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ORIGINAL PAPER

Metabolic responses to different immune challenges and varying resource availability in the side-blotched lizard (*Uta stansburiana*)

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Abstract The energetic cost of immunity depends on many factors, including the type of challenge, the timing of the response, and the state of the animal. We measured changes in the standard metabolic rates of side-blotched lizards (*Uta stansburiana* Baird and Girard, 1852) in response to different immune challenges and nutritional states. In the first experiment, lizards were randomly assigned to one of four treatments: lipopolysaccharide (LPS) injection (to stimulate the response to a pathogen), cutaneous biopsy (as a proxy to a superficial wound), both injection and biopsy, or neither (control). Four and five days later, we measured the standard metabolic rates of the lizards. In response to healing a cutaneous wound, lizards reduced metabolic rate and lost body mass. Healing rate was also inversely related to weight loss, but LPS had no effect on body mass or metabolic rate. In the second experiment, a new set of lizards were randomly assigned to a high-food or low-food diet and administered a cutaneous biopsy. As in the first experiment, we observed a reduction in metabolic rate after wounding; moreover, this decrease was positively correlated with the rate of healing. We observed higher rates of metabolism in lizards that ate more food, but food consumption was unrelated to the decrease in metabolic rate following the biopsy. These experiments demonstrate the dynamic nature of the immune response in response to immune challenge and the state of the organism.

Keywords Immune · Reptile · Metabolic rate · Cutaneous biopsy · Energetics · Stress

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Introduction

The immune system is a key adaptation of living things (Medzhitov et al. 2000), and is essential to the survival of individuals and persistence of species. The immune system can be highly complex, communicating within itself and with other physiological systems to respond adaptively to environmental challenges (Zimmerman et al. 2010). Investing energy in these responses can protect an animal from infection and improve chances of survival, but immune responses can be energetically costly (Barr et al. 1922; Demas et al. 1997; Råberg et al. 2000) and compete with other physiological processes for limited resources, which could potentially reduce fitness (Ahmed et al. 2002; French et al. 2007a; Jacot et al. 2004; Lochmiller and Deerenberg 2000; Mills et al. 2010; Sanz et al. 2004). Conversely, other physiological processes can divert energy from the immune system (Ahtiainen et al. 2005; French et al. 2007b; Svensson et al. 1998). In addition, the immune system can divert energy from one immune response to another (Neuman-Lee and French 2014). If biologists are to effectively evaluate immunity, we must understand how organisms respond to different immune challenges.

Resource fluctuations are a normal aspect of environmental conditions that animals have faced throughout evolutionary time (Andrén and Nilson 1983; Oro et al. 2013; White 1978), and should be considered when studying immunology in an ecological context. Classic

immunological studies have held organisms under tightly-controlled laboratory conditions, and although these studies are certainly useful and necessary, this reductionist approach may yield results that fall short in understanding natural responses and thus ecological relevance (Viney et al. 2005). Because trade-offs manifest according to resource availability (Reznick et al. 2000), researchers must consider the energetic state of an animal when evaluating the impacts of immune activation. For some animals, fighting an infection can be so energetically demanding that death occurs from starvation (Wobeser 2006). Alternatively, if an animal has a surplus of energy, trade-offs might not manifest immediately or at all (French et al. 2007b; Neuman-Lee et al. 2015; Norris and Evans 2000). Ecoimmunology focuses on immune responses under conditions more likely to be found in natural environments, thus integrating ecology and evolution (Demas et al. 2012).

The extent to which animals invest energy in immunity depends on not only the amount of energy available, but also the challenge being faced (Demas et al. 2011), as immunological prioritization likely varies across species and individuals (Ardia et al. 2011; Neuman-Lee and French 2014). For example, inflammatory agents such as lipopolysaccharide (LPS) have been used for decades in immunological research to simulate an (Nowell 1960), while other types of challenges, such as cutaneous wounds, reflect injuries that occur often in natural environments and require an integrative immune response that requires healing (Demas et al. 2011). Researchers have yet to compare how animals respond energetically to these different challenges and whether prioritization occurs when different challenges occur simultaneously. Furthermore, energy is often limiting in nature, and thus, animals may shift their investment of energy given their current energetic state, but it is unclear how this may influence their response to specific immune challenges.

To address these gaps in knowledge, we experimentally investigated the energetic consequences of immune challenges in side-blotched lizards (*Uta stansburiana* Baird and Girard, 1852). In Experiment 1, we studied the metabolic costs of mounting immune responses when animals were given an injection of LPS, a cutaneous biopsy, or both challenges. We hypothesized that LPS injections and cutaneous biopsies would increase the standard metabolic rates and that the combination of these treatments would additively affect metabolism. Considering that the glucocorticoid corticosterone is likely related to the immune response (Klasing et al. 1987; Padgett and Glaser 2003), we also expected a positive relationship with circulating corticosterone and metabolic rate. In the second experiment, we studied the effect of energetic state on an immune response. Food restriction is known to reduce immunocompetence (Alonso-Alvarez and Tella 2001; Fargallo et al. 2002), elicit life-history trade-offs (French et al. 2007a), and alter metabolic rates (Liang et al. 2015); therefore, we hypothesized that limited energy would alter how animals respond metabolically when healing wounds. We hypothesized that animals on low-food intake diets would heal slower and have slower metabolisms compared to animals on high intake diets. Taken together, the aim of these experiments was to understand how different immune responses and energetic states affect the energetics and efficacy of immunity.

Materials and methods

Experiment 1

Animals and overview

We measured the metabolic response to different immune challenges in adult, male side-blotched lizards, a widespread and common reptile in arid regions of the western United States (Stebbins 2003). Forty individuals were captured via noosing from Maricopa County, Arizona, USA in March 2014 under Arizona Game and Fish Department permit #SP667730. These lizards averaged 52.4 ± 0.3 mm in snout-vent length and 5.15 ± 0.09 g in mass (mean ± SEM). The lizards were collected in a haphazard manner, of similar body size and dewlap coloration and randomly assigned to treatment groups. The lizards were transported back to Arizona State University, where they were individually housed in 40 × 15 × 13 cm plastic terraria with paper substrate. The terraria were held inside environmental chambers which were programmed with a 12L:12D photoperiod at 36 °C for the light period and 20 °C for the dark. Lizards were fed crickets (Fluker Farms, Port Allen, Louisiana, USA) in excess daily, except for the 24 h prior to metabolic measurements when animals were fasted. The internal walls of the terraria were sprayed with water daily to raise humidity and provide droplets for lizards to drink. After 5 days of acclimation, each lizard was randomly assigned to one of four treatments in a 2 × 2 factorial design: LPS injection, cutaneous wound, both, or neither (control). The animals were of similar mass across treatments ($P = 0.38$) at the beginning of the study. At the termination of the study (Day 6), we collected blood from each animal's retro-orbital sinus within 3 min of handling. We centrifuged blood samples at 2200 RPM for 10 min to isolate plasma, which was then separated, frozen, and stored at −80 °C until assays were performed. A timeline of procedures can be found in Table 1. All procedures were approved by the Utah State University (USU) and Arizona State University (ASU) Institutional Animal Care and Use Committees (IACUC, USU protocol #2068, ASU protocol #14-1354NR).

Table 1 Timeline of procedures for Experiments 1 and 2

	Experiment 1	Experiment 2
Before day 0[a]		Feeding treatments
Day 0		Metabolic trial
Day 1	Biopsy/wound image	Biopsy/wound image
Day 2		Metabolic trial
Day 3		
Day 4	LPS injection/metabolic trial	Metabolic trial/wound image
Day 5	Metabolic trial	
Day 6	Blood sample/wound image	Blood sample/wound image

[a]Feeding treatments began 4 days before the initial metabolic trial for Experiment 2

Lipopolysaccharide Injection

On Day 4 of Experiment 1, each lizard received an injection of saline or LPS (2.5 µg g body mass^{-1}; L3129 Sigma-Aldrich, Saint Louis, Missouri, USA) from *Escherichia coli* diluted in phosphate-buffered saline (2.5 µg LPS/20 µl). The control was an identical mass-adjusted volume of saline. The dose of LPS injected was chosen based off previous research on lizards of similar size (López et al. 2009; Uller et al. 2006) and other reptiles (Deen and Hutchison 2001; do Amaral et al. 2002). Injections were administered at the same time of day when the animals were removed from their first metabolic trial (described in the following). Lizards were then returned to their individual enclosures and given 12 h before having their metabolic rates re-measured to assess the metabolic consequences of the LPS injection.

Cutaneous biopsy and measurements

We simulated wounding of lizards by surgically removing a small patch of skin on Day 1. Once lizards were anesthetized using isoflurane gas, a biopsy tool (Miltex Instrument Company, York, Pennsylvania, USA) was gently twisted against the dorsal surface of skin, anterior to the tail, to create a 3.5 mm circular wound. The piece of skin was then removed with forceps. Immediately after, the wound was photographed next to a ruler for scale. The wounds were photographed again at the end of the study, on Day 6. The images were used to calculate the wound area in ImageJ software program (NIH Imaging). When analyzing an image, the investigator was blind whether the lizard had received an injection of LPS. Lizards that did not receive a cutaneous wound (control animals) were handled and anesthetized as described above, except the plastic blunt end of the biopsy punch was pressed against the dorsal surface to simulate the handling required for a cutaneous biopsy. We placed each lizard back in its terrarium on top of a heating pad and waited until it was alert before returning the terrarium to an environmental chamber.

Metabolic measurements

We measured metabolic rates of lizards on Days 4 and 5 through closed-system respirometry. Days 4 and 5 were chosen as they represent the height of immune investment and a stress-sensitive period during the wound healing process (French et al. 2006; Neuman-Lee and French 2014). This timeframe also allowed us to examine the initial metabolic response to the immunological processes involved in the wounding healing (i.e., pre-LPS challenge) and not the metabolic response to the surgery procedure itself. The second metabolic measurement coincided with both the immunological response to LPS and peak immunological response to wound healing, thus allowing us to test the interactive effects of both immune challenges simultaneously. Lizards were fasted for 24 h preceding the metabolic trials to limit the metabolic response to digestion. To promote a quiescent state during measurements, lights in the environmental chambers were turned off an hour before the animals were moved to the test chambers. Each animal was quickly removed from its housing and placed in a glass chamber (473 ml), inside an incubator set at 36 °C. This temperature lies within the optimal range for side-blotched lizards (Waldschmidt and Tracy 1983). The inside of the incubator was dark, and the lizards were given an additional 2 h before measurements. Once lizards were inside glass chambers, the chambers were slowly filled with dry, CO_2-free air. Then, an automated sampling regime commenced, following the protocol described by Kolbe et al. (2014). Briefly, air from the chambers was pushed by a mass-flow regulator through an analyzer for carbon dioxide (LI-6252, LI-COR, Lincoln, Nebraska, USA) after passing through magnesium perchlorate to remove water. Then, air flowed through a column of Drierite® and Ascarite® before entering an analyzer for oxygen (Oxzilla, Sable Systems, Las Vegas, Nevada, USA). Because metabolic rate does not scale linearly with body mass, but more closely to the ¾ power of body mass (Kleiber 1947), milliliters of oxygen hr^{-1} were mass-adjusted for each lizard using the body mass$^{0.75}$, hereafter referred to as mass-adjusted metabolic rate. This is the dependent variable used to determine changes in metabolic rate.

Hormone and immune assays

To account for potential hormonal mediation of the lizards' responses to the immune challenges, we used radioimmunoassay to determine concentrations of circulating corticosterone, using a protocol modified from Moore (1986).

Corticosterone was extracted from the plasma using a solution of 30% ethyl acetate:isooctane and assayed in duplicate for (MP Biomedicals, Lot #3R3PB-19E). Hormone concentrations were adjusted using individual recoveries to account for any hormone lost during extraction. Inter-assay coefficient of variation was 8.5%.

We performed bactericidal assays, because these tests allow us to measure an organism's overall capacity to fight an infection, and are thus ecologically relevant (French and Neuman-Lee 2012). We followed the protocol outlined in French and Neuman-Lee (2012) to determine if the immune challenges affected the animals' innate immunocompetence. In brief, we combined a 1:5 plasma dilution with CO_2-independent media (plus 4 nM L-glutamine), 10^4 colony producing units of *E. coli* (EPowerTM Microorganisms #483-237-1, ATCC 8739, MicroBioLogics, St. Cloud, MN, USA), and agar broth on a 96-well microplate. We also included positive (media and bacteria with no plasma) and negative (media and no plasma or bacteria) controls to account for potential growth and ensure there was no contamination. We incubated the plate for 12 h and calculated absorbance with a microplate reader (300 nm, BioRad Benchmark, Hercules, CA, USA). Microbiocidal ability was calculated as 1 − (absorbance of sample/absorbance of positive controls) × 100. Inter-assay coefficient of variation was 2.59%.

Experiment 2

Animals and overview

Thirty-three adult male side-blotched lizards were captured via noosing from Washington County, Utah, USA in March 2015 under Utah State Department of Wildlife Resources COR #1COLL8382. Males were similarly sized at the beginning of the experiment, with snout-vent length averaging 49.5 ± 0.3 mm and mass 3.60 ± 0.10 g (mean ± SEM). The lizards were transported to Arizona State University in a temperature-controlled container within 3 days, where they were individually housed under identical conditions to the animals in Experiment 1. The animals were separated into two groups, which were offered different amounts of food. The animals were of similar mass between treatments ($P = 0.54$) at the beginning of the study. All animals underwent a cutaneous biopsy as an immune challenge on Day 1. Blood samples were collected from the retro-orbital sinus at the end of the experiment on Day 6. Cutaneous biopsies, metabolic measurements, radioimmunoassays, and bactericidal assays were all performed using the same methods outlined above in Experiment 1, and a timeline of procedures can be found in Table 1. All procedures were approved by the Utah State University (USU) and Arizona State University (ASU) Institutional Animal Care and Use Committees (IACUC, USU protocol #2068, ASU protocol #15-1413NR).

Feeding treatments

We randomly assigned animals to one of two feeding treatments: low- or high-food intake. Animals in the high intake group were offered two mealworms (Fluker Farms, Port Allen, Lousiana, USA) every other day. This schedule allowed for the lizards to be fasted the day prior to and during the metabolic measurements to prevent measuring a post-prandial metabolic response. We offered the low intake treatment group a single mealworm the day prior to the immune challenge, then every 4 days. Regardless of treatment, food was weighed before being offered to the lizards and removed the following day to determine intake. Although total intake ($t = -3.49$, $P < 0.01$) and mass lost ($t = 2.20$, $P = 0.04$) differed between treatments, feeding behavior varied with the individual lizards. Thus, most comparisons were made using food intake mass (g) as a continuous variable instead of a dichotomous level.

Hemolysis–hemagglutination assay

We performed a hemolysis–hemagglutination assay following an adaptation of the protocol of Matson et al. (2005), to assess biopsy or feeding treatment effects on that aspect of their immunocompetence. As opposed to the broader bactericidal assay, the hemolysis–hemagglutination assay focuses more on complement activity. Furthermore, given the negative effect of cutaneous biopsies on microbiocidal ability in Experiment 1 (see "Results"), we considered another immune measure warranted in Experiment 2. Briefly, 20 µl of plasma and 20 µl of phosphate-buffered saline were serially diluted (1:2) across a 96-well round (U) bottom microplate for resulting dilutions of 1:1 to 1: 2048. Six positive controls of deionized water (100% lysis) and six negative controls of phosphate-buffered saline (0% lysis) were present on each plate. Following the serial dilution, 20 µl of 1% washed sheep red blood cells (Hemostat SBH 100) in phosphate-buffered saline was added to every well. Plates were covered with Parafilm©, vortexed at 190 rpm for 60 s, and incubated at 37 °C for 90 min. Plates were then tilted for 20 min at 22 °C and visually scored for agglutination (Epson Perfection V750 Pro). Plates were then left at 22 °C for another 60 min to enable lysis. Following the final incubation, plates were centrifuged for 5 min at 500 rpm and the supernatant was aspirated and placed in a clean 96-well microplate to measure absorbance at 405 nm. To standardize results, hemolytic-complement activity (lysis) was expressed as CH_{50} units ml^{-1} serum, or the dilution of plasma that causes 50% lysis of sheep red blood cells. The mean intra-assay variation (SD) was ±0.2

titers for agglutination and ±0.1 titers for lysis. The mean inter-assay variation (SD) was ±0.4 titers for agglutination and ±0.3 titers for lysis.

Statistical analyses

For Experiment 1, we used a generalized linear model to estimate the effects of LPS injection and cutaneous biopsy on metabolic rate and mass loss. We used a normal distribution, because this fit the data best. We assessed whether interactions existed among main effects in our factorial design, and *P* values were generated from the full models using likelihood ratios. Percent mass lost and percent wound healed were correlated against each other.

Because feeding rates differed among individuals within each treatment in Experiment 2, regressions were used to measure the effect of intake (g) on other variables. The assumptions of normality and homogeneity of variance were fulfilled for these parametric tests. We also used regressions to assess the effect of wound healing rate on mass lost and metabolic rate. We used an alpha-level of $P = 0.05$, completed analyses in JMP 12.0.1™ (Statistical Analysis Software, Cary, North Carolina, USA), and created figures with GraphPad Prism 6 (GraphPad Software, San Diego California USA).

Results

Experiment 1

Overall, cutaneous biopsies affected the dependent variables in our study more than LPS injections did. Although there was no difference in metabolic rate between groups on Day 4 due to cutaneous biopsies ($\chi^2_1 = 0.75$, $P = 0.0.39$), biopsied lizards exhibited a reduction in metabolic rate between Days 4 and 5 that were 26.2 ± 6.2% ($\chi^2_1 = 4.42$, $P = 0.04$; Fig. 1) less than that of the controls. LPS injections had no significant effect on metabolic rate ($\chi^2_1 = 0.26$, $P = 0.61$; Fig. 2), and the effect of biopsy was similar for lizards injected with the LPS and those injected with vehicle solution ($\chi^2_1 = 1.677$, $P = 0.195$). Animals in the biopsied group lost 3.8% more body mass than the group that received LPS ($\chi^2_1 = 5.38$, $P = 0.02$; Fig. 2), which were similar to animals in the control group ($P = 0.56$). There was no effect of LPS injections ($\chi^2_1 = 0.34$, $P = 0.56$) and no interaction ($\chi^2_1 = 0.03$, $P = 0.87$). In addition, animals that healed larger percentages of their biopsies lost larger percentages of their body mass ($r = 0.51$, $P = 0.02$; Fig. 3). LPS injection had a marginal effect on microbiocidal ability, which was 20.49 ± 4.96% on average (±SEM) for the LPS treatment and 11.82 ± 1.43 for the biopsied animals ($\chi^2_1 = 3.65$, $P = 0.06$). Microbiocidal ability was not significantly affected by biopsies ($\chi^2_1 = 2.49$, $P = 0.12$), and the microbiocidal effect of LPS injection was similar for lizards that received a biopsy and those that did not ($\chi^2_1 = 2.46$, $P = 0.12$), indicating no strong interaction between treatments. Plasma corticosterone concentrations were similar across treatments ($\chi^2_1 = 3.98$, $P = 0.26$) and averaged 133.96 ± 17.10 ng ml^{-1} (±SEM). LPS injections did not affect the rate of wound healing ($\chi^2_1 = 0.09$, $P = 0.76$).

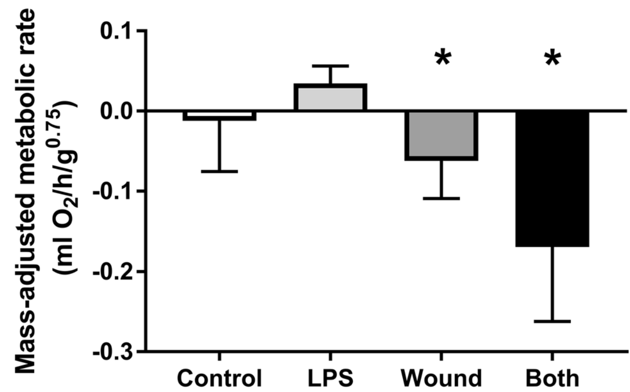

Fig. 1 Effect of treatment on the change in mass-adjusted metabolic rates in male side-blotched lizards in Experiment 1. *Bars* represent means (±SEM) of the change in metabolic rate between Days 4 and 5. Biopsied animals ($n = 20$) had significantly reduced metabolic rates ($\chi^2_1 = 4.42$, $P = 0.04$) compared to those without biopsies ($n = 20$). Animals receiving LPS injections ($n = 20$) did not show significant changes ($\chi^2_1 = 0.26$, $P = 0.61$) in metabolic rate to those not receiving injections. Significant differences ($P < 0.05$) designated with *asterisks*

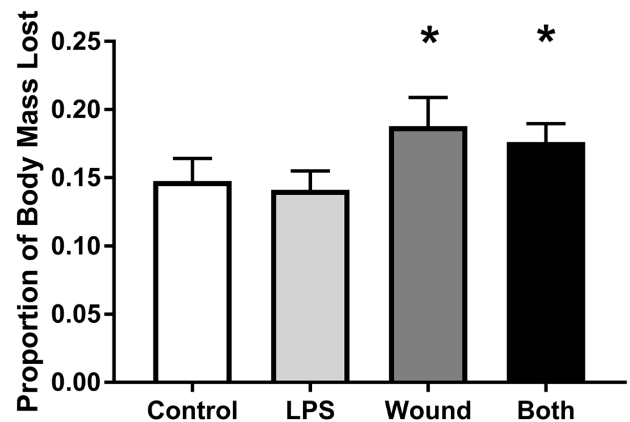

Fig. 2 Effect of treatment on the percentage of body mass lost in male side-blotched lizards in Experiment 1. *Bars* represent means (±SEM). Biopsied animals ($n = 20$) lost significantly more body mass ($\chi^2_1 = 5.38$, $P = 0.02$) compared to those without biopsies ($n = 19$). Animals receiving LPS injections ($n = 20$) did not show significant changes ($\chi^2_1 = 0.34$, $P = 0.56$) in mass lost to those not receiving injections ($n = 19$). Significant differences ($P < 0.05$) designated with *asterisks*

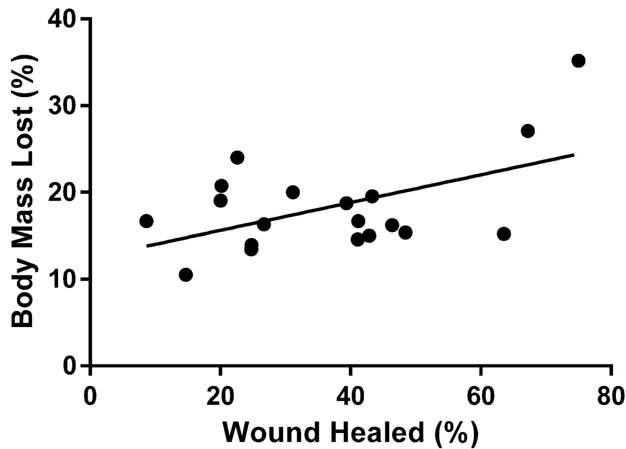

Fig. 3 Relationship of body mass percentage lost with the percentage of wound healed in male side-blotched lizards in Experiment 1. There is a positive correlation with mass lost and wound healing rate ($n=20$, $r=0.51$, $P=0.02$)

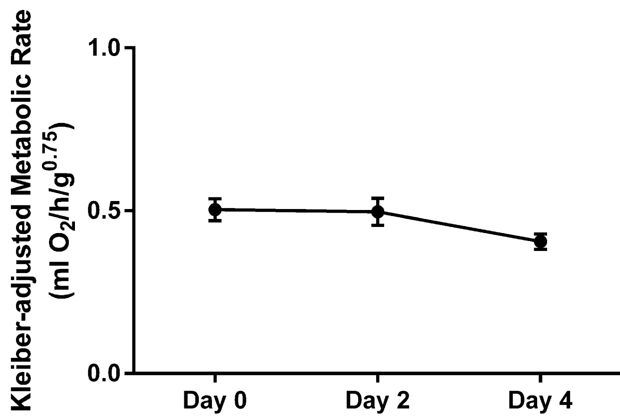

Fig. 4 Mass-adjusted metabolic rate through time in male side-blotched lizards for Experiment 2. Metabolic rate decreases between Days 2 and 4 in biopsied animals ($n=33$)

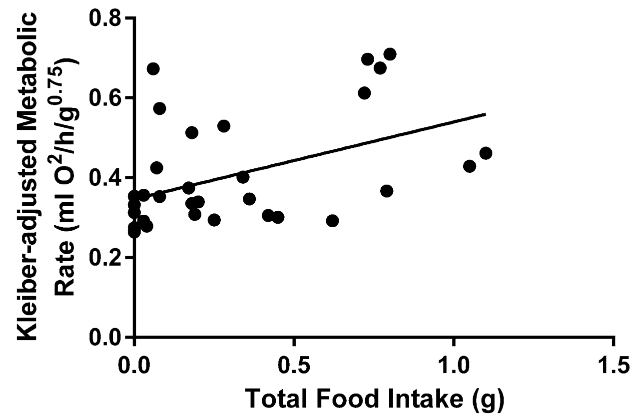

Fig. 5 Relationship with total food intake and the final metabolic rate on Day 4. The relationship increased from previous metabolic trials to a significant level ($R^2=0.44$, $F_{1,31}=24.96$, $P<0.01$)

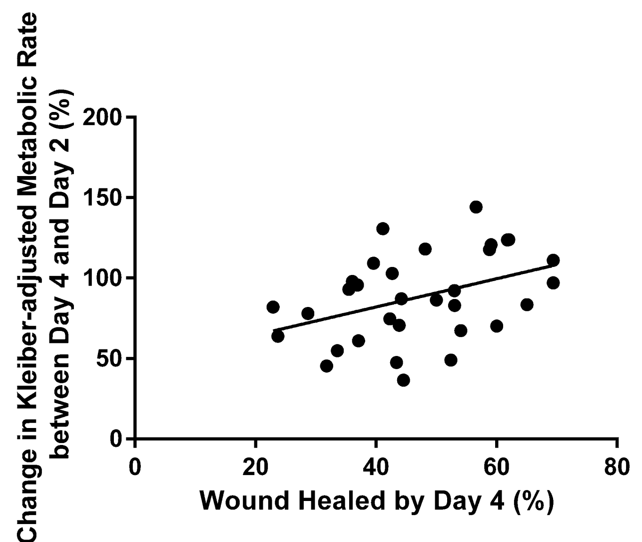

Fig. 6 Relationship between the percentage of the wound healed on Day 4 with the percent difference in mass-adjusted metabolic rate between Days 4 and 2 in Experiment 2. There was a significant relationship between wound healing and metabolic rate ($R^2=0.16$, $F_{1,30}=5.84$, $P=0.02$), regardless of feeding treatment

Experiment 2

Metabolic effects

As in Experiment 1, there was a reduction of metabolic rate in response to the cutaneous biopsies over time, by an average of 22.58% between Days 2 and 4 (Fig. 4), which is a critical time period for healing in this species (Neuman-Lee and French 2014). In addition, a positive relationship with food intake (g food eaten g body mass^{-1}) and metabolic rate developed throughout the study. Feeding treatment did not significantly affect the change in mass-adjusted metabolic rate throughout this study ($t=1.27$, $P=0.21$), so food intake was analyzed as a continuous variable. During the first measurement of metabolic rate, food intake had no significant effect on SMR ($R^2<0.01$, $F_{1,31}=0.30$, $P=0.59$). The relationship strengthened by the second measurement ($R^2=0.15$, $F_{1,31}=5.54$, $P=0.03$) and became highly significant by the third measurement ($R^2=0.44$, $F_{1,31}=24.96$, $P<0.01$), indicating that feeding affected metabolic rate later in the experiment (Fig. 5). There was also a positive relationship between wound healing rate and metabolic rate ($R^2=0.16$, $F_{1,30}=5.84$, $P=0.02$). Animals that healed larger percentages of their cutaneous biopsies on Day 4 had larger proportional difference between second and third metabolic runs, independent of food intake (Fig. 6).

Microbiocidal ability averaged (mean ± SEM) 15.31 ± 2.88% and was not correlated with food intake ($R^2 < 0.01$, $F_{1,24} = 0.02$, $P = 0.89$). Plasma corticosterone concentration was unrelated to food intake ($R^2 = 0.11$, $F_{1,16} = 1.89$, $P = 0.19$), averaging (mean ± SEM) 158.30 ± 28.49 ng ml^{-1}. In addition, unrelated to food intake was percent of wound healed by Day 4 ($R^2 = 0.07$, $F_{1,30} = 2.09$, $P = 0.16$), lysis and agglutination ($R^2 = 0.09$, $F_{1,27} = 2.60$, $P = 0.12$), and the change in Kleiber-adjusted metabolic rate ($R^2 = 0.02$, $F_{1,31} = 0.48$, $P = 0.49$).

Discussion

Contrary to our hypotheses, animals in both experiments reduced their energy expenditure after a cutaneous biopsy. In addition, contrary to our hypotheses, it does not appear that corticosterone is mediating this reduction. No change in metabolic rate was occurred in response to LPS injections in Experiment 1, but there was an effect of biopsies on weight loss, with biopsied animals losing more weight. In addition, animals that lost more weight tended to heal greater proportions of their wounds. There was a positive relationship between food eaten and metabolic rate that emerged during the metabolic trials after biopsies took place in Experiment 2, and increased throughout the healing process. In addition, the percentage of wound healed on Day 4 of Experiment 2 was related to the magnitude of reduction in metabolic rate between Days 2 and 4.

The general consensus has been that animals facing an immune challenge will upregulate their metabolism, and this has been observed in humans (Barr et al. 1922), rodents (Cooper et al. 1989; Demas et al. 1997; Derting and Virk 2005; Kristan and Hammond 2001; Magnanou et al. 2006), and birds (Martin II et al. 2003; Ots et al. 2001; Svensson et al. 1998). Our surprising results have several potential explanations. First, wound healing might deplete available energy stores in our lizards and left the animals in an energy-deprived state thereafter. This scenario would explain the lower metabolic rates later in the healing process. Fasting is known to decrease metabolic rates in this species (Roberts 1968), and thus, if wound healing is reducing stored energy over course of the study, it may be exacerbating already present fasting effects.

Alternatively, the decrease in metabolic rate and body mass might represent a compensatory response of other physiological functions to enable wound healing. The results of Experiment 1 support the idea that wound healing incurs significant costs. Within groups, animals that healed faster lost more body mass. Trade-offs are common in physiology, and there are multiple lines of evidence to support their presence during immune challenges. For instance, immune responses can reduce growth (Saino et al. 1998; Soler et al. 2003) or reproduction (French et al. 2007a; Råberg et al. 2000). In side-blotched lizards, it has also been demonstrated that individuals that healed biopsies faster in the early stages exhibited greater microbiocidal ability and stress reactivity later on, indicating prioritization (Neuman-Lee and French 2014). The lizards in the current study could have diverted energy from other functions in response to cutaneous biopsies in another instance of prioritization, with the net result of this shift manifesting as a lower metabolic rate.

Finally, the drop in metabolic rate during wound healing could have resulted from sickness behavior. Pro-inflammatory cytokines (especially interleukin-1, interleukin-6, and tumor necrosis factor α) increase during immune challenges such as wound healing (Barrientos et al. 2008; Werner and Grose 2003). These mediators promote lethargy and anorexia (Johnson 2002). Lizards could have behaved in this way when wounded. Because foraging is a risky behavior (Huey and Pianka 1981), sickness behavior may be especially important in a natural environment. Wounded lizards might benefit from resting and fasting in the wild to avoid predators and conspecifics. Short periods of fasting might also improve survival in animals undergoing an immune challenge, as Murray and Murray (1979) observed in laboratory-housed mice. Possibly, wounded lizards were not eating although food was available, which caused them to lose weight and downregulate their metabolic rates. However, because all animals were measured post-prandially, and at rest, this is an unlikely explanation. Although we see that in Experiment 1 biopsied animals lost more body mass than did control animals, we did not see a direct effect of wound healing on food intake in Experiment 2 (in which feeding was quantified, unlike Experiment 1), suggesting that animals were not responding via anorexia. Evidence in other immunological studies in ectotherms is also mixed. For example, cane toads decreased activity in response to LPS injections (Llewellyn et al. 2011), but bullfrog tadpoles injected with heat-killed *E. coli* increased activity when exposed to a predator (Lefcort and Eiger 1993). Foraging behavior and feeding can be affected by immune challenges, but it is also possible that food intake, and thus energy reserves, helps mediate the immune response.

Some studies of other ectotherms have been conducted, but no consensus on the metabolic costs of immunity or behavioral fever in these organisms has emerged. For example, painted turtles and box turtles (Monagas and Gatten 1983) and desert iguanas (Bernheim and Kluger 1976; Kluger et al. 1975) have exhibited increased body temperatures in response to bacterial injections, while armadillo girdled lizards (Laburn et al. 1981) and brown anoles (Cox et al. 2015) did not. Green anoles actually exhibited behavioral hypothermia (Merchant et al. 2008), which would reduce metabolic rate assuming there is not a higher

metabolic cost of the immune response that offsets this reduction. According to Deen and Hutchison (2001), factors, such as infection intensity and energy reserves, combine to determine an animal's immune response. When cane toads (Sherman and Stephens 1998) and green iguanas (Malvin and Kluger 1979) were not given thermal choices (similar to the animals in our experiments), their metabolic rates did not change, which might explain our nonsignificant effect of LPS injections on metabolic rates. Possibly, the animals in the present study did not respond to the LPS, but this is unlikely because we observed increased microbiocidal ability in the LPS-treated lizards, and we used doses consistent with those inducing physiological effects in other reptiles, including lizards (Deen and Hutchison 2001; do Amaral et al. 2002; López et al. 2009; Uller et al. 2006). To ensure our doses were adequate, we performed a subsequent study with twice the dose used in Experiment 1 (5 µg LPS/20 µl) to determine if the dose was too small, and found similar results (Smith et al., unpublished data). Different immune challenges have different associated costs, and it is possible that LPS challenges do not require the same metabolic responses that were observed with cutaneous biopsies, or lizards that had been previously wounded were prioritizing wound healing over their response to LPS. Given that lizards that only received LPS (and no biopsy) displayed to metabolic difference when compared to controls, this explanation is unlikely. It is also possible that the lizards in this study responded to LPS after their second metabolic trial, and future studies would benefit from more metabolic trials as the animals fully recover from these challenges.

A multitude of factors must be put into context when evaluating the metabolic costs of immunity, and factors such as lineage, energetic state, behavioral response to the challenge, and the type of challenge likely inform the nature of the immune response. Although sickness behavior might explain some of our surprising results, it is more likely that physiological trade-offs also occurred concomitantly. Future studies should attempt to measure the metabolic costs of immunity competing for resources with other physiological processes, such as growth or reproduction.

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Compliance with ethical standards

Conflict of interest No one involved with these experiments have any competing interests.

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