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RESEARCH ARTICLE

Exposure to fluctuating temperatures leads to reduced immunity and to stress response in rattlesnakes

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ABSTRACT

Ectothermic organisms often experience considerable variation in their body temperature throughout the circadian cycle. However, studies focusing on the measurement of physiological traits are usually performed under constant temperature regimes. This mismatch between thermal exposure in the field and experimental conditions could act as a stressor agent, as physiological functions are strongly influenced by temperature. Herein, we asked the question whether constant thermal regimes would cause a stress response and impact the immunity of the South American rattlesnake (*Crotalus durissus*) when compared with a fluctuating thermal regime. We addressed this question by determining heterophil:lymphocyte (H:L) ratio, plasma bacteria-killing ability (BKA) and corticosterone (CORT) levels in snakes kept under a constant temperature regime at 30°C, and under a fluctuating regime that oscillated between 25°C at night and 35°C during the day. The experiments had a mirrored design, in which half of the snakes were subjected to a fluctuating-to-constant treatment, while the other half was exposed to a constant-to-fluctuating treatment. The shift from constant to fluctuating thermal regime was accompanied by an increase in plasma CORT levels, indicating the activation of a stress response. Exposure to a fluctuating thermal regime at the onset of the experiments induced a decrease in the BKA of rattlesnakes. H:L ratio was not affected by treatments and, therefore, the shift between thermal regimes seems to have acted as a low-intensity stressor. Our results suggest that removal from temperatures close to the snake's preferred body temperature triggers a stress response in rattlesnakes.

KEY WORDS: Thermal regime, Ectothermic, Snakes, Corticosterone, Innate immunity, Heterophil:lymphocyte ratio

INTRODUCTION

Environmental temperature is an important abiotic factor influencing living organisms, as it broadly affects behavioral and physiological traits (Huey and Stevenson, 1979; Kingsolver and Woods, 1997; Angilletta et al., 2002). The influence of environmental temperature on behavioral and physiological functions is particularly important in ectothermic animals, as their body temperature is primarily dependent on the thermal environment (Angilletta et al., 2002; Andrade, 2016). Thus, not surprisingly, the effects of temperature on a variety of physiological traits, such as metabolism (Dorcas et al., 2004; Gavira and Andrade, 2013; Andrade, 2016) and growth (Niehaus et al., 2012), have been extensively studied in ectothermic animals (Angilletta et al., 2002).

The influence of temperature on different physiological parameters is often tested by subjecting animals to constant temperature regimes over a period of many consecutive days (Secor, 2009; Andrade, 2016; Saxon et al., 2018). However, under natural conditions, the body temperature of ectothermic animals (Niehaus et al., 2012; Kingsolver et al., 2015; Colinet et al., 2015), snakes included (Tozetti and Martins, 2008; Gomes and Almeida-Santos, 2012; Andrade, 2016; Brischoux et al., 2016), may exhibit a considerable degree of variation along the circadian cycle. Thus, subjecting ectothermic animals to constant temperature regimes during experiments can be quite different from what they usually experience under natural conditions. This discrepancy may lead to bias in data acquisition and hampers ecological interpretations. Indeed, differences in thermal regime are known to affect metabolism (Gavira and Andrade, 2013; Stahlschmidt et al., 2015), growth rate (Niehaus et al., 2012; Kingsolver et al., 2015) and thermal tolerance (Zatsepina et al., 2000; Manenti et al., 2018). Therefore, it is possible that the absence of temperature fluctuations by denying a thermally variable environment or the possibility of thermoregulation could be perceived as a stressor by ectothermic animals (Niehaus et al., 2012; Andrade, 2016; Fabrício-Neto et al., 2019).

Exposure to stressful situations triggers the activation of the hypothalamic–hypophysis–adrenal/interrenal axis (HHA/HHI) in vertebrates, resulting in the secretion of glucocorticoids (GCs) into the bloodstream (Wingfield et al., 1998; Wingfield, 2013). The effects of this increase in GC levels can be adaptive or deleterious (Dickens et al., 2010; Lucas and French, 2012) depending on the concentration and temporal pattern (Martin, 2009; Dickens et al., 2010). A short-term increase in GCs may improve the immune response during the post-stress recovery (Dhabhar and McEwen, 1999; Sapolsky et al., 2000) and may also increase the mobilization of energy reserves (Durant et al., 2008; Preest and Cree, 2008). However, long-term exposure to stressors can lead to a chronic elevation of GC levels in the bloodstream, resulting in deleterious effects on reproduction, growth rates and the immune response (Guillette et al., 1995; Sapolsky et al., 2000; French et al., 2007a; Dickens et al., 2010).

In reptiles, changes in environmental temperature may also be associated with changes in GC levels (Dupoué et al., 2013; Jessop et al., 2016), which can in turn affect diverse physiological attributes, such as metabolism (DuRant et al., 2008; Preest and Cree, 2008), digestive performance (Bonnet et al., 2013), reproductive behavior (Brischoux et al., 2016) and immune response, e.g. plasma bacteria-killing ability (BKA) (Neuman-Lee et al., 2015). The release of GCs also leads to an increase in the ratio between circulating heterophils and lymphocytes (H:L ratio) (Davis and Maerz, 2008; Davis et al., 2008). Changes in GC and leukocyte

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profile in the bloodstream show different temporal dynamics in response to a stressor, such that an increase in H:L ratio usually requires longer exposure to the stressor agent (Seddon and Klukowski, 2012; Sparkman et al., 2014) or a higher intensity stress stimulus (Assis et al., 2015). The magnitude of the stress response in reptiles generally has a positive correlation with plasma levels of corticosterone (CORT), which is the main GC in this group (Romero, 2004; Lind et al., 2018), and can affect the immune response in a complex manner in different vertebrates (Martin, 2009). In snakes, such a stress response is known to be triggered by handling, restraint (Kreger and Mench, 1993; Schuett et al., 2004; Sykes and Klukowski, 2009), translocation (Heiken et al., 2016), water deprivation (Dupoué et al., 2014) and acute temperature changes (Dupoué et al., 2013). In this sense, the determination of H:L ratio, CORT levels and BKA may indicate the potential stress caused by exposure of snakes to different temperature regimes.

Accordingly, the aim of the present study was to investigate whether and how differences in thermal regimes may affect parameters associated with the stress and immune response in the South American rattlesnake (*C. durissus*). Our hypothesis is that the exposure of rattlesnakes to a constant thermal regime will act as a stressor as this species is known to experience a daily variation in body temperature of up to 10°C (Tozetti and Martins, 2008; Andrade, 2016). We determined plasma CORT levels and H:L ratio as stress indicators, while BKA was used as an immune parameter. We predicted that under a constant thermal regime, CORT levels will increase after acute exposure, H:L ratio will increase after chronic exposure, and BKA will increase after short-term exposure, but will decrease after longer periods of exposure. In contrast, we predicted that exposure to the fluctuating thermal regime will not affect the investigated variables.

MATERIALS AND METHODS

Animals

The South American rattlesnake (*Crotalus durissus* Linnaeus 1758) is a large-bodied member of the Viperidae family, with a wide geographical distribution, found mainly in the Brazilian Cerrado domain (Campbell and Lamar, 1989). *Crotalus durissus* experiences considerable variation in body temperature (Tozetti and Martins, 2008; Gomes and Almeida-Santos, 2012; Andrade, 2016), which makes this species a good candidate for studying the effect of different thermal regimes on physiological stress and innate immunity.

We used 24 adult individuals of *C. durissus* (15 males and 9 females) collected in the state of São Paulo, Brazil. The snakes were kept in captivity up to 2 years before experimentation and were maintained individually in transparent plastic boxes (480×270×133 mm, 8.6 l) with holes for ventilation, lined with corrugated cardboard and provided with a water bowl. Cages were kept in a room under natural photoperiod and with air temperature varying between 22 and 28°C, in the Laboratory of Animal Physiology, Universidade Estadual Paulista, Rio Claro, SP, Brazil. Snakes were fed with mice (*Mus musculus*) every 15 days, except during experimentation, in which case they were fasted for 15 days prior to the trials.

Permits for animal collection and maintenance were issued by ICMBIO (no. 22028-1 and 35081-3). The experiments were conducted with the approval of the Ethics and Animal Use Committee (protocol no. 6613/2016) of the Instituto de Biociências, Universidade Estadual de São Paulo, Rio Claro, SP, Brazil.

Experimental protocol

Snakes were assigned to one of two treatments: (1) constant-to-fluctuating (CF) and (2) fluctuating-to-constant (FC). The CF treatment comprised a 12 day period in which the temperature was kept constant at 30°C, followed by a change to a fluctuating thermal regime composed of 12 h:12 h thermoperiods set to 25°C (18:00 h to 06:00 h) and 35°C (06:00 h to 18:00 h), which lasted for another 12 days (Fig. 1A). The FC treatment replicated the duration, temperature and regime of the CF treatment, but the order of exposure to the constant and fluctuating regimes was switched (Fig. 1B). Snakes were randomly allocated to the experimental treatments, maintaining the proportion between males and females in both treatments (CF: 8 males and 4 females; FC: 7 males and 5 females). The constant temperature of 30°C was chosen for three reasons: (1) this temperature is near the preferred body temperature of *C. durissus* during activity (32.4°C; see Gavira, 2017); (2) in a previous study, we found that the same thermal regimes herein adopted influenced the resting metabolic rate of *C. durissus* (Fabrício-Neto et al., 2019); and (3) the fluctuating thermal regime simulated a daily amplitude variation of 10°C, which is known to be experienced by *C. durissus* in natural (Tozetti and Martins, 2008) and semi-captivity conditions (Andrade, 2016).

We measured the snakes' body mass (Mars AS5500C, ±0.01 g) and snout–vent length (SVL, ±0.1 cm) to calculate their body condition index (BCI), as BCI can affect GC levels in snakes (Lind and Beaupre, 2014). The index was represented by the standardized residuals of a linear regression between SVL and body mass (both data $\log_{10}$ transformed) (Brusch and DeNardo, 2017).

At the beginning of the experiments, snakes were transferred in their plastic boxes into a climatic chamber (ELETROlab, model 122FC), in which the environmental temperature was controlled, and photoperiod was set to a 12 h light (06:00 h to 18:00 h):12 h dark (18:00 h to 06:00 h) period. Humidity was monitored inside the plastic boxes and maintained between 70% and 80%. Blood samples (0.5 ml) were collected from the snakes on days 2, 10, 14 and 22 of the experimental treatments (Fig. 1). To avoid the effect of the circadian variation in CORT levels, all snakes were sampled in the same time period (between 12:00 h and 15:00 h). On blood collection days, the daily increment in temperature of the fluctuating thermal regime, set from 25 to 35°C, was kept at 30°C for a period of 6 h coincident with the sampling period, which ensured that the body temperature of the snakes, at the moment of blood collection, was the same as the constant treatment (Fabrício-Neto et al., 2019). All experiments were carried out between January and March of 2018.

Blood sampling and sample treatment

Blood samples were collected through cardiocentesis of non-anesthetized immobilized animals, with heparinized 22 G and 30 mm needles and 3 ml syringes (see Isaza et al., 2004; Dyer and Cervasio, 2008; Brown, 2010; Johnson, 2011; Bonnet et al., 2016). After restraint in acrylic tubes (Johnson, 2011), snakes were placed in dorsal recumbency and the heart was located through visual inspection of heartbeats. The heart was then immobilized between the fingers of the collector and, after the site had been cleaned with 70% alcohol, the needle was inserted one or two ventral scales below the heart, aiming to puncture the ventricle. In all cases, blood samples were collected in under 5.7 min (mean 3.36± 0.91 min, range 1.50–5.67 min) and by the same person (A.F.-N.).

Immediately after collection, two blood smears were prepared for the later analysis of the leukocyte profile (two drops of blood, 40–80 µl). The remaining blood sample was transferred to a test tube

Journal of Experimental Biology (2019) 222, jeb208645. doi:10.1242/jeb.208645

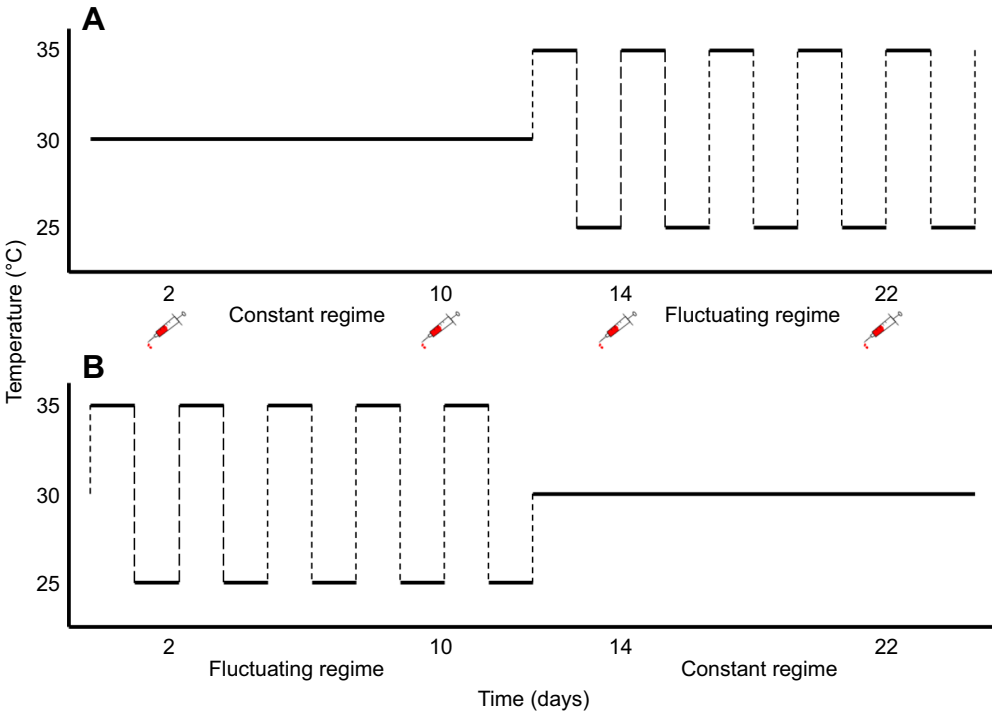

Fig. 1. Experimental treatments to which *Crotalus durissus* individuals were exposed. (A) The constant-to-fluctuating (CF) treatment, in which snakes were exposed to a constant thermal regime from days 1 to 12, and then to a fluctuating thermal regime from days 12 to 24. (B) The fluctuating-to-constant (FC) treatment, in which snakes were exposed to a fluctuating thermal regime from days 1 to 12, and then to a constant thermal regime from days 12 to 24. On blood collection days, the daily increment in temperature of the fluctuating thermal regime, set from 25 to 35°C, was kept at 30°C for a period of 6 h coincident with the sampling period, which ensured that the body temperature of the snakes, at the moment of blood collection, was the same as the constant treatment. Blood samples were collected after 2, 10, 14 and 22 days of exposure.

and centrifuged for 3 min at 4000 rpm. The centrifuged plasma was carefully harvested and divided in two aliquots, one for the CORT assay and one for the BKA assay. Plasma samples were frozen at −80°C until being transported (on dry ice) to the Department of Physiology of the University of São Paulo, São Paulo, SP, Brazil, for analysis, and this stocking period did not exceed 20 days.

CORT assay

CORT samples were initially extracted with ether (Barsotti et al., 2017; Madelaire et al., 2019): 10 µl of plasma was placed into a test tube and 3 ml of ethyl ether was added, and the resulting mixture was agitated for 30 s and then centrifuged at 4°C for 9 min at 1800 rpm. The tubes containing the samples were then held at −80°C for 9 min and the liquid phase transferred to a new test tube, which was kept in an evaporation chamber for removal of ether for approximately 48 h.

CORT was assessed through ELISA kits (Cayman Chemical, cat. no. 501320). Prior to the assay, samples were resuspended in buffer solution. Next, 50 µl of calibrator and sample was added to each well of the 96-well plate in duplicate; 50 µl of tracer and 50 µl of antiserum specific for CORT were added, and the plate was incubated overnight at 4°C. The plates were then washed 5 times with wash buffer (Cayman Chemical, cat. no. 400062) and 200 µl of Ellman reagent (Cayman Chemical, cat. no. 400050) was added to each well, and then incubated in an orbital shaker for 1 h 45 min. CORT concentration was determined with a spectrophotometer (Spectra Max 250, Molecular Devices) with wavelength 412 nm. The mean intra- and inter-assay coefficients of variation were 8.1% and 13.6%, respectively.

Validation of the use of the corticosterone assay kit (Cayman Chemical, cat. no. 500655) for rattlesnakes was conducted with a parallelism test, using a different set of plasma samples from a different group of *C. durissus* kept under the same general conditions as the experimental animals. Pooled plasma samples (250 µl) from males (n=5) and females (n=3) were mixed with 5 ml of ethyl ether for CORT extraction and processed following the same procedures mentioned above (Barsotti et al., 2017; Madelaire et al., 2019). These pooled samples were then resuspended and diluted in EIA buffer. The top standard of the CORT kit and the pooled plasma samples were used for serial dilution (1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 and 1:128 for standards; and 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024 and 1:2048 for pooled plasma samples) and assayed on the same plate. The standard and sample curves were plotted on x–y axes, and the 50% binding point was used as indicator of the best dilution factor to run the samples. The standard and sample curves were parallel (Fig. S1), corroborating the functionality of the assay for rattlesnakes. The best dilution factor for pooled plasma of *C. durissus* males and females was 1:256 and 1:512 (Fig. S1), respectively.

BKA assay

The BKA of *C. durissus* plasma was assessed *in vitro* by exposure of suspension solutions of *Escherichia coli*, a known pathogen presenting ecological relevance for snakes (Brusch and DeNardo, 2017), to rattlesnake plasma (modified from French and Neuman-Lee, 2012; de Assis et al., 2013). First, *E. coli* pellets (derived from ATCC 8739, MicroBioLogics, cat. no. 24311) were resuspended in 1 ml of sterile phosphate-buffered saline (PBS). Then, 100 µl of this solution was added to 5 ml of sterile trypticase broth (TSB broth), and this mixture was kept overnight at 37°C for growth of the bacteria. The next day, the concentration of bacteria was estimated from the optical density obtained using a plate spectrophotometry apparatus (Spectra Max 250, Molecular Devices; wavelength: 600 nm, 96-well ELISA plate) and serial dilutions with PBS were used to obtain the working concentration of microorganisms (1×10^6 microorganisms ml^{-1}). Plasma samples were diluted in a sterile PBS solution (10 µl of plasma in 190 µl of PBS), and 10 µl of the *E. coli* working solution (1×10^4 microorganisms ml^{-1}) was added. The mixture was agitated, partitioned and incubated for 1 h at 37°C, which is the optimal temperature for *E. coli* growth. To determine the positive control of each assay, 10 µl of the working solution of *E. coli* was added to 200 µl of sterile PBS, and the negative control was

Journal of Experimental Biology (2019) 222, jeb208645. doi:10.1242/jeb.208645

determined with 210 µl of sterile PBS, both incubated under the same conditions and in the same plate as the plasma samples. After incubation, 500 µl of TSB was added to all samples and the resulting solution agitated and transferred, in duplicate, to a 96-well culture plate (300 µl per well), which was then incubated at 37°C for 2 h. At the end of the incubation period, we started a sequence of four readings of the optical density of the samples in the spectrophotometer with intervals of 1 h. The antimicrobial activity of the plasma was calculated as: 1−(optical density of the sample/optical density of the positive control), which represents the proportion of dead microorganisms in the plasma containing solutions in relation to the positive control. For the calculations, we used the optical density data at the initial moment of the exponential growth phase of *E. coli* in the positive control, because this represents the maximum growth of the bacteria and, consequently, the highest index of bactericidal capacity of the samples.

Leukocyte profile

Blood smears were prepared by adding two drops of freshly collected blood onto glass microscopy slides in duplicate. The smears were allowed to dry up for 30 min, and then fixed with methyl alcohol for at least 2 min and stained with Giemsa's solution (20% diluted) for 25 min. The leukocyte profile was determined under optical microscopy with a magnification of 1000× (Olympus CX41) with the aid of immersion oil. Based on cell morphology (see Kindlovits et al., 2017), the first 100 leukocytes found in the smear were classified as monocytes, lymphocytes, heterophils, azurophils and basophils. To assess the H:L ratio, the number of heterophils was divided by the number of lymphocytes (Seddon and Klukowski, 2012; Sparkman et al., 2014; Assis et al., 2015).

Data treatment and statistical analysis

To test whether CORT levels, BKA and H:L ratio (dependent variables) were affected by exposure to the two treatments, time of exposure, sex of the animals, time for blood collection or BMI, we submitted our data to mixed linear modeling using the LMER function (package 'lme4'; Bates et al., 2015; http://CRAN.R-project.org/package=lme4), and a series of models was proposed to explain the results. The treatment to which the animals were exposed (categorical variable with two levels), the sex of the animals (categorical variable with two levels), the time of exposure to the treatment (categorical variable with four levels) and time for blood collection and BMI (continuous variables) were defined as fixed factors. Some models also included interaction terms between the fixed factors (Table 1). As our experimental design involved repeated measures, all proposed models included snake identification (ID) as a random factor. The models were submitted to Akaike information criterion (AIC) selection, in which each competitive model receives a ΔAIC and a weight, and the model with the lowest ΔAIC is selected as the most accurate to describe the results (package 'bbmle'; Bolker et al., 2009; http://CRAN.R-project.org/package=bbmle) (Burham and Anderson, 2002). We also considered Akaike weight in the explanatory power of the models with ΔAIC≤2.0 (Burham and Anderson, 2002). Further, we examined the significance of the fixed effects in the selected models [using the 'summary(model)' function in R], considering significant factors with *t*-values higher than 2 and smaller than −2.0 (Luke, 2017). As CORT levels can affect BKA (Assis et al., 2015) and H:L ratio (Davis et al., 2008; Sparkman et al., 2014), we also performed another modeling stage, including CORT concentration as a fixed factor, maintaining BKA and H:L ratio as dependent variables.

Table 1. Fitted models to assess the relationship between dependent and independent variables

Model	Description
1	DV∼Treatment+(1|ID)
2	DV∼Treatment+Sex+(1|ID)
3	DV∼Treatment+Time+(1|ID)
4	DV∼Treatment+Time+Sex+(1|ID)
5	DV∼Treatment×Sex+(1|ID)
6	DV∼Treatment×Time+(1|ID)
7	DV∼Treatment×Sex+Time+(1|ID)
8	DV∼Treatment×Time+Sex+(1|ID)
9	DV∼Treatment+Time×Sex+(1|ID)
10	DV∼Null model

Dependent variables (DV) were corticosterone (CORT), bacteria-killing ability (BKA) and heterophil:lymphocyte (H:L) ratio; independent variables were treatment, time of exposure and sex. Animal ID was used as a random effect term.

Prior to any analyses, data were checked for normality and BKA data had to be SEN transformed to meet this assumption. All analyses were performed using R software (version 3.5.0, R Core Team, https://www.R-project.org/).

RESULTS

The descriptive analysis of all dependent variables is presented in Table S1. Time for blood collection and BMI presented *t*-values between −0.001 and 0.001 in all models; therefore, these variables were removed from the models. Models selected for each dependent variable are presented in Table 2.

Variation in plasma CORT levels was not explained by sex, but rather by an interaction between treatment and time of exposure (t=2.11; Fig. 2, Table 3). Rattlesnakes from the CF treatment exhibited increased CORT levels in comparison to those under the FC treatment on day 14, i.e. 2 days after thermal regimes were switched.

Males presented higher BKA in comparison to females (sex: t=2.24; Fig. 3, Table 3). Snakes from the FC treatment presented lower BKA compared with snakes under the CF treatment throughout the experiment (treatment: t=−3.64; Fig. 3, Table 3), and time of exposure was not a significant factor.

Variation in H:L ratio was not affected by treatment, time of exposure or sex (null models selected in all comparisons; see Table 2, Fig. 4).

Plasma CORT levels were not associated with variation in BKA and H:L ratio (null models selected in all comparisons).

DISCUSSION

Considering that prior to the experiments, snakes were maintained under a natural thermal regime that fluctuated with the circadian cycle, the initial transition from the maintenance condition to the

Table 2. Models selected through the AIC for CORT, BKA and H:L ratio in rattlesnakes (*Crotalus durissus*)

Dependent variable	Selected model	AIC	ΔAIC	d.f.	Weight
CORT	8	788.9	0.0	11	0.82
BKA	2	198.5	0.0	5	0.4
BKA	5	198.9	0.5	6	0.31
BKA	1	199.2	0.8	4	0.27
H:L ratio	10	−101.2	0.0	3	0.95

Models are described in Table 1. BKA was assessed at 37°C. AIC, Akaike information criterion; d.f., degrees of freedom. Weight indicates the robustness of the model in the explanation of the data.

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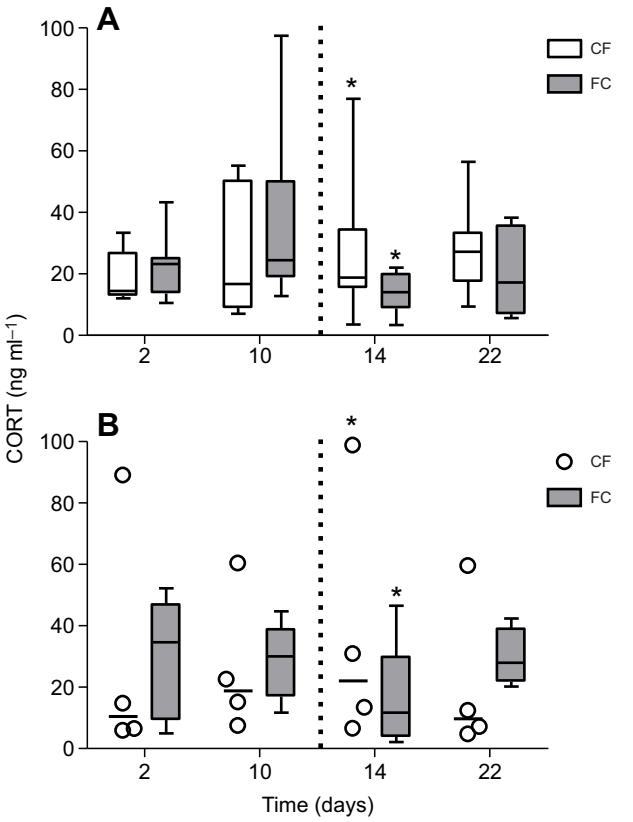

Fig. 2. Corticosterone (CORT) levels in *C. durissus* exposed to the CF and FC treatments. Data are for (A) males (CF *n*=8 and FC *n*=7) and (B) females (CF *n*=4 and FC *n*=5).The vertical dotted line marks day 12, on which the shift between thermal regimes occurred. The asterisks denote statistical differences between treatments (*t*=2.11).

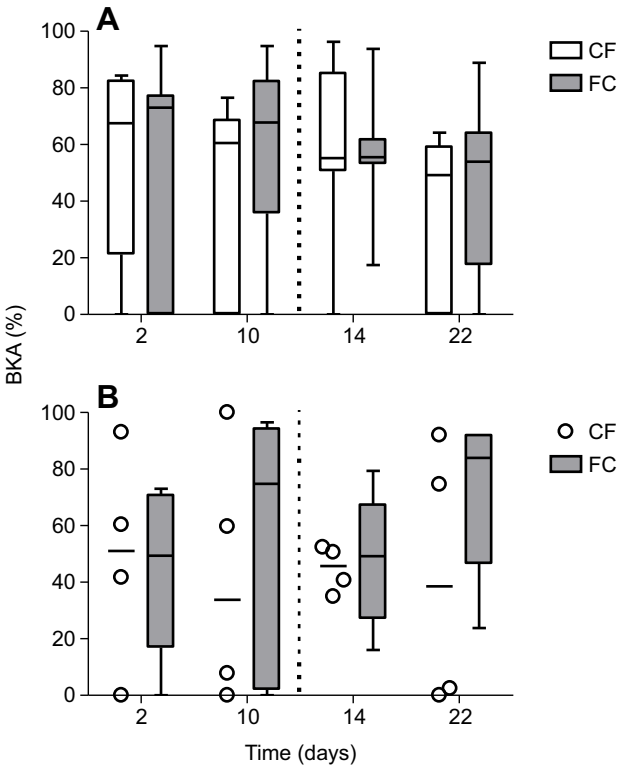

Fig. 3. Bacteria-killing ability (BKA) of *C. durissus* exposed to the CF and FC treatments. Data are for (A) males (CF *n*=8 and FC *n*=7) and (B) females (CF *n*=4 and FC *n*=5). The vertical dotted line marks day 12, on which the shift between thermal regimes occurred. Snakes exposed to the FC treatment presented a lower BKA response in comparison to snakes from the CF treatment (*t*=−3.64), and males presented a higher BKA response in comparison to females (*t*=2.24).

different experimental thermal regimes may have been more drastic for the CF than for the FC treatment. This possibility, however, is ruled out by the fact that there were no considerable differences between treatments when snakes transitioned from their maintenance condition to the initial exposure, regardless of whether it was fluctuating or constant. Instead, the most prominent responses were observed later when the initial exposure was mirror-switched between treatments, and only for the CF treatment. Indeed, contrary to our expectation, the maintenance of *C. durissus* under a constant thermal regime was not associated with chronic stress and immunosuppression. Instead, the change in thermal regime from constant to fluctuating was found to be the trigger of an acute stress response, which attenuated and vanished within 10 days. Perhaps a constant thermal regime, stable and predictable, facilitated snake acclimation, causing the change to the more complex fluctuating thermal regime to be perceived as an acute stressor agent (Sapolsky et al., 2000), even though this change brought the animals to a condition closer to a more natural condition.

The acute shift from a constant to a fluctuating thermal regime resulted in increased plasma CORT levels in rattlesnakes, while such an effect was absent in snakes transitioning from a fluctuating to a constant regime. A possible explanation for this result is that the *C. durissus* preferred body temperature is close to 30°C (~32.4°C; Gavira, 2017), while the temperatures of the fluctuating thermal regime (25 and 35°C) take the snakes far from this. Thus, even if the change from constant to fluctuating happens toward a more natural condition, it also removes the snakes from their thermal preference and could be perceived as a potential stressor agent. Indeed, acute exposure to suboptimal temperatures has been found to act as stressor agent in Children's python, *Antaresia childreni* (Dupoué et al., 2013). Additionally, there is evidence that high temperatures may lead to decreased CORT levels in caimans (Moleón et al., 2018), lizards (Dupoué et al., 2018) and snakes (Dupoué et al., 2013; Claunch et al., 2017). In this context, snakes in the CF treatment, which were previously exposed to constant conditions at a relatively warm temperature (30°C), may have their CORT levels lowered and, at the transition to the fluctuating thermal regime (12th day) had their CORT response inflated. Thus, the low temperatures (25°C) of the fluctuating regime may also explain the increase in CORT levels noticed in snakes transitioning from constant-to-fluctuating thermal regimes. This acute response may allow the

Table 3. *t*-values of significant fixed effects from selected models explaining variance in CORT and immune function after exposure to the two treatments: CF and FC

Selected model	Fixed effects	*t*-value
8 (CORT)	Intercept	3.36
	Treatment CF×Time of exposure (14 days)	2.11
2 (BKA)	Intercept	0.19
	Treatment FC	−3.64
	Sex M	2.24
5 (BKA)	Intercept	−0.75
	Sex M	2.66
1 (BKA)	Intercept	2.35
	Treatment FC	−3.75

CF, constant-to-fluctuating; FC, fluctuating-to-constant; M, male.

Journal of Experimental Biology (2019) 222, jeb208645. doi:10.1242/jeb.208645

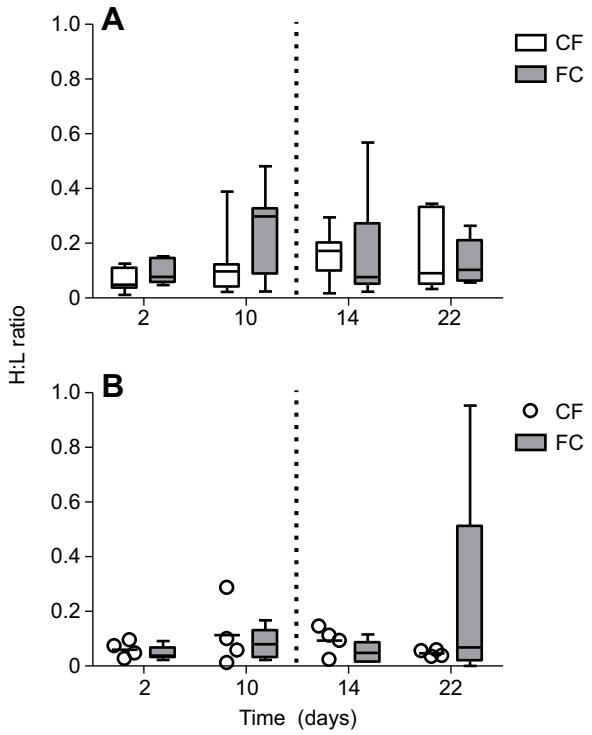

Fig. 4. Heterophil to lymphocyte (H:L) ratio of *C. durissus* exposed to the CF and FC treatments. Data are for (A) males (CF *n*=8 and FC *n*=7) and (B) females (CF *n*=4 and FC *n*=5). The vertical dotted line marks day 12, on which the shift between thermal regimes occurred.

snakes to cope with the change in environmental temperature by recruiting behavioral and physiological adjustments (Sapolsky et al., 2000; Romero, 2004; Dickens et al., 2010), including the triggering of a heat-seeking response (Preest and Cree, 2008). For instance, some snakes display a shift in metabolic rate (Gavira and Andrade, 2013; Stahlschmidt et al., 2015) and immune function (Stahlschmidt et al., 2017) after exposure to new thermal environments. Thus, the increase in plasma CORT levels may play a role in modulating these shifts in order to maintain organism homeostasis as thermal environment changes (Sapolsky et al., 2000; Dupoué et al., 2013; Telemeco and Addis, 2014).

Rattlesnakes exposed to the FC treatment presented lower BKA than snakes exposed to the CF treatment throughout the experiment. As previously discussed, 30°C is near the thermal preference for *C. durissus*, and BKA might be maximized at this temperature. Additionally, evidence suggest that caimans exposed to high temperatures present an increased immunological response in comparison to animals that were exposed to lower temperatures (Moleón et al., 2018). Therefore, it is plausible that rattlesnakes initially acclimated to constant 30°C would present higher BKA. However, in the fluctuating regime, the optimal temperature (~30°C) only occurs briefly twice a day, i.e. when the temperature is increasing from 25°C to 35°C and decreasing from 35°C to 25°C, which leaves the snakes exposed to suboptimal temperatures for the remaining of the time. Similar to our BKA results, the exposure to fluctuating thermal regimes in amphibians caused a decrease in immune response (Raffel et al., 2006; Raffel et al., 2013). Snakes initially subjected to the fluctuating thermal regime presented lower BKA that lasted for the entire experiment duration. This result shows that the effects of initial exposure to the fluctuating thermal regime, i.e. the lowering of BKA, persisted for 10 days after the thermal regime had been changed to constant. Although we lack information on the time course of the BKA response in rattlesnakes, different phenotypic variables often have different activation/deactivation times in response to stressors (Martin, 2009). The temporal effects of thermal regimes on immune parameters clearly deserve further investigation.

We found that males had higher BKA values than females in both treatments throughout the experiment. This result partially diverges from those found in corn snakes (*Panterophis guttatus*), in which males had higher levels of peak agglutination than females after exposure to a fluctuating thermal regime, but not prior to it (Stahlschmidt et al., 2017). High levels of estrogens can be associated with immunosuppression (Szwejser et al., 2017), and reproduction, especially vitellogenesis, imposes a high energetic cost that can compromise immunocompetence (French et al., 2007b). Thus, considering that the timing of our experiments coincided with the second stage (active phase) of vitellogenesis for *C. durissus* (Almeida-Santos and Orsi, 2002), we think it is plausible that such a conflict may explain the blunted BKA response of female rattlesnakes in our study.

Contrary to our predictions, we did not find any evidence that plasma CORT levels, thermal regime, time of exposure or sex influenced H:L ratio. Although H:L ratio and plasma CORT levels have been used as proxies for the stress response, the temporal response dynamics presented by each isolated variable can be quite distinct. Thus, differences in the temporal development of diverse stress response parameters complicate the correspondence among them, as previously reported for lizards (Seddon and Klukowski, 2012) and snakes (Sparkman et al., 2014). Indeed, the change in H:L ratio is a more conservative stress index, as it requires longer times of exposure to the stressor (Sparkman et al., 2014), while CORT levels can respond and even return to pre-stressor levels more quickly (Davis et al., 2008; Sparkman et al., 2014). Finally, it is also possible that more intense stressors are necessary to promote changes in the H:L ratio than in CORT in ectotherms (Assis et al., 2015). In this sense, the absence of effects on H:L ratio suggests that, while the shift from the constant to the fluctuating thermal regime was an intense enough stressor to trigger an increase in CORT, it may not have been intense or long enough to cause heterophilia and/or lymphopenia (Davis et al., 2008).

There is a growing interest in considering thermal variability in studies focusing on physiological measures (Vasseur et al., 2014), with some studies describing the importance of incorporating thermal variability into experimental design (see Morash et al., 2018). In this sense, fluctuating thermal regimes have been suggested as more realistic approaches for testing temperature-related challenges faced by ectotherms (Niehaus et al., 2012; Andrade, 2016; see also Burggren, 2019). Changes from fluctuating to constant temperatures do not seem to cause the stress response in *C. durissus* under the experimental conditions used in this study. In this sense, it is possible that the constant temperature used (30°C) was close to the optimal for this species and, perhaps, exposure to the constant regime at lower or higher temperatures could have resulted in a stress response (see Dupoué et al., 2013; Jessop et al., 2016). In contrast, the transition from a constant thermal regime to a fluctuating thermal regime increased plasma CORT levels in rattlesnakes. We suggest that this response might be related to the departure from the snake's preferred body temperature under the fluctuating thermal regime. The initial exposure of snakes to a fluctuating regime promoted a reduced BKA, which was also influenced by sex, possibly through a differential modulation by sexual hormones and reproductive stage, as females presented decreased BKA in comparison to males in both treatments.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
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