contrary to our prediction, we found that when accounting for body mass, costs of immune activation increase with lifespan, where longer-lived species of a given size experience greater costs than short-lived ones (Figure
1.2). We also found that body mass predicts the extent of functional costs of immune activation, but only when accounting for lifespan. Also contrary to our initial predictions, we found that small animals for their lifespan experience relatively greater costs than animals larger than predicted for their lifespan (Figure 1.3), and that costs have a very shallow scope compared to other processes that also scale with mass (e.g., metabolic rate, cell number). Lastly, we found that time to maturity, even when accounting for body mass, did not significantly predict immune costs.

Studies have previously demonstrated that activation of the immune system can result in subsequent decrements in physical performance, breeding success and other physiological functions (Bonneaud et al. 2003; Bonneaud et al. 2004; Cox & Calsbeek 2010; Bowers et al. 2015). Such decrements, because of the direct or indirect implications they have for host survival and fitness, can be viewed as a cost. While the patterns of immune activation costs we have found in our meta-analysis may be influenced by the overrepresentation of vertebrates (mostly birds), we were still able to demonstrate the trend that costs are ubiquitous, although highly variable across the animal kingdom (Figure 1.1). It would be insightful for future studies to expand upon our finding and broaden our knowledge base of immune costs beyond the most commonly studied animal groups.

Across animal taxa, we found that costs of immune activation tend to increase with lifespan (Figure 1.2). Although we cannot distinguish which costs are associated with specific or non-specific responses with our current dataset, such a finding may be due to the tendency for short-lived species to lack the adaptive immune responses often seen in longer-lived animals, because they are unlikely to see repeated infections over a short lifetime and/or they do not live long enough to benefit from the investment in responses that are more antigen-specific and less
damaging (Lee 2006). For example, once exposed to an antigen, the reaction of the immune system is highly conserved across a diverse range of taxa; antigens are identified via preserved pathogen-associated molecular patterns (PAMPs), initiating nonspecific responses (e.g., antimicrobial peptides) (Medzhitov & Janeway 1997; Siva-Jothy, Moret & Rolff 2005). In animals possessing an open circulatory system, the rapidity of this response is key to preventing free movement of antigens throughout the body cavity (Siva-Jothy, Moret & Rolff 2005). However, in vertebrates, the adaptive immune response is also initiated, resulting in the production of specialized cells (Medzhitov & Janeway 1997; Janeway Jr & Medzhitov 1998) that can be beneficial to hosts likely to experience multiple exposures to the same antigen, because they minimize the costs of immune activation by reducing inflammatory collateral damage (Råberg et al. 2002b; Schmid-Hempel 2011). Thus, despite the initial cost of developing long-term immune memory, costs of subsequent exposure should be lower (Råberg et al. 2002a). However, short-lived species may not have evolved specific responses, or have the resources available to develop them and may also be less likely to experience the payoff of developing such responses because they are unlikely to suffer repeated exposures. It is also possible that when faced with an immune challenge, short-lived species with few opportunities to reproduce are increasing reproductive effort, thus making it appear as though they suffer smaller functional costs than long-lived species (Terminal Investment Hypothesis) (Williams 1966; Bowers et al. 2015). Future studies would be able to further elucidate this idea by developing datasets where it was possible to separate reproductive, performance and metabolic costs of immune activation. Additionally, we found that functional costs of an immune response were higher in animals that were small for their lifespan and were negligible among the animals that were large for their lifespan. The relationship between body size (relative to lifespan) and immune function may
occur because in all animals, once exposed to a pathogen, non-specific immune responses are triggered as a first line of defense. In small animals, which also have a higher mass-specific metabolic rate (ratio of average metabolic rate of a cell to cell size) (Savage et al. 2007), this response may result in greater oxidative damage and higher cell turnover as a result of their higher cellular metabolic rate (Speakman 2005; Savage et al. 2007). Our finding that relative body size influences immune costs is important, as it demonstrates that body size is important even in large animals, when longevity is controlled. Hence, despite a smaller surface area available for infection than larger animals, smaller animals may experience an increased cost of immune activation as a direct result of the increased metabolic turnover of the short-lived immune cells involved in the initial response to infection.

It is also possible that immune costs are inversely related to body mass because the risk of infection might decrease with animal size because although larger animals have more surface area that risks parasite exposure (Brown et al. 2004; Wiegel & Perelson 2004), their greater tissue volume may act as a physically larger barrier to cross for parasites to successfully colonize. If so, then parasite exposure in a small animal may have a greater potential for successful infection and it may be more beneficial for small animals to attempt to eradicate infection and cope with the subsequent costs of a strong immune response to decrease the risk of a potentially debilitating infection (Siva-Jothy, Moret & Rolff 2005). It would be interesting for future studies to further explore the relationship between host size and ability of parasites to successfully colonize. Additionally, the shallow scaling of immune activation costs with mass may indicate that there has been strong selection to minimize costs in all taxa, regardless of size.

Altogether, the results of our meta-analysis show relationships between body mass and lifespan and the costs of immune activation, however, it is yet unclear how these patterns affect variation.
in immune responses seen in nature. It would be informative for future studies to elucidate how immune costs shape immune responses, and provide insight into how species of different body size and lifespan respond to emerging pathogens.

Acknowledgments

We thank Holly Kilvitis, Sarah Burgan, Stephanie Gervasi and Douglas Baron for comments on earlier drafts of this manuscript, and Jennifer Everhart for help collecting data. We also thank members of the NSF RCN in Ecoimmunology for multiple insightful discussions over the course of this project. Funding was provided by NSF RCN in Ecoimmunology grant (0947177), NSF IOS 0920475 and 1257773 to LBM, NSF DBI 1262545 to MJL and NIH grant 1R15HD066378-01 to JLG. KLB was supported by ARC Future Fellowship FT140100131.

Literature cited


Figure 1.1: Immune activation is costly among animals; all Hedges’ $d$ effects are negative. Forest plot of pooled effect sizes (Hedges’ $d$) among various taxonomic groups. Pooled effects are based on conventional (blue) and phylogenetic (orange) random-effects meta-analyses, and the number of effect sizes pooled among each group is provided in parentheses. Among all the major taxonomic groups, the conventional meta-analysis model compared to the phylogenetic model was the better fit (among all animals: LR $\chi^2 = 10.78$, $p < 0.001$; among taxonomic groups of vertebrates and invertebrates: LR $\chi^2 = 7.99$, $p < 0.001$; and among taxonomic classes: LR $\chi^2 = 4.96$, $p < 0.001$).
Figure 1.2: Lifespan (average days to death corrected by mass is a significant predictor of immune costs such that longer-lived animals experience higher immune costs. Higher costs are shown as more negative, indicating a decrease in function, performance or fitness as a result of immune activation. Random-effects meta-regression lines are based on conventional (blue) and phylogenetic (orange) models, and are derived from a meta-regression that simultaneously modeled mass and lifespan as cost predictors (non-significant intercept was not included in this meta-regression model). The phylogenetic meta-regression compared to the conventional model was the better fit (LR $\chi^2 = 0.22$, $p = 0.318$).
Figure 1.3: Body mass (grams) corrected for lifespan is a significant predictor of immune cost such that costs are largest for the smallest animals. Higher costs are shown as more negative, indicating a decrease in function, performance or fitness as a result of immune activation. Random-effects meta-regression lines are based on conventional (blue) and phylogenetic (orange) models, and are derived from a meta-regression that simultaneously modeled mass and lifespan as cost predictors (non-significant intercept was not included in this meta-regression model). The phylogenetic meta-regression compared to the conventional model was the better fit (LR $\chi^2 = 0.22$, $p = 0.318$).
Table 1.1: Studies included in the meta-analysis that met our inclusion criteria. Studies must have included manipulations using an immune stimulant that did not include live infections; treated animals must have been compared to a control group that was either unmanipulated or procedural (e.g., sham, saline); reported study outcomes that measured a fitness, performance, or other functional response (e.g., activity, food intake, growth rate, mass change, egg production, egg size, gonad size, breeding effort, individual survival, recruitment rate, dispersal behavior).

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Table 1.2: Sources of mass and life history information (average life expectancy, average time to maturity) if the information was not available in the source paper.

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CHAPTER 2: HIGHWAY TO THE DANGER ZONE: EXPOSURE-DEPENDENT COSTS OF IMMUNITY IN A VERTEBRAE ECTOTHERM

Note to Reader:

This chapter has been previously published: Brace, A.J., Sheikali, S, Martin, L.B.. Highway to the danger zone: exposure-dependent costs of immunity in a vertebrate ectotherm. *Functional Ecology* 29:924-903. See Appendix A for the PDF of the published document and Appendix B to see permission from the publisher.
CHAPTER 3: COSTS OF IMMUNITY PREDICT PROTECTION FROM MALARIA PARASITES IN BROWN ANOLE LIZARDS

Authors
Amber J. Brace, Marshall D. McCue, and Lynn B. Martin

Abstract
Exposure to parasites and subsequent immune system activation often causes physiological changes, which can ultimately result in tradeoffs within hosts. These costs of immunity are likely a factor in the wide variation in immune responses seen in nature. However, despite all we know about immune costs, the relationship between costs and any resultant benefits in terms of better protection from parasites remains relatively undefined. Here, we examined host- and parasite-mediated costs of immunity and the relationship between costs and protection from parasites by exposing brown anole lizards (*Anolis sagrei*) to live or killed malaria parasites. Specifically, we measured oxidation of glucose and inferred host costs of immunity by how much glucose was not oxidized. Deficiency in glucose oxidation is an ideal cost surrogate for hosts because glucose is critical to host performance but excessive removal of circulating glucose can result in torpor and death. Moreover, circulating glucose is also the primary fuel source for malaria parasites, without which the parasites cannot replicate. As such, we predicted that hosts that oxidized less glucose would experience the greatest benefits in terms of low parasite burdens. We found that malaria parasite-exposed lizards oxidized less glucose than controls. However, lizards infected with live malaria parasites did not oxidize glucose differently than killed-parasite infected lizards, suggesting the presence of a parasite-mediated
mechanism to circumvent host-driven glucose sequestration. Most importantly, among infected lizards, low glucose oxidation (i.e., high sequestration) was protective; lizards that oxidized the least glucose also had lower malaria parasite burdens. Interestingly, we also found that smaller animals tended to be more tolerant of malaria parasites than larger animals. Altogether, our results show that brown anoles that experience high costs of immunity upon exposure to malaria parasites also experience greater protection. Such findings add to our understanding of the relationship between immune costs and benefits within hosts and might lend insight into how individual-level processes can drive disease dynamics within communities.

Introduction

When animal hosts are exposed to most parasites, physiological changes occur, including initiation of the acute phase response (Klasing 1998; Lochmiller & Deerenberg 2000; Adelman & Martin 2009), which is typically accompanied by sickness behaviors such as fever, lethargy, anorexia, and resource sequestration (Hacker, Rothenburg & Kluger 1981; Lochmiller & Deerenberg 2000). Because immune responses are so resource-intensive, and accompanying sickness behaviors often result in lost opportunities (e.g., foraging, reproduction), immune activation often leads to tradeoffs with other processes (Klasing 1998; Soler et al. 2003; Uller, Isaksson & Olsson 2006; Brace, Sheikali & Martin 2015). Such costs of immune activation are argued to be a factor in the widespread variability in immune responses and subsequent protection from parasites seen among species, populations, and individuals. Ultimately, costs of immunity also affect host-parasite interactions and coevolution (Martin, Weil & Nelson 2008; Adelman 2014; Staley & Bonneaud 2015). Indeed, models have demonstrated that tradeoffs (i.e., costs) experienced by both the host and parasite strongly influence the outcomes of host-parasite interactions due to the relationship between costs of immune activation and the
magnitude of immune response (Best, White & Boots 2009; Boots et al. 2009). For example, if immune costs increase linearly with magnitude of immune response, then strong responses would exact high costs on hosts. As costs increase, host fitness may suffer, and selection may favor hosts with more moderate immune responses that presumably experience lower costs of immunity. Because of a less robust immune response, these hosts may then maintain some level of parasite infection without losing fitness (i.e., tolerate infection), thereby raising infection risk in other individuals and potentially increasing parasite prevalence within the community. These predictions rest on the common assumption that a positive relationship exists between costs of immunity and protection from parasites, yet this relationship remains poorly defined (Adelman 2014; Staley & Bonneaud 2015). To better understand how costs of immunity affect host-parasite interactions and ultimately the consequences for members of the communities in which these interactions occur, the relationship between costs of immunity and resultant benefits needs to be characterized.

Here, we examined the relationship between cost of immune activation and resultant benefits experienced by the host by exposing brown anole lizards (*Anolis sagrei*) to parasites that cause malaria. Malaria parasites are ubiquitous protozoans known to be detrimental to nearly all of their hosts including reptiles, and have contributed to millions of human deaths and the decline of multiple bird populations (van Riper III et al. 1986; Schall 1996; Sachs & Malaney 2002; Vardo, Wargo & Schall 2005). To disentangle host- and parasite-mediated costs, we first measured costs in individuals exposed to both live and killed malaria parasites by quantifying glucose oxidation during the earliest stage of the host-parasite interaction; a time when the most pronounced immune costs should occur (12-36 hours post-exposure) (Klasing 1998; Merchant et al. 2008; LeGrand & Alcock 2012). We then followed the course of malaria infection over a 7
week period in the live-exposed animals to determine whether and how cost of immunity immediately following exposure related to protection from or tolerance of malaria parasites. To measure cost of immune activation, we dosed individuals with glucose labelled with a heavy stable isotope (i.e., $^{13}$C-Glucose). When organisms ingest glucose, it is either stored as lipids or glycogen, or oxidized to form ATP, which is immediately useable for physiological work (Newgard et al. 1983; McCue & Welch 2015). When labelled glucose is used to form ATP, the $^{13}$C marker forms $^{13}$CO$_2$ as a byproduct of the oxidation process. It is then possible to track how much labelled glucose is immediately transformed into energy by quantifying the amount of $^{13}$C present in exhaled breath. Concomitantly, it is then possible to infer costs of immunity following malaria exposure in terms of how much glucose was not metabolized, and instead likely sequestered (i.e., stored as lipids or glycogen) (McCue 2011). Resource sequestration is commonly seen during bacterial infections where iron is removed from circulation. Removal of too much iron can result in anemia, and can thus be costly to hosts, but it can also cause a beneficial decrease in bacterial growth (Hacker, Rothenburg & Kluger 1981). Malaria parasites rely on circulating glucose as their main energy source (Tuteja 2007), and glucose sequestration from the blood can slow or stop parasite replication, which explains the long-term use of quinine to treat malaria in humans as it lowers circulating glucose by increasing insulin levels. Indeed, quinine and similar drugs are among the most effective ways to treat malaria in humans in some parts of the world (Davis et al. 1993). However, severe reductions in circulating glucose (hypoglycemia) can cause tremors, torpor, and death (White et al. 1983; Campbell 1996) and even modest declines can decrease physical performance and other activities. Yet, when exposed to malaria parasites, the sequestration of glucose may reduce parasite replication. As such, we predicted that brown anoles exposed to both live and killed malaria parasites would
react to the threat or perceived threat by experiencing higher costs in terms of lower glucose oxidation than the control group, however, we expected that costs would be highest in animals infected with live parasites. Such a decrease in oxidation would limit the amount of circulating glucose available to the parasites. We also predicted that within the live parasite group, individuals that experienced high costs would also experience lower parasite loads compared to individuals that experienced low costs.

As our interests in assessing the relationship between the costs of immunity and resultant benefits were motivated by consequences for other members of the host community, we also evaluated whether immune costs were related to how hosts tolerated malaria infection (Adelman 2014; Brace, Sheikali & Martin 2015). When infected with malaria parasites, hosts can suffer from multiple symptoms, which may include but are not limited to, kidney failure caused by the build-up of toxic by-products from the parasite’s digestion of hemoglobin, and anemia, which can be severe (Barsoum 2000; Tjitra et al. 2008). The latter symptom can cause low blood oxygen levels and decreased stamina and physical performance, which is one of the most commonly studied in lizards (Schall 1990a; Schall 1996). Decreased performance may increase chances of predation and lower an individual’s ability to compete for resources. Anemia and low blood oxygen levels occur when the asexual life stage of the parasite (merozoites) invade red blood cells and reproduce until the cell ruptures, releasing new asexual forms that then infect other red blood cells. To compensate for red blood cell loss, hosts will prematurely release immature red blood cells into circulation to recoup diminished oxygen transport capability (Schall 1990c). Subsequently, relationships between immature red blood cells and parasite burden along with body mass and parasite burden represent particularly insightful, functional metrics of malaria parasite tolerance that can be repeatedly and non-destructively measured in
such a small host. We predicted that hosts that experienced lower costs of immunity (i.e., oxidized more glucose) upon malaria exposure would exhibit greater tolerance to infection than those that oxidized less glucose. This is because individuals that require a greater amount of oxygen to function may be unable to sequester glucose when faced with infection but may be better able to replenish red blood cells destroyed by malaria parasites because they are already functioning at a higher rate. Additionally, by not sequestering glucose, these individuals would not consume energy reserves to compensate for the decrease of circulating glucose, which may allow them to better maintain their body mass. In these individuals, the costs of sequestering glucose may be too high and outweigh the costs of a persistent infection (Brace, Sheikali & Martin 2015).

**Materials and Methods**

**Study species, husbandry and experimental infections**

Brown anoles are small, semi-arboreal lizards native to Cuba that have established successful populations throughout Florida, the Caribbean, and Hawaii. Across their introduced range in Florida, brown anoles have been found to be infected with *Plasmodium floridense*, a widespread saurian malaria parasite (Schall 1990c; Schall 1990b; Vardo, Wargo & Schall 2005). Between February and March 2015, wild brown anoles were captured on the University of South Florida campus (28.05º N, 82.41º W). At capture, morphological measurements (body mass, snout-vent length (SVL)) and sex were determined. Only putatively mature individuals (greater than 34mm SVL; (Lee et al. 1989)) were included in the study. Also at capture, two drops of blood were collected from each individual via toe clip under isoflurane anesthesia to test for the presence of *Plasmodium* infection; blood smears were made with one drop, then stained with Giemsa stain and examined under the 1000X for the presence/absence of parasites in red blood
cells (Vardo, Wargo & Schall 2005). The other blood drop was placed on filter paper and used for *Plasmodium*-specific PCR analysis to confirm the absence of infection. Any individuals found to be positive for *Plasmodium* at capture were not included in this study. Individuals were then semi-randomly assigned (i.e., sexes were equally distributed) to one of three treatment groups (live parasite (LP), killed parasite (KP) or control (CTL)) and housed in size- and sex-matched pairs in 23x13x8 cm ventilated plastic containers containing branches and leaves collected from the area they were captured and *ad lib* access to water. Anoles were kept at room temperature (23ºC) on a 12-12 light/dark schedule and were each fed 2-4 live vitamin-enriched crickets twice per week.

Approximately two weeks post-capture, experimental *Plasmodium* exposures were performed using a modified protocol (Telford Jr 1972). In short, on the day of exposures, blood smears from previously infected (donor) individuals were made as described above and *Plasmodium* burden was quantified by counting the number of parasites per 1,000 red blood cells and estimating the number of parasite per µL of blood by multiplying the aforementioned malaria parasite burden ratio by 1,000 (Telford Jr 1972). To collect a sufficient amount of infected blood for exposures, blood had to be pooled from several donors. The estimated number of parasites per µL was averaged among all donors. To perform the exposures, individuals from the LP group were given an intra-peritoneal (IP) injection of donor blood mixed with saline containing approximately 200,000 *Plasmodium* parasites (25 µL total injection volume) (Telford Jr 1972). The KP group received 25 µL total injection volume of heat-killed parasites IP; parasites were killed by heating the blood-saline suspension to 95ºC for 20 minutes (Friesen & Matuschewski 2011). The CTL group received a 25 µL of sterile saline IP. The dose of approximately 200,000
Plasmodium parasites was found to successfully infect the majority of brown anole individuals in a previous study (Figure 3.6).

Twelve hours post-exposure, all individuals were weighed to the nearest 0.01 gram, given an oral dose of 2 mg 99% $^{13}$C-D-Glucose (hereafter called $^{13}$C-glucose; Cambridge Isotope Labs, Tewksbury, MA, USA) and placed into 60 mL syringes for 24 hours for breath CO$_2$ sample collection. Prior to the collection of each CO$_2$ sample, syringes were sealed for 60 minutes to allow for CO$_2$ accumulation to levels sufficient for isotope measurement (~2%) (McCue & Welch 2015). Over a period of 24 hours, CO$_2$ samples from each individual were collected every 2 hours for 12 hours and then every 4 hours for the remaining 12 hours by injecting 10 mL of air from the sealed 60 mL syringes into an Exetainer tube (Labco Limited, Lampeter, Ceredigion, UK). After each sample was collected, syringes were left open to ventilate between each sampling period. When CO$_2$ sampling was complete, individuals were returned to their housing containers, where weekly for 5 weeks and then again at week 7, they were weighed and had a blood sample collected as described above to monitor immature red blood cell count (iRBC) and Plasmodium parasite burden. All procedures met guidelines for the use and care of animals in research and were approved by USF IACUC (WIS0668).

Isotope analysis

Breath analysis was performed at St. Mary’s University, San Antonio, TX. The total CO$_2$ in each breath sample was used to calculate the rate of CO$_2$ production (i.e., VCO$_2$) using equation 4.21 from (Lighton 2008). The amount of $^{13}$C in the breath samples was measured using a HeliANPlus nondispersive spectrometer (Fischer Analysen Instrumente GmbH; Germany) interfaces with a FANas autosampler. Vials containing reference gases with known $^{13}$C-concentrations were measured in triplicate between every n = 20 unknown samples and
expressed in terms of atom percent (At%) according to equation 2 of (McCue & Welch 2015).

We calculated instantaneous and cumulative rates of $^{13}$C-glucose oxidation using VCO$_2$ ad At% according to equations 2 and 3 of (Khalilieh, McCue & Pinshow 2012).

**Burden and immature red blood cell quantification**

To quantify malaria burden, blood smears were made on glass slides and stained as described above. Once dry, smears were examined under the 1,000X objective of a light microscope and number of parasites per 1,000 red blood cells were counted (10 fields of approximately 100 red blood cells). Burden was expressed in terms of number of parasites per 1,000 red blood cells (Vardo-Zalik & Schall 2008). To quantify immature red blood cells (iRBC), three fields of approximately 100 red blood cells were examined and the number of iRBC per field was recorded. The number of iRBC per field was then averaged among the three fields (Vardo-Zalik & Schall 2008).

**DNA extraction and PCR analysis**

For PCR analysis for malaria presence, DNA was extracted from blood samples using the DNeasy spin column extraction protocol with the following modifications: for the digestion step, dried blood dots were incubated with 180 µL of vertebrate lysis buffer and 5 µL of Proteinase K overnight; for the washing step, an additional wash with 750 µL of PE buffer was performed; for the elution step, only 20 µL of elution buffer was used. Nested PCR reactions were performed using extracted DNA and primers specific to the *Plasmodium* cytochrome b gene according to (Vardo, Wargo & Schall 2005). The product of the second PCR reaction was then visualized on a 1% agarose gel; the presence of a band indicated an individual was infected with *Plasmodium*. 
Statistical analyses

We first used linear mixed models (LMMs) to assess whether glucose oxidation over the 24 hour sampling period following exposure was predicted by parasite exposure type (live parasite (LP), killed parasite (KP), or control (CTL)), time since glucose administration (time), or body mass at time of infection (mass) as well as two-way interactions between exposure type and time and exposure type and body mass. We included body mass at time of infection in our analyses because mass affects nearly every aspect of physiology (West, Brown & Enquist 1997; Gillooly et al. 2001) and likely plays an important role in how well hosts protect themselves from parasitic infection. For example, larger lizards tend to have greater energetic reserves (Cox et al. 2010) and may be better able to store, rather than immediately oxidize exogenous glucose. Greater energetic reserves also may enable larger individuals to mount a more effective immune response (Meylan, Haussy & Voiturion 2010). Prior to analysis, glucose oxidation was log plus 1- transformed, and mass was log-transformed to achieve normality.

To analyze whether cost or body mass at time of exposure predicted cumulative malaria parasite burden (i.e., resistance), we used an LMM with individuals from the LP group only. We also included latency to visible infection in the model because we found that individuals that became infected later in the study typically had lower burdens.

To assess tolerance of malaria parasites, we used change in iRBCs and change in mass from weeks 3 to 7 as our measures of fitness, as this was the time period during which the majority of animals became visibly infected and experienced the greatest increases in burden. As such, it should also be the time period during which animals suffer the greatest decrements to fitness. To determine whether we could measure the ability of lizards to limit damage caused by malaria parasites, we first used simple linear regressions to define the slope of change in burden, iRBCs
and body mass from weeks 3 to 7 for each individual. We then used MANOVA to examine whether there was a relationship between change in iRBCs and burden and/or body mass and burden. As we wanted to focus on individual measures of tolerance, we then determined individual tolerance estimates by using simple linear regressions to define the slope of the change in iRBCs in relation to the change in parasite burden from week 3 to 4, week 4 to 5, and week 5 to 7. These individual tolerance estimates were then used in LMMs to examine whether glucose oxidation predicted individual tolerance to malaria parasites.

Results

Effects of malaria parasite exposure on glucose oxidation

We found that all treatment groups increased glucose oxidation (time: $F_{8, 294}=105.454, P<0.0005$; Figure 3.1) and oxidized the similar amounts of glucose over the 24 hour sampling period (treatment * time: $F_{16, 294}=0.411, P=0.979$). However, we found a significant effect of treatment on cumulative glucose oxidation ($F_{2, 294}=5.053, P=0.007$), indicating that whereas all groups ultimately oxidized the same amount of glucose, they differed in the rate of oxidation (Figure 3.1). A Bonferonni post-hoc analysis showed that the KP group oxidized less glucose than the CTL group ($P=0.008$), indicating a higher cost (i.e., lower oxidation) in the KP group early in the sampling period. The LP group did not differ from either the KP ($P=0.117$) or the CTL groups ($P=0.832$). We also found that individuals that were heavier on the day of parasite exposure oxidized more glucose than lighter individuals ($F_{1, 294}=92.290, P<0.0005$). Additionally, we found a significant interaction of treatment and body mass ($F_{2, 294}=8.543, P<0.0005$) such that body mass only affected glucose oxidation in the control group as this group had the only slope that differed significantly from zero (CTL slope=1.094, $P=0.018$; LP slope=0.428, $P=0.172$; KP slope=0.448, $P=0.139$; Figure 3.2); heavy individuals from the LP and KP groups oxidized less
glucose than heavy control individuals while there was no apparent difference among treatment groups among lighter individuals.

Resistance to malaria parasites

Within the LP group, we found that individuals that oxidized less glucose had lower cumulative malaria burdens (F1, 86=8.535, P=0.004; Figure 3.3). We also found a marginally non-significant effect of body mass such that heavier lizards at the time of parasite exposure tended to have lower cumulative burdens than lighter individuals (F1, 86=3.259, P=0.075; Figure 3.4). Lastly, we found that individuals with a longer latency to visible infection had lower cumulative burdens (Parameter Est= -1.460, SE=0.233, F1, 86=39.128, P<0.0005).

Tolerance of malaria parasites

Change in iRBCs was significantly related to changes in malaria parasite burden within individuals over the course of infection (F1=19.253, P=0.007; Figure 3.7A). However, we found no such relationship between change in body mass and burden (F1=0.648, P=0.457; Figure 3.7B). We subsequently used change in iRBCs while infected to ask whether glucose oxidation and/or other host factors predicted individual tolerance of malaria parasites. Using individual tolerance estimates, we found that glucose oxidation did not predict tolerance (F1, 60= 0.117, P=0.733), however, animals that were lighter on the day of exposure tended to be more tolerant than heavy individuals, although this effect was marginally non-significant (F1, 60=3.549, P=0.064; Figure 3.5).

Discussion

We predicted that when exposed to live malaria parasites, brown anoles would experience greater costs of immune activation (oxidize less glucose) than when exposed to killed and control infections. We also expected that individuals that experienced greater costs would have lower
parasite burdens, likely as a result of reducing circulating glucose and thus removing the primary resource that fuels malaria parasite replication. We found partial support for our first hypothesis, as KP individuals oxidized less glucose than the CTL group early in our sampling period (Figure 3.1). Importantly, in support of our second prediction, we found that within the LP group, individuals that sequestered the most glucose following malaria parasite exposure also had lower parasite burdens over 7 weeks (Figure 3.3). Additionally, we found that animals that were heavier on the day of parasite exposure tended to have lower parasite burdens, however, this result was marginally non-significant (Figure 3.4). Lastly, we found that costs of exposure to/infection with malaria parasites did not predict individual tolerance of malaria parasites, although animals that were lighter on the day of exposure tended to be more tolerant (Figure 3.5).

**Glucose sequestration as a protective mechanism**

Resource sequestration in response to infection can limit the proliferation of invading parasites (Hacker, Rothenburg & Kluger 1981; Parrow, Fleming & Minnick 2013). Yet, resource sequestration can be detrimental to hosts, as seen in cases of anemia resulting from severe reduction in plasma iron (Hacker, Rothenburg & Kluger 1981). Depletion of glucose can also be costly to hosts, as it decreases a readily available source of energy and can lead to hypoglycemia, a dangerous condition that can result in torpor and death in reptiles (Campbell 1996). However, if hosts respond to malaria exposure by oxidizing less glucose, the availability of this resource would be limited to the parasites and the probability of the malaria parasites successfully colonizing an individual may be reduced. Our finding that individuals in the KP group oxidized less glucose than the CTL group early in the sampling period indicates that brown anoles are indeed responding to malaria parasite exposure by reducing glucose oxidation. Interestingly,
reduction in glucose oxidation appears to be the most pronounced in the KP group 2 to 10 hours post-glucose administration (14-24 hours post-infection), the time during which the malaria parasites are making their way to the liver where they will replicate before being released into the bloodstream. This is also the time during which individuals are likely to be mounting an acute phase response in an effort to limit or eliminate the infection (Klasing & Barnes 1988; Merchant et al. 2008). Because of the detrimental effects of prolonged and/or severe reduction of circulating glucose, individuals may only be able to reduce glucose oxidation for a short period of time following exposure, or risk suffering the effects of hypoglycemia. Importantly, our finding that animals in the LP group that oxidized less glucose (i.e., experienced a higher cost of immunity) also experienced lower burdens over 7 weeks further supports the idea that glucose sequestration, at least over a short period of time, is a protective mechanism against malaria parasite infection in brown anole lizards. It would be informative for future studies to examine glucose sequestration following exposure to malaria parasites in greater resolution, for example, by continuously sampling individuals for 48 hours following exposure. Such a study would allow us to determine the window during which reduced glucose oxidation is most likely to occur and is most beneficial to the host. It would also be interesting to see if differences in circulating glucose that occur naturally in hosts affect an individual’s probability of becoming infected and to see if fluctuations in circulating glucose can be induced by exposure to malaria parasites. Interestingly, our additional finding that glucose oxidation in the LP group did not differ from either the CTL or KP groups may indicate that the malaria parasite we used has evolved a mechanism by which it can slow or stop host-mediated reduction of glucose oxidation. Such an evasion of resource sequestration has been seen in several pathogenic bacteria that have evolved enzymes that circumvent iron sequestration in mammals (Fischbach et al. 2006).
Contrary to our predictions, glucose oxidation did not predict the ability of brown anole lizards to tolerate malaria parasites, which may be due to the low number of individuals in which we could measure tolerance (n=7). Yet, it is also possible that even with a larger sample size that costs in terms of glucose oxidation may be unrelated to individual tolerance of malaria parasites. However, we did find a marginal effect of body mass such that lighter individuals were better able to produce iRBCs to counter the negative effects of parasite-mediated destruction of RBCs (i.e., were more tolerant). One explanation for this may be because animals with lower body masses also tend to have higher cellular turnover rates, which causes high production of reactive oxygen species (ROS) (Speakman 2005; Savage et al. 2007). Because ROS can be damaging to red blood cells (Smith 2012), it is possible that lighter individuals that have high cellular turnover are naturally better able to replace damaged RBCs, whether the RBCs are damaged by ROS or malaria parasites. While the majority of studies on differences in cellular turnover rates are across species spanning a wide range of body sizes, it is reasonable to expect that differences, although much smaller, also exist among individuals of varying body size within a population. It would be interesting for future studies to explore whether cellular turnover affected an individual’s ability to tolerate malaria infection.

Costs of parasite exposure and benefits of protection

While previous work has shown that costs of immunity are an important influence on how hosts respond to parasite exposure (Best, White & Boots 2009; Adelman 2014; Brace, Sheikali & Martin 2015), without an understanding of the relationship between immune costs and resultant benefits in terms of protection from parasites, we cannot make accurate predictions about how immune costs may affect the ecology and evolution of host-parasite relationships. If selection favors more moderate immune responses to compensate for high costs of immunity, then
individuals with moderate responses may be increasing the exposure risk to others within the population (May & Anderson 1983; Brace, Sheikali & Martin 2015). The main purpose of this study was to determine whether and how costs of immunity at the individual-level may affect the movement of parasites through communities (Adelman 2014).

We found that in brown anole lizards, high costs of immunity resulted in better protection in terms of lower parasite burdens. However, because better protection from parasites comes with the potentially life-threatening cost of reducing glucose oxidation, it is unlikely that all individuals are capable of protecting themselves from infection. Indeed, those that do not reduce glucose oxidation are more likely to have higher burdens. Our finding that only heavy animals exposed to malaria parasites (alive and dead) had lower glucose oxidation than CTL animals (Figure 3.2), and our marginal result that lighter individuals tended to have higher burdens indicates that the ability to lower glucose oxidation in response to malaria parasite exposure may be limited by an individual’s resource reserves and/or by their physiology. For example, brown anole lizards use abdominal fat bodies to support themselves in times of decreased resource availability or increased nutritional demand. These fat bodies tend to be larger in heavier animals (Derickson 1976; Cox et al. 2010), potentially providing them with alternative resources to be able to handle a reduction in glucose oxidation. Moreover, small animals tend to consume more resources to maintain homeostasis than large animals (Savage et al. 2007), perhaps making it impossible for them to reduce glucose oxidation in response to malaria parasite exposure. If this is indeed the case, then there may be a body size refugium from infection and small individuals may be disproportionately contributing to the prevalence of malaria parasites within their communities by maintaining relatively high burdens. Our additional marginal finding that smaller animals tended to be more tolerant of malaria parasites further supports this idea because
small individuals are not only maintaining higher burdens, but are also able to cope with the
damage caused by the parasites and may be less likely to succumb to predation as a result of
decrements in physical performance from low RBCs.

As malaria parasites are ubiquitous across the range of brown anole lizards and infect multiple
anole lizard species (Telford 1975; Telford Jr 1984; Vardo, Wargo & Schall 2005), our findings
have implications for communities that harbor introduced populations of brown anoles, such as
Florida. It would be informative for future studies to examine whether brown anoles are
significantly affecting the prevalence of malaria in native anole species across their introduced
ranges. Indeed, a previous study examining experimental coccidian infections in an introduced
sparrow species (*Passer domesticus*) and a native congener (*Passer griseus*) in Kenya found that
the introduced sparrow harbored considerable more parasites while maintaining the same level of
fitness as the native species (Coon *et al.* 2014).

Conclusion

Our study was able to determine successfully that high costs of immunity result in greater
protection from parasites at the level of individual hosts. Additionally and importantly, we were
able to identify that sequestration of glucose may be a mechanism for brown anole lizards to
resist successful colonization by malaria parasites. In all, our characterization of the relationship
between host costs of immunity and resultant benefits in terms of protection from parasites could
enable us to better predict which individuals within a population may be contributing the greatest
to the prevalence of malaria parasites and ultimately to better predict the outcomes of host-
parasite interactions.
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Figure 3.1: All treatment groups increase glucose oxidation and oxidize the same amount of glucose over the 24 hour sampling period. However, a significant effect of treatment on mean cumulative glucose oxidation revealed that the killed parasite (KP) group differed in oxidation rate; they oxidized less glucose early in the sampling period than the control (CTL) group. The live parasite (LP) group did not differ from either the CTL or KP groups.
Figure 3.2: Individuals with higher body mass on the day of malaria parasite exposure oxidized more glucose than individuals that were lighter on the day of exposure. However, heavy LP and KP individuals oxidized less glucose than heavy CTL individuals.
Figure 3.3: Within the LP group, individuals that experienced a higher cost (lower glucose oxidation) also experienced lower cumulative parasite burdens over 7 weeks.
Figure 3.4: Within the LP group, individuals that were heavier on the day of malaria parasite exposure tended to have lower cumulative burdens than lighter individuals, although this effect was marginally non-significant.
Figure 3.5: Within the LP group, individuals that were lighter on the day of exposure tended to be more tolerant (higher increase in iRBCs in relation to increase in burden) than individuals that were heavier, although this effect was marginally non-significant.
Figure 3.6: Results from a pilot study examining the efficacy of varying malaria parasite doses at causing infection over 5 weeks shows that lizards dosed with ~25,000, ~50,000, or ~100,000 live parasites were not infected within this time frame. When dosed with ~150,000 parasites, 2 out 4 individuals were infected at 5 weeks and when dosed with ~200,000 parasites, 3 out of 4 individuals were infected. As the majority of animals became visibly infected within 5 weeks when exposed to ~200,000 parasites, we used this dose in our current study.
Figure 3.7: (A) When examining whether we could measure the ability of individuals to limit damage caused by malaria parasites, we found a significant relationship between change in iRBCs and burden from weeks 3 to 7. (B) No relationship existed between change in body mass and burden from weeks 3 to 7. As such, we used iRBCs as our fitness proxy to determine individual tolerance metrics.
APPENDIX A: Highway to the danger zone: exposure-dependent costs of immunity in a vertebrate ectotherm

Note to Reader:

This chapter has been previously published: Brace, A.J., Sheikali, S., Martin, L.B. Highway to the danger zone: exposure-dependent costs of immunity in a vertebrate ectotherm. *Functional Ecology* 29(7): 924-930. See Appendix A for the PDF of the published, open-access document.
**Highway to the danger zone: exposure-dependent costs of immunity in a vertebrate ectotherm**

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**Summary**

1. Parasite exposure often causes innate immune activation, resulting in trade-offs among physiological processes and strong selection on the parasite. Costs of immune activation vary widely among and within host populations though, likely dependent on the evolutionary history of host–parasite interactions and the environments in which they occur. For hosts, degree of exposure may drive the magnitude of costs incurred and subsequently whether hosts resist or tolerate infections.

2. If costs increase concomitantly with exposure, a threshold may exist where the expense of parasite resistance becomes prohibitive and parasite tolerance becomes favourable. Here, we characterized exposure-dependent costs of an innate immune response in brown anoles (*Anolis sagrei*) by tracking allocation of an isotopically labelled essential amino acid (\(^{13}\)C-leucine), to the liver and gonads. To elicit immune responses, we used lipopolysaccharide (LPS), a strongly immunogenic molecule from *Salmonella* spp.

3. We found that both sexes paid dose-dependent costs of *Salmonella* LPS-induced immune activation, but costs were experienced differently by the sexes, likely due to differences in life history. Males allocated more leucine to their livers in response to higher LPS doses. In females, a tendency for increased costs in response to dose was only revealed when leucine allocation ratios between lymphoid and reproductive organs were considered. We also found that regardless of dose, males always allocated more leucine to their gonads than females. Lastly, and perhaps most interestingly, cost functions in both sexes were linear, but with shallow slopes, indicating modest costs of immune activation in response to *Salmonella* LPS in this species.

4. Altogether, our results demonstrate that costs of immunity are dose dependent in this introduced lizard species, sexes experience costs differently. Characterization of relationships between host exposure and costs of immune activation such as these can facilitate predictions about how parasites might circulate through communities.

**Key-words:** *Anolis sagrei*, dose–response, introduced species, parasite tolerance, parasite virulence, resource allocation

**Introduction**

The innate immune response rapidly detects and eliminates diverse parasites upon exposure. Although beneficial, these responses are often costly to hosts in a variety of ways. Upon innate immune activation, many hosts increase metabolic rates and production of immune proteins involved in the acute phase response (Lochmiller & Deerenberg 2000). In addition to these direct costs, hosts also often suffer decreases in growth (Lochmiller & Deerenberg 2000; Soler *et al.* 2003), reproductive success (Bonneaud *et al.* 2003; Uller, Isaksson & Olsson 2006) and/or physical performance (Cox & Calsbeek 2010; Cox *et al.* 2010). Such costs can lead to trade-offs, which are sometimes mitigated by abundant resources (Ruiz *et al.* 2010); however, as resources in most natural environments are limited, costs of immunity may be difficult to avoid. Indeed, the diversity of immune defences present in nature has been argued to exist partly because of such heterogeneous immune costs (Viney, Riley & Buchanan 2005; Martin, Weil & Nelson 2008). Several studies have demonstrated that the magnitude of parasite exposure can also be an important driver of the costs of immunity that hosts pay (Paulo *et al.* 2010; Nystrøm & Dowling 2014). For example, case fatality...
rates during the 1918 human influenza outbreak appear to have been influenced by the number of contacts with infected individuals (Paulo et al. 2010). In other words, in response to flu in humans and other parasites by other vertebrate hosts, it is likely that the cost of an immune response is mediated by the history and extent of interactions between hosts and parasites and the environments in which both occur.

Surprisingly, the relationship between host exposure and costs of immunity remains relatively unknown. The shape of such relationships (e.g. linear, decelerating or accelerating functions) is integral to characterize though because such information would facilitate predictions about the evolution of parasite virulence and transmission and thus foreshadow how parasites might circulate through communities (Best et al. 2012; Boots, Donnelly & White 2013; Adelman 2014). For instance, if host immune costs increase rapidly with increasing exposure, a threshold may be reached where the host can no longer afford the expense. In such a case, strong selection for tolerance would occur (Schmid-Hempel 2011), and certain tolerant hosts that maintain high parasite burdens would subsequently increase the risk of exposure in other organisms by shedding large numbers of parasites into the environment.

Here, we investigated whether and how exposure to an immunogenic parasite component affected costs of innate immunity in Anolis sagrei (brown anoles) by examining critical nutrient allocation among various organs. We used concentrations of lipopolysaccharide (LPS) from Salmonella spp., a naturally occurring pathogen in this species (Hoff & White 1977) to induce immune responses. LPS, a pathogen-associated molecular pattern (PAMP), is a conserved, highly immunogenic component of Gram-negative bacteria cells walls that is recognized by the innate immune system (Gao, Jeong & Tian 2008). Upon recognition, it stimulates an acute phase response, one of the most protein- and calorie-intensive immune responses, often accompanied by fever and sickness-related behaviours such as lethargy and anorexia (Klasing 1998). The major benefit of using a PAMP such as LPS for an immune challenge is that the immune system has evolved to respond to such molecules, so activation of the immune response can occur without the added effects of a replicating pathogen, which could obscure whether costs are attributable to hosts, parasites or their interactions (Sheldon & Verhulst 1996).

To measure the costs of an immune challenge, we quantified allocation of $^{13}$C-1-L-leucine to an immune tissue (liver) and reproductive tissue (gonad). The liver was the tissue of choice because lymphoid tissue is very diffuse; no single organ serves a solely immunological function. Furthermore, the liver is integral to the production of acute phase proteins (e.g. serum amyloid A and C-reactive protein) in response to recognition of a pathogen (Klasing & Korver 1997) as well as some of the leucocytes expressing the pattern recognition receptors (PRRs) responsible for recognizing PAMPs such as LPS (Gao, Jeong & Tian 2008). The liver also comprises a large proportion of vertebrate metabolism. We used leucine because it is an essential amino acid, is a component of virtually every protein, including those associated with resistance (e.g. haptoglobin) and breeding (vitellogenin, sperm production), but it cannot be synthesized by the body, and thus is a valuable commodity for infected and healthy individuals. As such, an exogenous dose can be given and allocation tracked via measurement of the attached $^{13}$C tracer, serving as a proxy for protein synthesis in tissues (McCue et al. 2011).

Due to the involvement of the liver in the acute phase response, especially in the production of acute phase proteins as a result of parasite exposure and subsequent PAMP recognition by the immune system (Klasing & Korver 1997; Gao, Jeong & Tian 2008; Zimmerman, Vogel & Bowden 2010), we predicted that allocation to the liver would increase with LPS dose. Such an increase would indicate a greater immune cost with higher exposure levels. As female A. sagrei experience comparatively higher physiological cost of reproduction than males (Cox & Calabreck 2010; Cox et al. 2010), we expected allocation to their livers to be less pronounced. Coupled with the increase in allocation to the liver, we also predicted that allocation to the gonads in both sexes would decrease with increasing magnitude of LPS exposure. As ectotherm immune responses are closely tied to ambient temperature (Mondal & Rai 2001; Zimmerman, Vogel & Bowden 2010), we conducted our study at two temperatures commonly experienced by the host across their range and at the location of their capture in Tampa, Florida (20 and 30 °C). We predicted greater nutrient allocation to both tissues at 30 °C, as this is within the range of their mean body temperature when sampled in their native and introduced ranges (Lister 1976), meaning that this temperature likely falls within their ideal thermal performance range. Additionally, metabolic rate and thus protein turnover should be greater at higher temperatures (Gatten 1974), leading to greater leucine incorporation in the tissue. We had no predictions about the shape of the immune cost functions, but evaluated whether they were best described as linear or more complex. We found that both sexes paid dose-dependent costs of Salmonella LPS exposure, but female costs were marginally non-significant and were only revealed when leucine allocation ratios between lymphoid and reproductive organs were considered. More importantly, cost functions were linear, but with shallow slopes, indicating modest costs of Salmonella-induced immune activation in this species.

**Materials and methods**

**STUDY SPECIES**

*Anolis sagrei* are small, semi-arboreal lizards native to Cuba that have successfully established populations in Jamaica, Little Cayman and Florida. Their success may be partially attributable to their ability to tolerate substantial changes in habitat (and associated temperature differences) by shifting their preferred thermal ranges based on the available habitat in their new range (Lister 1976).
This species breeds continuously throughout the summer months (Lee 1985), during which females lay an egg approximately every 7 days. Females experience large physiological costs of reproduction, including decrements in both physical performance and immunity (Cox & Calsebe 2010; Cox et al. 2010). This suggests that processes requiring additional resources (such as immune activation) would likely come at the expense of resources that would have been used for reproduction (i.e. a trade-off). Additionally, male *A. sagrei* are particularly aggressive during breeding season, often engaging in physical altercations while defending territories or mates. Such aggressive behaviour suggests that in addition to the direct cost of reproduction (e.g. spermatogenesis), males also experience indirect costs (e.g. territory defence through the maintenance of competitive ability), meaning that investment in immune activation may shift resources away from processes integral for reproductive success. This species is of ecological concern in Florida because they are so abundant, yet little is known about how their immune responses might affect disease cycles in their introduced territories. If *A. sagrei* experience high costs of immune activation in response to high doses of *Salmonella* LPS, they may tolerate infection, leading to increased parasite burdens and subsequently increased transmission risk for other organisms with which they cohabitate. Indeed, this species is thought to have facilitated the introduction of some intestinal parasites in Hawaii, another introduced range (Goldberg & Bursey 2000).

**HUSBANDRY AND TREATMENT**

Wild *A. sagrei* were captured by hand or noose between June and July 2013 on the University of South Florida campus, Tampa, FL (28°05′N, 82°41′W). Body mass, snout–vent length (SVL) and sex were determined at the time of capture. Only individuals with an SVL greater than 34 mm were collected to ensure that all individuals were within the reproductive size range (Lee et al. 1989). Individuals were housed together in size-matched, mixed treatment groups of 3–5 individuals in ventilated 23 × 13 × 8 cm plastic containers with access to water and branches and leaves collected from the natural environment. Plastic housing containers were placed in Styrofoam temperature control chambers set to either 20 or 30 °C, depending on the temperature group to which individuals were randomly assigned at capture. Also upon capture, individuals were randomly assigned at capture. Also upon capture, individuals were within the reproductive size range (Lee 1989). Individuals received an intraperitoneal injection of *Salmonella* spp. LPS (Sigma-Aldrich, Milwaukee, WI, USA) or phosphate-buffered saline (PBS) according to their assigned treatment group. Concurrently, all individuals received an oral dose of 4 mg of 99% 13C-L-leucine (hereafter, 13C-leucine), suspended in 40 µL of sunflower oil (Cambridge Isotope Labs, Cambridge, MA, USA; modified from McCue et al. (2011); Coon et al. (2014). After treatments, all individuals were returned to their respective housing and climate control containers. Twenty-four hours later, individuals were euthanatized by rapid decapitation under deep isoflurane anaesthesia. Six individuals did not receive LPS or doses of 13C-leucine and were used to measure background 13C values, as tissue 13C can vary based on diet (Rubenstein & Hobson 2004). Immediately after euthanasia, entire livers, gonads and any developing eggs were removed and kept at −40°C until lyophilized and prepared for isotope analysis (McCue et al. 2010; Coon et al. 2014). All procedures met guidelines for the use and care of animals in research and were approved by USF IACUC (W0059).

**ISOTOPE ANALYSIS**

Isotope mass spectrometry was performed at the University of South Florida PaleoLab in St. Petersburg, FL, using a Thermo Delta+XL stable isotope mass spectrometer (Thermo Scientific, Waltham, MA, USA). The 13C-leucine values measured in the liver and gonads were recorded as δ13CVPDB, which is the difference between the sample and an industry 13C standard ( Slater, Preston & Weaver 2001; Coon et al. 2014). The naturally occurring background δ13C was calculated from the average tissue values from the six individuals not dosed with leucine. The background values were then used to calculate the increase in δ13C from the exogenous tracer in all individuals that received labelled leucine (enrichment values). The enrichment values were corrected for individual organ mass by dividing them by the dry weight of the whole organ. The enrichment values are expressed in terms of log 13C, which are a proxy for leucine allocation. We also compared 13C-leucine allocation in the liver relative to the gonads by dividing the organ mass-adjusted enrichment values for the liver by the adjusted values for the gonads for each individual. This unitless ratio was log plus 2-transformed to achieve normality (hereafter, called liver to gonad ratio).

**STATISTICAL ANALYSES**

We used general linear models (GLMs) to assess possible nutrient costs of LPS exposure. Enrichment values were log plus 2-transformed to achieve normality for statistical analysis. 13C-leucine allocation to the liver and gonads was analysed separately to determine whether LPS dose (a continuous predictor), ambient temperature, SVL and sex were significant predictors of allocation. The two-way interactions of LPS dose with sex and with temperature, as well as the interaction of temperature and sex, were also included in models. We then analysed how LPS dose, temperature and SVL affected liver to gonad ratios, but analysed each sex separately because of large differences in gonad size and because the above analyses indicated strong differences between sexes. All statistics were performed using *spss* 22.0 (IBM, Armonk, NY, USA), and figures were created in *Graphpad prism v.5.0* (GraphPad Software, Inc., San Diego, CA, USA).

**Results**

13C-leucine allocation

LPS dose alone did not predict 13C-leucine allocation to the liver (*F*1,103 = 0.039, *P* = 0.843) or the gonads (*F*1,102 = 2.773; *P* = 0.099), but this was because of a strong sex by tissue interaction and a strong effect of sex overall on allocation. In the liver, sexes responded differently to LPS dose (sex*LPS dose: *F*1,103 = 5.165; *P* = 0.025); males increased 13C-leucine allocation, whereas females showed no change (Fig. 1a, see Table S1A, Supporting Information). This sex difference was not seen in the gonads (sex*LPS dose: *F*1,102 = 0.107; *P* = 0.745; Fig. 1b, Table S1B), but males allocated significantly more 13C-leucine allocation to the gonads than females (*F*1,102 = 7.099, *P* = 0.009; males: mean = 2.039, SD = 0.484, *N* = 53; females: mean = 1.081, SD = 0.452, *N* = 49; Fig. 1b, Table S1B). Temperature, independent of sex or dose, was a significant predictor of 13C-leucine allocation to the liver (*F*1,103 = 6.484; *P* = 0.012; Fig. S1a, Supporting Information).
but not the gonads ($F_{1,102} = 0.00/C1_{255}; P = 0.615$; Fig. S1b), with greater allocation at 30 than 20°C (liver 30°C: mean $= 2.076$, SD $= 0.335$, $N = 52$; liver 20°C: mean $= 1.807$, SD $= 0.416$, $N = 51$). In the gonads, SVL was a significant predictor of $^{13}$C-leucine allocation ($F_{1,102} = 24.144$, $P < 0.0005$; Table S1B) with larger individuals allocating more $^{13}$C-leucine. This pattern was not observed in the liver (SVL: $F_{1,103} = 2.225$, $P = 0.139$; Table S1A).

RELATIVE $^{13}$C LEUCINE ALLOCATION

In males, temperature, SVL and their interaction had significant effects on the liver to gonad ratio of $^{13}$C-leucine (temperature: $F_{1,47} = 28.269$, $P < 0.0005$; SVL: $F_{1,47} = 15.098$, $P < 0.0005$; temperature x SVL: $F_{1,47} = 25.974$, $P < 0.0005$; Table S2A); larger males and males at the lower temperature had lower ratios. Temperature and SVL had no effect on liver to gonad ratios in females though (temperature: $F_{1,46} = 0.091$, $P = 0.764$; SVL: $F_{1,46} = 0.023$, $P = 0.881$; Table S2B). However, LPS dose and its interaction with SVL had marginally non-significant effects on female liver to gonad ratios (LPS dose: $F_{1,46} = 3.341$, $P = 0.075$; LPS dose x SVL: $F_{1,46} = 3.168$, $P = 0.083$; Table S2B); female liver to gonad ratios tended to increase with LPS dose (Fig. 2a), and larger females had smaller liver to gonad ratios at high LPS doses. LPS dose did not predict liver to gonad ratios in males ($F_{1,46} = 0.076$, $P = 0.785$; Fig. 2b).

**Discussion**

We hypothesized that increasing exposure (i.e. *Salmonella* LPS dose) would incite greater and greater costs of innate immunity in *A. sagrei*, and we expected that such costs would be larger in males than females, but were unsure whether such cost functions would be linear or more complex. We found that innate immune cost functions, when detectable, were linear with fairly shallow slopes and that cost functions differed between the sexes such that males allocated more $^{13}$C-leucine to the liver at high LPS doses (Fig. 1a) but did not shift $^{13}$C-leucine from gonads to livers (Fig. 2). Females, by contrast, tended to bias resources to their livers and away from gonads with increasing LPS dose.

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**Fig. 1.** Male and female *Anolis sagrei* differ in $^{13}$C-leucine allocation to the liver in response to *Salmonella* LPS. Males increase allocation with dose ($\beta = 0.186 (\pm 0.077$ SE)) (a) while females do not (b). Males and females do not differ in $^{13}$C-leucine allocation to the gonads in response to LPS. However, males (c) allocate more than females (d) irrespective of LPS dose. Error bars represent SEM. Data points offset to avoid overlap.
Exposure-dependent immune costs in an ectotherm

Our results indicate that the costs of an innate immune response in A. sagrei increase linearly with LPS exposure. However, the slope of the cost functions was either shallow (e.g. increase in allocation to the liver in males) or marginally non-significant (e.g. liver to gonad allocation ratios in females), suggesting that the costs of innate immunity are modest and perhaps inconsequential in the host–parasite interaction. We hesitate in claiming that our results are reflective of the responses of this host to all infections. First, A. sagrei is an invasive species at our capture location (Florida, USA), and invasive species tend to have altered immune responses in their introduced ranges (Lee, Martin & Wikelski 2005), perhaps to preserve resources for processes that favour successful establishment and expansion, including reproduction (Lee & Klasing 2004). If A. sagrei damp immune responses to preserve reproductive effort, shallow cost functions are not surprising. Further, as the study was conducted when animals were actively breeding, cost functions may be shallower than at many other times of year. The finding that larger individuals allocated more to their gonads supports this possibility; large individuals may be more successful at acquiring mates but are penalized in terms of their ability to afford an immune response.

Another possible interpretation of shallow cost functions is that the innate immune response itself was weak. Previous research has shown altered/reduced immune responses to bacteria in invasive species in their new ranges (Lee, Martin & Wikelski 2005; Martin et al. 2010). These results might occur because introduced hosts lose the native parasites that necessitate strong responses, because conditions for some parasites in the new range are unfavourable (Enemy Release; Torchin et al. 2003), or because introduced hosts are selected or induced plastically to damp some immune responses and thus avoid damage caused by chronic inflammation that can result from novel parasite exposures (Lee & Klasing 2004). The likely result of such changes is decreased costs of immune activation in these individuals, which may be advantageous because resources that would have gone towards immune responses can instead be allocated to traits that facilitate successful establishment and spread, such as reproduction. As we did not measure immune parameters in these individuals, we cannot address this possibility directly.

A possible explanation of modest immune costs in females lies in our use of the complete reproductive tract of each female, including all eggs present, in isotope analysis. As females of this species lay an egg every 7 days, multiple eggs in varying stages of development were present in each female’s reproductive tract. Even though 13C-leucine uptake in the gonads was corrected for dry mass of the tissue, it is possible that eggs in early development were able to take up 13C-leucine, but more fully developed (shelled) eggs were metabolically independent from the adult female. Such an occurrence could obscure increased allocation or account for some of the variation seen in allocation to the gonads in females because of the differential ability of eggs to take up the tracer. Additionally, the presence of eggs in varying stages of development in females may have contributed to the variation in leucine incorporation to the liver as the liver is also involved in the production of yolk precursor protein (vitellogenin). This variation may be


Fig. 2. LPS dose did not affect liver to gonad ratios in Anolis sagrei males (a). However, liver to gonad ratios tended to increase with LPS dose in females [β = 28.354 (± 10.434 SE)] (B). Error bars represent SEM. Data points offset to avoid overlap.

doses (Fig. 2), although this outcome was marginally non-significant. 13C-leucine allocation to the gonads was not affected by LPS dose in either sex; however, males always allocated more 13C-leucine to their gonads than females (Fig. 1b). Interestingly, temperature had little impact on costs of immunity in either sex. We did find that 13C-leucine allocation to the liver (but not the gonads) was higher at 30°C than at 20°C, but independent of Salmonella LPS dose. This response may be reflective of the ability of A. sagrei to adapt to the climates in their introduced ranges such that even temperatures outside their thermal preference (e.g. 20°C) (Lister 1976) do not alter costs of immune activation. Below, we discuss our results and specifically how they might influence our understanding of community disease dynamics as well as the evolution of host resistance and tolerance.

QUANTIFYING COSTS OF IMMUNE ACTIVATION

Our results indicate that the costs of an innate immune response in A. sagrei increase linearly with LPS exposure. However, the slope of the cost functions was either shallow (e.g. increase in allocation to the liver in males) or marginally non-significant (e.g. liver to gonad allocation ratios in
obscuring increased allocation to the liver in response to immune activation by Salmonella LPS, making it appear as although females are only experiencing modest costs.

A final possibility for the low costs of immunity in A. sagrei involves their co-evolutionary history with Salmonella bacteria. As Salmonella is a naturally occurring parasite of this species (Hoff & White 1977), A. sagrei may have evolved other mechanisms to mitigate costs of immunity; indeed, if exposure is quite common and the history of interaction between species is long, substantial immune costs might only manifest at very high exposures. If so, we would expect to see greater costs at higher concentrations of Salmonella LPS than we gave. However, we chose our doses based on a concentration known to cause altered behaviour in Anolis lizards (Merchant et al. 2008); without dose–response studies such as ours, it is not possible to know at what level of infection an organism will respond.

COSTS OF IMMUNE ACTIVATION, RETURN ON INVESTMENT AND THE EVOLUTION OF VIRULENCE

Our major motivation in this study was to quantify the shape of the immune cost function of A. sagrei to Salmonella sp. and thus to begin to understand how the mechanisms underlying host–parasite interactions might affect host-population ecological and evolutionary dynamics (Adelman 2014). We hypothesized above that if costs of immune activation increase with magnitude of exposure to an immunogenic parasite component, a threshold might exist where the costs of sterilizing immunity might outweigh the costs of enduring a persistent, low-level infection. As exposure to some common, generalist parasites, such as Salmonella, is very likely, in some contexts, selection might favour parasite tolerance over resistance, especially if tolerance incurs a lower cost to the host. To our knowledge, the costs of tolerance have yet to be measured in any species.

Here, we found that costs of Salmonella LPS exposure increase linearly for both sexes, for males in the liver (Fig. 1a) and for females in the liver at the expense of the gonads (Fig. 2). The linear cost function suggests that when exposed to Salmonella bacteria, A. sagrei might tolerate high levels of infection as the cost of resisting infection is likely to become unmanageable. At the population level, tolerant hosts might increase exposure risk of Salmonella bacteria to other individuals, maintaining high prevalence in this species (Hoff & White 1977; May & Anderson 1983) and further selecting for reduced immune costs at the individual level (Schmid-Hempel 2011) (in the form of either tolerance or mechanisms mitigating innate immune costs, which might not be mutually exclusive). These conditions in the focal, invasive species may consequently increase exposure risk to native reptile populations, including Anolis carolinensis (green anoles).

One critical assumption to the above arguments is that the cost of an immune response translates directly into increased effectiveness of parasite control. If we are to employ cost functions as a way to utilize host physiology to predict or understand natural disease cycles, we will also need to evaluate whether individuals obtain good returns on their immune investment. In our study, it is possible that the females had unmeasurable immune costs because they mount very effective immune responses without experiencing a large cost. It is also possible that large individuals are able to mount more efficient immune responses (i.e. responses that require fewer resources) and are thus able to mount these responses without sacrificing resources for reproduction. Such strategies would enable individuals to maintain both health and reproductive success (Schmid-Hempel 2011). One way to address this problem is to include infection with live parasites as a treatment in future studies. Such studies would make it possible to determine whether greater costs of immune activation result in increased protection from parasites and therefore an increased ability to resist an infection. In turn, such studies would help us to determine which individuals in a population (e.g. males or females) are most likely to spread infection and thus better predict how certain species, populations or members of populations may be affecting parasite prevalence and transmission.

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Data accessibility

All data can be accessed via Dryad (http://datadryad.org/); doi: 10.5061/dryad.8n74q (Brace, Sheikali & Martin 2014).

References


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Supporting Information

Additional Supporting information may be found in the online version of this article:

Fig. S1. (a) Temperature increased 13C-leucine allocation to the liver independently of sex, whereas (b) 13C-leucine allocation to the gonads was much greater in males than females independent of temperature.
Table S1. (A) GLM analysis of 13C-leucine allocation to the liver. (B) GLM analysis of 13C-leucine allocation to the gonads.
Table S2. (A) GLM analysis of 13C-leucine liver to gonad ratios for males. (B) GLM analysis of 13C-leucine liver to gonad ratios for females.
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