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Thermal regime effects on the resting metabolic rate of rattlesnakes depend on temperature range

Ailton Fabrício-Neto[a,*], Rodrigo Samuel Bueno Gavira[b], Denis Vieira Andrade[a]

[a] Departamento de Zoologia, Instituto de Biociências, Universidade Estadual Paulista, 13506-900, Rio Claro, São Paulo, Brazil
[b] Centre d'Etudes Biologiques de Chizé, CNRS, 79360, Villiers-en-Bois, France

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ABSTRACT

While ectothermic organisms often experience considerable circadian variation in body temperature under natural conditions, the study of the effects of temperature on metabolic rates are traditionally based on subjecting animals to constant temperature regimes. Whether data resulting from constant-temperature experiments accurately predicts temperature effects under more natural fluctuating temperature regimes remains uncertain. To address such possibility, we measured the resting metabolic rates of the South American rattlesnakes (*Crotalus durissus*) under constant and circadian fluctuating thermal regimes in a range of temperatures. Metabolic rates measured at constant 20 °C and 25 °C did not differ from the rates measured at fluctuating regimes with corresponding mean temperatures. However, the difference between thermal regimes increased with temperature, with the metabolic rate measured at constant 30 °C being greater than that measured at the fluctuating thermal regime with corresponding mean temperature. Therefore, our results indicate that thermal regime effects on rattlesnakes' metabolism is dependent on temperature range. Broadly, our results highlight the importance of considering multi-factorial attributes of temperature variation in the exam of its effects over functional traits. Such approach provides a more solid support for inferences about temperature effects on the life history, ecology and conservation of ectothermic organisms.

1. Introduction

Temperature is one of the most influential physical parameters affecting the functioning of living organisms (Huey and Stevenson, 1979; Huey, 1982; Kingsolver and Woods, 1997; Angilletta et al., 2002). Such influence is evident in ectothermic animals whose body temperature (Tb) is fundamentally associated to the thermal characteristics of the environment (Andrews and Pough, 1985; Angilletta et al., 2002; Niehaus et al., 2012). Thus, a pervasive approach in eco-physiological investigations of ectothermic organisms is the examination of temperature effects on different functional attributes. Traditionally, this examination involved the comparison of animals kept at different constant temperatures throughout the duration of the experiments (Andrade, 2016), which, in some cases, may extend for multiple days (e.g., Songdahl and Hutchison, 1972; Rismiller and Heldmaier, 1991; Milsom et al., 2008; see also Secor, 2009; Andrade, 2016). However, more recently, a growing body of evidences indicates that aspects of thermal variability, other than average temperature, may substantially influence functional traits (Niehaus et al., 2012; Colinet et al., 2015; Stahlschmidt et al., 2015). This realization is potentially consequential to the study of ectothermic organisms because they often exhibit considerable circadian fluctuations in Tb (Lutterschmidt et al., 2002; Basson and Clusella-Trullas, 2015; Kingsolver et al., 2015; Tattersall et al., 2016). Therefore, the ecological relevance of testing ectothermic animals under constant thermal regimes may be questionable (Paaijmans et al., 2010; Gavira and Andrade, 2013; Boher et al., 2016).

The bias arising from the gap between experimental and natural conditions in thermal regime is uncertain, but can lead to mislead interpretations of the consequences of temperature variation on life history attributes, ecology and conservation. In the context of global climatic changes, for example, it has been suggested that the increase of global mean temperatures is not the main risk for species survival, but rather the increase of thermal variability (Williams et al., 2012; Vasseur et al., 2014; Bozinovic et al., 2016). Indeed, insects were found to have their thermal tolerance reduced under fluctuating thermal regimes, indicating that these organisms were rendered more vulnerable to thermal constraints when experiencing a higher variability in environmental temperature (Colinet et al., 2015; Bozinovic et al., 2016). In another example, Paaijmans et al. (2010) found that, in addition to differences in means, daily fluctuations in temperature affected parasite

* Corresponding author.
E-mail addresses: afabrineto@hotmail.com (A. Fabrício-Neto), denis.v.andrade@unesp.br (D.V. Andrade).

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infection and development, besides traits associated to malaria transmission in the host mosquito. Therefore, inferring broad interpretations based on the exam of temperature effects on functional attributes, such as in the context of climate change, is likely to benefit from adequate consideration of the potential effects of thermal variability (Ragland and Kingsolver, 2008; Bozinovic et al., 2016).

Metabolic rate determination provides an integrative measure of energy expenditure, allowing a quantitative approach to questions relative to energy budget (Bennett, 1978, 1980; Andrade et al., 2005). As ectotherms exhibit considerable variation in Tb and metabolic rates varies with it, the exam of temperature on metabolic rates have been widely studied in these organisms (Angilletta, 2009; Williams et al., 2012; Bozinovic et al., 2013; Foucreau et al., 2016). However, most of these studies were conducted under constant temperature regimes (Colinet et al., 2015; Andrade, 2016). Nevertheless, thermal regime is known to influence the metabolism of ectothermic animals (Bozinovic et al., 2013; Gavira and Andrade, 2013; Stahlschmidt et al., 2015), which may carry on important consequences for energetic considerations. For example, a non-feeding dormant ectotherm which depends on stored energy reserves to survive periods of seasonal inactivity may have its survival compromised if temperature variability accelerates the pace of energy use (see Williams et al., 2012). These observations reiterate the importance of incorporating different descriptors of temperature variability while examining its consequences on functional attributes of ectothermic organisms (see also Colinet et al., 2015; Bozinovic et al., 2016).

In the present study, we aimed to investigate the potential influence of thermal regime on the rates of energy metabolism in an ectothermic vertebrate, the South American Rattlesnake (*Crotalus durissus*). Accordingly, we estimated resting metabolic rates (RMR) by measuring the rates of oxygen consumption in snakes kept under constant temperature regimes and under circadian fluctuating thermal regimes with equivalent mean temperatures. We repeated this comparison at different temperature levels to test whether potential differences between thermal regimes were influenced by temperature range. We anticipate that constant thermal regimes will result in higher RMR than fluctuating ones of equivalent mean temperature, and that this effect will become more pronounced at higher temperatures. Previous findings indicate that constant temperature regimes can trigger a stress response in ectothermic organisms (Gangloff et al., 2016; Jessop et al., 2016; Telemeco et al., 2017) and, consequently, lead to increases in metabolic rates (DuRant et al., 2008; Colinet et al., 2015). Indeed, a constant thermal regime was associated with higher metabolic rates in a Crotalinae snake (Gavira and Andrade, 2013) in comparison to a daily fluctuating thermal regime of equivalent mean temperature. The South American Rattlesnake is an adequate experimental subject to investigate the questions approached in our study as its Tb exhibits daily variations of up to 10 °C under natural and experimental conditions (Tozetti and Martins, 2008; Andrade, 2016).

2. Materials and methods

2.1. Animal care

Adult male snakes (n = 13) were collected at three cities (Itu, Rio Claro, São Paulo) in São Paulo state, southeastern Brazil, and kept for about two months in captivity before measurements. At the lab, each snake was measured (snout-vent length, SVL; ± 0.1 cm) and, before every trial, weighed (body mass, BM; ± 0.01 g). The mean snakes' BM was 450.50 ± 12.36 g (ranging from 194.46 to 707.60 g) and the SVL 82.3 ± 2.3 cm (ranging from 66.7 to 101.7 cm). Snakes were kept individually in wooden cages (30 × 29 × 27 cm) provided with side holes for ventilation, a glass front door, and corrugated cardboard for lining. Maintenance cages were kept in a room with controlled temperature (25 ± 2 °C), at the Universidade Estadual Paulista (UNESP), in the city of Rio Claro, São Paulo state, Brazil, under natural photoperiod. Animals were monthly fed with mice (*Mus musculus*) and water was provided *ad libitum*. Only animals that appeared to be healthy and were not undergoing skin shedding were used. Prior to the measurements, snakes were fasted for at least 15 days to ensure they were post-absorptive. The permissions for animal collection and maintenance were issued by the Brazilian Institute of the Environment and Renewable Natural Resources (IBAMA, process numbers: #22028-1 and #35081-3). The experiments were conducted under approval of the Ethics Committee on Animal Use (CEUA, protocol number: #1492), from the Instituto de Biociências, Universidade Estadual Paulista, Rio Claro, SP, Brazil.

2.2. Experimental protocol

Resting metabolic rates were determined under the constant temperatures of 15 °C, 20 °C, 25 °C, 30 °C, and 35 °C, and under fluctuating temperature regimes composed by the following temperature combinations: 15–25 °C, 20–30 °C, and 25–35 °C. For the fluctuating regimes, temperature oscillations were set at a 12:12 h cycles, with the higher temperature period coinciding with daylight (from 06:00 to 18:00 h) and the lower temperature with nighttime (from 18:00 to 06:00 h). The time course of temperature change for the fluctuating thermal regimes is illustrated at Fig. 1 (see also Table 1). Trials lasted for 4 consecutive days and every individual snake was measured in every experimental treatment in a randomized sequence. Between trials, snakes were fed and kept in their maintenance cages for a minimum period of 15 days to recover. Temperatures were controlled by a BOD-type climatic chamber (model 122FC, Eletrolab, São Paulo, SP, BRA), being periodically checked with a maximum and minimum thermometer (model 5201.03, Incoterm, São Paulo, SP, BRA), accepting a variation of ± 1 °C. All RMR measurements occurred between July of 2013 and May of 2015.

2.3. Variation of Tb under fluctuating thermal regimes

In order to determine the time course variation in body temperature

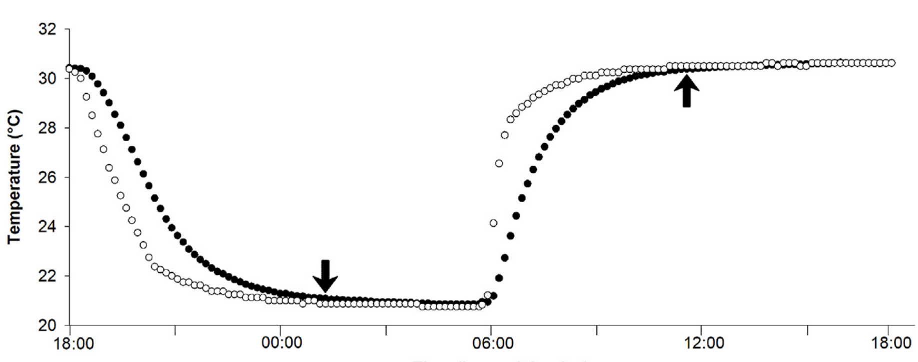

Fig. 1. Example of a 24 h cycle of a fluctuating thermal regime used in our experiment (20–30 °C). The arrows indicate the approximate hours in which RMR measurements started for each thermoperiod. The white circles represent the climatic chamber temperature, and the black circles represents the snakes (n = 4) body temperature.

Table 1
Time for the thermal equilibration between the climatic chamber and the rattlesnakes' Tb during the fluctuating thermal regimes. Chamber temperature was switched from hot to cold (cooling) and from cold to hot (warming) thermoperiods at 12 h intervals (see text for details). Values are presented as mean ± SE (n = 4).

Thermal regime (°C)	Equilibration time for cooling (h)	Equilibration time for warming (h)
15–25	6.46 ± 0.24	4.46 ± 0.38
20–30	6.46 ± 0.3	4.71 ± 0.25
25–35	5.71 ± 0.23	4.42 ± 0.25

under the fluctuating thermal regimes, we surgically implanted four snakes with temperature data loggers (iButton Thermochron, model: DS1922L-F50, precision: 0.0625 °C, Maxim Integrated Products, Sunnyvale, CA, USA) and submitted them to the experimental fluctuating thermal regimes, as described above. These snakes were not used for metabolic measurements, but had body masses similar to those that were (overall mean = 501.30 ± 30.43 g, range = 412.58–549.30 g; $F_{1,104} = 1.301$, $P > 0.2$).

Data loggers were previously impermeabilized by dipping them in liquid synthetic rubber coating (Plasti Dip International, Performix Brand, Blaine, MN, USA) and sterilized (alcohol dipping), and then implanted intraperitoneally, approximately in the middle of the snout-vent length of the snakes. Animals were anesthetized with isoflurane gas inhalation and then partially placed in restraining tubes (*i.e.*, with their first third inside the tubes) for safe handling. Implantation area was treated with povidone-iodine solution (10%) and locally injected with 2% lidocaine hydrochloride (Anesthetic L; Eurofarma-Pearson). When animals no longer responded to pinching of the skin, a small incision (2–3 cm) was made on the left side of the medial portion of the snakes' body, at the area where dorsal and ventral scales meet. Through this incision, the temperature data logger, was advanced into the snake's body cavity, while being secured in place by a small piece of suture fastened to the body wall. The incision was then sutured close and snakes were treated with a broad-spectrum antibiotic (enrofloxacin; Baytril, 10 mg kg^{-1}). They were let to recover for at least 7 days before being submitted to the fluctuating temperature regime as previously described. At the end of experiments, data loggers were surgically removed under the same general procedure just described for implantation.

2.4. Respirometry

Resting metabolic rates were estimated by the rates of oxygen consumption ($\dot{V}O_2$) measured with an intermittently closed respirometric system. After weighing, snakes were transferred individually into hermetically sealed respirometry containers (700–2000 ml), which were kept inside the climatic chamber for temperature control. In the respirometry system, an air pump connected to a mass flow meter (SS4 Subsampler, Sable Systems, Las Vegas, NV, USA) generated a constant flow (150 ml min^{-1}) of ambient air. Airflow was directed to a multiple flow controller (RM8 Multiplexer, Sable Systems), set to intercalate periods of 60 min in which the chambers were ventilated with outside air (open phase), with periods of 10 min in which the air contained in the chambers was recirculated (closed phase) through an oxygen analyzer (PA-1, Sable Systems). Excurrent flow was passed through a drying column (silica gel) before entering the gas analyzer. The decline in air fractional concentration of O_2 during the closed phase was monitored using a data acquisition interface (UI-2, Sable Systems) and used to calculate the rates of oxygen consumption. As our system intercalated open- and closed-phases while rotating among the seven respirometric chambers, we were able to obtain one $\dot{V}O_2$ measurement, for each snake, at every 70 min. $\dot{V}O_2$ was normalized to standard temperature and pressure, using equations provided by Vleck (1987) under the assumption of a respiratory quotient of 0.8. All data acquisition and instrument control were carried out under Datacan V software (Sable Systems).

2.5. Treatment and data analysis

From the four days of each trial duration, we discarded the first 24 h to account for snake's habituation within the respirometric chamber and used the remaining 72 h to estimate the RMR. Although, we did not monitor snakes' movement inside the respirometric chambers, a few rare $\dot{V}O_2$ data points were identified as outliers and were not used for RMR calculations. These data points were typically more than 70% off the prevalent steady-state $\dot{V}O_2$ measurements at the time and could be associated to bursts of movement in attempt to escape from confinement or to long periods of apneia (see Wang and Abe, 1994). Previous to any of the RMR calculations just described, we verified that RMR within each treatment did not vary along the days that composed each trial (OneWay RM Anova, $P > 0.2$ in all cases) and neither with the circadian cycle (Paired T-Test, $P > 0.05$ in all cases).

Following the initial data treatment and tests just described, RMR was calculated as the average of all the $\dot{V}O_2$ measurements taken during the last three days of each of the constant temperature trials. Due to the nature of the fluctuating thermal regimes, RMR calculations were more elaborated. First, since under the fluctuating thermal regimes snakes took several hours to reach temperature equilibration, we restrict our RMR estimations to the $\dot{V}O_2$ measurements taken after the period required for thermal equilibration, which usually were under 6 h after each temperature change (see Fig. 1, Table 1). After thermal equilibration was reached, we averaged the remaining $\dot{V}O_2$ measurements, until the next change in temperature, to estimate a given RMR value. This procedure yielded three RMR measurements for each temperature composing the fluctuating thermal regime, which equated for the 3 cycles of temperature fluctuation along the 72 h that each trial lasted. These values were posteriorly averaged in two means, one for each temperature, and one grand-mean that was accepted as representative of the mean RMR value corresponding to the mean temperature of a given fluctuating thermal regime.

We used a Oneway ANOVA test for Repeated Measures to identify differences among temperatures and thermal regimes. Whenever a statistical difference was found, we used a *post-hoc* Student-Newman-Keuls test for multiple comparisons. Before the statistical tests, we evaluated the parametric premises of normality (Shapiro-Wilk) and equality of variances (Levene's test). Results are presented as mean ± standard error and the significance level was set at $P < 0.05$. Statistical analyzes were done with the software SigmaPlot version 12.5.

3. Results

Under the fluctuating thermal regimes, temperature changes inside the climatic chambers (Ta) were stabilized within 2 h, regardless of temperature being elevated or lowered (Table 1). These changes in Ta were followed by longer changes in Tb, which differed with the direction of temperature change. In general, new Tb equilibrium was attained around 4.5 h after temperature start to be elevated and around 6.2 h after temperature lowering (see Table 1). However, the average temperature for the total 72 h period of each trial duration were essentially identical to their corresponding constant thermal regime (respectively: 20.45 ± 0.02 °C and 20.49 ± 0.09 °C for 20 °C trials; 25.29 ± 0.20 °C and 25.25 ± 0.25 °C for 25 °C trials; and 29.99 ± 0.03 °C and 30.02 ± 0.03 °C for 30 °C trials; Paired T-Tests: $P > 0.2$ for all cases).

The body masses of the rattlesnakes used for metabolic measurements (see Animal Care section for mean and range) did not differ among treatments ($F_{7,94} = 1.635$, $P > 0.1$).

RMR under constant regimes differed among all temperatures

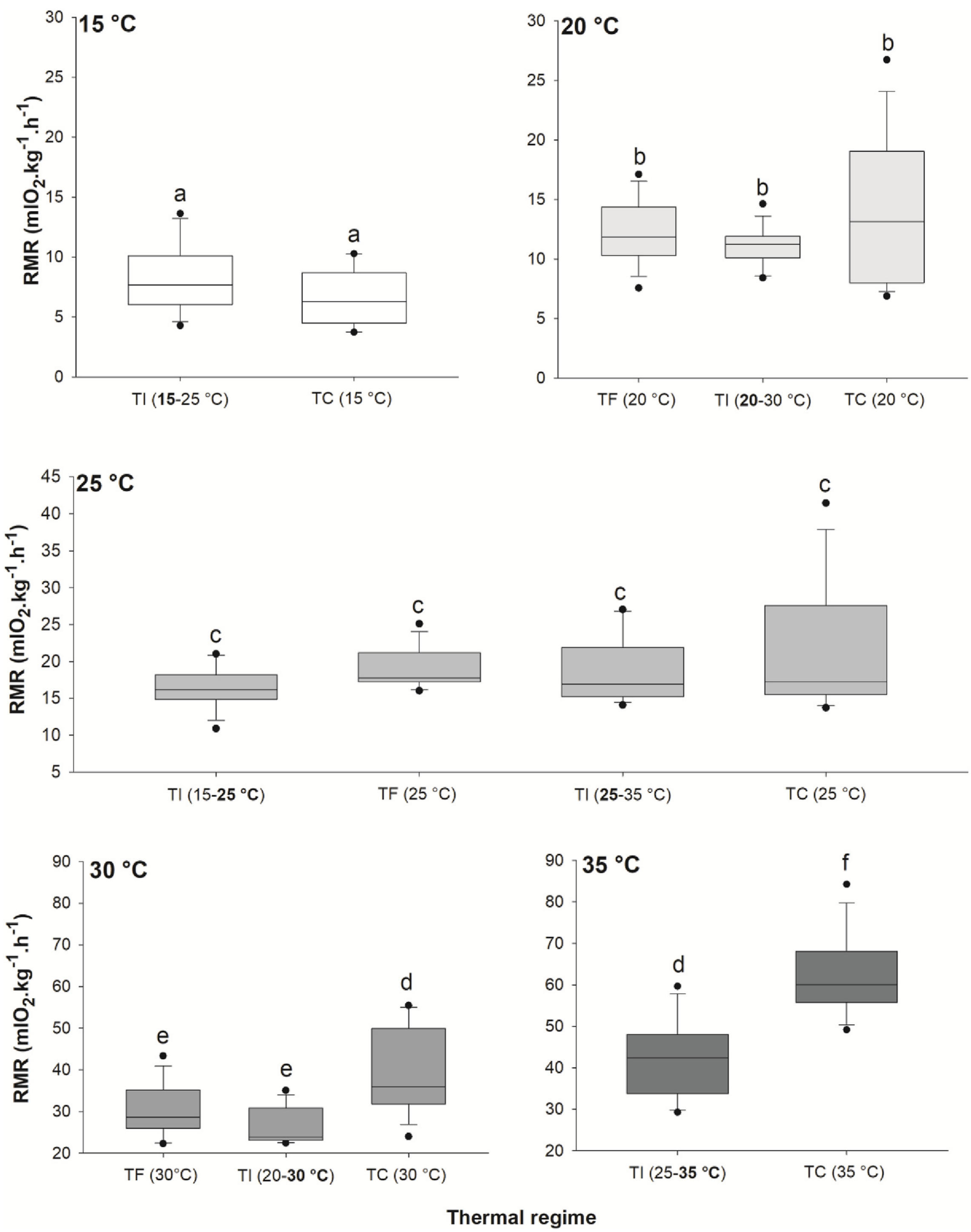

Fig. 2. Variation of the resting metabolic rates (RMR) of rattlesnakes measured at constant (TC) and fluctuating (TF) thermal regimes, as well at the isolated thermoperiods (TI, indicated as bold values; see text for details) composing the TF regimes, at temperatures of 15, 20, 25, 30 and 35 °C. Different letters indicate statistical difference among treatments (n = 13).

($F_{4,58}$ = 133.784, P < 0.001; Pairwise comparisons: 15 °C *vs* 20 °C, P = 0.011; 20 °C *vs* 25 °C, P = 0.005; 25 °C *vs* 30 °C, P < 0.001; 30 °C *vs* 35 °C, P < 0.001), with a positive effect of temperature over RMR. RMR determined at the 15 °C isolated from the 15–25 °C fluctuating regime did not differ from RMR measured at constant 15 °C ($F_{1,22}$ = 2.046, P = 0.180, Fig. 2). RMR measured at constant 20 °C did not differ from the mean RMR averaged for its corresponding fluctuating regime (i. e., 15–25 °C) and neither from that measured at 20 °C for the 20–30 °C fluctuating regime ($F_{2,36}$ = 1.676, P > 0.2 for all cases, Fig. 2). Similarly, RMR measured at constant 25 °C did not differ from the mean RMR averaged for its corresponding fluctuating thermal regime (i.e., 20–30 °C) and neither from the RMR measured at 25 °C for the 15–25 °C and 25–35 °C fluctuating regimes ($F_{3,48}$ = 2.501, P > 0.07 for all cases, Fig. 2). The RMR measured at constant 30 °C was significantly greater than that averaged for its corresponding fluctuating regime (i. e., 25–35 °C) and from that measured at 30 °C for the 20–30 °C fluctuating regime ($F_{2,36}$ = 10.141, $P \leq 0.005$ for both cases, Fig. 2). RMR measured at constant 35 °C was greater than that measured at 35 °C for the 25–35 °C fluctuating regime ($F_{1,22}$ = 29.568, $P < 0.001$, Fig. 2).

4. Discussion

Crotalus durissus metabolism increased with temperature at rates well within the limits commonly reported for reptiles (see Bennett, 1982), including viperid snakes (e. g., Cruz-Neto and Abe, 1994; Beaupre and Zaidan, 2001; Dorcas et al., 2004; Gavira and Andrade, 2013). The temperature-induced increment in metabolism is an almost universal feature present in ectothermic organisms (Schulte, 2015; Sinclair et al., 2016), and this general trend was evident in our study irrespective of thermal regime. However, the magnitude of this temperature dependence of RMR was influenced by the temperature interval. While the factorial increment in metabolism for the 20–25 °C interval was identical for both thermal regimes (1.57), the increment observed from 25 to 30 °C under constant conditions (1.83) was greater than that occurring between the averaged corresponding fluctuating thermal regimes (1.59). Consequently, the RMR measured at constant 30 °C was greater than that averaged for the equivalent fluctuating regime of 25–35 °C. This difference can be almost entirely ascribed to a less than expected increment in metabolism during the 35 °C thermoperiod. Indeed, the RMR measured under this thermoperiod was 32% lower than that measured at the same temperature under constant regime. On the other hand, for the 25 °C thermoperiod, the drop in metabolism averaged only 8.6%, in comparison to constant 25 °C. Thus, the effect of constant thermal regimes in causing an elevation in metabolism compared to fluctuating thermal regimes seems to be at play only at relatively warm temperatures in rattlesnakes.

A positive correlation between corticosterone levels and metabolic rates has already been reported in lizards (DuRant et al., 2008; Preest and Cree, 2008). Also, the rise in glucocorticoid levels promotes intermediary metabolism, such as glucose mobilization (Sapolsky et al., 2000; Romero, 2004) and, in the absence of compensatory mechanisms, increments in whole organism metabolism (DuRant et al., 2008). Thus, although our study did not access the proximal determinants involved in the metabolic response of the rattlesnakes, we suspect that submitting them to a 30 or 35 °C constant regime may have trigged a thermal stress response (see Niehaus et al., 2012) and an accompanying elevation in metabolism. Indeed, rattlesnakes preferred body temperature vary daily between 32.4 °C when active and 18.5 °C during inactivity (Gavira, 2017). Therefore, it seems plausible that subjecting them to constantly warm conditions may have resulted in the triggering of attempts to escape from the respirometric chambers, with increased locomotor activity, and other metabolic costly processes being recruited as part of a stress response (Gangloff et al., 2016; Telemeco et al., 2017). Under the fluctuating thermal regime, even if a stress response had occurred during the 35 °C thermoperiod, it had a shorter duration and was likely alleviated by the alternation with the intervening periods of low temperature. Such reasoning agrees with that stated by Niehaus et al. (2012) that, in a fluctuating environment, the thermal optimal for each physiological process is likely to be accessed along the course of the day, thereby minimizing a potential thermal stress caused by a constant temperature. It seems to us that the interplay among thermal regime, stress response, and physiological parameters in ectothermic organisms clearly deserves further investigation.

Similarly to what we found in *C. durissus*, the viperid snake *Bothrops alternatus* exhibited an increased metabolic rate under a constant temperature regime of 25 °C compared to the corresponding fluctuating regime varying from 20 to 30 °C (Gavira and Andrade, 2013). Although *C. durissus* and *B. alternatus* overlap in greater portions of their geographic distribution, the area of occurrence of *B. alternatus* extends toward austral regions in which *C. durissus* is absent (Sawaya et al., 2008). Also, differences in thermal preference and tolerance indicate that *B. alternatus* is a species associated to colder conditions than *C. durissus* (Gavira, 2017). If that is the case, the interplay among temperature range, thermal regime and metabolic variation previously discussed for *C. durissus* might also be applicable to *B. alternatus*, but occurring at a lower temperature range for this species. Thus, the temperature level in which thermal regimes cause a significant effect in RMR may vary in consonance with interspecific differences in the thermal biology attributes and life-history traits (see also Jessop et al., 2016). Indeed, different aspects of temperature variability, other than differences in means, have been reported to induce important changes in many life-history traits. These reports include changes in developmental time (Ragland and Kingsolver, 2008), hatching success (Ji et al., 2007), and some phenotypic characteristics of the progeny (Pétavy et al., 2004; Folguera et al., 2009), besides influencing seasonal changes in metabolism (Milsom et al., 2008; Paaijmans et al., 2010; Krams et al., 2011; Williams et al., 2012). Our findings that thermal regime influences the metabolism of rattlesnakes at higher temperatures may also be consequential to life history attributes. Higher rates of metabolism demand more feeding activity, which may not be supported by ecological proximates, such as prey availability, or be accommodated by the animal's capacity in processing the food (see Pough, 1983). In combination with that, energy to support basic resting metabolism could be diverted from other relevant life history traits such as growth and reproduction (DuRant et al., 2007). The investigation of detailed energetic budgets under different thermal regimes constitutes a worth and poorly explored approach in the study of ectothermic organisms.

Temperature variation can lead to energetic constraints that compromises animal performance, fitness and, ultimately, survival (Sinervo et al., 2010; Bozinovic et al., 2013; Colinet et al., 2015). Consequently, a number of studies have incorporated energetic considerations in forecasting the consequences of future climate change scenarios, especially related to global warming, to animal life (Paaijmans et al., 2010; Thompson et al., 2013; Schulte, 2015; Bozinovic et al., 2016; Sinclair et al., 2016). Pivotal to the accuracy of such investigations is a realistic assessment of the temperature-induced effects on metabolism. In this regard, metabolism (Williams et al., 2012; Bozinovic et al., 2013; Gavira and Andrade, 2013; Stahlschmidt et al., 2015; Foucreau et al., 2016, present study) seems to agree with other functional traits (Niehaus et al., 2012; Williams et al., 2012; Colinet et al., 2015), in which other aspects of thermal variability, besides differences in means, are found to be influential. Our study adds that, at least in consideration to thermal regime, such responses are likely to be mediated by temperature range. Therefore, broad ecological and biogeographical inferences based on the thermal dependency of functional traits performance are likely to be improved by refining our approach in the exam of the effects of temperature variability.

Compliance with ethical standards

Conflicts of interest

The authors declare no conflict of interest.

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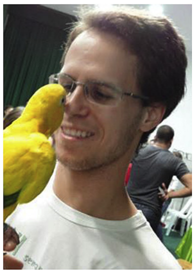

Ailton Fabrício Neto Graduation in Ecology in 2015, currently Masters candidate in Zoology at the São Paulo State University, Brazil. My research interests encompass ecophysiology and zoology, specifically questions about differences in thermal regimes, acclimation and stress response of ectotherms.

Rodrigo S. B. Gavira Graduation in Biological Sciences, Master's degree and PhD in Zoology, São Paulo State University (UNESP), Brazil. Currently, I am a post-doctoral researcher at the Centre d'Études Biologiques de Chizé (CEBC/CNRS), France, where I investigate the potential of hydro- and thermoregulation to influence ecological responses to climate change on Squamata reptiles.

Denis V. Andrade Associate Professor at São Paulo State University, Biosciences Institute, Zoology Department, Rio Claro, SP, Brazil. I am broadly interested in how animals function, how they interact with the environment, and how they have evolved.