RESEARCH ARTICLE

The Metabolic Cost of Mounting an Immune Response in Male Brown Anoles (*Anolis sagrei*)

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ABSTRACT

The tradeoff between reproduction and survival is central to life-history theory and is thought to reflect underlying energetic tradeoffs between reproduction and self-maintenance. Immune responses to parasites and pathogens are important components of self-maintenance in many species, but whether these defenses impose significant energetic costs has only been tested in a handful of organisms. We tested for a metabolic cost of mounting an immune response in the male brown anole (*Anolis sagrei*), a lizard in which we have previously shown that reproduction causes a marked reduction in immune response to the novel antigen phytohaemagglutinin (PHA). We treated captive male anoles with a subcutaneous injection of either PHA, which induces an immune response that manifests as localized swelling, or saline vehicle as a control. Prior to injection and at 24, 48, and 72 hr post-injection, we measured swelling at the site of injection and whole-animal resting metabolic rate (RMR) using stop-flow respirometry. Although we detected a robust swelling response to PHA at 24, 48, and 72 hr post-injection, mean RMR did not differ between treatments at any of these time points. However, within the PHA treatment group, RMR increased with the extent of swelling, suggesting a variable metabolic cost that scales with the magnitude of the induced immune response. Although individual anoles varied considerably in the extent to which they responded to PHA challenge, our results suggest that an immune response can impose a substantial metabolic cost (potentially as much as 63% above baseline RMR) for individuals that do respond maximally. *J. Exp. Zool. 323A:689–695, 2015.* © 2015 Wiley Periodicals, Inc.

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The adaptive benefits of maintaining an effective immune system are clear, but mounting an immune response can also impose costs. For example, an extreme immune response to an antigen can be just as damaging as the initial infection (i.e., autoimmune disease; Raberg et al., '98; Schmid-Hempel, 2003). Moreover, if immune defenses are energetically costly, then an unnecessarily large immune response could usurp resources from other important functions (Lochmiller and Deerenberg, 2000; Schmid-Hempel, 2003). The metabolic costs associated with immune function are likely to vary within and among individuals due to the type and magnitude of immune response, and among species due to differences in metabolism, in the types of parasites and pathogens encountered, and in the immune system itself. However, the metabolic costs associated with any aspect of immune defense have only been investigated in a handful of species (Svensson et al., '98; Martin et al., 2003; Eraud et al., 2005; Downs et al., 2013). Moreover, several of these studies suggest that measures of immune function commonly employed by eco-immunologists have little or no associated metabolic costs

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(Boughton et al., 2007; Meylan et al., 2013; Merlo et al., 2014). Consequently, the integration of immune function within energetic models for reproductive investment and life-history evolution remains partial.

One of the most commonly employed assays for assessing immune function in wild vertebrates is the phytohaemaglutanin (PHA) assay (e.g., Tella et al., 2002; Amo et al., 2007; Cox et al., 2009; Vinkler et al., 2012). This assay method uses subcutaneous injection of a carbohydrate-binding lectin protein (PHA) derived from red kidney beans (*Phasolus vulgaris*) to challenge the vertebrate immune system with a novel antigen (Vinkler et al., 2010). The response to PHA is typically assessed by measuring the extent of localized swelling at the site of injection. This localized swelling is the result of an inflammatory response caused by invasion of the surrounding tissue by various components of the innate and acquired immune system, including lymphocytes, heterophils, thrombocytes, and macrophages (Martin et al., 2006; Vinkler et al., 2010). The interpretation of this swelling response is complex, and some have cautioned against the typical assumption that a larger swelling response is indicative of higher immunocomptence per se (Kennedy and Nager, 2012). Nonetheless, the ease with which this assay can be conducted on wild vertebrates has led to its widespread use in a variety of taxa, including mammals, birds, and lizards (Tella et al., 2002; Amo et al., 2007; Cox et al., 2009; Vinkler et al., 2012). Though some studies have documented a respiratory cost associated with immune response to PHA (Martin et al., 2003), others have found no change in metabolic rate following PHA challenge (Boughton et al., 2007; Merlo et al., 2014).

We measured the energetic cost of mounting an inflammatory response to PHA in the brown anole (*Anolis sagrei*), a small Caribbean lizard that has emerged as a model species in evolutionary ecology. Previous research on brown anoles has used the PHA assay to test for physiological costs of reproduction (Cox and Calsbeek, 2010), physiological divergence among morphs (Calsbeek et al., 2008; Cox and Calsbeek, 2011), immunosuppression by androgens (Cox et al., 2009), and sex differences in the fitness costs of mounting an immune response (Calsbeek and Bonneaud, 2008). However, the magnitude of the metabolic cost associated with mounting an immune response to PHA is not known for this species, which complicates interpretations about the functional and adaptive significance of many of these results. In the present study, we challenged adult male brown anoles with PHA and compared them to a saline-injected control group to (i) measure the time course of the swelling response to PHA over 72 hr, (ii) test for treatment differences in resting metabolic rate (RMR) over this same time course, and (iii) characterize the relationship between the magnitude of the swelling response and RMR to assess the metabolic cost of mounting an immune response.

METHODS

Study Animals

We collected adult male brown anoles (*Anolis sagrei*) from the island of Great Exuma (23°29′N, 75°45′W) in The Bahamas in January 2012. We chose to focus on males because their larger body size (three times more massive than females) results in more repeatable measurements of metabolism and immune response. Additionally, reproductive cycling in female anoles could confound both measurement and interpretation of metabolism and immune response. We housed these animals individually in plastic cages ($29 \times 19 \times 18$ cm, Lee's Kritter Keeper, Lee's Aquarium and Pet Products, San Marcos, CA) maintained at 29°C and 65% relative humidity. We fed all animals three times per week with crickets (*Gryllus assimilis*) supplemented with Fluker's Calcium with Vitamin D_3® and Fluker's Reptile Vitamin with Beta Carotene® (Fluker's, Port Allen, LA). We sprayed the sides of each cage twice daily with deionized water. Each cage included a perch (PVC pipe), a hammock (fiberglass screen), carpet substrate, and a potted plant. Each cage was illuminated by two ReptiSun 10.0 UVB bulbs (ZooMed, San Luis Obispo, CA) set on a 12L:12D photoperiod.

Immune Challenge

We took initial measurements of foot thickness for each lizard at 24 hr and 6 hr prior to treatment. We measured foot thickness to the nearest 0.1 mm between the first and fifth phalange on the left hind foot using a dial caliper. Individuals in the control group then received an injection of 0.01 mL phosphate-buffered saline (PBS) at this same location in the left hind foot, whereas the immune-challenged group received 0.1 mg lyophilized PHA (PHA-P, L8754; Sigma–Aldrich, Inc., St. Louis, MO) dissolved in 0.01 mL PBS. We then measured post-treatment foot thickness at 24, 48, and 72 hr following injection. At each time point, we calculated foot thickness for each individual as the mean of three successive measurements to minimize measurement error.

Resting Metabolic Rate

We measured resting metabolic rate (RMR) at 24 and 6 hr prior to injection, and again at 24, 48, and 72 hr following injection using stop-flow respirometry (Lighton, 2008) with a Field Metabolic System (FMS, Sable Systems, Las Vegas, NV). We fasted lizards for 2 days prior to measurement and throughout the time course of the experiment. We weighed each animal to the nearest 0.01 g and placed it in one of eight 300-mL Plexiglass respirometry chambers (G115, Qubit Systems, Kingston, ON, Canada) housed in a temperature cabinet set at 30 ± 0.2°C (PTC-1 cabinet and PELT-5 temperature controller, Sable Systems). We routed air through a Drierite column and then through each chamber using an 8-channel multiplexer calibrated for stop-flow operation (RM-8, Sable Systems). We

designed our stop-flow sampling so that chambers remained closed for 50 min, during which time lizards depleted O_2 and produced CO_2. We then pushed air through the chamber at 1,000 mL/min using a Mass Flow System sensor and pump (MFS, Sable Systems) into a manifold attached to the Field Metabolic System, which pulled air at 200 mL/min and sampled water vapor pressure, CO_2 concentration, and O_2 concentration. We sampled each chamber every hour for 7 hr, yielding seven estimates of O_2 consumption per animal, per day. We automated sampling and data collection using Expedata software (v. 1.6.4 Sable Systems), and calculated O_2 consumption (VO_2) after correcting for water vapor pressure, CO_2 concentration, and the amount of time that the chamber was closed (Lighton, 2008). Prior to the experiment, we calibrated the O_2 sensor using the fixed-span mode with ambient air flowed through a Drierite column (Lighton, 2008). We calibrated the CO_2 sensor with pure nitrogen (zero oxygen) and custom span gas (0.05% CO_2, 99.5% nitrogen, product number NI CD5000C-Q from GTS-WELCO, Morrisville, PA). We calibrated the water-vapor sensor using zero-humidity nitrogen and water-saturated ambient air (Lighton, 2008). We collected all RMR measurements during the same time of the day (1,000–1,700 hr) and considered the lowest of the seven sequential measures of O_2 consumption per day as the best estimate of RMR for that individual on that day.

Statistical Analyses

We tested for an effect of PHA treatment on immune response by analyzing foot thickness using a repeated-measures design with PHA treatment (among-subjects effect), time (within-subjects effect), body mass (covariate), and two-way interactions between time and both PHA treatment and body mass. We tested for an effect of PHA treatment on metabolism using an analogous repeated-measures design with RMR as the response variable. We $\log_{10}$ transformed body mass, foot thickness and RMR prior to analysis. Because individuals in the PHA treatment varied considerably in the magnitude of their swelling response, we also assessed the energetic cost of immune response by regressing RMR on proportional foot swelling (calculated by dividing the difference between pre- and post-treatment foot thickness by pre-treatment foot thickness) at each post-treatment time point. We included body mass as a covariate in these analyses. We conducted these analyses separately for each treatment group and predicted that we would only observe significant positive correlations in the PHA group. All statistical analyses were conducted in JMP v. 9.0.1 (SAS Institute).

RESULTS

PHA challenge stimulated a significant swelling response relative to the control group (Fig. 1A and Table 1). This immune response peaked at 24 hr post-treatment (40% elevation in foot thickness relative to pre-treatment levels), remained high at 48 hr (38% elevation), and began declining by 72 hr (23% elevation; Fig. 1A).

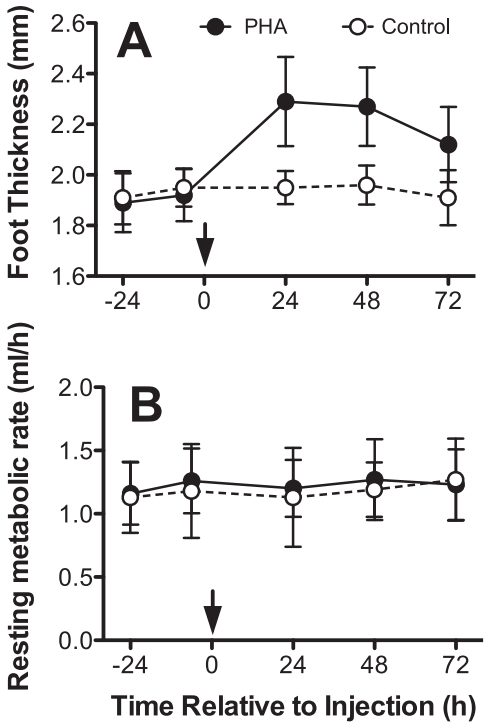

Figure 1. Changes in (**A**) foot thickness (immune response), and (**B**) resting metabolic rate over time for PHA-treated and control male brown anoles. Arrows indicate time of injection for PHA or saline vehicle as a control. Data are least-squares means ± SE from repeated-measures ANCOVA models with body mass as a covariate.

By contrast, resting metabolic rate (RMR) did not differ as a function of PHA treatment, time, or the time-by-treatment interaction (Fig. 1B and Table 1). Although mean RMR did not differ between treatment groups at any time point, variance in RMR was high within both groups, and RMR correlated with the magnitude of the swelling response across individuals within the PHA treatment at both 48 and 72 hr post-treatment (Table 2). By contrast and as predicted, RMR was unrelated to the magnitude of swelling, which was minimal, in the control group (Fig. 2A and Table 2). When averaging RMR and proportional swelling for each individual across the three post-treatment measurements (Table 2), variance in the magnitude of swelling explained about a quarter of the variance in RMR within the PHA-treated group (Fig. 2B). The slope of this regression corresponds to an increase of approximately 63% in RMR from those individuals that did not mount any discernible swelling response to PHA (predicted RMR = 0.95 mL O_2 h^{-1}) to those that responded maximally to PHA challenge (40% increase in foot thickness; predicted RMR = 1.55 mL O_2 h^{-1}).

Table 1. Summary of the effects of PHA treatment on foot thickness or RMR as the response variables in two separate, repeated-measures analyses including treatment and body mass as among-subjects effects and time as a within-subjects effect.

		Response variable			
		Foot thickness		RMR	
Effect	df (error)	*F*	*P*	*F*	*P*
Treatment	24	**21.71**	**<0.0001**	0.02	0.88
Time	27	**2.59**	**0.0467**	0.77	0.55
Mass	26	**27.75**	**<0.0001**	0.27	0.61
Time × treatment	27	**19.76**	**<0.0001**	0.39	0.81
Time × mass	27	2.17	0.0846	0.73	0.57

Body mass, foot thickness, and RMR were $\log_{10}$ transformed prior to analysis. Bold values indicate significance at $P < 0.05$.

DISCUSSION

Male brown anoles in our study had resting metabolic rates (RMR) that were positively correlated with the extent of their localized swelling response to PHA, suggesting that metabolic expenditure increases with the magnitude of immune response. This interpretation is consistent with previous findings that testosterone alters the relationship between growth and swelling response to PHA (Cox et al., 2009). This is also broadly consistent with other empirical studies that have detected a metabolic cost of mounting an immune response (Svensson et al., '98; Martin et al., 2003; Eraud et al., 2005; Downs et al., 2013). However, despite a robust swelling response in the PHA-injected group (Fig. 1A), we found no difference in mean RMR between control and PHA-injected groups (Fig. 1B). Below, we discuss these results with particular emphasis on the importance of considering individual variation in immune function and its associated metabolic costs.

There are several possible sources of variation in our measurement of RMR and the swelling response to PHA. First, it is difficult to consistently administer precise doses of PHA to the small footpads of brown anoles, such that experimental variation in actual PHA dose could contribute to some of the wide range of swelling responses (0–40% increase in foot thickness) that we observed within the PHA-injected group. This would be consistent with observed dose-dependent responses to other immune challenges (e.g., lipopolysaccharide derived from *Salmonella*) in brown anoles (Brace et al., 2015). Variance in swelling response to PHA presumably also reflects inherent differences in immunocompetence among individuals, which is the conventional interpretation of this assay (Kennedy and Nager, 2012). Irrespective of the causes of this variance in immune response to PHA, we found that metabolic rate increased sharply and significantly with the magnitude of the swelling response

Table 2. Summary of relationships between RMR and foot swelling within each treatment at each of four time points relative to injection, and for the mean of each measure across three post-injection time points (24–72 hr).

			Foot swelling		Body mass	
Time point (hr post-injection)	Treatment	df (error)	*F*	*P*	*F*	*P*
−6 (pre-injection)	Control	24	0.08	0.777	3.56	0.071
	PHA	27	2.08	0.161	3.40	0.076
24	Control	26	0.04	0.847	0.50	0.488
	PHA	27	0.58	0.454	0.49	0.492
48	Control	27	1.45	0.240	0.03	0.864
	PHA	27	**9.42**	**0.005**	0.11	0.738
72	Control	27	1.31	0.236	**5.21**	**0.031**
	PHA	27	**8.36**	**0.008**	0.00	0.998
24–72 (mean)	Control	27	0.05	0.817	0.194	0.663
	PHA	27	**9.17**	**0.005**	0.502	0.485

Statistical results are from separate general linear models for each treatment and time point, with RMR as a response variable and foot swelling and body mass as independent variables. Body mass, foot swelling, and RMR were $\log_{10}$ transformed prior to analysis. Bold values indicate significance at $P < 0.05$.

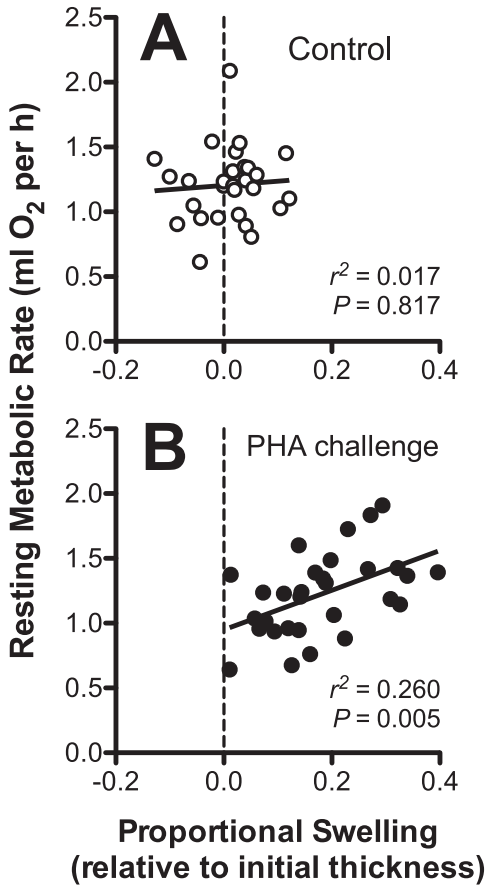

Figure 2. Correlations between post-treatment averages for resting metabolic rate and proportional foot swelling (change in foot thickness divided by pre-injection foot thickness) of (**A**) control and (**B**) PHA-treated male brown anoles. Statistics are from models with log-transformed variables and a body mass covariate.

across individuals. Those individuals that responded maximally to PHA challenge (40% increase in foot thickness) had metabolic rates approximately 63% greater than those individuals who did not mount any discernable response (Fig. 2B), which suggests a fairly substantial metabolic cost associated with localized swelling in response to PHA.

Nonetheless, RMR varied substantially among individuals, comparable to other studies of small ectothermic vertebrates (Sears, 2005; Secor et al., 2007; Streicher et al., 2012). This combination of inherent variance in RMR and in the magnitude of the immune response to PHA may have prevented us from detecting mean differences in RMR between treatment groups in the present study. Most previous research on the metabolic cost of an immune response has focused on endothermic birds and mammals, which have mass-specific metabolic rates that are up to two orders of magnitudes greater than those of the ectothermic lizards in our study (Pough, '80; Nagy et al., '99). In both birds and mammals, multiple species show evidence of an increase in metabolic rate associated with mounting an immune response (Ots et al., 2001; Derting and Compton, 2003; Martin et al., 2003; Downs et al., 2013). However, not all birds and mammals exhibit detectable increases in metabolic rate in response to an immune challenge (Boughton et al., 2007; Merlo et al., 2014), similar to results from a previous study in another lizard, *Zootoca vivipara* (Meylan et al., 2013). Likewise, when we compared mean metabolic rates between control and PHA treatment groups, we did not detect a metabolic cost associated with mounting an immune response to PHA. However, our analysis of individual variation in swelling response and metabolic rate suggests not only that there is a metabolic increase associated with an immune response, but also that this response can be fairly costly for individuals that mount a maximal immune response, at least for males. An explicit consideration of individual variation in the magnitude of the observed response to immune challenge may, therefore, be essential to detecting a metabolic response to an immune challenge.

Although our research has focused on the metabolic cost of mounting an immune response, there are multiple factors that can constrain an immune response and structure the nature of tradeoffs between the expression of immunity and reproductive investment (or other life history traits; Adelman, 2015). While our work focused on metabolism, the costs of an immune response can also be incurred in other currencies, such as biologically important molecules (Klasing and Barnes, '88; Klasing, 2004), behavior (Hart, '88), and damage to host tissues (Raberg et al., '98; Graham et al., 2005). Beyond metabolic and other tradeoffs, the ultimate cost of an immune response can also be structured by physiological regulatory networks, which can constrain the allocation of resources to an immune response within any given tissue (Cohen et al., 2012; Martin and Cohen, 2015). Our work suggests that an energetic cost associated with an immune response can play a role in mediating the tradeoff between reproductive investment and immune function in male brown anoles, and understanding both metabolic and other costs of and constraints upon immune function can give insight into how investment in physiological systems is dynamically allocated to maximize fitness in nature.

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