Behavioral Responses to Immune-System Activation in an Anuran (the Cane Toad, *Bufo marinus*): Field and Laboratory Studies

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ABSTRACT

The challenges posed by parasites and pathogens evoke behavioral as well as physiological responses. Such behavioral responses are poorly understood for most ectothermic species, including anuran amphibians. We quantified effects of simulated infection (via injection of bacterial lipopolysaccharide [LPS]) on feeding, activity, and thermoregulation of cane toads *Bufo marinus* within their invasive range in tropical Australia. LPS injection reduced feeding rates in laboratory trials. For toads in outdoor enclosures, LPS injection reduced activity and shifted body temperature profiles. Although previous research has attributed such thermal shifts to behavioral fever (elevated body temperatures may help fight infection), our laboratory studies suggest instead that LPS-injected toads stopped moving. In a thermal gradient, LPS-injected toads thus stayed close to whichever end of the gradient (hot or cold) they were first introduced; the introduction site (rather than behavioral thermoregulation) thus determined body temperature regimes. Shifts in thermal profiles of LPS-injected toads in outdoor enclosures also were a secondary consequence of inactivity. Thus, the primary behavioral effects of an immune response in cane toads are reduced rates of activity and feeding. Thermoregulatory modifications also occur but only as a secondary consequence of inactivity.

Introduction

Most behavioral traits are highly labile, with organisms capable of switching rapidly from one behavior to another in response to cues from the environment (including from other organisms) or in response to changes in physiological state. Often, such behavioral responses to physiological state differ among and within taxa. For example, pregnancy elevates mean selected body temperatures of females in some reptile species but reduces it in others (Beuchat and Ellner 1987; Daut and Andrews 1993), and food limitation either may stimulate active foraging behavior or result in inactivity depending on seasonal environmental conditions (Bull et al. 1996).

One inevitable event in the life of any organism is an immune response, stimulated by pathogens or parasites. Vertebrates possess both cellular and humoral immune defenses (Knox et al. 1994; Alberts et al. 2007) that can be costly to the host in terms of both energy and morbidity (Lochmiller and Deerenberg 2000). During an immune response, chemical messengers activate behavioral strategies as well as physiological defense processes (Larson and Dunn 2001; Janeway et al. 2005). An animal that has upregulated its immune system to combat infection or parasites may exhibit associated behavioral modifications for three reasons. First, the pathogen may irritate or debilitate the organism such that it cannot carry out its usual behaviors (Hatalski and Lipkin 1997). Second, the pathogen may manipulate host behavior in ways that enhance pathogen fitness (e.g., by increasing pathogen transmission rates; Hatalski and Lipkin 1997). Third, behavioral modifications may assist the host's immune system to fight the pathogen (e.g., behavioral elevation of body temperatures in ectotherms may assist the fighting of infection). Cytokine-induced "sickness behaviors," often incorrectly interpreted as manifestations of debilitation, provide an adaptive, albeit nonspecific, response to assist immune defense (Hart 1988). Many of the behavioral changes commonly associated with illness (e.g., inactivity, reduced sociality, anorexia, fever; Rau and Putter 1984; Edwards 1988) are responses of this third type.

In vertebrates, activation of an immune response often is associated with reduced activity and food intake and increased body temperature (Hart 1988; Aubert 1999; Larson and Dunn 2001). Reduced activity and feeding rates enhance immune function by allowing maximal energy allocation to immune defenses instead of movement and digestion (Aubert 1999), and the elevated temperature (fever) enhances immune processes such as macrophage receptor expression and phagocytotic action (Kluger et al. 1998; Hasday et al. 2000). Fever is primarily generated metabolically by endotherms, whereas ectotherms rely on behavioral shifts to change their thermal regimes (Hart 1988; Hasday et al. 2000). Despite this divergence in mechanisms, fever in response to immune challenge has been reported in all ectothermic and endothermic vertebrate classes and in some invertebrates (Vaughn et al. 1974; Reynolds et al.

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Table 1: Species of anuran reported to exhibit a behavioral fever when subject to pyrogenic stimulation

Species	Pyrogen	Reference
Bombina bombina	*Pseudomonas aeruginosa*	Cabanac and Cabanac 2004
Bufo marinus	*P. aeruginosa*	Cabanac and Cabanac 2004
B. marinus	LPS	Sherman et al. 1991; Sherman and Stephens 1998
Bufo paracnemis	LPS	Bicego and Branco 2002; Bicego et al. 2002
Hyla cinerea	*Aeromonas hydrophila*	Kluger 1977
Rana aurora	*Candida humicola*	Lefcort and Blaustein 1995
Lithobates catesbeiana	*A. hydrophila*	Casterlin and Reynolds 1977; Lefcort and Eiger 1993
Rana esculenta	*Mycobacterium xenopi*, *Mycobacterium ranae*	Myhre et al. 1977
Pelophylax pipiens	*A. hydrophila*	Casterlin and Reynolds 1977

Note. LPS = lipopolysaccharide.

1976; Casterlin and Reynolds 1977; Bronstein and Conner 1984; Sherman et al. 1991; Kluger et al. 1998).

We studied the behavioral correlates of immune activation in cane toads *Bufo marinus*, Linnaeus 1758, within their introduced range in tropical Australia by injecting toads with bacterial lipopolysaccharide (LPS) to trigger the body's defenses against bacterial attack (Sweet and Hume 1996). In an invasive species such as the cane toad, sickness behaviors are likely to play an important role in nonspecific defense against novel pathogens. Thus, we predict that toads faced with immune challenge will reduce both feeding and activity and also that LPS-injected toads will develop a behavioral fever. These behavioral responses may have negative consequences, such as desiccation (Szczepanska-Sadowska et al. 1979) and a reduced ability to disperse, but are likely to increase the individual's chance of survival.

Material and Methods

Specimen Collection and Husbandry

Cane toads *Bufo marinus* (see Pramuk 2006 for alternative nomenclatural designation as *Rhinella marina* and Frost et al. 2006 for reallocation to *Chaunus marinus*) are large bufonid anurans native to Central and South America but introduced to Australia (and many other countries) to control insect pests (Lever 2001). We collected cane toads of both sexes and body masses 45–400 g on the Adelaide River floodplain of the Northern Territory (NT; 131°18′E, 12°37′S). We used these animals to study the effect of immune challenge on (*a*) feeding rates in the laboratory and (*b*) movement patterns and selected body temperatures in field enclosures. To investigate thermoregulatory behavior in laboratory thermal gradients, we obtained toads (both sexes, 21–64 g) from a captive breeding program. The parents of these animals had been collected in Cairns (Queensland). All animals were housed in plastic bins (1.2 m × 1.2 m × 0.9 m) with access to water and dry ground. Captive toads routinely were fed crickets and cockroaches (3 toad^{-1} d^{-1}, 5 d wk^{-1}) but were fasted for 2 d before commencing any experiment, to control for possible effects of feeding on other traits.

Pyrogen

Anurans of several species exhibit behavioral fever when challenged with gram-negative bacteria such as *E. coli* (Table 1). To stimulate an immune response, we used LPS from the outer wall of gram-negative bacteria. Because LPS contains lipid A (a bacterial component readily recognized by a host's immune defenses), injection with LPS activates both innate and humoral immune processes of a vertebrate host (Kluger 1991; Gulig 1996; Klasing 2004). LPS (from *E. coli* Serotype 0111:B4; Sigma-Aldrich) was purified by phenol extraction and diluted with phosphate-buffered saline (PBS) for injection into toads. All toads were injected into their dorsal lymph sac (so that injected fluids would circulate around the body) using a 29-gauge needle at a volume of 0.002 mL g^{-1} body mass. Experiments were conducted at the University of Sydney Tropical Ecology Research Facility, on the Adelaide River floodplain (NT).

Effect of Immune Challenge on Feeding Rates

Because toads in the wild often congregate in suitable areas for feeding (Zug and Zug 1979), we used two toads per enclosure (one control, one LPS-injected) to incorporate competitive effects as would occur in nature. One toad from each of 10 mass-matched pairs was injected with LPS at a concentration of 0.002 mg g^{-1} body mass in a PBS solution of 1 mg LPS mL^{-1}. The other toad in the pair was injected with an equivalent volume of PBS. Six hours after injection the two toads were placed into the feeding arena (1.2 m × 1.2 m, with 0.9-m-high walls). Two minutes later we added 10 crickets and recorded the number eaten by each toad over the following 10 min. Similar feeding trials were repeated at 30 h after injection and at every 24 h thereafter for 9 d postinjection. Further trials were conducted

at 14, 21, and 29 d postinjection. The same two toads always competed against each other. Toads were not fed during the period in which feeding trials were conducted except for the food they consumed during the trials.

Effect of Immune Challenge on Hydration Status

Eight toads (5 female, 3 male) were placed in each of four open-topped outdoor enclosures (4 m × 5 m) that provided access to water, open and thickly grassed areas, piles of grass clippings, and burrows. Toads were allowed to explore enclosures for two nights before being recaptured and weighed. They were then injected with either LPS at a dose of 0.002 mg g^{-1} body mass in a 1 mg mL^{-1} solution or an equivalent amount of PBS ($n = 4$ toads per enclosure) before being replaced in the enclosures and left for 7 d. Changes in body mass over the course of a trial were recorded and used as an index of hydration status, because fluctuations in mass over this timescale primarily reflect water balance rather than changes in energy reserves (Claussen 1969; Shirreffs 2003; Prates and Navas 2009). The trial was then repeated (with 32 different toads) over another 7-d period.

Effect of Immune Challenge on Movement Patterns

Effects of LPS injection on movement patterns were assessed in the same trials as described above for hydration effects. Either two or four individuals in each enclosure (one or two of each control and treatment) had a cotton spool attached to their waist; the other end of the cotton was tied at the release point such that the spool unraveled as the toad moved. Each morning, we measured the length of unraveled cotton to determine the distance each toad had traveled the previous night (Miles et al. 1981; Seabrook and Dettmann 1996).

Effect of Immune Challenge on Thermoregulatory Behavior in the Laboratory

We constructed thermal gradients from 100-mm-diameter PVC pipe, 1.5 m long, closed off at both ends. One end was heated to 42°C (using thermostatically controlled heating wire) whereas the other end was kept at 15°C (by pumping cold water around the outside of the pipe). To maintain 100% humidity inside the gradients throughout all trials, we ran a cloth wick along the inside of each gradient, with the end of the wick (outside the gradient) in a container of water. Wicks were removed and washed in 10% bleach before being rinsed in fresh water between each trial. The experimental room was maintained at 25.0° ± 1.0°C and 40% humidity.

Before running trials, we placed thermochron iButtons (Maxim Integrated Products, Sunnyvale, CA) at 100-mm intervals along the floor of each gradient to record temperature every minute for 1 h. These data allowed us to quantify the spatial pattern of temperatures along the gradient so that any shift in a toad's temperature could be interpreted in terms of how far the animal had to move to experience that shift.

To measure mean selected temperatures, we placed one toad into each gradient at 1900 hours (Central Standard Time) and left the animal without disturbance for 23 h. The temperature of each toad was recorded every 15 min by means of a thermochron iButton attached to the toad's ventral surface by a waist belt. Calibration studies showed that temperatures monitored in this way were highly correlated with internal body temperatures of the toad ($n = 9$ toads, total $n = 4{,}743$ pairs of simultaneously recorded data points of internal vs. external temperature; per toad, $r^2 = 0.96$ to 0.99; for combined data, $r^2 = 0.96$; mean disparity between internal and external temperature = 0.05°C, SE = 0.006). We ignored temperature data for the first 2 h of each trial to minimize effects of handling stress and exploratory behavior. Gradient temperatures throughout each trial were monitored by thermochrons.

Sixteen toads were injected with PBS immediately before being put into the thermal gradients. Eight toads were placed into the hot end of the gradient and another eight into the cold end (one toad per gradient). After 23 h, all toads were removed and injected with LPS at a concentration 0.02 mg LPS g^{-1} body mass (10 × the dose used in other experiments; Sherman et al. 1991), then monitored in the gradients for a further 23 h. This dose was chosen to replicate a previous study (Sherman and Stephens 1998) and because pilot studies indicated no behavioral fever at a dose of 0.002 mg LPS g^{-1} body mass. Pilot studies also showed that controls (injected with a second dose of PBS instead of LPS) experienced no significant change in thermal behavior from repeated injection (data not shown).

Effect of Immune Challenge on Thermoregulatory Behavior in the Field

We used thermochron iButtons attached to waist belts to measure thermal profiles of toads during the field-enclosure trials (see above). Miniature radio transmitters attached to the same waist belts enabled us to relocate the toads (see Phillips et al. 2006 for methods). Body temperatures were recorded every 15 min for 7 d but combined into 2-h averages per toad for analysis. We also deployed iButtons in a range of thermally distinctive habitat types (open ground, long grass, underground burrow) to quantify thermal availability.

Because the thermal opportunities for toads are more limited in enclosures than in nature, we monitored body temperatures of six free-ranging toads near the research station. These six toads were paired according to body mass. One toad from each pair was injected with LPS at 0.002 mg g^{-1} body mass in a 1 mg mL^{-1} solution with PBS, whereas the other received an equivalent volume of PBS. We then attached radio transmitters as well as iButtons to their waist belts, released the animals simultaneously, and recaptured them 7 d later. For analysis, temperature data (one reading every 15 min) were collated into 2-h averages as above.

Statistical Analysis

Our data conformed to the distributional assumptions of parametric testing. Repeated-measures ANOVAs (rmANOVAs) were conducted using the statistical program JMP version 7.0 (SAS Institute 2007) to assess effects of LPS versus saline (PBS) injection on patterns of toad feeding, hydration, movement, and thermoregulation. Treatment (LPS vs. PBS) was used as the factor, and time was the repeated measure. For analyses of thermoregulation in the laboratory, our analyses included "starting end" (i.e., whether the toad was initially placed at the hot vs. cold end of the gradient) as an additional factor.

Results

Effect of Immune Challenge on Competition and Feeding

From the 10 pairs of toads tested from 6 h postinjection, LPS-injected toads ate fewer crickets than did saline-injected (control) competitors over most of the 4-wk test period (main effect, $F_{1,16} = 35.36$, $P < 0.001$). The significant interaction between time and treatment ($F_{12,192} = 3.32$, $P = 0.0002$) reflects an initial similarity in feeding rates followed by a divergence between LPS-injected and PBS-injected toads and a decline in feeding rates of control animals toward the end of the trial period (Fig. 1).

Effect of Immune Challenge on Hydration Status and Movement Patterns

Changes in body mass over the first 7 d postinjection did not differ significantly between LPS-injected and control toads in outdoor enclosures (rmANOVA, interaction between treatment and time, $F_{1,26} = 0.32$, $P = 0.58$).

PBS-injected controls moved more than LPS-injected toads over the 7-d test period (rmANOVA, main effect, $F_{1,19} = 16.05$, $P = 0.0008$; Fig. 2). The effect of treatment on movement changed over time (interaction, $F_{6,114} = 2.69$, $P = 0.018$). Control and treatment toads moved similar distances on nights 1 and 7 ($P = 0.65, 0.30$, respectively), but in the intervening period (nights 2–6), control toads moved farther (mean ± SE = 35.6 ± 3.9 m) than LPS-injected toads (4.4 ± 0.7 m; $P < 0.0001$; Fig. 2).

Thermoregulatory Response to Immune Challenge

LPS-injected toads (28.6° ± 0.7°C) were warmer than controls (25.4° ± 0.7°C; $P = 0.003$) when all data from toads in the thermal gradients were pooled. However, the effect of treatment (LPS vs. PBS) on temperature selection depended on whether the toad was initially placed into the cold versus the hot end of the gradient at the beginning of the trial (interaction treatment × starting end from rmANOVA, $F_{1,26} = 4.60$, $P = 0.042$). LPS-injected toads placed into the hot end of the gradients selected higher temperatures than controls (mean values of 29.8° ± 0.8°C and 24.7° ± 0.8°C; $F_{1,13} = 18.3$, $P = 0.0009$; Fig. 3*a*), whereas there was no significant effect of treatment on temperature selection when toads were placed in the cold end of the gradients ($F_{1,13} = 0.3$, $P = 0.60$; Fig. 3*b*). Using temperature-position correlations to infer distances moved, LPS-injected toads moved less than the controls (1.37 ± 0.20 m vs. 4.21 ± 0.56 m; $P < 0.0001$). Within each treatment, the

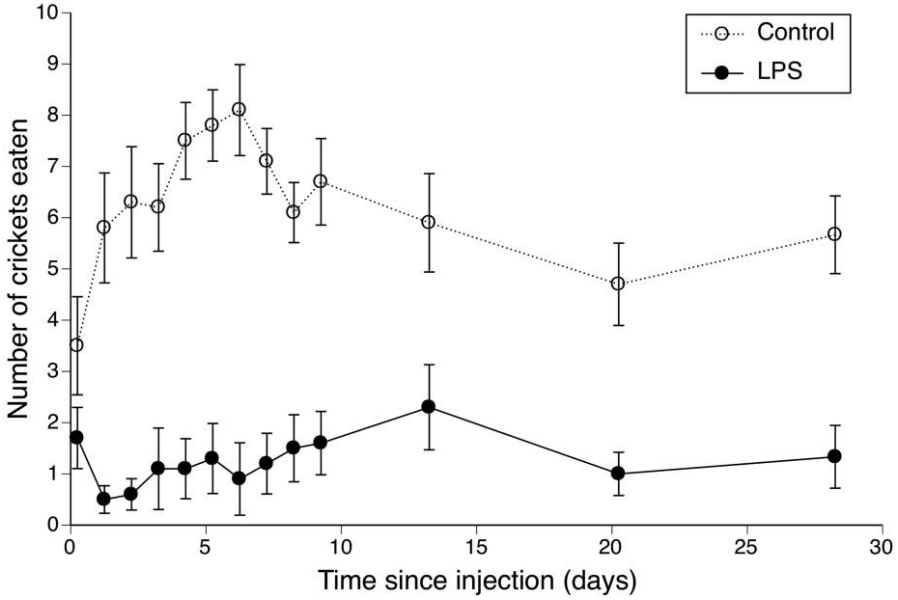

Figure 1. Mean number of crickets eaten by cane toads *Bufo marinus* in competitive feeding trials. Control and lipopolysaccharide-injected toads competed against each other to feed on a maximum of 10 crickets. Trials were repeated on a number of occasions over 4 wk. Values represent means ± 1 SE.

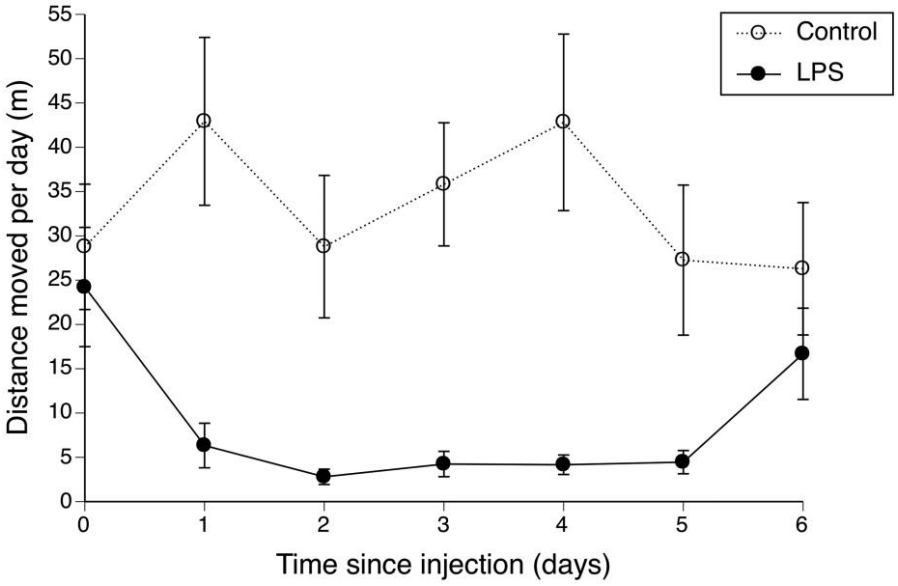

Figure 2. Mean distance moved each night by both control and lipopolysaccharide-injected cane toads *Bufo marinus* in field enclosures over seven consecutive nights. Graph represents mean ± 1 SE.

mean distance moved by a toad was not significantly affected by the end of the gradient in which it was initially released (LPS: 1.46 ± 0.37 m [hot] vs. 1.28 ± 0.18 m [cold], $P = 0.66$; PBS: 4.26 ± 1.01 m [hot] vs. 4.16 ± 0.55 m [cold], $P = 0.93$).

Thermal data from toads in the outdoor enclosures were similar to those from the thermal gradients. Treatment affected body temperatures, with LPS-injected toads selecting higher mean temperatures than PBS-injected controls (rmANOVA, $F_{1,51} = 5.25$, $P = 0.026$; Fig. 4). An interaction between treatment and time was also apparent ($F_{76,3876} = 2.73$, $P < 0.0001$) with no significant difference between treatments for the first 40 h; thereafter, LPS-injected toads exhibited higher temperatures than did controls. The thermal difference between treatments was most evident at two time periods during the diel cycle (the night and midafternoon [1400–1600 hours]; Fig. 4).

Thermal regimes of free-ranging toads were similar to those in the field enclosures. Although LPS-injected toads exhibited higher temperatures than controls in most time periods (Fig. 5), the difference was not statistically significant ($F_{1,3} = 8.68$, $P = 0.06$).

Discussion

Sick animals typically exhibit behavioral changes (Hart 1988), many of which are stimulated on a molecular level by cytokines produced as part of an immune response (Inui 2001; Larson and Dunn 2001; Johnson 2002). The sickness behaviors exhibited by cane toads in their introduced Australian range are similar to those shown by other vertebrate taxa (McCarthy et al. 1984; Hart 1988; Johnson et al. 1993; Inui 2001). When systemically injected with LPS, toads experience a reduction in activity, anorexia, and a shift in the diel cycle of body temperatures. The hydration status of toads was not affected by LPS injection.

The only attribute we measured that was apparently not affected by LPS injection was hydration status; over a 1-wk period in outdoor enclosures, there was no significant difference in rates of body mass change between LPS-injected and control toads. This result ran counter to our a priori prediction based on previous studies showing that activating an immune response often affects water balance. In endotherms, immune challenge is associated with a reduction in thirst and thus water intake (Szczepanska-Sadowska et al. 1979; Hart 1988). In amphibians, a lack of movement may reduce access to waterbodies for rehydration (Putnam and Hillman 1977). In our study, the immune-challenged toads were able to seek shelter in damp places and thus reduce water loss (Putnam and Hillman 1977; Prates and Navas 2009). Additionally, change in body mass may provide only a crude estimate of hydration status (Maughan et al. 2007); future work could usefully employ more sophisticated measures, such as urine and plasma osmolalities or hematocrits (Saris et al. 2003; Shirreffs 2003).

LPS injection strongly reduced activity levels in cane toads, both in laboratory thermal gradients (67% average reduction in distances moved) and in outdoor enclosures (88% average reduction in distances moved). Reduction in activity and social behavior is often associated with infection (Rau and Putter 1984; Edwards 1988; Johnson and Von Borell 1994) and commonly reflects a cytokine-induced reduction in psychological motivation to be active (Aubert 1999). Reduced activity may entail survival benefits. While sick, mobility may be reduced, and thus predator escape strategies may be compromised (Bau-

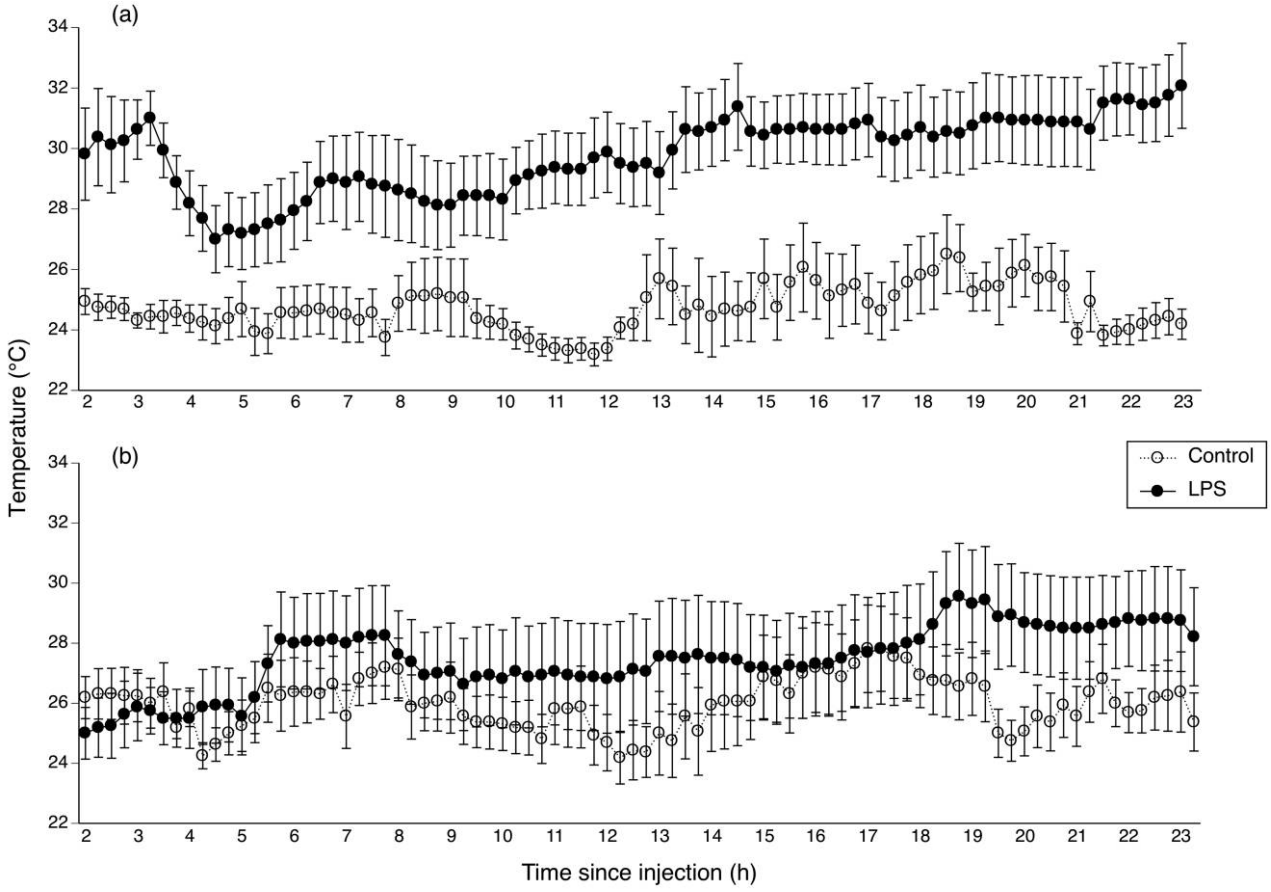

Figure 3. Body temperatures selected by cane toads *Bufo marinus* injected with phosphate-buffered saline (control) or lipopolysaccharide (LPS) in thermal gradients over a 23-h period. Immediately after injection, toads were introduced into the hot end (*a*) or the cold end (*b*) of the gradient. Values depict means ± 1 SE.

wens and Thoen 1981; Singal et al. 2004). Thus, a reduction in movement and therefore an increase in the time spent sheltering could help to reduce risks of predation. Because of the paucity of toad predators in their Australian range, it is more likely that reduction in movement elicits benefits in the form of reduced desiccation, as noted above, and in the conservation of energy. Energy saved could be reallocated into immune defense (Hart 1988; Martin et al. 2008).

Reduction in feeding is another common response to immune challenge (Hart 1988; Inui 2001; Larson and Dunn 2001). Because of increases in metabolic demands during immune challenge (Demas et al. 1997; Sherman and Stephens 1998), anorexia may seem maladaptive. However, by reducing food intake, animals can inhibit pathogenic multiplication by maintaining lower plasma iron concentrations (Elin and Wolff 1974; Bullen 1981; Hart 1988; Weinberg 2009). The effect of immune-system activation on food intake may be influenced by three major pathways. First, a loss of appetite following immune challenge is mediated by the neurocytokine interleukin-1β (IL-1β; Bretdibat et al. 1995; Inui 2001). During immune challenge, cellular production of IL-1β increases, resulting in a decrease in appetite (Johnson 2002). Second, a decrease in activity and foraging behavior leads to a reduction in food intake (Hart 1988; Larson and Dunn 2001; Szelenyi and Szekely 2004). In field-enclosure trials and feeding trials, LPS-injected toads moved less than PBS-injected conspecifics and were less attentive to prey items. Third, a toad's feeding rate depends on its ability to outcompete conspecifics (our rationale for designing the feeding-rate studies as competitive trials). Even if they attempted to feed, sick toads were outcompeted by healthy conspecifics.

In nature, all three of the above factors are likely to reduce rates of feeding by sick toads. LPS-injected toads often ignored easily accessible prey items, suggesting that an intrinsic lack of interest in food (perhaps coupled with a lack of the movements needed to bring toads into contact with potential prey) is the most important reason why an immune response reduces food intake. Thus, even if food is available within close proximity, a lessened desire to feed would result in reduced food intake. One puzzling aspect of our results was the long duration of feeding-rate suppression following the immune challenge; future work could usefully measure toad feeding rates under different conditions (e.g., solitary vs. in groups) to evaluate the causal basis for this long-lasting reduction in food intake rates.

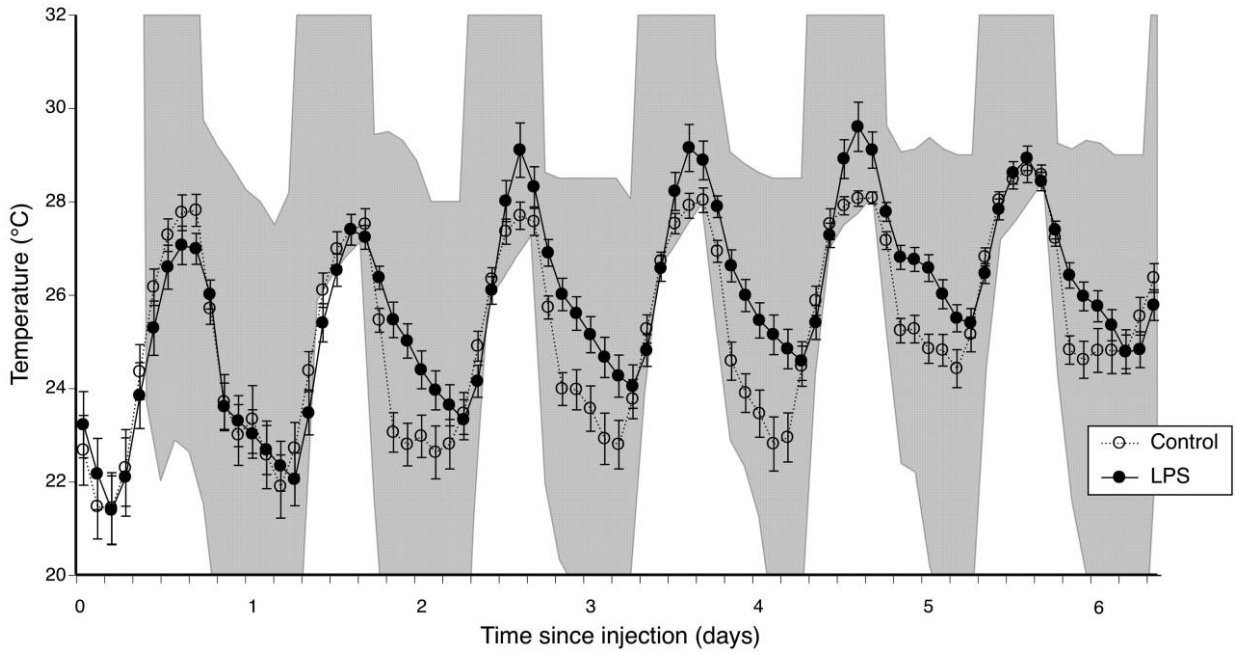

Figure 4. Average 2-h body temperatures experienced by cane toads *Bufo marinus* injected with phosphate-buffered saline (control) or lipopolysaccharide (LPS) in field enclosures over a 7-d period. Values depict means ± 1 SE. Background shading represents the environmental temperatures as determined by a series of thermochron iButtons.

The fourth variable we investigated was body temperature. Fever is probably the most commonly reported symptom of an immune challenge (Kluger et al. 1998; Hasday et al. 2000). Our pilot studies showed no significant elevation in body temperatures of toads injected with LPS at a lower dose (0.002 mg g^{-1} body mass) than that with which the trials were conducted (0.02 mg g^{-1} body mass) despite published reports of other toad species exhibiting fever at one-tenth of this dose (Bicego and Branco 2002; Bicego et al. 2002). In light of our pilot results, we adopted an LPS dose of 0.02 mg g^{-1} body mass to replicate a previous study on fever in cane toads from their native range (Sherman et al. 1991). Consistent with the report by Sherman et al. (1991), LPS-injected toads in our study selected higher temperatures than did controls but only if the toads were placed at the hot end of the gradient when the trials commenced.

Although at first sight the thermal-gradient results appear to demonstrate behavioral fever in response to injection of LPS, a methodological problem falsifies that interpretation. In a laboratory thermal gradient (as in nature), an animal's thermal regimes are affected not only by active thermoregulatory behavior but also by levels of activity. Because we initially placed toads at the hot end of the thermal gradient, a reluctance to move about would result in relatively high temperatures, regardless of the toad's thermal "preference." In both our laboratory and field-enclosure trials, LPS injection substantially reduced activity. To test this alternative explanation for the thermal effect of LPS injection, we introduced toads to the cold rather than the hot end of the gradient. If the measured thermal elevation results from active thermoregulation, this change in methodology should have little effect on toad temperatures during the main part of the trial. If the thermal elevation induced by LPS is a by-product of reduced activity, however, the effect of treatment on temperatures should disappear or be reversed. The lack of a significant febrile response in toads started at the cold end of the gradient suggests that the major force driving results was inactivity, not thermoregulation. Regardless of which end of the gradient they were placed, LPS-injected toads moved about 600 mm from their release point within the first 2 h (at which time we started recording temperatures) and then remained near there.

These results cast doubt on the uncritical use of laboratory thermal gradients to characterize selected temperatures in ectotherms and especially to suggest the presence of a febrile response to infection. This standard methodology has been used to demonstrate behavioral fever in many taxa of reptiles, amphibians, fishes, and invertebrates (e.g., Reynolds et al. 1976; Bronstein and Conner 1984; Sherman et al. 1991; Bicego et al. 2002; Merchant et al. 2008). A previous study on reptiles in thermal gradients demonstrated the same kind of artifact: an apparent shift in mean selected temperatures (induced by ingesting a large prey item) may be an artifact of reduced activity levels (Wall and Shine 2008). Given these examples, in both an amphibian and a reptile, future studies using thermal gradients should routinely compare results of animals introduced to either end of the gradient. Alternatively, laboratory studies on thermal preference should begin with the animal positioned in the middle of the thermal gradient at the acclimation temper-

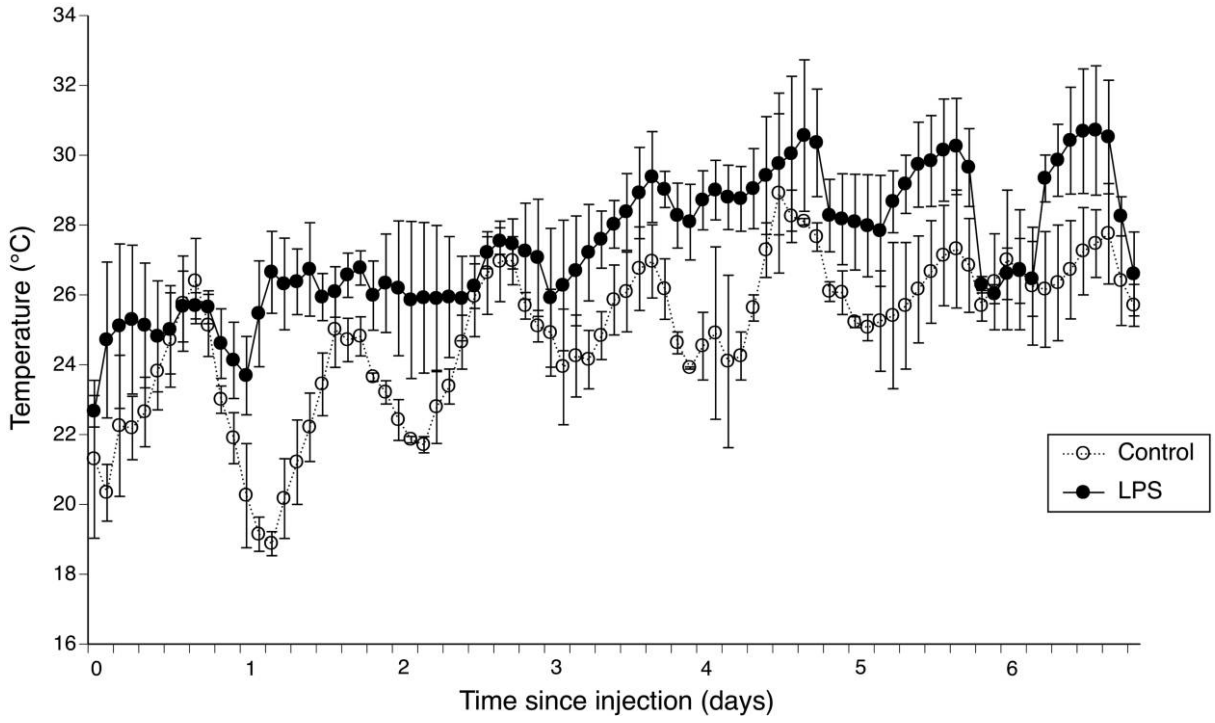

Figure 5. Average 2-h body temperatures experienced by free-ranging cane toads *Bufo marinus* injected with phosphate-buffered saline (control) or lipopolysaccharide (LPS) released on the Adelaide River floodplain (Northern Territory) over a 7-d period. Values depict means ± 1 SE.

ature to examine preferences for warmer or cooler conditions at the ends of the gradient. Previous studies reporting behavioral fever in toads in laboratory thermal gradients have placed their animals in the center of the gradient at the beginning of trials (Sherman et al. 1991; Bicego and Branco 2002; Bicego et al. 2002), which should have minimized experimental artifacts. However, the central position in the gradient (especially in spatial terms, if the gradient is nonlinear) may well be above or below the acclimation temperature; that is, the toads' thermal preferendum may not be at exactly the midpoint of the range provided in the gradient. Thus, researchers should pay careful attention to the possibility that an experimental animal's body temperatures are affected by apparently superficial details of methodology, such as the exact thermal conditions at the site where the animal is placed at the beginning of a trial.

Importantly, confounding activity levels and thermoregulation can complicate interpretation of field data as well as thermal-gradient studies. At first sight, our results from outdoor-enclosure studies suggest that LPS-injected toads select higher temperatures than do controls (Fig. 4). The thermal-gradient studies, in combination with distances moved by toads in outdoor enclosures, suggest instead that LPS-injected toads simply ceased to move around. One consequence of that immobility was that they remained inside their shelters at night, and that immobility was the primary reason why LPS-injected toads remained warmer than the control toads that moved into the (cooler) open to forage at night. A second consequence was that the LPS-injected animals did not move about during daylight hours to actively avoid high temperatures inside their retreat sites, unlike the control toads (Fig. 4).

Our data do not demonstrate that cane toads lack a behavioral fever; LPS injection does result in an elevation of body temperatures, both in outdoor enclosures and in free-ranging toads (Figs. 4, 5), but that thermal shift is a by-product of reduced activity rather than a thermoregulatory tactic per se. Because infected cane toads may derive a fitness benefit from higher temperatures (e.g., by increased levels of certain macrophage receptor expressions, enhanced phagocytotic action, and increased pathogen recognition; Roberts 1991; Hasday et al. 2000), these thermal shifts may have adaptive significance. The mechanism by which such thermal shifts are generated (i.e., active thermoregulatory behavior vs. a passive consequence of inactivity) does not affect the possibility that the end result enhances organismal fitness. Interestingly, however, the toads' apparent reliance on an indirect pathway to generate higher body temperatures would work only in relatively hot climates. As toads invade cooler regions, the indirect nature of this pathway might result in infected (inactive) animals experiencing cooler rather than warmer temperatures (i.e., in a cold environment, high temperatures are achievable only by actively seeking heat). Hence, the nature of the mechanistic pathway by which infection modifies body temperature may directly affect the success or failure of this invasive species across a range of thermal environments.

The effects of infection on toad activity have a further implication for the species' invasion biology. Cane toads have

dispersed at increasingly rapid rates across tropical Australia because spatial selection has resulted in the accumulation of genes for rapid dispersal at the invasion front (Travis and Dytham 2002; Phillips et al. 2010). Any trait that results in a toad dispersing less rapidly will tend to be more common in toads from long-colonized areas than at the vanguard of the invasion (Phillips et al. 2006, 2008). Our data show that reduced activity (and hence, dispersal) will be a major consequence of activating the immune system. Thus, any toads at the invasion front that are faced with an immune challenge are likely to fall behind. We may then expect that the only toads at the invasion front are those that are not, and have never been, faced with serious immune challenges. This situation could result in very little disease transmission at the invasion front, and thus toads at the invasion front may be able to survive with lower immunocompetence than conspecifics from well-established populations. Other theoretical work has generated similar predictions (albeit for different reasons; Lee and Klasing 2004; Phillips et al. 2010), suggesting that invasive species may offer ideal models to investigate evolutionary pressures on investment into immune function.

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