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ANIMAL SCIENCE

Effects of an immune challenge on the thermal preferences of adult and newborn *Liolaemus* lizards from Patagonia, Argentina

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Abstract: Body temperature has relevant effects on the immune response. Here, we characterized the thermal biology and health condition of the viviparous lizard *Liolaemus kingii* from Patagonia (Argentina), by studying field body temperatures, presence of injuries or ectoparasites, body condition (BC), and individual immune response capacity with the phytohemagglutinin (PHA) skin-swelling assay. In addition, we analyzed the effects of injections of a bacterial endotoxin (lipopolysaccharide; LPS) on the preferred temperature (T_p) and BC of adult males and newborns. The PHA treatment caused detectable thickening at 2 and 20 hours post-assay in males, indicating a significant immune response related to an increase in cellular activity. LPS-challenged lizards thermoregulated accurately and at stable body temperatures within the 50% interquartile of T_p (T_{set}) over the 72-hour period while the control group showed a more variable and lower T_p. Exposure to LPS negatively affected the BC of newborns, whereas it did not affect the BC of adult males. LPS challenges, used as a proxy of pathogen exposures to study lizard behavioral thermoregulation, constitute a practical approach to assess the immunological constraints lizards from high-latitude regions may face due to global warming and anthropogenic disturbances.

Key words: body condition, immune challenge, *Liolaemus kingii*, lipopolysaccharide, thermoregulation.

INTRODUCTION

The resilience of animal populations to novel host-pathogen interactions is governed by their physiological capacity to adjust to the new challenges (Graham et al. 2011). Apart from avoiding diseases, animals generally use two strategies to survive an infection: fever and hypothermia (Romanovsky & Székely 1998, Rakus et al. 2017). The increase in body temperature (fever) is the first and most widespread mechanism to enhance the animal immune response during disease. Fever, or febrile response (*sensu* Romanovsky et al. 2005), has been known in warm-blooded animals since Hippocratic times (Atkins 1982) but was only identified in ectothermic animals about four decades ago (Kluger 1979).

Lizards were the first ectothermic vertebrates reported to show behavioral fever (Vaughn et al. 1974, Kluger et al. 1975, Bernheim & Kluger 1976). Fever was later confirmed in fishes (Reynolds et al. 1976, Covert & Reynolds 1977), amphibians (Casterlin & Reynolds 1977a, Kluger 1977), turtles (Monagas & Gatten 1983), and snakes (Burns et al. 1996). In invertebrates, it was first reported for crayfish (Casterlin & Reynolds 1977b), followed by other groups such as insects (Bronstein & Conner 1984, Stahlschmidt & Adamo 2013).

Behavioral fever amplifies the innate immune response increasing host survival (Kluger 1986, Elliot et al. 2005, Boltaña et al. 2013). However, its physiological demands compete for resources with other activities such as reproduction (French & Moore 2008) and growth (Uller et al. 2006). Even though fever occurs mostly when there are no immediate threats of a substantial energy deficit (Romanovsky & Székely 1998), it may not always be beneficial to the individual as it can cause immunopathologies in host tissues (Graham et al. 2005).

A more attenuated response involving hypometabolism and hypothermia (Romanovsky & Székely 1998, Ganeshan et al. 2019) incurs in lower physiological cost, while maintaining the overall host fitness (Smith & French 2017). Hypothermia was generally thought to represent a thermoregulatory failure of the animal immune system (Steiner & Romanovsky 2019). Hypothermia is now known in several species (Romanovsky et al. 2005), with evidence that it can be more advantageous than fever (Liu et al. 2012). Behavioral hypothermia is a widespread response to immune challenges in many lizards such as *Iguana iguana* (Deen & Hutchison 2001), *Anolis carolinensis* (Merchant et al. 2008), *Sceloporus occidentalis bocourtii* (Megía-Palma et al. 2020), and *Liolaemus sarmientoi,* one of the southernmost lizards of the world, that lives in the cold temperate environment of Patagonia, Argentina (Duran et al. 2020).

Therefore, fever and hypothermia can be seen as two thermometabolic responses to systemic inflammation, each representing a trade-off between the costs of thermoregulation (Hallman et al. 1990, Ortega et al. 1991, Merchant et al. 2008) and the benefits derived from the control of body temperature (Zamora-Camacho et al. 2015). Whereas behavioral fever ensures an active attack against the infectious agent, regulated hypothermia ensures the defense of the host's vital systems (Romanovsky et al. 2005, Bicego et al. 2007).

Moreover, animal strategies to fight diseases are generally context specific (Viney et al. 2005, Smith & French 2017), including the stage of the life cycle of the host. Although it has been known for almost a century that the immune system of newborns differs in many ways from that that of adults, the impact of the life stage at the moment of infection (Fedson 2018) remains under-explored. Pioneering research of Glenny et al. (1925) in Guinea pigs, followed by work of Barr et al. (1953), Howie et al. (1953), and Kerr & Robertson (1954) on lambs and calves, demonstrated the lack of immune response to intramuscular injection of antigens in newborns. Thus, neonatal immune T-cells develop tolerance when exposed to antigens, which seems to be regulated by the environment in which T-cells develop early in life (Ridge et al. 1996), although the exact mechanism still needs to be investigated (Gensollen et al. 2016). Lipopolysacharide (LPS), a component of the outer membrane of Gram-negative bacteria, is a potent endotoxin capable of activating the vertebrate immune system. Pre-pubertal mice challenged with LPS exhibited, along with improved survival, a higher percentage of weight loss compared to post-pubertal mice (Joachim et al. 2017), evidencing differences according to the life stage of the host at the moment of infection.

In the present study, we characterize the thermal biology and the health status of a natural population of *Liolaemus kingii*. In addition, we explored the effects of LPS exposure on the preferred body temperature (T_p) and on the body condition of both adults and newborns in the laboratory. We hypothesize that the immune challenge will affect behavioral thermoregulation and body condition, and that the effects in newborns will differ from

that in adults. We predict that in response to an LPS challenge, adults will select higher T_p than non-challenged individuals and maintain their body condition, whereas newborns would compromise their body condition at expenses of maintaining high body temperatures to enhance the innate immune response.

MATERIALS AND METHODS

Species and collection area

The genus *Liolaemus* has a wide range, extending north to the Andes of Peru and south to Tierra del Fuego in Argentina and Chile (from 10°S to 54°30'S, and from sea level to 5000 m above sea level (masl); Schulte et al. 2000, Aparicio & Ocampo 2010). *Liolaemus* species show great adaptive plasticity in their physiological responses to a high diversity of environments and climates (Labra et al. 2009, Ibargüengoytía et al. 2010). *Liolaemus kingii* is abundant in southwestern Chubut Province and throughout Santa Cruz Province (Argentina; Breitman et al. 2014), and is classified as "not threatened" (Abdala et al. 2012, Breitman et al. 2014). This species is considered a robust lizard with a mean snout-vent length (SVL) of approximately 100 mm, an insectivorous diet, and a viviparous mode of reproduction (Ibargüengoytía et al. 2002, Scolaro 2005). Field work was carried out in February, 2017, in western Chubut Province (43°S, 70°W; 630 m asl), an area characterized by low mean annual temperatures, and great daily and seasonal thermal amplitude (Paruelo et al. 1998). This site features sparse sub-bush vegetation (*Verbena*, *Nassauvia*, *Chuquiraga spp.*) and scarce pastures dominated by genera *Stipa* and *Poa* (Scolaro 2005).

A total of 28 adult individuals (21 males and 7 pregnant females) were captured by hand or loop when they were active between 1000 and 2000 h. Immediately after capture, the body temperature (T_b) was measured (TES 1303, ± 0.03 ºC digital thermometer) using a thermocouple (TES TP-K01, 1.62 mm diameter) inserted approximately 10 mm inside the cloaca. Body temperatures were taken by grasping the body from the neck to the hips with three fingertips within 10-sec of capture to prevent heat transfer from the operator's hands. Lizards were kept in individual cloth bags in a thermally isolated terrarium to maintain a stable temperature and were provided with water once a day until they reached the laboratory. Captures were authorized by the Wildlife Service of the Province of Chubut (Permit # 03588/16 MP; Disposition # 48/08). We followed the Guidelines for the Use of Live Amphibians and Reptiles in Field and Laboratory Research of the American Society of Ichthyologists and Herpetologists (ASIH), the Herpetologists' League (HL), and the Society for the Study of Amphibians and Reptiles (SSAR), as well as the regulations detailed in Argentinean National Law # 14346.

Laboratory conditions and experiments

Assessment of health status and reproductive state of lizards

Lizards were brought to the laboratory (a greenhouse with natural light and automatic control of ambient temperature and ventilation) and were examined to detect injuries or ectoparasites such as mites, and to determine the tail status (intact, cut, or regenerated). We recorded SVL (digital gauge ± 0.02 mm, CA-01, Lee Tools, Guangzhou, Guangdong, China), body mass (BM, 100 g spring scale ± 0.5 g; Pesola AG, Baar, Switzerland), and sex (males were distinguished by precloacal pores). Female reproductive status (pregnant) was detected by palpation. All pregnant females gave birth in laboratory. The SVL and BM of the neonates were measured immediately after birth. These

newborns were used in the lipopolysaccharide immune challenge described below.

Lizard maintenance

Lizards were housed individually in open-top fibreboard terraria (100 × 20 × 17 cm) supplied with a refuge, water *ad libitum* and a 75-W incandescent bulb energized daily 1000h to 1700h. They were kept in the same terraria while conducting experiments in the laboratory. They were fed daily with mealworm larvae (*Tenebrio molitor*) dusted with vitamins and calcium (ReptoCal, Tetrafauna™), and were observed to ensure they were feeding.

Preferred body temperature (T_p)

The first thermoregulation trial (initial T_p) was executed on the first day in the laboratory as soon as we returned from the field (2 to 3 days after capture). A thermal gradient (17 - 40 °C) was constructed with the 75-W incandescent bulb placed over one end of each terrarium. Thermoregulation trials were performed during the hours of activity in their natural environment (1000 to 2000 h). Body temperature of each lizard was measured using an ultra-thin (0.08 mm) thermocouple fixed to the abdomen with hypoallergenic adhesive tape, which does not alter locomotory ability nor does it interfere with defecation during the experiments. Thermocouples were connected to a Data Acquisition Module (USB-TC08, OMEGA) to record body temperature every 10-sec for approximately a 4-hour period.

Mean preferred body temperatures, lower and upper boundaries of the 50% interquartile of T_p (T_{set}), and maximum ($T_{p\ max}$) and minimum preferred temperature ($T_{p\ min}$) were calculated for each lizard ($N_{\ males}$ = 21) following the methodologies of Ibargüengoytía et al. (2010) and Medina et al. (2011).

To determine whether the thermocouple on the abdomen is a good proxy of the core temperature, we performed a calibration experiment by placing a lizard in a terrarium (15 × 20 × 20 cm) provided with an infrared 150-W lamp. We adhered a thermocouple to its abdomen and inserted another one approximately 10-mm inside its cloaca, both fixed in place with hypoallergenic adhesive tape. During a 2-hour test, the lamp was moved to different heights to generate different temperatures throughout the calibration, while body temperature was recorded every 2 min. Thermocouples placed in the abdomen and within the cloaca recorded similar T_bs (Simple Linear Regression, $F_{1,47}$ = 5440.99, P < 0.001, R^2 = 0.992, lower 0.950 and upper 1.003 confidence interval boundaries).

Assessment of local inflammatory response: phytohemagglutinin (PHA) skin-swelling assay

The delayed-type hypersensitivity (DTH) test is a cell-mediated immune responsiveness assessment tool broadly used in animals such as birds (Smits et al. 1999), lizards (Svensson et al. 2001) and amphibians (Clulow et al. 2015). Subcutaneous injection of phytohemagglutinin (PHA) triggers a series of physiological reactions that produce local inflammation at the injection site, related to the increase in cellular activity (Chandra & Newberne 1977, Roitt et al. 1996, Clulow et al. 2015). This reaction increases the proliferation of polyclonal T-cells, causing an inflammation which is used as a standard index of immunocompetence (Zimmerman et al. 2010).

The PHA test was performed only in males 2 days after the initial-T_p trial to avoid interfering with the thermoregulation behavior. The thicknesses of the right and left posterior sole pad were measured with a digital thickness gauge (constant pressure Mitutoyo 700-118-20 CAL, ± 0.01 mm). Following the methodology of Huyghe et al. (2010), immediately after these measures,

20 µl of PHA solution (4 mg of PHA 0.1 mg L-8754 Sigma-Aldrich, St. Louis, MO, USA per ml of PBS) was injected into the posterior right sole pad (treatment) and the same volume of phosphate buffered saline (PBS) was injected into the left posterior sole pad (control). The thickness of the right and left posterior sole pads were measured 2h, 20h and 48h after injections. The swelling in response to PHA (treatment) or PBS (control) was estimated from the proportional increase in thickness in the posterior sole pads before and after the injection. The PHA test does not cause any negative health effects and the reaction stimulated by the PHA disappears within 48h after the injection, as has been previously shown in other lizard species (Cabido 2009, Iglesias-Carrasco et al. 2016, Duran et al. 2020).

Lipopolysaccharide (LPS) immune challenge

Following the completion of the PHA test, lizards were acclimatized for 20 days under laboratory conditions before starting the LPS immune challenge. The acclimation period offered all lizards similar environmental and feeding conditions and prevented possible interactions with the initial-T_p trial and the PHA test. During this time, six females gave birth to three offspring each, except one gave birth to two. The newborns were also supplied with a refuge and water *ad libitum*, and were fed with mealworm larvae (*Tenebrio molitor*) once a day.

After the acclimation period, three out of the 21 adult males and six out of the 20 newborns were randomly selected and set aside for another study. Thus, 18 adult males were randomly split into two groups. One group (treatment, N = 9) was injected intra-peritoneally with *Escherichia coli* 0111:B4 LPS (L2630, Sigma-Aldrich, St. Louis, MO, USA; 2.5 µg endotoxin/g of body mass, dissolved to a concentration of 0.5 mg/ml in sterile saline). The other group (control, N = 9) was injected intra-peritoneally with sterile PBS to account for possible effects of either or both injections, and handling. The dose supplied was calculated based on each individual BM following previous studies on lizards with body sizes similar to *L. kingii* (Deen & Hutchison 2001, Uller et al. 2006, López et al. 2009, Duran et al. 2020). Similarly, the 14 newborns were randomly assigned to either a treatment group (N = 7, LPS) or a control group (N = 7, PBS).

Five thermoregulation trials were performed to determine the possible variation of T_p with time: the day before the injection (24h before); and 2h (2h Post), 24h (24h Post), 48h (48h Post), and 72h (72h Post) post-injection. Following the same methodology used to obtain the initial-T_p, these trials lasted 6 hours per day and were performed over successive days from 1000 to 1600 h to simulate part of the time in which lizards are active in their natural environment. We calculated the daily mean $T_{p\,i}$ for each individual i (24h before and 2h, 24h, 48h, and 72h post-injection of LPS or PBS). Lizards were fed daily after the completion of each thermoregulation trial.

Statistical analyses

We used the statistical software programs Sigma Plot 11.0® and R (R Core Team 2021). The body condition (BC) was estimated calculating the scaled mass index ($\widehat{M}_i$) of each individual as an estimator of stored (fat) energy (*sensu* Peig & Green 2009, 2010) as:

$$\widehat{M}_i = BM_i \times \left[SVL_0 / SVL_i\right]^{b^{SMA}}$$

where BM_i and SVL_i are the mass and SVL of the individual, SVL_0 is the arithmetic mean SVL of the population, and b^{SMA} exponent is the standardized major axis slope from the regression of ln(BM) on ln(SVL) for the population (Peig & Green 2009, 2010). The b^{SMA}

exponent was calculated using the package *lmodel2* (Legendre 2015) in R (R Core Team 2021). The BC of adult males was calculated on the first day in the laboratory ($BC_{1\ males}$), at the end of the acclimation period ($BC_{2\ males}$), and on the last day of the LPS challenge ($BC_{3\ males}$). The BC in newborns was calculated at birth ($BC_{1\ newborns}$), right before ($BC_{2\ newborns}$) and at the end ($BC_{3\ newborns}$) of the LPS challenge.

We used a Paired *t*-test and One-Way Repeated Measures Analysis of Variance (One-Way RM ANOVA) to detect changes in body condition over time in the laboratory in adult males and newborns. The dependence between the inflammatory response to PHA injection and initial body condition ($BC_{1\ males}$) were analyzed by simple regressions. We used a *t*-test to compare BC and T_p before the experiments between the LPS and PBS groups in adult males and also in newborns.

We applied a linear mixed modeling approach to evaluate the effects of treatment on the thickness of sole pads and T_p over time using the package *lme4* (Bates et al. 2015) and, for *post hoc* tests, the package *emmeans* (Russell 2019) in R software (R Core Team 2021). For the phytohemagglutinin skin-swelling assay, thickness was the response variable, treatment (PHA or PBS), time and their interaction were the fixed effects, and individual identity was the random variable. For the LPS immune challenge, T_p was the response variable, treatment (LPS or PBS), time, age and their interaction were the fixed effects, and individual identity was the random variable. The statistical significance of the individual identity in both analyses was assessed by likelihood ratio tests based on restricted maximum likelihood (PHA analysis: $\chi^2_{[1]}$ = 46.99, -2018344190 $P < 0.001$; LPS analysis: $\chi^2_{[1]}$ = -311.04, -2018344190 $P < 0.05$) using the function *rand* of the *lmerTest* package (Kuznetsova et al. 2017). In both analyses, -2018344190 *P*-values for fixed effects were obtained using type III sums of squares based on Satterthwaite approximation for denominator degrees of freedom (Kuznetsova et al. 2017). In both cases, we started our analyses with a global model that included all variables and their interactions. To avoid overfitting, because our sample size was small, model comparisons were based on the corrected Akaike Information Criterion (AICc) and were conducted with the function *dredge* of *MuMIn* package in R (Legendre & Legendre 1998, Burnham & Anderson 2002). According to this function, models are ranked according to their AICc values, and the model with the lowest AICc is considered the best, whereas those with an AICc value difference less than 2 with the AICc value of the best model are considered models with substantial support (Burnham & Anderson 2002).

Assumptions of normality and homogeneity of variance were tested with the Shapiro-Wilk's test and with the Levene test, respectively. When the assumptions of normality and/or homogeneity of variance were not met, we used the corresponding non-parametric test, such as Mann–Whitney rank–sum tests. Means are given with ± 1 standard error (SE).

RESULTS

Field body temperatures (T_b) and initial preferred body temperatures (initial-T_p)

The mean T_b for adult males was 30.61 ± 0.54 °C (27.30 - 36.50 °C, $N = 21$). The mean initial-T_p calculated in the laboratory 2 or 3 days after capture and before the acclimation period was 34.93 ± 0.31 °C ($T_{p\ max}$ = 39.43 ± 0.29 °C and $T_{p\ min}$ = 30.41 ± 0.57 °C), with a set-point of T_p (T_{set}) ranging between 33.60 to 36.43 °C ($N = 21$). The T_b for adult males was significantly lower than the selected T_p in laboratory (*t*-test, t_{40} = -4.551,

$P < 0.001$). Since all newborns were born in laboratory, we did not have equivalent initial-T_p data for them.

Health status of individuals

We observed no injuries or ectoparasites in the captured lizards and only 3 males had a regenerated tail. Mean values of the morphological variables (SVL and BM) in adult males, pregnant females and newborns, as well as the mean values of the body condition (BC) in adult males and newborns are presented in Table I.

The BC of adult males (used for LPS experimentation) did not change in the laboratory during acclimation ($BC_{1\ males}$ *vs* $BC_{2\ males}$; Paired *t*-test, $t_{17} = 0.495$, $P = 0.627$, $N = 18$). Newborns did not change their BC (post-natal compared to onset of LPS-experiment; $BC_{1\ newborn}$ *vs* $BC_{2\ newborn}$; Paired *t*-test, $t_{13} = 1.916$, $P = 0.078$, $N = 14$).

Phytohemagglutinin (PHA) skin-swelling assay.

The inflammatory response to PHA injection did not show significant associations with body condition ($BC_{1\ males}$) before injection (Simple Linear Regression: $F_{1,20} = 0.0002$, $P = 0.989$), nor 2h after (Linear Regression: $F_{1,20} = 0.071$, $P = 0.794$), 20h after (Linear Regression: $F_{1,20} = 0.254$, $P = 0.620$) or 48h after PHA injections (Linear Regression: $F_{1,20} = 1.315$, $P = 0.266$).

The comparison of right versus left sole pad thicknesses over time in males showed a significant effect of treatment factor (PHA *vs* PBS), time factor (prior injections *vs* 2h, 20h, and 48h post-injection) and the interaction of treatment *x* time (Table II). Right and left sole pad thicknesses did not differ prior to injections (Tukey test, $t_{PHA\ vs\ PBS} = -1.225$, $P = 0.923$, $N = 21$), 2h afterwards ($t_{PHA\ vs\ PBS} = 0.895$, $P = 0.986$) or 48h after injection ($t_{PHA\ vs\ PBS} = 2.835$, $P = 0.094$; Fig. 1). However, the PHA treatment caused detectable thickening of 0.14 mm at 20h compared to the left sole pad at the same time (Tukey test, $t_{PHA\ vs\ PBS} = 3.936$, $P < 0.005$; Fig. 1). The comparison of the treated right-side sole pad over time showed they became 0.11 mm thicker at 2h and 0.13 mm thicker at 20h after injection (Tukey test, $t_{before\ vs\ 2h} = 3.110$, -2018344187 $P < 0.045$, $t_{before\ vs\ 20h} = 3.702$, $P < 0.005$, $N = 21$) but not at 48h after injection ($t_{before\ vs\ 48h} = 0.578$, $P = 0.999$; Fig. 1).

Effect of LPS on T_p and BC in males and newborns

The body condition ($BC_{2\ males}$ measured before the LPS-PBS experiment) and the initial-T_p were not different between the males assigned to the treatment (LPS) and control (PBS) groups (*t*-test $_{BC}$, $t_{16} = 0.125$, $P = 0.902$; *t*-test $_{initial\text{-}Tp}$, $t_{16} = 1.188$, $P = 0.252$). The newborns assigned to the treatment (LPS) and control (PBS) groups did not differ in neither the BC_2 before the experiment (*t*-test, $t_{12} = 0.349$, $P = 0.733$) nor the T_p obtained 24h before the injections (Mann–Whitney test, $U = 17.000$, $P = 0.371$).

Table I. The mean ± standard error (SE), range, and sample size (*N*) of body mass (BM, g) and snout-vent length (SVL, mm) for adult males, pregnant females, and newborns, and body condition the first day in the laboratory (BC_1) for adult males and newborns of *Liolaemus kingii* are presented.

L. kingii	*N*	BM ± SE (range)	*N*	SVL ± SE (range)	*N*	BC_1
Adult males	21	7.77 ± 0.39 (4.90-11.60)	21	67.37 ± 1.25 (58.24-81.38)	18	7.77 ± 0.23 (6.28-9.91)
Pregnant females	7	8.32 ± 0.35 (7.20-9.70)	7	66.45 ± 1.41 (62.26-72.47)		
Newborns	20	1.01 ± 0.02 (0.81-1.25)	20	31.94 ± 0.23 (30.10-34.50)	14	1.02 ± 0.09 (0.82-1.18)

There were significant main effects of treatment (LPS *vs* PBS) and time (24h before and 2h, 24h, 48h, and 72h post-injection), and their interaction in T_p (Table III). Individuals in the LPS-treatment had a mean T_p 2.69 °C higher than individuals of PBS-control at 2h after injection (Tukey test, $t_{\text{2h LPS vs 2h PBS}} = -4.006$, $P < 0.005$), but at all other times they did not show differences (Tukey test, $P > 0.05$). The comparison of the effect of either LPS or PBS on T_p over time showed that individuals of the LPS-treatment did not show differences and maintained their T_p over time (Tukey test, $P > 0.05$; Fig. 2). In contrast, individuals of the PBS-control decreased 3.09 °C the T_p 2h post-injection (Tukey test, $t_{\text{24h before vs 2h post}} = -5.088$, $P < 0.001$). And then recovery their T_p values, increasing 1.99 °C T_p 24h post-injection (Tukey test, $t_{\text{2h post vs 24h post}} = -3.279$, $P < 0.043$), and 2.48 °C T_p 48h post-injection (Tukey test, $t_{\text{2h post vs 48h post}} = -4.006$, $P < 0.005$), and 2.89 °C T_p 72h post-injection (Tukey test, $t_{\text{2h post vs 72h post}} = -4.635$, $P < 0.005$; Fig. 2).

The body condition did not vary with time in males injected with LPS nor PBS (Table IV). There were also no differences between the two groups (LPS *vs* PBS) in the body condition after experiments ($BC_{\text{3 males}}$: *t*-test, $t_{16} = 0.211$, $P = 0.835$). On the other hand, the body condition of LPS-challenged newborns decreased with time; in particular, $BC_{\text{2 newborn}}$ was higher than $BC_{\text{3 newborn}}$ (Table IV). Newborns of the control group showed no change in BC during the experiments (Table IV).

Table II. Significance of the fixed effects of the best generalized mixed model of the phytohemagglutinin skin swelling-assay over time (prior injections and 2h, 20h, and 48h post-injection) between treatments (PHA and PBS) for adult males of *Liolaemus kingii*.

	SS	MS	Num	DenDF	F value	Pr(>F)
Hours	0.34350	0.114499	3	140	9.1088	≤0.001
Treatment	0.13037	0.130371	1	140	10.3715	≤0.005
Treatment x hours	0.19434	0.064779	3	140	5.1534	≤0.005

Note: SS= sum of squares, MS= mean squares, DenDF= degrees of freedom of the denominator are indicated.

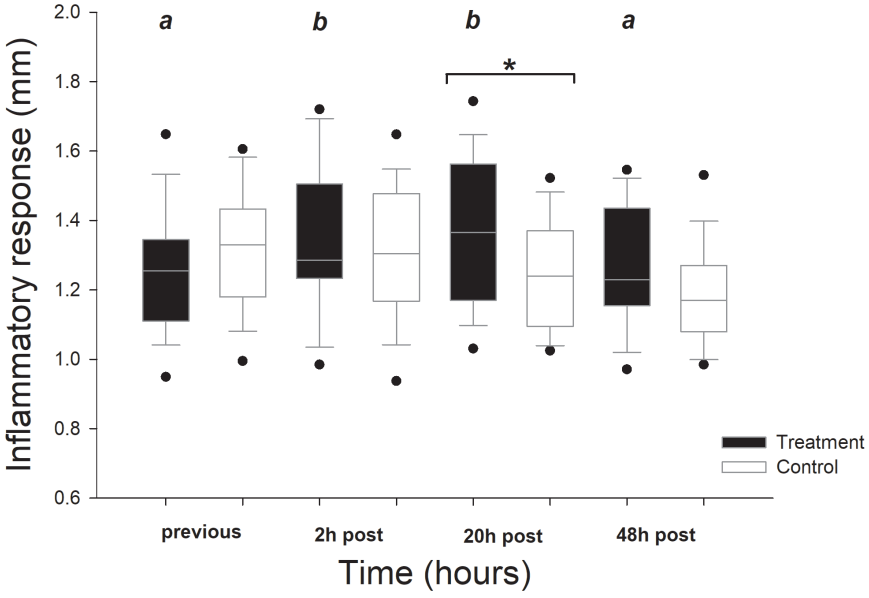

Figure 1. Box plot of the effect of the injection on the right posterior sole pad (PHA, treatment; black box) and on the left posterior sole pad (PBS, control; white box) in adult males of *Liolaemus kingii* performed to analyze the inflammatory responses during the experiment. The medians, 5%, 25%, 75% and 95% percentiles of the frequencies are indicated. Asterisks indicate significant differences between the treatment and control groups ($P < 0.05$). The letters indicate the differences among the four treatment groups: previous, 2h, 20h, and 48h after the injection of PHA ($P < 0.05$).

Table III. Significance of the fixed effects of the best generalized mixed model of the preferred body temperature (T_p) over time (24h before and 2h, 24h, 48h, and 72h post-injection) between treatments (LPS and PBS) for individuals of *Liolaemus kingii*.

	SS	MS	Num	DenDF	F value	Pr(>F)
Time	48.796	12.199	4	120	4.1260	≤0.005
Treatment	21.387	21.387	1	30	7.2335	≤0.05
Treatment x hours interaction	43.630	10.908	4	120	3.6892	≤0.01

Note: SS= sum of squares, MS= mean squares, DenDF= degrees of freedom of the denominator are indicated.

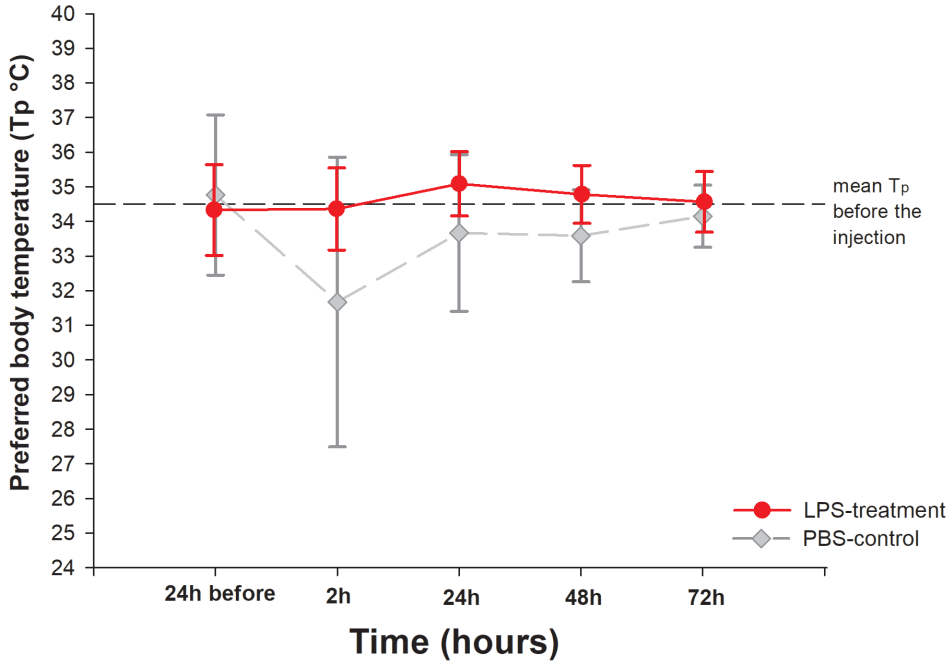

Figure 2. Mean preferred body temperatures (T_p) and their standard deviations during LPS-treatment or PBS-control over time (24h before and 2h, 24h, 48h, and 72h post-injection) for individuals of *Liolaemus kingii*. Dashed line indicates the mean T_p before injection.

DISCUSSION

The absence of injuries or ectoparasites, as well as the homogeneous body condition observed in the captured individuals, suggest that the wild population is in good health. In addition, the PHA assay in adult males showed a temporary and localized inflammatory response related to a stimulation of T-cell proliferation (Roitt et al. 1996, Martin et al. 2006), thus confirming the immunocompetence of *L. kingii* as described in other lizards (Cabido 2009, Iglesias-Carrasco et al. 2016, Duran et al. 2020).

Our results on thermoregulation are in agreement with an early review of the topic, which considered that most physiological processes progress optimally near T_p (Dawson 1975), including immune responses for which temperatures above T_p, not only present a challenge to enzymatic function, but also were shown to reduce non-specific leukocyte activity and antibody titers in several reptile species (Dawson 1975, Zimmerman et al. 2010). By providing controlled environments with thermal gradients, LPS-challenged *L. kingii* behaviorally thermoregulated within the set point of T_p more precisely, and at higher temperatures, than sham-challenged individuals (Fig. 3a). This included the LPS-challenged newborns which, as observed in some juvenile iguanas (*Iguana iguana*; Deen & Hutchison 2001, < 1 yr old), were able to develop different T_p than the

Table IV. The body condition in adult males and newborns of *Liolaemus kingii*, treated (LPS) and control (PBS), the first day in the laboratory (BC_1), right before (BC_2), and at the end (BC_3) of the immune challenge, are presented and compared.

L. kingii	*N*	BC_1	BC_2	BC_3	One-Way RM ANOVA	
Adult males (LPS)	9	7.77 ± 1.16	7.66 ± 0.94	7.71 ± 0.83	$F_{8,26}$ = 0.195	P = 0.825
Adult males (Control)	9	7.76 ± 0.81	7.71 ± 0.49	7.78 ± 0.50	$F_{8,26}$ = 0.052	P = 0.949
Newborns (LPS)	7	1.03 ± 0.04	0.96 ± 0.07	0.86 ± 0.07	$F_{6,20}$ = 11.406	P = 0.002*****
Newborns (Control)	7	1.01 ± 0.13	0.98 ± 0.11	0.92 ± 0.13	$F_{6,20}$ = 2.444	P = 0.129

Note: (*) posteriori test. Holm-Sidak method, $t_{BC2\ vs\ BC3}$ = 2.713, P = 0.019, $t_{BC1\ vs\ BC2}$ = 2.048, P = 0.063.

sham-control group. Thus, our study found that *L. kingii* newborns have the capacity to raise and maintain their temperature within a narrow range as a response to the bacterial pyrogen LPS (Fig. 3b). However, little is known about the characteristics of the immune responses in newborn reptiles (Brown & Shine 2016) or the changes in physiology and behavior in the face of an immune challenge. In mammals, for instance, newborns were unable to develop fever physiologically in response to bacterial pyrogens but did so behaviorally when they were provided with a thermal gradient (behavioral fever; Satinoff et al. 1976, Kleitman & Satinoff 1981).

Most males and some newborns of the sham-challenged group significantly decreased their T_p 2h after the PBS injection. Inflammatory processes, both sterile and infectious, occur after activation of toll-like receptors (TLRs; Beutler 2004) through the recognition of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) on leukocytes (Nourshargh & Alon 2014). Neutrophils constitute the first line of defense during an infection or tissue injury by regulating the adaptive immune response through B-cell and T-cell activation (Hidalgo et al. 2019). Research on sterile tissue injury in mice has shown that accumulation of neutrophils at the damage site occurs during the first hour, with those present at the injury site forming clusters (Ng et al. 2011). On the contrary, the injection of LPS activates recognition of PAMPs by TLRs with neutrophils continuing to migrate to the infected site producing dynamic clusters showing a swarming behavior (Chtanova et al. 2008). Those studies demonstrated that DAMPs as well as PAMPs regulate inflammation controlling neutrophil infiltration (Ng et al. 2011). Therefore, in the absence of pathogens, early neutrophil infiltration can activate vasodilation mechanisms such as production of nitric oxide (Skovgaard et al. 2005), thus inducing short-term hypothermia as it was observed here in the behavioral thermoregulation of sham-challenged *L. kingii*.

Maintaining body temperature within the set point of T_p can be advantageous to avoid the costs of thermoregulation. Most importantly, metabolic costs include increasing metabolic rates about 10% every 1 °C increment (Kluger 1979, Boltaña et al. 2013), and consequently the depletion of fat bodies (Huey 1974, Adolph & Porter 1993). Other costs include greater exposure to predators as they spend more time outside shelters for thermoregulation (Herczeg et al. 2008, Zamora-Camacho et al. 2016), leaving less time to allocate to reproduction, feeding, social interactions, and other functions. Although there are examples of lizard species reaching up to 2 °C above the mean T_p in response to LPS exposures, *e.g. Dipsosaurus dorsalis* (Vaughn et al. 1974), *Callopistes maculatus* (Hallman et al. 1990),

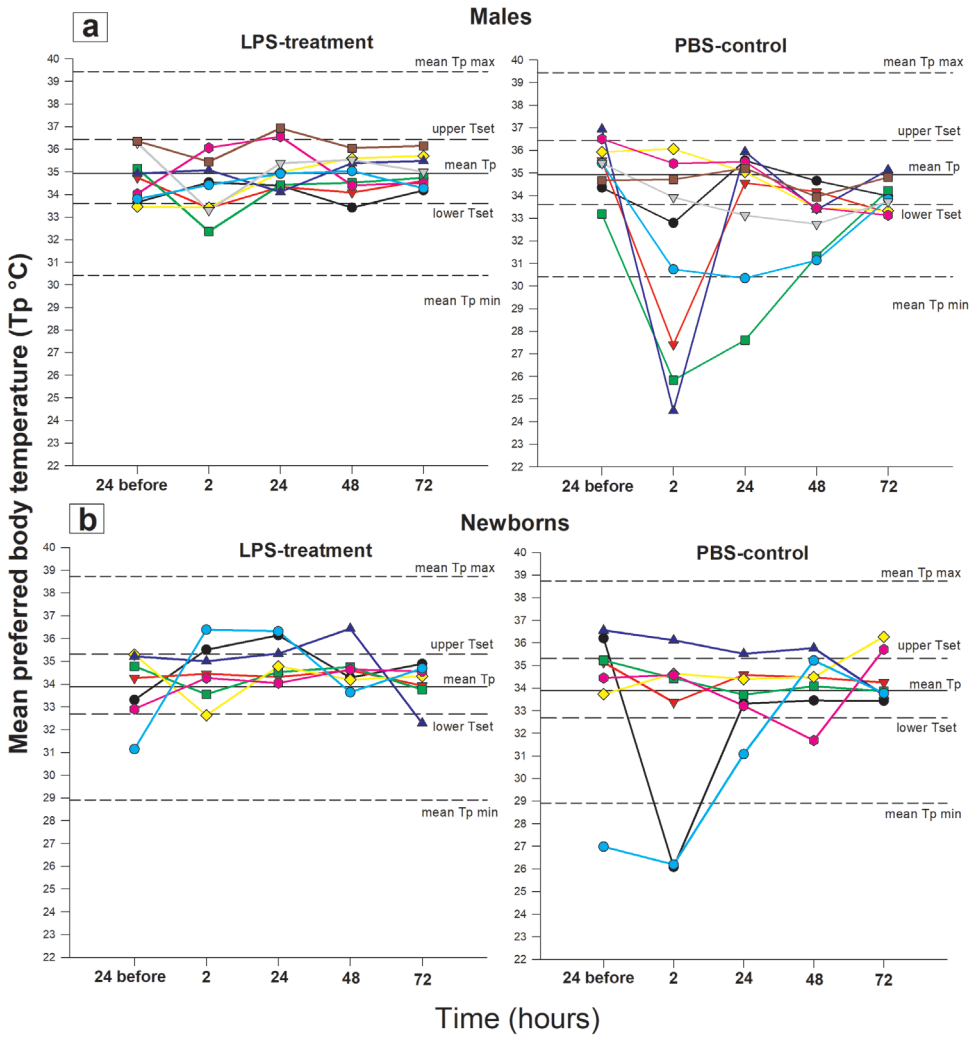

Figure 3. Mean preferred body temperatures (T_p) of each of the treated individuals (LPS, treatment; on the left) and control individuals (PBS, control; on the right) over time (h) for males (a) and newborns (b) of *Liolaemus kingii*. The mean T_p, upper T_{set}, lower T_{set}, mean $T_{p\ max}$, and mean $T_{p\ min}$ recorded before the trials are shown.

Agama agama (Ramos et al. 1993), and *Oplurus cyclurus* (Muchlinski et al. 1995); other species do not increase T_b nor develop hypothermia after the injection of a pyrogen, as shown in the armadillo lizard *Cordylus cataphractus* (Laburn et al. 1981), the lizard *Anolis equestris* (Muchlinski et al. 1995), and the alpine lizard *Psammodromus algirus* (Zamora-Camacho et al. 2016). http://3cn.cima.fcen.uba.ar/index1.php. In the present study, LPS-challenged lizards raised their body temperature but maintained it within the limits of their T_{set}, avoiding maximum critical temperatures ($CT_{max;}$ Fig. 3). In this way, lizards under a pathogen threat may enhance their immune response, while at the same time maintaining enzymatic functions, locomotor performance and, ultimately, securing their survival (Angilletta 2009).

Changes in body temperature of a few degrees °C above normothermia as a consequence of pyrogenic infection have a significant energy cost (Sherman & Stephens 1998). Adult males did not show significant changes in BC while in captivity (from capture to 72h after the LPS experiment); nor did BC differ between control and treatment lizards. On the other hand, newborns treated with LPS (unlike controls) showed a significant decrease in BC over time. This indicates an allocation of fat reserves to the immune response, pointing out

the newborns' vulnerability to pathogens and the consequent selective pressure that pathogens exert on natural populations. Present results confirm our prediction since LPS-challenged newborns seem to have compromised their body condition at expenses of maintaining body temperatures within a narrow range of T_{set}.

Environmental constraints on thermoregulation seem to play a key role in the immune responses of *Liolaemus*. The only study that focused on thermoregulatory responses to LPS in *Liolaemus* genus, has been carried out in a phylogenetically close related species *L. sarmientoi* (Duran et al. 2020). Both *L. kingii* and *L. sarmientoi* demonstrated a large gap between the T_b they can attain in the field, and the T_p obtained in a thermal gradient in the laboratory. *Liolaemus kingii* inhabits milder environments (air temperature 10.28 °C) than the southernmost *L. sarmientoi* (8.64 °C) (mean air temperatures during the activity period of lizards, September to April, were obtained from CONICET historical data registered for studies of global warming for Argentina, 3CN database, http://3cn.cima.fcen.uba.ar/index1.php from 1960 to 2010). Therefore, environmental differences appear to have shaped distinct evolutionary pathways for thermal responses to immune challenges: hypothermia in *L. sarmientoi* (Duran et al. 2020) or maintenance of high and stable T_p in *L. kingii*.

Environmental changes caused by anthropogenic impact, such as the use of the land for animal production or mineral extraction, as well as climate warming could trigger niche tracking, challenging resident populations with new pathogens threats requiring an acceleration of adaptive evolution for population viability. The present study shows the capacity of lizards to cope with immune challenges by means of behavioral thermoregulation, but also points out that such thermoregulatory demands can be detrimental to the body condition of newborns.

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Fernando Duran conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft. Jorgelina M. Boretto conceived and designed the experiments, performed the experiments, authored or reviewed drafts of the paper, and approved the final draft. Leandro A. Becker authored or reviewed drafts of the paper, approved the final draft. Nora R. Ibargüengoytía conceived and designed the experiments, performed the experiments, authored or reviewed drafts of the paper, approved the final draft.