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(ÁREA: ZOOLOGIA)

Termorregulação, balanço hídrico e metabolismo energético de viperídeos Neotropicais (Serpentes: Crotalinae)

RODRIGO SAMUEL BUENO GAVIRA

Tese apresentada ao Instituto de Biociências do Câmpus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Doutor em Ciências Biológicas (Zoologia).

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TÍTULO DA TESE: **Termorregulação, balanço hídrico e metabolismo energético de viperídeos neotropicais (serpentes: crotalinae)**

AUTOR: RODRIGO SAMUEL BUENO GAVIRA
ORIENTADOR: DENIS OTAVIO VIEIRA DE ANDRADE

Aprovado como parte das exigências para obtenção do Título de Doutor em CIÊNCIAS BIOLÓGICAS (ZOOLOGIA), pela Comissão Examinadora:

Prof. Dr. DENIS OTAVIO VIEIRA DE ANDRADE
Departamento de Zoologia / Instituto de Biociências de Rio Claro - SP

Prof. Dr. ARIOVALDO PEREIRA DA CRUZ NETO
Departamento de Zoologia / Instituto de Biociências de Rio Claro - SP

Prof. Dr. CLÉO ALCANTARA COSTA LEITE
Departamento de Ciências Fisiológicas / UNIVERSIDADE FEDERAL DE SAO CARLOS

Prof. Dr. GUILHERME GOMES
Departamento de Física e Ciência Interdisciplinar / USP - Instituto de São Carlos / SP

Prof. Dr. FABIO CURY DE BARROS
Programa de Ecologia e Evolução / Universidade Federal de São Paulo - UNIFESP

Rio Claro, 20 de julho de 2017

DEDICO ESTA TESE...

AOS MEUS PAIS CELSO E JACIRA,

À MINHA ESPOSA FRANCIELE,

AO MEU FILHO DANIEL,

PELO AMOR, CONFIANÇA E APOIO.

"...If you falter in a time of trouble, how small is your strength!"

(Proverbs 24:10)

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SUMÁRIO

RESUMO DO TRABALHO

A temperatura afeta dramaticamente todos os processos fisiológicos dos organismos e, portanto, a maioria dos animais investe tempo e energia consideráveis na termorregulação. Animais ectotérmicos regulam a temperatura corpórea (T_b) principalmente com base em fontes externas de calor e por meio de ajustes comportamentais. Como consequência, ectotermos experimentam flutuações muito maiores da T_b do que a grande maioria dos animais endotérmicos. A variação da T_b tem impactos profundos nas capacidades funcionais das ectotermias e pode resultar em importantes consequências ecológicas. Em ectotermos, a taxa metabólica de repouso (TMR) e a perda de água evaporativa (PEA) são em geral diretamente relacionadas com a T_b, podendo ainda ser afetadas por características térmicas e disponibilidade de água dos diferentes hábitats. Ademais, a termorregulação nos ectotermos é determinada por um equilíbrio entre os custos e benefícios. Os ajustes no comportamento termorregulatório para compensar as condições adversas do ambiente podem acarretar numa alta demanda energia e tempo para os animais, enquanto que a regulação T_b pode resultar em benefícios associados à otimização de vários processos fisiológicos a uma determinada temperatura. Assim, particularmente para Squamata, os animais exibirão uma temperatura corpórea preferida (T_{pref}) na faixa térmica que melhora suas atividades. Estendendo-se abaixo e acima da faixa de T_{pref}, ectotermos irão atingir um limite térmico inferior (CT_{min}) ou superior (CT_{max}), nos quais o desempenho e, consequentemente, a sobrevivência serão comprometidos. Em serpentes, tanto a T_{pref} quanto os limites de tolerância térmica (LTT) variam intra e interspecificamente, sendo influenciados por muitos fatores, como por exemplo as condições ambientais do hábitat dos animais. Portanto, T_{pref} e LTT são atributos centrais da biologia termal de organismos ectotérmicos, incluindo serpentes, uma vez que podem refletir diferenças históricas e/ou adaptativas. Para abordar os aspectos ecofisiológicos envolvidos na termorregulação, balanço hídrico e metabolismo energético acima comentados, examinamos a T_{pref}, CT_{min} e CT_{max}, bem como os efeitos da temperatura (15, 25 e 35°C) sobre TMR e PEA em quatro serpentes Neotropical Crotalinae da América do Sul (*Bothrops alternatus*, *B. jararaca*, *B. moojeni* e *Crotalus durissus*). Estas espécies de serpente são filogeneticamente relacionadas e ocupam áreas geográficas que se sobrepõem extensivamente, mas apresentam diferenças consideráveis em termos de microhábitats preferidos e outros atributos biológicos. Em geral, *B. jararaca* e *B. moojeni* ocupam principalmente áreas florestadas, enquanto *B. alternatus* e *C. durissus* habitam

regiões de áreas abertas. Os resultados mostraram que tanto a TMR quanto a PEA aumentaram com a temperatura em todas as espécies. Tanto em 15 e 35°C, *B. jararaca* e *B. moojeni* apresentaram PEA semelhante, porém maiores que *B. alternatus* e *C. durissus*. TMR a 25°C não diferiu entre as espécies. *Crotalus durissus* exibiu as menores taxas de PEA dentre todas as espécies, em qualquer temperatura. As espécies de *Bothrops* apresentaram taxas de PEA semelhantes a 15 e 25°C. A 35°C, no entanto, *B. alternatus* exibiu taxas mais baixas de PEA do que espécies congêneres. Todas as espécies de serpentes tiveram T_{pref} à noite maior do que T_{pref} durante o dia. Interespecificamente, T_{pref} diurna não diferiu entre as espécies, enquanto que durante a noite *B. jararaca* e *C. durissus* selecionaram temperaturas mais altas do que *B. alternatus* e *B. moojeni*. T_{pref} noturna em *B. alternatus* foi maior do em *B. moojeni*. *Bothrops alternatus* exibiu o menor CT_{min} entre todas as espécies, enquanto *B. jararaca* apresentou valores de CT_{min} mais baixos do que *B. moojeni*. Os valores de CT_{max} para *B. alternatus* e *C. durissus* foram semelhantes, mas superiores aos de *B. jararaca* e *B. moojeni*. Nossos dados mostraram que TMR e PEA das serpentes foram congruentes com o tipo de ambiente ocupado pelos animais, do ponto de vista da fitofisionomia. As espécies de serpentes de áreas abertas (*B. alternatus* e *C. durissus*) exibiram taxas mais baixas de PEA do que as espécies florestadas (*B. jararaca* e *B. moojeni*). Além disso, *C. durissus*, seguido de *B. alternatus*, seria menos vulnerável a um potencial estresse térmico causado pelo potencial aquecimento climático em comparação com *B. moojeni* e *B. jararaca*, uma vez que, em temperaturas extremas, essas espécies não apresentariam muito um alto gasto de energia e seriam menos suscetíveis à dessecação. Do mesmo modo, tanto para T_{pref} quanto LTT nossos resultados pareceram ter relação com o tipo de ambiente ocupado pelas serpentes. As espécies de áreas abertas (*B. alternatus* e *C. durissus*) apresentaram maior CT_{max} e foram mais tolerantes ao calor do que as espécies florestadas (*B. jararaca* e *B. moojeni*). Entre as espécies de *Bothrops*, *B. alternatus* mostrou ser a espécie mais tolerante ao frio. Em conclusão, todas as serpentes selecionaram temperaturas menores durante o dia, o que poderia proporcionar uma economia de energia durante o período de inatividade. CT_{max} de *C. durissus* e *B. alternatus* indicou que essas espécies seriam menos vulneráveis ao estresse térmico causado pelo aquecimento do clima em comparação com *B. moojeni* e *B. jararaca*.

INTRODUÇÃO GERAL

É consenso que a temperatura é um dos fatores que afeta de forma mais significativa os processos fisiológicos dos organismos (Cossins and Bowler, 1987; Angilletta et al., 2002). Em decorrência, muitos animais investem considerável energia e tempo a fim de regular a temperatura corpórea (T_b) (Pough et al., 2001). Particularmente, nos ectotérmicos terrestres, alterações na T_b podem afetar diversos processos fisiológicos, como a taxa metabólica (Andrade et al., 1997; Secor, 2009; Gavira e Andrade, 2013), perda evaporativa de água (PEA) (Bennett e Licht, 1975; Dmi'el, 1985; Dmi'el, 2001; DeNardo et al., 2004; Tracy et al., 2008) entre outros. No caso dos répteis, a T_b é regulada principalmente por meio de ajustes comportamentais e escolha de micro-hábitats (Cowles e Bogert 1944; Patterson e Davies, 1982; Huey et al., 1989; Krohmer, 1989; Grant, 1990; Peterson et al., 1993), embora ajustes fisiológicos também ocorram (Dzialowski e O'Connor, 1999; Tattersall et al., 2004; Stuginski et al., 2011). Por sua vez, uma das principais consequências da ectotermia é que a regulação da T_b é amplamente influenciada pela disponibilidade de nichos térmicos adequados no ambiente (Angilletta, 2009) e, como tal, os organismos ectotérmicos geralmente apresentam flutuações muito maiores na T_b do que a grande maioria dos endotérmicos (Huey, 1982; Angilletta et al., 2002). Nesse sentido, os ajustes termorregulatórios para compensar condições adversas podem demandar elevado gasto de energia e o tempo (Huey e Slatkin, 1976; Lelièvre et al., 2011). Em contrapartida, a regulação da temperatura corporal pode resultar em benefícios associados à otimização de vários processos fisiológicos a uma dada temperatura ou intervalo térmico (Huey e Bennett, 1987; Hertz et al., 1993; Angilletta et al., 2002, Blouin-Demers e Nadeau, 2005; mas ver Fitzgerald et al., 2003). Neste sentido, muitas espécies de Squamata, quando em condições permitidas, exibirão uma temperatura corpórea preferida (T_{pref}) numa faixa termal que melhora o seu desempenho (Huey, 1982; Angilletta, 2009).

Assim como a regulação da T_b, a manutenção do balanço hídrico também é fundamental. Em muitos grupos animais, a PEA está correlacionada com a disponibilidade de água e/ou umidade do habitat, refletindo, portanto, na ocupação do habitat (Belasen et al., 2017). De fato, no caso dos Squamata, animais de regiões mais áridas apresentam PEA menores que os de regiões mais úmidas (Dmi'el, 1972; Mautz, 1982; Guillon et al., 2014). A ocupação de hábitat de uma determinada espécie, bem como sua distribuição geográfica, é resultado da interação entre fatores ecológicos e evolutivos, incluindo competição intra- e/ou interespecífica, disponibilidade de recursos, fatores históricos, entre outros (Case e Taper, 2000; Case et al., 2005). Para o grupo dos répteis, o hábitat ocupado por algumas espécies está intimamente relacionado com a temperatura do ambiente (Huey, 1987; Campbell e Solórzano, 1992; Krebs, 1994; Gaston, 2003; Blouin-Demers and Weatherhead, 2001). Consequentemente, com o aquecimento do clima, diversas espécies de ectotérmicos estão sendo forçadas a procurar hábitats altenativos (i.e., com temperaturas mais amenas), a fim de evitar o estresse térmico (Araújo et al., 2006; Moreno-Azócar et al., 2012; ver também Seebacher et al., 2015). De fato, muitas destas espécies vêm apresentando uma drástica diminuição tanto na sua riqueza quanto na densidade, sendo que algumas já se encontram inclusive ameaçadas de extinção (Araújo et al., 2006; Foufopoulos et al., 2011; ver também Huey et al., 2009). Por exemplo, estima-se que daqui cerca de 60 anos, 20% das espécies de lagartos estarão extintas em razão do aquecimento climático, principalmente devido severas restrições no período de atividade destes animais, relacionadas dentre outras causas ao superaquecimento e/ou desidratação (Sinervo et al., 2010). Mesmo considerando que a temperatura influencie funções fisiológicas e, consequentemente, o desempenho dos ectotérmicos, são ainda relativamente poucos os estudos que têm tentado incorporar parâmetros fisiológicos na tentativa de se entender padrões de distribuição geográfica e ocupação de habitat por esses animais (Kearney e Porter, 2004; 2009; Navas, 2006; Titon e Gomes, 2012; 2015).

As serpentes neotropicais do grupo *Bothrops* fazem parte de um conjunto aparentemente monofilético de cerca de 50 espécies, ocorrendo em praticamente toda a América Latina (Hoge e Romano-Hoge, 1981; Campbell e Lamar, 1989; Greene, 1992; Alencar et al., 2016). Dentro do gênero *Bothrops*, aproximadamente sete linhagens são reconhecidas e as espécies aqui estudadas, *B. alternatus*, *B. jararaca* e *B. moojeni*, são representantes dos grupos *'alternatus'*, *'jararaca'* e *'atrox'*, respectivamente (Carrasco et al., 2012). De uma forma geral as serpentes *Bothrops* ocupam campos florestais e possuem hábito terrestre (Campbell e Lamar, 2004), embora algumas espécies possam ser encontradas em áreas abertas, apresentar hábito arbóreo, ou mesmo exibir uma variação ontogenética na utilização do micro-hábitat (Amaral, 1921; Campbell e Lamar, 2004; Sazima, 1992; Martins et al., 2002). No Brasil, o gênero *Bothrops* é considerado o grupo de maior importância médica, responsável por cerca de 90% das 20.000 ocorrências anuais de acidentes ofídicos (Ribeiro e Jorge, 1997; Melgarejo, 2009), com taxa de letalidade próxima a 0,3% dos casos (Araújo et al., 2003). Entretanto, de acordo com Sandrin et al. (2005), tais informações são transmitidas à população com uma série de problemas conceituais, o que leva a exacerbar a real nocividade das serpentes e, consequentemente, o medo natural das pessoas em relação às serpentes, acarretando possível matança e declínio desses animais. Somando-se a isto, a questão da perda e degradação de hábitats é considerada uma das principais causas da ameaça de extinção entre os répteis, uma vez que grande parte destes vertebrados não é capaz de sobreviver em ambientes demasiadamente alterados por ação antrópica (Martins e Molina, 2008; Marques, 1998; Marques et al., 2010). Por exemplo, segundo o Livro Vermelho do Estado de São Paulo das 29 espécies do grupo *Bothrops* (aqui incluso o gênero *Bothrocophias*; Bérnils e Costa, 2011) pertencentes à herpetofauna nacional, cinco estão ameaçadas de extinção, sendo que duas (*B. alcatraz* e *B. insularis*) são consideradas como "Criticamente Ameaçadas" (Marques et al., 2009; IUCN, 2015). Portanto, uma melhor compreensão sobre a biologia destas serpentes, incluindo fatores determinates de sua distribuição no habitat, é fundamental para a preservação e conservação tanto das espécies como do hábitat em que estão inseridas.

Devido a isto, a presente tese teve como objetivo central investigar diversos parâmetros fisiológics, até então desconhecidos, para serpentes viperídeas do gênero *Bothrops*, como fisiologia termal, balanço hídrico e metabolismo energético. As espécies de serpentes aqui estudadas foram *B. alternatus*; *B. jararaca;* e *B. moojeni*, escolhidas tanto pela sua disponibilidade quanto pelo fato de representarem linhagens distintas dentro do gênero. Também por estes motivos, incluímos no trabalho uma quarta espécie, a cascavel (*Crotalus durissus*), a qual foi considerada como grupo externo das espécies eleitas dentro do gênero *Bothrops* (Alencar et al., 2016). Assim, investigamos nas quatro espécies de serpente os efeitos da temperatura sobre a taxa metabólica de repouso e perda evaporativa de água, bem como a preferência térmica e as tolerâncias térmicas mínima e máxima.

As três espécies de *Bothrops* selecionadas para o desenvolvimento do presente projeto apresentam ampla distribuição geográfica com algumas áreas de sobreposição (Fig. 1). A distribuição de *B. jararaca* se dá mais ao leste do país, da Bahia até o Rio Grande do Sul, bem como nordeste do Paraguai e norte da Argentina. *Bothrops moojeni* ocorre numa faixa mais central do Brasil, do Piauí ao Paraná, se extendendo ao norte da Argentina e leste da Bolívia e do Paraguai. *Bothrops alternatus* é a espécie que se distribui mais ao sul dentre as três, presente nas regiões centro-oeste, sudeste e sul do Brasil, se estendendo até o Uruguai, Paraguai e norte da Argentina (Campbell e Lamar, 2004). Quanto ao micro-hábitat, tanto *B. jararaca* quanto *B. moojeni* são encontradas em regiões florestadas, sendo esta última quase sempre associada a matas de galeria (Sazima, 1992; Nogueira et al., 2003; Sawaya et al., 2008; Barbo et al., 2011; Pires et al., 2012). Ademais, embora próxima a corpos de água, *B. moojeni* é frequentemente encontrada em áreas abertas de Cerrado (Martins et al., 2002; Nogueira et al., 2003), ao passo que que *B. jararaca* raramente está ligada a tais regiões não florestadas (Sazima, 1992). *Bothrops alternatus*, por sua vez, é mais comum no campo sujo, próximos a áreas ripárias, podendo estar tanto no solo como sobre a vegetação (Sazima, 1992; Martins et al., 2001; Sawaya et al., 2008). Além disso, *B. alternatus* também é frequentemente encontrada em formações abertas, incluindo áreas agrícolas, suburbanas e perturbadas (Lema, 2002; Campbell & Lamar, 2004; Sawaya et al., 2008). No caso da cascavel sul-americana, *Crotalus durissus*, apresenta ampla distribuição na América do Sul sobrepondo, inclusive, a distribuição geográfica das três espécies de *Bothrops* inclusas neste estudo (Fig. 1). No Brasil, esta serpente habita os cerrados do Centro-oeste, as regiões áridas e semi-áridas do Nordeste e os campos e áreas abertas no Sul, Sudeste e Norte (Campbell e Lamar, 2004; Tozetti e Martins, 2008).

Desta forma, ao longo do desenvolvimento deste trabalho, buscamos responder as seguintes questões:

(i) O aumento da temperatura eleva as taxas metabólica e a perda evaporativa de água das serpentes?

(ii) Espécies que habitam ambientes próximos a corpos d'água apresentam maiores taxas de perda de água?

(iii) Espécies de ambientes com temperaturas mais amenas selecionam temperaturas mais baixas em laboratório?

(iv) Espécies de ambientes com menor variação de temperatura (e.g., áreas florestadas) exibem menores tolerâncias térmicas do que espécies de áreas abertas?

(v) Espécies que habitam ambientes mais secos (e.g., áreas abertas e afastadas de regiões ripárias) apresentam menor PEA em comparação às espécies de locais mais úmidos?

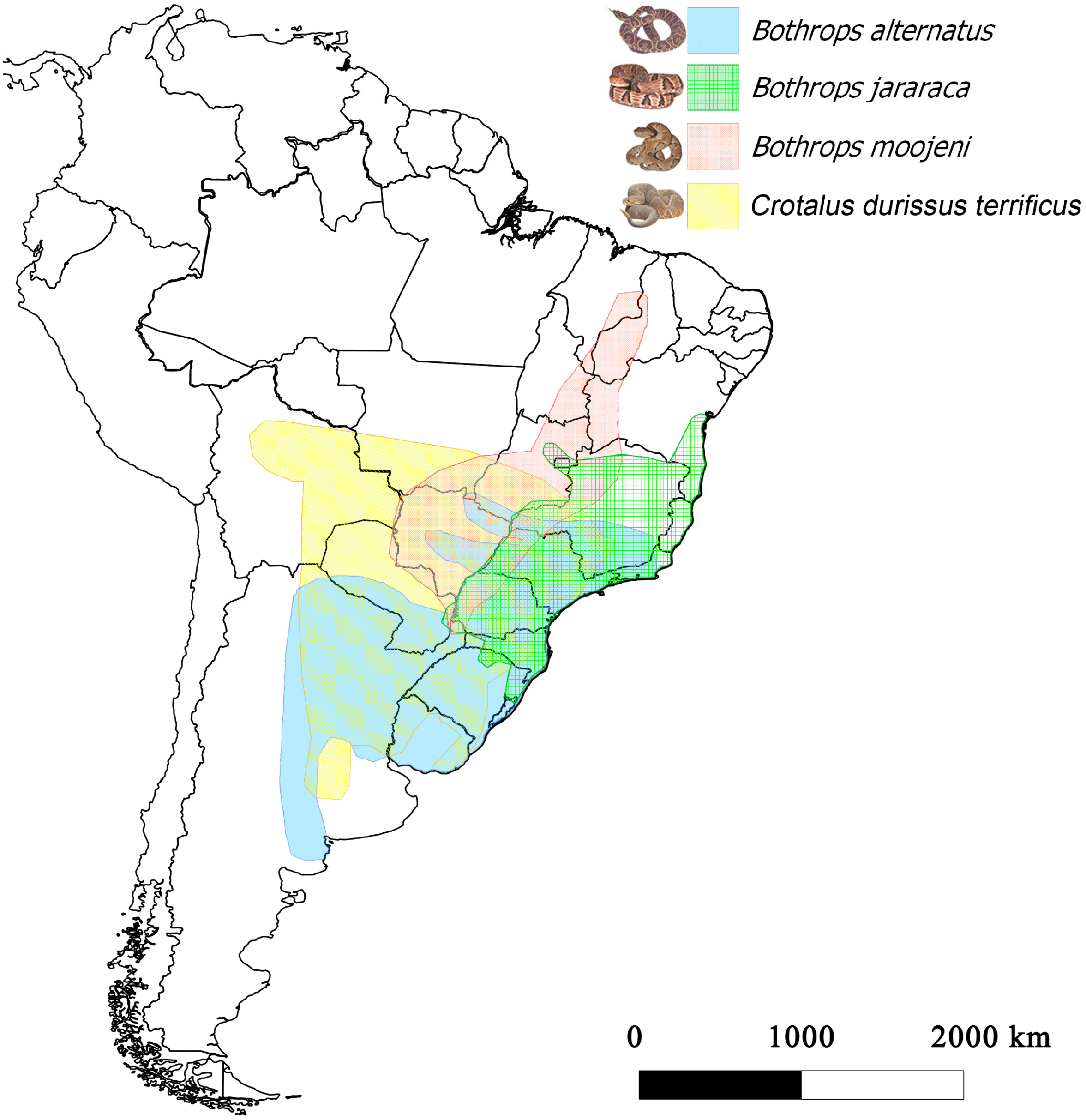

Figura 1. Mapa da distribuição geográfica das quatro espécies de serpente utilizadas no presente trabalho (adaptado de Campbell e Lamar, 2004).

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CAPÍTULO I

Resting metabolic rate and evaporative water loss in Neotropical pitvipers (Serpentes: Crotalinae)

RODRIGO S. B. GAVIRA[1*] & DENIS V. ANDRADE[1]

[1]Departamento de Zoologia, Instituto de Biociências, Universidade Estadual Paulista "Júlio de Mesquita Filho". Avenida 24-A, 1515. 13506-900. Rio Claro, SP, Brazil.

*Corresponding author: rodgavira@gmail.com

ABSTRACT

In ectotherms, resting metabolic rate (RMR) and evaporative water loss (EWL) are typically directly related to T_b, and may also vary interespecifically and with thermal characteristics and water availability of different habitats. Thus, in order to address the ecophysiological aspects involved with the variation in metabolism and evaporative water loss in a group of snakes, we examined the effects of temperature (15, 25, and 35°C) on RMR and EWL in four Neotropical Crotalinae snakes (*Bothrops alternatus*, *B. jararaca*, *B. moojeni*, and *Crotalus durissus*) from South America. These snakes are phylogenetic related and occupy geographical areas that overlap extensively, but present considerable differences in terms of preferred microhabitats and other life history attributes. In general, *B. jararaca* and *B. moojeni* occupy mainly forested areas, whereas *B. alternatus* and *C. durissus* are open area dwellers. For all species, RMR and EWL increased with temperature. For *B. jararaca* and *B. moojeni*, RMR was greater than those measured for *B. alternatus* and *C. durissus* at 15 and 35ºC. However, at 25ºC, no difference in RMR was found among snake species. *Crotalus durissus* had the lowest rates of EWL among all species investigated, regardless temperature. Among the *Bothrops* no difference in EWL rates occurred at 15 and 25°C. However, at 35°C, *B. alternatus* exhibited lower rates of EWL than congeneric species. Broadly, differences in RMR and EWL seems to be consistent with differences in microhabitat occupancy.

KEYWORDS: Metabolism; Water balance; Crotalinae snakes; Habitat occupancy.

1. INTRODUCTION

Body temperature (T_b) regulation is central for the adequate functioning of physiological processes, survival and, ultimately, fitness (Cossins and Bowler, 1987; Bradshaw, 1997; Angilletta et al., 2002; Barros et al., 2010; Titon et al., 2010; Clusella-Trullas et al., 2011; Lemoine and Burkepile, 2012). Accordingly, most animals invest considerable time and energy in thermoregulation, that branches onto two major divisions: endothermy and ectothermy. Endothermic animals generally regulate T_b at fairly constant and high levels at the expenses of endogenous heat production, derived from metabolic activity (Cossins and Bowler, 1987; Buckley et al., 2012; Rezende and Bacigalupe, 2015). On the other hand, ectothermic animals regulate their body temperature primarily on the basis of external heat sources explored by means of behavioral adjustments (Cowles and Bogert, 1944; Angilletta et al., 2002). Hence, one of the major consequences of ectothermy is that T_b regulation is largely influenced by the availability of adequate thermal niches in the environment (Angilletta, 2009) and, as such, ectothermic organisms usually experience much larger fluctuations in body temperature than the vast majority of endotherms (Huey, 1982; Angilletta et al., 2002).

Body temperature variation has profound impacts on the functional capabilities of ectotherms and can result in important ecological consequences (Wieser, 1973; Cossins and Bowler, 1987; Angilletta et al., 2002). In general, as body temperature increases, within critical limits, the rate of most physiological processes augments concurrently (Angilletta, 2009; Dillon et al., 2010). Thus, understandably, a vast literature have focused on the exam of temperature influence on a different organismal processes (Angilletta et al., 2002; Huey et al., 2012; Andrade, 2016). Among these processes, some are more integrative than others and, most likely, metabolic rate is one of the most integrative aspects of the biology of ectotherms that is often examined (McNab, 2002). Indeed, metabolic determinations allow for the assessment of the sum of the energy expenditure derived from all physiological systems at work under a given set of circunstances (Dorcas et al., 2004; McCue, 2006; Lighton, 2008). Obviously, as these circumstances vary, so does the level of metabolic activity and, therefore, the metabolic consequences associated to a number of organismal and environmental variables is largely documented (e.g., Huey, 1982; Angilletta, 2009; Huey et al., 2012; Seebacher et al., 2012; Basson and Clusella-Trullas, 2015; see also Secor, 2009 and references therein). For comparative purposes, however, a parameterization of the metabolic determinations is instrumental. In this regard, the most basic aspect to be examined on the metabolism of an ectothermic organism refers to the minimum cost for the maintenance of physiological system at their lowest level of activity, often referred as the resting metabolic rate (RMR). RMR is determined in resting and post absorptive animals, measured out of their activity period and at a specified temperature (Bennett and Dawson, 1976; McCue, 2006). In snakes, RMR may account from 10 up to 45% of the yearly energy expenditure (Congdon et al., 1982; Secor and Nagy, 1994; Beaupre, 1995), being considerably affected by temperature (Al-Johany and Al-Sadoon, 1996; Wang et al., 2003; Gavira and Andrade, 2013a; Lourdais et al., 2013). Therefore, studies focusing on the determination of RMR, as well as the factors affecting it, particularly temperature, are central to promote a better assessment of the energetic requirements and potential trade-offs of ectothermic organisms (Hailey and Davies, 1986; Zaidan, 2003; Aubret et al., 2015).

The maintenance of adequate concentration levels of bodily water and solutes is essential for animal life. Central to this is the balancing between water gains and losses and, in this regard, one important avenue to be considered in terrestrial animals is the potential risk of evaporative water loss (EWL) through the integument and respiratory surfaces (Dmi’el and Zilber, 1971; Hattingh, 1972; Lillywhite, 2006; Cooper and Cruz-

Neto, 2009). Similar to metabolism, temperature has the potential to influence importantly the rates of EWL. Temperature may exerts its influence directly, by affecting the air water vapor capacitance and, therefore, the potential for evaporation (Lillywhite, 2006), and indirectly, since the metabolic increment associated with temperature (commented above) is, in most cases, associated to a concurrent increase in ventilation, which results in higher levels of EWL from the respiratory surfaces (Lillywhite and Maderson, 1982; Dean and Gratz, 1983). For example, in snakes EWL rates increase with T_b, as expected (e.g., Dmi'el, 1972; 1985; Mautz, 1982a; Hailey and Davies, 1986; Guillon et al., 2014; Dupoué et al., 2015) and this increase is often accompanied by the increment in lung ventilation (Lillywhite and Maderson, 1982; Dean and Gratz, 1983; Guillon et al., 2014). Thus, osmoregulation, in general, and EWL, in particular, is intimately integrated with body temperature regulation, metabolism, and other physiological processes as well (Dmi'el, 1972; Withers and Cooper, 2014). Finally, EWL susceptibility is known to vary among species, which is generally thought to reflect adaptation to environmental contingencies, especially in thermal characteristics and water availability, of different habitats (Elick and Sealander, 1972; Roberts and Lillywhite, 1980; Mautz, 1982a; Peterson et al., 1993; Lahav and Dmi'el, 1996; Winne et al., 2001; Moen et al., 2005; Guillon et al., 2014), a pattern that seems also valid for snakes (Gans et al., 1968; Cohen, 1975; Spotila and Berman, 1976; Lillywhite and Sanmartino, 1993; Lillywhite et al., 2009).

In order to address the ecophysiological aspects involved in metabolism and EWL variation commented above, herein we examined the effects of temperature on RMR and EWL in a group of Crotalinae snakes from the Neotropical region. These snakes have a degree of phylogenetic relatedness relatively well established (Alencar et al., 2016), occupy geographical areas that overlap extensively (Campbell and Lamar, 2004), but present considerable differences in terms of preferred microhabitats and other life history attributes (Sazima, 1992; Martins et al., 2001; Oliveira and Martins, 2001; Sawaya et al., 2008). The snakes elected to carry out the study were 3 species of the *Bothrops* genus (*B. alternatus*, *B. jararaca*, and *B. moojeni*) and the assigned outgroup for them, the South-American rattlesnake, *Crotalus durissus*. The Neotropical pitvipers from the *Bothrops* genus compose an apparently monophyletic clade of about 50 species, occurring throughout diverse landscapes in Central and South America (Hoge and Romano-Hoge, 1981; Campbell and Lamar, 2004; Greene, 1992; Alencar et al., 2016). Within the *Bothrops* genus, approximately seven lineages are recognized and the species herein studied, *B. alternatus*, *B. jararaca*, and *B. moojeni*, are representative of the '*alternatus*', '*jararaca*', and '*atrox*' groups, respectively (Carrasco et al., 2012). *Bothrops jararaca* occurs mainly in eastern Brazil, as well as northeast Paraguay and northern Argentina, whereas *B. moojeni* is found in from central-western to the south of Brazil, extending to northern Argentina and east of Bolivia and Paraguay. *Bothrops alternatus* is the southernmost snake species, occupying the regions central-west, south-eastern and southern of Brazil, being also found in Uruguay, Paraguay and northern Argentina (Campbell and Lamar, 2004). In relation to the microhabitat, both *B. jararaca* and *B. moojeni* are primarily found in forested areas, the latter being often associated with gallery forests (Sazima, 1992; Nogueira et al., 2003; Sawaya et al., 2008; Barbo et al., 2011; Pires et al., 2012). In addition, while *B. moojeni* may also be found in open areas of Brazilian Cerrado (Martins et al., 2002; Nogueira et al., 2003), *B. jararaca* is mostly common in areas of Atlantic Forest, seldom found in open areas (Sazima, 1992; Hartmann, et al., 2009; Sousa et al., 2012). *Bothrops alternatus* inhabits regions of open savanna ("campo sujo") close to riparian areas (Sazima, 1992; Martins et al., 2001; Lema, 2002; Campbell and Lamar, 2004; Sawaya et al., 2008). *Crotalus durissus* occurs mainly in open formations (Colli et al., 2002; Marques et al., 2004; Bastos et al., 2005; Tozzetti and

Martins, 2008) and its geographic distribution overlaps those of all three *Bothrops* species (Campbell and Lamar, 2004; Wüster et al., 2005). In general, *C. durissus* is largely acknowledged as a more generalist and tolerant species in comparison to *Bothrops*, with some indications that deforestation may indeed benefit its populations (Bastos et al., 2005; Duarte and Menezes, 2013). Thus, on the basis of the differences in macro habitat preference and use among the different species herein investigated, we predict that rates of metabolism and water loss will vary concurrently. In particular, we expect that EWL rates will be lower and less affected by temperature in *B. alternatus* and *C. durissus*, particularly in the latter, compared to *B. jararaca* and *B. moojeni*. Similarly, we expect that *C. durissus* and *B. alternatus* exhibit lower RMR and less sensitivity to temperature change than *B. jararaca and B. moojeni.*

2. MATERIAL AND METHODS

2.1. Study animals

We used fifty-two both sexes snakes (*Bothrops alternatus* = 10; *B. jararaca* = 14; *B. moojeni* = 15; and *Crotalus durissus* = 13). Snakes were collected in several locations of São Paulo state, southern Brazil and also received from donations by other São Paulo state research institutes.

Animals were housed at the Laboratório de Fisiologia Animal Comparada, at Universidade Estadual Paulista (UNESP), Rio Claro municipality, São Paulo state, Brazil. They were placed individually in wooden cages with glass front and side holes for ventilation (30 x 29 x 27 cm), lined with corrugated cardboard kept in room with temperature controlled at approximately 26°C (± 2°C). Animals were fed monthly with a single mouse (*Mus musculus*) and had free access to water. Only apparently healthy animals and not undergoing ecdysis were used in the experiments.

All snakes were fasted for a period of 15 to 20 days before the experiments to avoid any influence of digestion on the experiments (see Andrade et al., 2005; Gavira et al., 2013b; Dupoué et al., 2015). All animals were obtained under a license for capture and transport issue by the "Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis" (IBAMA, process number and 22028-1). Procedures were approved by the "Animal Use Ethic Committee" (CEUA) from Instituto de Biociências, Universidade Estadual Paulista (protocol number 1655, March 12th 2014).

2.2. Respirometry

Resting metabolic rate was estimated from the oxygen consumption rates ($\dot{V}O_2$) of snakes at 15, 25, and 35°C. After weighing, animals were placed in circular respirometric chambers hermetically sealed (PVC with an acrylic cover; Fig. 2), with two holes for inlet and outlet air flow, and volume suitable to the snake's size (500–2000 ml). For $\dot{V}O_2$ determination, it was used a system of an intermittent-flow closed respirometry, in which seven animals were measured simultaneously (Fig. 2). Basically, an air pump connected to a mass flow meter (SS4 Subsampler, Sable Systems) generated a constant air flow (150 $ml.min^{-1}$). This flow was then directed to a multiple flow controller (Multiplexer v2.0, Sable Systems) programmed to switch periods of 60 minutes in which the chambers were ventilated with outside air (open phase), with periods of 10 minutes (closed phase) in which the air contained in the chambers was recirculated through an oxygen analyzer (PA-1, Sable Systems). Finally, all this apparatus was attached to a universal interface (UI-2 Data Acquisition Interface, Sable Systems) connected to a computer, where the readings of O_2 percentage were recorded.

The fall in O_2 concentration was always linear, and the slope of the regression between these two variables (i.e., *b* value) was used for the calculation of $\dot{V}O_2$. As it was used data acquisition rate of 1 Hertz for each regression (closed phase of 10 minutes) we obtained 600 individual samples every record. However, the initial 100 and the last 10 samples of each period were discarded, and thus, for each $\dot{V}O_2$ calculation, it was used regressions of 490 samples, in general with r^2 value greater than 0.8. Hence, the drop in the fractional concentration of O_2 recorded during the closed phase allowed it to determine the snakes $\dot{V}O_2$, estimated from milliliters of O_2 uptake per hour, normalized to 1 kg body mass (see McCue, 2006).

Fasted snakes remained in the respirometry for 72 hours, of which the first 24 hours were dedicated for the animals' acclimatization. Thus, RMR values were estimated as the average of oxygen consumption during the last two days in the respirometry. At the end of RMR experiment, snakes retuned to their maintenance boxes, where they were fed and remained for 20 days, until the beginning of EWL trials (see item 2.3). Experimental temperature was controlled by keeping the animals inside a BOD climatic chamber (122FC, Eletrolab), whose temperatures were periodically checked by a maximum and minimum thermometer (5201.03, Incoterm), accepting up to ± 1°C of variation. In order to avoid an overestimation of RMR (Andrade et al., 2005) due to digestive processes, snakes were fasted for a period of 15 to 20 days.

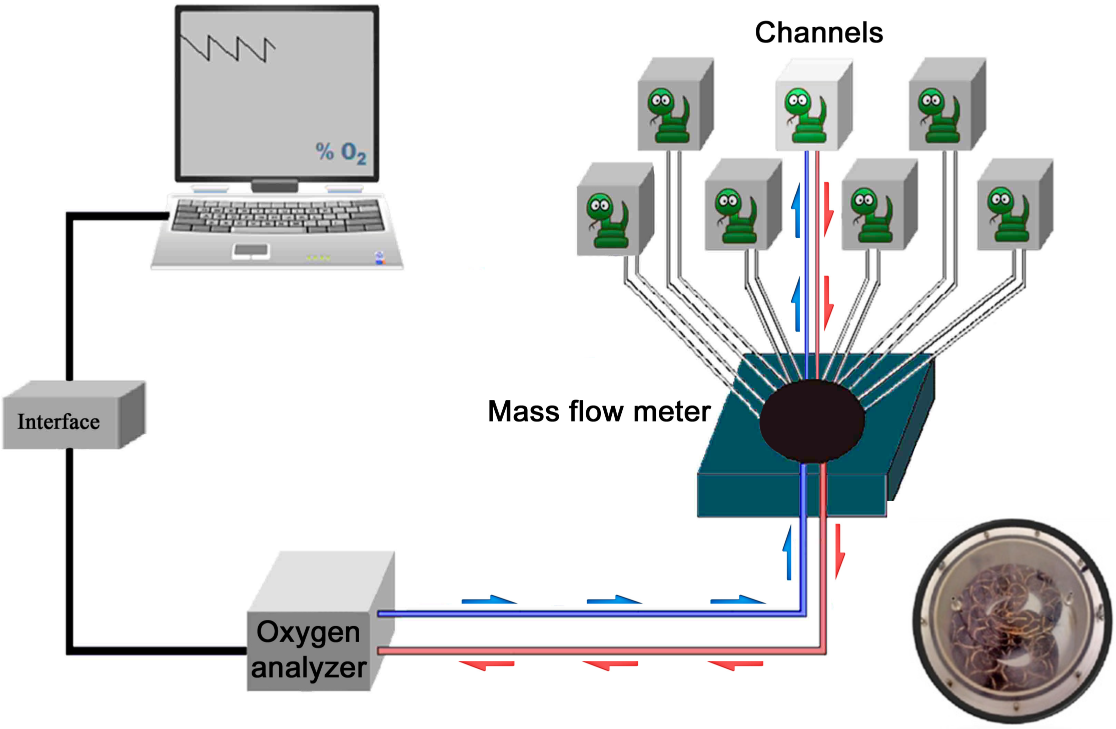

Figure 2. Schematic representation of the respirometry system used for metabolic measurements. The arrows indicate the direction of airflow before (blue) and after (red) passing the respirometric chamber during $\dot{V}O_2$ measurements. The drop in the fractional concentration of O_2 inside the chambers was monitored by an oxygen analyzer and recorded by a computerized data acquisition system. System was set to automatically measure one snake (i.e. channel) each ten minutes (closed phase). Snakes in the channels that were not being measured (open phase) received constant flow of ambient air. In the lower right corner, a snake inside the respirometric chamber.

2.3. Evaporative water loss

For estimating the total evaporative water loss, snakes were weighted and placed individually in circular PVC chambers with volume compatible to their body size (1000 ml – 2000 ml). Each snake was measured at the temperatures of 15, 25, and 35°C, respectively, using an automated open-flow system (Fig. 3), which consisted of a set of air pump associated with a mass flowmeter (SS4 Subsampler, Sable Systems), responsible for the air supply to the apparatus. Air flow rate was regulated at 767 or 1,534 ml.min^{-1} for chambers of 1000 and 2000 ml, respectively, which ensured a 99% of air turnover within the chamber every 6 minutes (Lasiewski et al., 1966). Before reaching the animal´s chamber, air flow was directed to a container filled with silica gel (Kitasato flask, ~ 2000ml), for water vapor removal. Usually, the relative humidity (RH) of the excurrent airflow after passing the silica container was at 1% (± 1%). The dry airflow was then directed to the animal chamber and to a flow-through water vapor analyzer (RH-300, Sable Systems) (Fig. 3). EWL was estimated by the difference in RH of the airflow before and after passing through the chamber containing the animals (Winne et al., 2001) and was expressed by the hourly amount of water efflux per body mass unit (mg $H_2O.g^{-1}.h^{-1}$).

Evaporative water loss data were continuously recorded through a computerized data acquisition system (UI-2, Sable Systems). All EWL apparatus, except for the computer and the interface, were kept inside a climatic chamber (122FC, Eletrolab) at the experimental temperature.

EWL trials usually lasted from 40 minutes (small specimens) to 1 hour (large ones) per individual, when snakes exhibited a period of steadystate readings lasting for at least 10 minutes, typically during the last half of the measurement period. Finally, at the end of the records, the surface temperature of the snakes was recorded with an infrared thermometer (TN2, TSI). In order to avoid an overestimation of EWL (Dupoué et al., 2015) due to digestive processes, snakes were fasted for a period of 15 to 20 days.

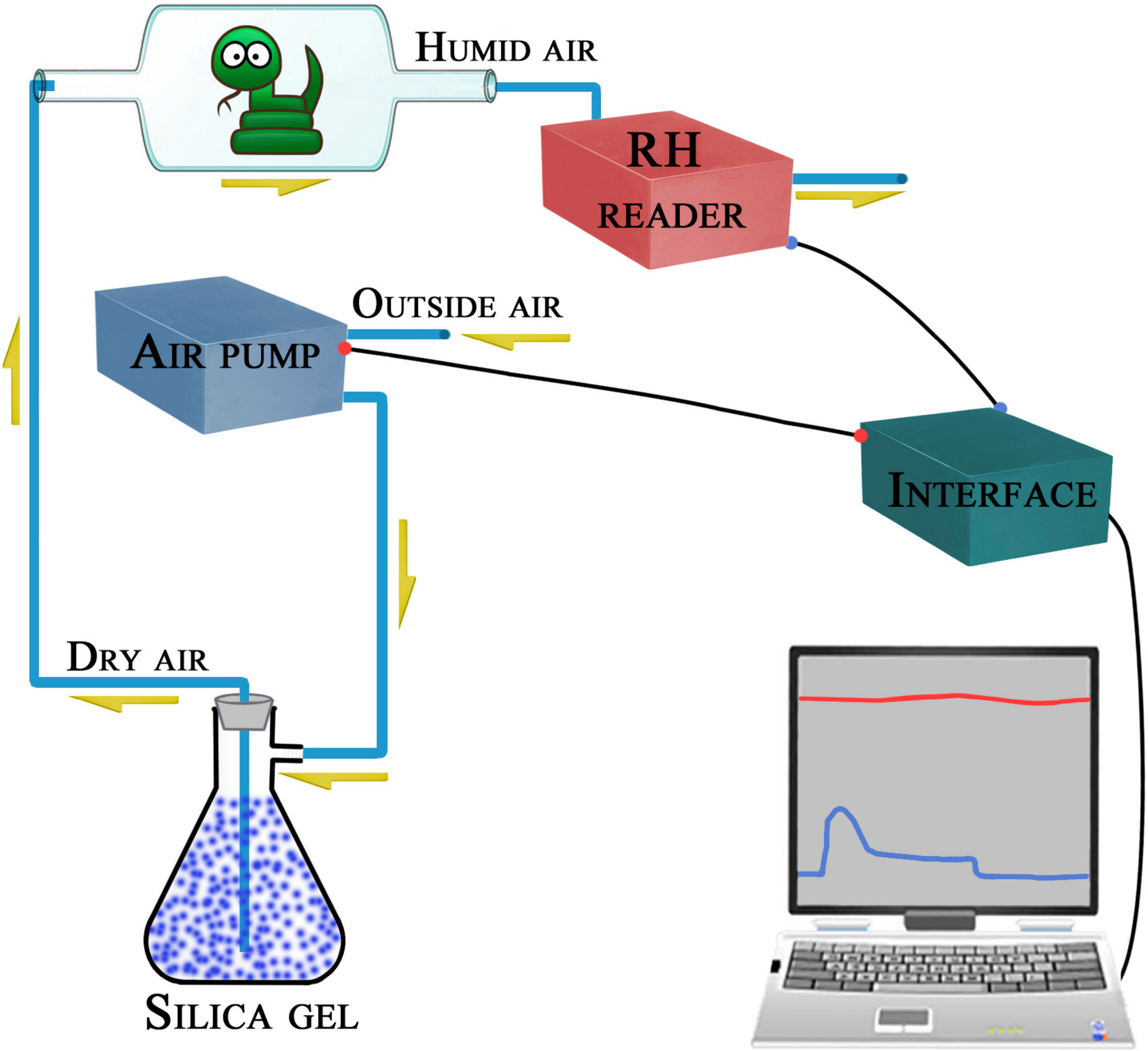

Figure 3. Schematic representation of the experimental setup used to measure evaporative water loss. Arrows indicate airflow direction. Computer screen shows the airflow rate (in red) and the relative humidity (in blue). Air pump pulled outside air and forced it through a Kitassato flask filled with silica gel and, thereafter, to the chamber containing the snake and, finally, through the humidity analyzer, whose reading was recorded with a computerized data acquisition system.

2.4 . Data analyses

Although we are aware that conventional statistical approach disregards the effect of common ancestry on data independence among different species (Harvey and Pagel, 1991), we did not perform a phylogenetically informed analysis of our results due to the low number of species investigated (see also Li et al., 2009; Qu et al., 2011; Guillon et al., 2014; Watson and Burggren, 2016; Gienger et al., 2017). Our approach was based on the fact that little or no phylogenetic signal can be detected in sample sizes with *n* less than twenty species (Blomberg et al., 2003; Revell, 2010; Münkemüller et al., 2012).

All snakes were measured repeatedly at the temperatures of 15, 25, and 35°C, for both EWL and RMR experiments. To compare the temperature effects on RMR and EWL intraespecifically we used one-way ANOVA test for repeated measures. The RMR and EWL data were first analyzed via a two-way analysis of covariance (ANCOVA), using temperature and species as factors, and body mass as the covariate. Once it was determined that mass was not a significant covariate, this variable was dropped from the model and the analyses were run for the temperature and species factors using a two-way ANOVA with temperature and species as the factors, followed by Holm-Sidak test for *post hoc* multiple comparisons. For analysis of thermal sensitivity (i.e., Q_{10}) in both RMR and EWL, for each species, we used a Student's t-test between Q_{10} values estimated at 15–25°C and 25–35°C intervals. We also compared Q_{10} values for RMR and EWL among the four species for each experimental temperature, and for that, we used a two-way ANOVA with temperature interval and species as the factors, followed by Holm-Sidak test for *post hoc* multiple comparisons. Before the statistical tests, we evaluated the parametric premises of normality (Shapiro-Wilk) and equality of variances (Levene's test). When we observed lack of normality and/or homoscedasticity of variance, the data were logarithmized and the test repeated. All tests followed Zar (1999), and the results are presented as mean ± standard error. The significance level was set at $P < 0.05$.

3. RESULTS

3.1. Resting metabolic rate

Snakes' body masses did not differ among temperatures intraespecifically (*B. alternatus*, $F_{2,18} = 1.61$; *B. jararaca*, $F_{2,26} = 3.14$; *B. moojeni*, $F_{2,26} = 0.03$; *C. durissus*, $F_{2,22} = 0.14$; $P \geq 0.109$ for all species; Table 1). Interespecifically, *C. durissus* had higher body mass than *B. alternatus* and *B. moojeni* ($F_{3,146} = 6.15$; $P < 0.001$). In spite of these difference, the analyses of covariance indicated that RMR was not affected by it ($F_{3,146} = 0.053$; $P = 0.656$). RMR increased with temperature in all species (*B. alternatus*, $F_{2,18} = 224.08$; *B. jararaca*, $F_{2,26} = 300.26$; *B. moojeni*, $F_{2,26} = 292.52$; *C. durissus*, $F_{2,22} = 258.05$; $P < 0.001$ for all species; Fig. 4; Table 1). Interespecifically, at 15°C, *B. alternatus* and *B. moojeni* presented similar RMR ($P = 0.854$), however, higher than those found in *B. jararaca* and *C. durissus* ($P < 0.001$; Table 1). At 25°C, RMR did not differ among species ($P \geq 0.365$). At 35°C, *B. jararaca* and *B. moojeni* showed similar RMR ($F_{6,138} = 3.23$; $P = 0.151$) but higher than *B. alternatus* ($P < 0.05$ and $P < 0.01$, respectively) and *C. durissus* ($P < 0.01$ and $P < 0.001$, respectively; Table 1).

Bothrops alternatus ($t_{18} = -2.31$; $P < 0.05$) and *B. moojeni* ($t_{26} = -2.38$; $P < 0.05$) had Q_{10} values in the 15–25°C interval lower than those in the 25–35°C, whereas *B. jararaca* ($t_{26} = 0.95$; $P = 0.349$) and *C. durissus* ($t_{22} = 0.70$; $P = 0.494$) presented no differences between these both temperature intervals (Table 1). Interespecifically, *B. jararaca* showed higher Q_{10} than *B. alternatus* ($P < 0.01$) and *B. moojeni* ($P < 0.05$) in the 15–25°C interval ($F_{3,92} = 2.81$).

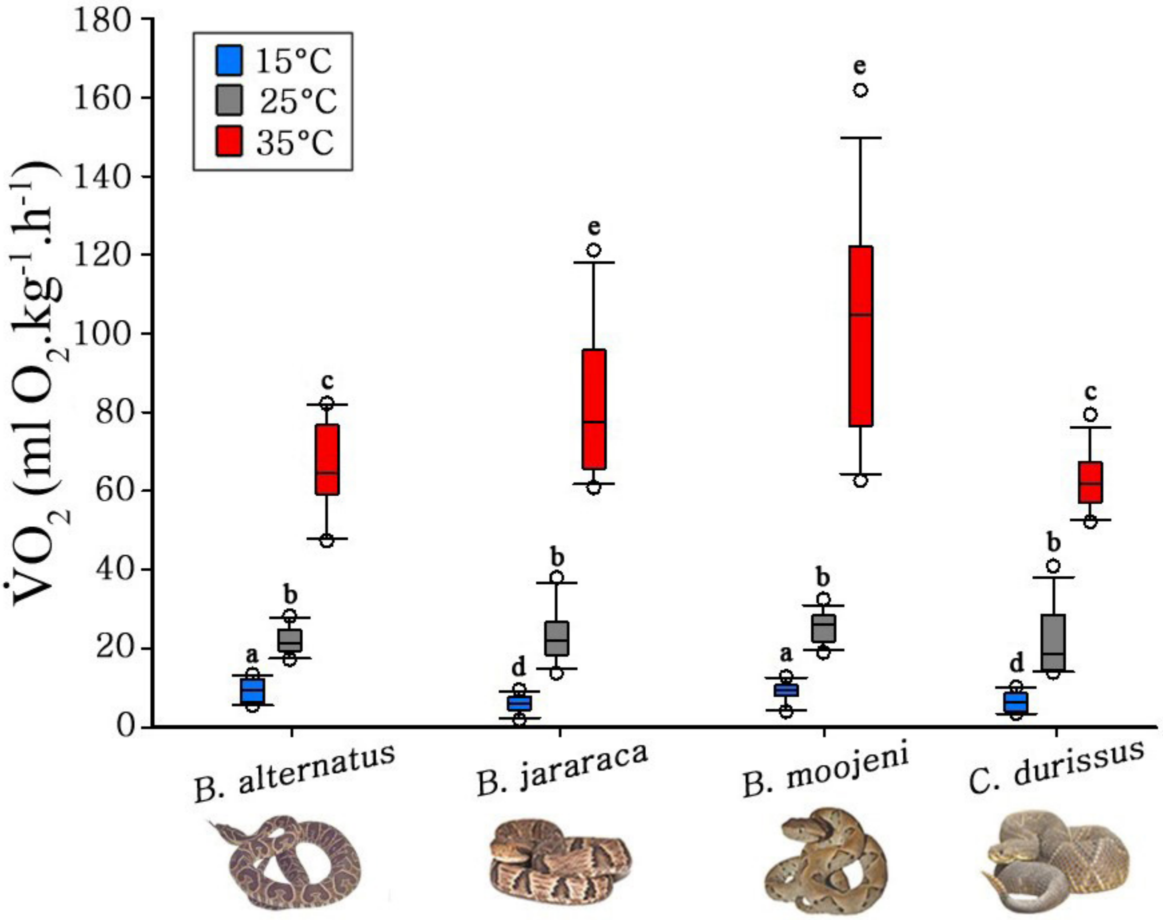

Figure 4. Resting metabolic rates (oxygen uptake rates, $\dot{V}O_2$) of four Viperidae snakes measured at 15, 25, and 35°C. Different letters indicate significant differences among temperatures and species. Horizontal lines represent the median, boxes represent lower and upper quartiles, and whiskers the minimum and maximum values. Empty circles, above and below the whiskers, denote outliers.

Table 1. Body mass, oxygen consumption rates, and respective Q_{10} of four Viperidae snakes measured at 15, 25, and 35°C.

	n	**Body mass (g)**	**RMR (ml $O_2 . kg^{-1} . h^{-1}$)**			**Q_{10_RMR}**	
			15°C	**25°C**	**35°C**	**15 – 25°C**	**25 – 35°C**
B. alternatus	10	337.91±27.45 (169.05–632.20)	9.43±0.94 (5.51–13.27)	21.80±1.03 (17.17–28.15)	66.51±3.58 (48.00–82.90)	2.47±0.20 (1.54–3.40)	3.08±0.17 (2.25–3.92)
B. jararaca	14	367.60±37.29 (76.96–1005.40)	5.95±0.58 (1.78–9.44)	23.18±1.87 (13.64–37.90)	80.91±5.25 (58.38–118.76)	4.44±0.56 (2.15–9.47)	3.69±0.28 (1.65–5.30)
B. moojeni	14	289.50±20.57 (59.10–675.30)	9.19±0.68 (3.92–12.76)	25.43±1.05 (18.90–32.43)	102.00±8.00 (62.59–161.75)	3.11±0.41 (1.63–7.39)	4.04±0.29 (2.62–5.76)
C. durissus	12	451.46±17.51 (213.97–707.60)	6.46±0.68 (3.41–10.14)	21.69±2.46 (13.83–40.89)	62.67±2.12 (52.12–79.31)	3.56±0.36 (1.94–5.85)	3.23±0.30 (1.49–4.72)

Data are shown as mean ± SE (minimum and maximum values).

3.2. Evaporative water loss

Snakes' body masses did not differ among thermal treatments intraespecifically (*B. alternatus*, $F_{2,18} = 3.45$; *B. jararaca*, $F_{2,24} = 2.70$; *B. moojeni*, $F_{2,20} = 2.99$; *C. durissus*, $F_{2,18} = 1.82$; $P \geq 0.054$ for all species; Table 2). Interespecifically, *C. durissus* had higher body mass than *B. moojeni* and *B. jararaca* ($F_{3,128} = 5.65$; $P < 0.001$ in both cases). Analyses of covariance indicated that EWL of snakes was affected by the differences in body masses among species, being that larger individuals had lower EWL ($F_{3,128} = 5.23$; $P < 0.005$). In any species, rates of EWL increased with temperature (*B. alternatus*, $F_{2,18} = 102.52$; *B. jararaca*, $F_{2,24} = 267.86$; *B. moojeni*, $F_{2,20} = 354.13$; *C. durissus*, $F_{2,18} = 106.29$; $P < 0.001$ for all species; Fig. 5; Table 2). Regardless of the temperature, *C. durissus* showed the lowest rates of EWL compared to the other three species ($P < 0.05$ in all cases), except at 15°C when it did not differ from that of *B. alternatus* ($P = 0.240$; Fig. 5; Table 2). At 35°C, the rates of EWL in *B. alternatus* were lower than in all the other congeners ($P < 0.05$ in both cases), but higher than *C. durissus* ($F_{6,120} = 1.30$; $P < 0.001$; Fig. 5; Table 2).

Bothrops jararaca ($t_{24} = -2.98$; $P < 0.01$) and *B. moojeni* ($t_{20} = -4.63$; $P < 0.001$) had a EWL Q_{10} value estimated for the 15–25ºC temperature interval lower than that attained between 25–35ºC. Interespecifically, all four species showed no differences in Q_{10} for 15–25°C interval ($P \geq 0.298$). At 25–35°C interval, *C. durissus* showed Q_{10} values lower than those exhibited by *B. moojeni* ($F_{3,80} = 1.19$; $P < 0.01$).

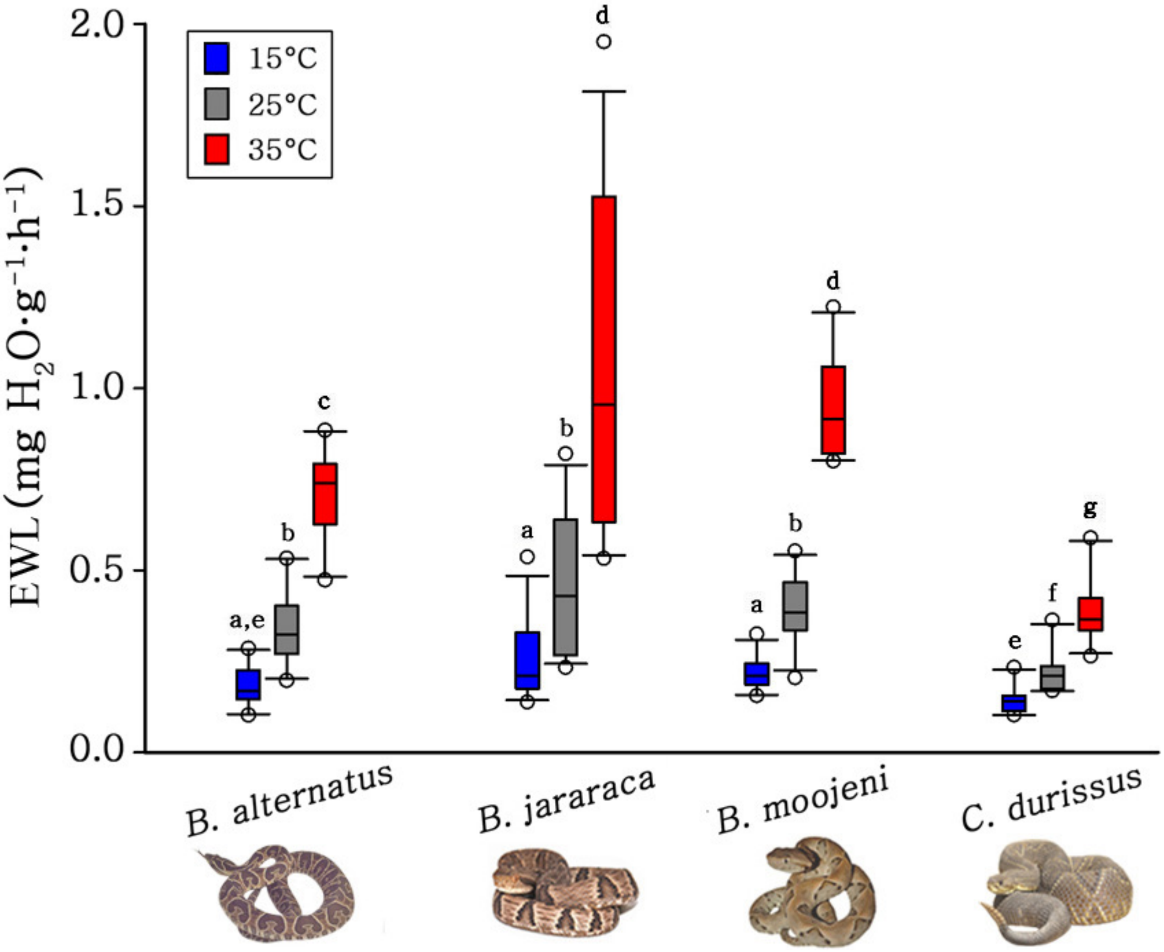

Figure 5. Evaporative water loss rates in four Viperidae snakes measured at 15, 25, and 35°C. Different letters indicate significant differences among temperatures and species. Horizontal lines represent the median, boxes represent lower and upper quartiles, and whiskers the minimum and maximum values. Empty circles above and below the whiskers denote outliers.

Table 2. Body mass, evaporative water loss, and respective Q_{10} of four Viperidae snakes measured at 15, 25, and 35°C.

	n	**Body mass (g)**	**EWL (mg H_2O . g^{-1} . h^{-1})**			**Q_{10_EWL}**	
			15°C	**25°C**	**35°C**	**15 – 25°C**	**25 – 35°C**
B. alternatus	10	346.26±29.55 (172.38–607.60)	0.181±0.017 (0.102–0.285)	0.341±0.034 (0.196–0.532)	0.711±0.040 (0.473–0.884)	1.99±0.23 (1.17–3.48)	2.20±0.15 (1.21–2.87)
B. jararaca	13	328.76±32.64 (78.26–702.50)	0.262±0.032 (0.138–0.536)	0.465±0.054 (0.232–0.819)	1.051±0.129 (0.532–1.951)	1.82±0.14 (1.31–3.25)	2.27±0.10 (1.74–3.27)
B. moojeni	11	270.74±17.33 (149.28–520.80)	0.215±0.014 (0.155–0.324)	0.388±0.029 (0.204–0.552)	0.958±0.043 (0.199–1.223)	1.80±0.09 (1.31–2.26)	2.56±0.16 (1.92–4.01)
C. durissus	10	433.12±25.09 (144.25–703.30)	0.141±0.012 (0.102–0.233)	0.220±0.018 (0.168–0.363)	0.391±0.029 (0.264–0.589)	1.58±0.08 (1.16–2.10)	1.80±0.08 (1.51–2.15)

Data are shown as mean ± SE (minimum and maximum values).

4. DISCUSSION

Rates of resting metabolism and evaporative water loss increased with temperature in all four snake species studied, and we found significant differences in these traits among the four Viperidae snakes studied. In general, all snake species exhibited RMR equivalent to other Viperidae snakes (Cruz-Neto and Abe, 1994; Secor and Nagy, 1994; Andrade et al., 1997; Beaupre and Zaidan, 2001; Zaidan, 2003; Zaidan and Beaupre, 2003; Tsai et al., 2008; Stuginski, 2014), which forage by ambush and typically present lower metabolic rate than that reported for active foragers (Secor and Nagy, 1994; Secor and Diamond, 2000; McCue and Lillywhite, 2002; Zaidan and Beaupre, 2003; Ott and Secor, 2007). RMR at 25°C was similar among all snake species and differences in RMR were found only at lower and higher temperatures. At 15°C, *B. alternatus* and *B. moojeni* exhibited higher RMR than *B. jararaca* and *C. durissus*, what may be related to differences in their thermal biology. *Bothrops alternatus*, besides being the southernmost species (Campbell and Lamar, 2004), occupies habitats characterized by open savanna (Lema, 2002; Sawaya et al., 2008) and is found active in the field at air temperatures up to 13°C (Nogueira et al., 2003). *Bothrops moojeni* is nocturnally active at air temperatures ranging from 18.5 to 25.0°C (Nogueira et al., 2003), and has the lowest preferred body temperature of species measured (see Capítulo II). Furthermore, *B. moojeni* is known to occupy both forest and open (but always close to riparian woodlands) areas, in which the environmental temperature is often below 15°C during dry season (i.e., May to beginning of September; França and Braz, 2013). In contrast, although geographic distribution of the four species is broadly overlapping, *B. jararaca* and *C. durissus* are commonly found in warmer microhabitats. For instance, *B. jararaca* occurs in forested habitats (Sazima, 1992; Barbo et al., 2011), whose climate is typically characterized by substantial precipitation, high relative humidity, and mean environmental temperatures mild and less variable than in open areas (Joly et al., 1999; Colombo and Joly, 2010; Hartmann, et al., 2009; Marques et al., 2004; 2015), whereas *C. durissus* occurs mainly in the hot and dry Cerrado domain (Colli et al., 2002; Sawaya et al., 2008), although it may experience very low temperatures in there during dry season (Colli et al., 2002; Tozetti and Martins, 2013). Thus*, B. moojeni* and *B. alternatus* are likely to experience low temperatures more frequently than *B. jararaca* and *C. durissus*. Indeed, higher Q_{10} values from 15°C (the lowest temperature) to 25°C in *B. alternatus* and *C. durissus* (Table 1) reinforces this idea, since thermal sensitivity of metabolic rate in Squamata is also mediated by different operative temperatures which animals experience in their thermal niches (Bakken, 1992; Beaupre and Zaidan, 2001; Zaidan, 2003).

According to what we found at 15°C, snakes' RMR at 35°C seemed to be congruent with the thermal niche in which animals interact in their habitat. *Bothrops moojeni* and *B. jararaca* presented the highest $\dot{V}O_2$ at 35°C, respectively about 37% and 19% greater than other snakes here studied. As mentioned above, *B. moojeni* is found mainly associated with gallery forests and marshes (Sawaya et al., 2008), and active at air temperatures between 18.5 and 29.5°C (Nogueira et al., 2003). *Bothrops jararaca* occurs in Atlantic rainforests, which typically present mild to high temperatures throughout the year (Marques et al., 2001; 2004; Hartmann, et al., 2009) and do not go through rigorous thermal variation. In this regard, *B. jararaca*, and particularly *B. moojeni*, usually experiment environmental temperatures in nature much lower than 35°C, what could have triggered the exceptionally high RMR for both species at this temperature. Indeed, *B. jararaca* and *B. moojeni* have the lowest heat tolerance among four snake species herein measured (see Capítulo II), thus reinforcing our idea that physiological traits may reflect habitat occupancy.

Mean Q_{10} values for metabolic rates in most snake species typically range from 1.5 to 3.0 (Lillywhite, 1987). For Crotalinae species, however, Q_{10} varies between 1.8 and 4.8 (Beaupre and Duvall, 1998; Beaupre and Zaidan, 2001; McCue and Lillywhite, 2002; Zaidan and Beaupre, 2003; Dorcas et al., 2004; Gavira and Andrade, 2013a; Leite et al., 2014; Dupoué et al., 2015). Our focal species had similar mean values of Q_{10}, which ranged from 2.4 to 4.0 and compare well to those for other Crotalinae species. Furthermore, high Q_{10} in Crotalinae may imply energetic constraints to these snakes, since environmental thermal variation have profound impacts on their maintenance cost (Dorcas et al., 2003; Stuginski, 2014). Thermal sensitivity were similar in all snake species herein measured except for the forest-dweller *B. jararaca* which showed high sensitivity to temperature variation from 15 to 25°C. As we previously expected, *C. durissus* had the lowest RMR among species at low and, as well as *B. alternatus*, at high experimental temperatures. In conclusion, these open area dwellers constantly face high variation in the environmental temperature and experience extreme temperatures more frequently than forested species (Hartmann, et al., 2009; Marques et al., 2004; 2015). Indeed, although all of our snake species increased RMR with T_b, such increment was much more pronounced in forested species. Accordingly, Gunderson et al. (2015) stated that species living in warm regions, such as tropical forests, might be less resilient to environmental thermal variation, especially to extremely high temperatures.

All snake species studied increased EWL with temperature. Indeed, with only very few exceptions (see Dawson et al., 1966), EWL in reptiles is temperature dependent, being that the warmer the T_b, the higher water efflux rates (Dmi'el, 1972; Mautz, 1982b; Eynan and Dmi'el, 1993; Lillywhite, 2006). Rates of EWL of our focal species were in accordance to those reported for several snake species (Dmi'el, 1972; 1985; Mautz, 1982a; Hailey and Davies, 1986; Guillon et al., 2014; Dupoué et al., 2015). *Bothrops* snakes showed similar rates of EWL at 15°C and 25°C, but were significant different at the highest experimental temperature (i.e., 35°C). Not many studies have investigated the EWL of different Squamata species in different temperatures. In these investigations, except for desert or aquatic species (Dmi'el, 1972; Lillywhite et al., 2009; Aubret and Sarraude, 2015; Cox and Cox, 2015), differences in animals' EWL tend to be more conspicuous as temperature increases (Dmi'el, 1972; Eynan and Dmi'el, 1993; Lahava and Dmi'el, 1996; Dmi'el, 1998; Dupoué et al., 2015). Such response may be related to the fact that the animals' adaptation to avoid dehydration risk is more evident, and indeed essential, in high temperatures, in which the integument of organisms is more vulnerable to water efflux due to the higher vapor pressure and activity of water molecules (Lillywhite, 1987; Angilletta, 2009). In congruence to this, at 35°C rates of EWL were lower in open area dwellers (*C. durissus* and *B. alternatus*) in comparison to the forested species (*B. jararaca* and *B. moojeni*), underpinning the congruence between the physiology of the snake species and habitat occupancy.

In fact, snake species from open areas exhibited lower rates of EWL than forested species in all experimental temperatures (15, 25, and 35°C). As predicted, *C. durissus*, which occupy the driest habits among all four species (Colli et al., 2002; Sawaya et al., 2008) and exhibited the lowest EWL rates in any temperature, tended to maintain constant low Q_{10} values with the temperature increment (Table 2). Indeed, Roberts and Lillywhite (1980) found that snakes from xeric habitats present lower thermal sensitivity in EWL compared to more mesic species. In addition, rates of EWL in snakes are known to be influenced by climatic conditions of animals' habitat, being that EWL is typically inversely related to habitat aridity, i.e., snakes from different regions have EWL rates proportional to the available water in the habitats from which they occur (Gans et al., 1968; Cohen, 1975; Spotila and Berman, 1976; Lillywhite and Sanmartino, 1993; Dmi'el, 1998; Winne et al., 2001; Moen et al., 2005; Guillon et al., 2014). Although *B. alternatus* may occupy more humid areas than *C. durissus* (Lema, 2002; Sawaya et al., 2008), both species are distributed in regions with open forest vegetation, comprising areas of Brazilian savanna-like, the Cerrado (sensu stricto), with drier climatic characteristics (Colli et al., 2002; Sawaya et al., 2008; Tozetti and Martins, 2008). On the other hand, *B. jararaca* and *B. moojeni* presented the highest rates of EWL at the highest temperature. Both species inhabit forested areas (Sazima, 1992; Barbo et al., 2011) with average rainfall and relative humidity higher than in Cerrado domain (Joly et al., 1999; Colombo and Joly, 2010; Hartmann, et al., 2009; Marques et al., 2004; 2015).

Cutaneous and respiratory evaporation are the main route of water loss in terrestrial ectotherms (Dmi'el and Zilber, 1971; Blamires and Christian, 1999). Respiratory water losses appear to be variable among different snake species, accounting in average for 21-44% of the total loss (Prange and Schmidt-Nielsen, 1969; Dmi'el, 1972; 2001). The increase in metabolic rate with temperature is usually associated with an increment in EWL rates due to the accompanying increase in lung ventilation and respiratory evaporation (Dmi'el, 1972; Lillywhite and Maderson, 1982; Dean and Gratz, 1983; Guillon et al., 2014). For example, the Viperidae snakes *Daboia* (*Vipera*) *palaestinae* and *Cerastes cerastes* exhibited higher ventilatory and EWL rates when T_b was increased (Dmi'el, 1972). Hence, it is plausible that the high RMR at 35°C in *B. moojeni* and *B. jararaca,* in comparison to *B. alternatus* and *C. durissus*, was accompanied by high ventilatory rates, what led to an increment of respiratory water efflux and, therefore, higher rates of water loss.

A possible caveat of our outcomes could be a potential influence of phylogeny on the results that, presently, we were unable to isolate. In this regard, the most distantly related species, *C. durissus*, was the one in which some of the most marked differences were found. However, even within the more closely related species of the *Bothrops* genus, significant differences were apparent and seem to be associated with differences in their ecological attributes and habitat occupied. Clearly, a more solid interpretation about the factors involved on the evolution of thermal biology in Neotropical Viperidae snakes will demand the exam of a greater number of species. The second aspect that may confound some of our results and interpretations are differences in body size. In fact, body size variation among the different snake species herein studied was considerably lesser than the variation presented in the body size of viperid snakes at equivalent life stages (Martins et al., 2001; Sawaya et al., 2008). However, the fact that body size still exerted an influence in the parameters examined in our study indicates, unequivocally, the importance of acknowledging the influence of this fundamental organismal attribute while trying to understand patterns of association between functional and ecological traits (Bini et al., 1998; Olalla-Tárraga et al., 2009; Terribile et al., 2009; Gouveia, Andrade, et al., submitted). Therefore, the potential influence of this variable was incorporated in our analyses and, therefore, we were able to weigh its relevance while discussing our results, especially at EWL, in which larger individuals had lower rates than smaller ones. Such findings are typical for most animals, given the relatively large surface area-to-volume ratio of smaller organisms make them more susceptible to dehydration (Christian, 1978; Mautz, 1982a; Dmi'el, 1985; Agugliaro and Reinert, 2005; Tracy et al., 2010). However, although body size of *C. durissus* was slightly larger than *B. jararaca* and *B. moojeni*, we believe that such differences in body size among snake species did not alter our main conclusions, specially because all *Bothrops* species had similar body size.

Although factors such as environmental temperature variation, ecotone areas, predation risk, and prey availability might be contributing to the habitat occupancy of snakes, the physiological parameters herein evaluated showed congruence to the type of environment occupied from the point of view of phytophysiognomy. Indeed, the relation of vegetation structure and habitat selection is relatively well established in several snake species (Blouin-Demers and Weatherhead, 2002; Row and Blouin-Demers, 2006; Lelièvre et al., 2011). In conclusion, we acknowledge that our climatic description of snakes' habitat was narrow, based on few studies of some spots of their geographic distribution. Thus, further investigation regarding the bioclimatic conditions of snake's distribution, such as the relation between environmental thermal variation and average precipitation with habitat selection, may provide better understand of how physiological limits constrain animals' occupancy. Anyway, our results evidence that differences in RMR and EWL exhibited by our Crotalinae snakes were related to the physical characteristics of their environment, being that species from open areas were less sensitive to temperature change and had lower RMR and EWL than forested snake species. Finally, *C. durissus*, followed by *B. alternatus*, would be clearly less vulnerable to a potential thermal stress caused by climatic warming in comparison to *B. moojeni* and *B. jararaca*, since at extreme high temperatures those species would not present very high energy expenditure and would be less susceptible to desiccation.

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CAPÍTULO II

Thermal preference and tolerance in Neotropical pitvipers (Serpentes: Crotalinae)

RODRIGO S. B. GAVIRA[1*] & DENIS V. ANDRADE[1]

[1]Departamento de Zoologia, Instituto de Biociências, Universidade Estadual Paulista "Júlio de Mesquita Filho". Avenida 24-A, 1515. 13506-900. Rio Claro, SP, Brazil.

*Corresponding author: rodgavira@gmail.com

ABSTRACT

Preferred body temperature (T_{pref}) and critical thermal limits (CT_{min} and CT_{max}) are central attributes of the thermal biology of ectothermic organisms, and probably reflect historical and/or adaptive differences. In this regard, the exam of thermal preference and tolerance in different snake species of known degree of relatedness and differing in habitat occupancy and some other biological traits, might be particularly enlightening. Herein, we select four Neotropical Crotalinae snakes (*Bothrops alternatus*, *B. jararaca*, *B. moojeni*, and *Crotalus durissus*) to investigate possible differences in T_{pref}, CT_{min}, and CT_{max}. These snakes are phylogenetic relatedness and occupy geographical areas that overlap extensively, but present considerable differences in terms of preferred microhabitats and other life history attributes. In general, *B. jararaca* and *B. moojeni* occupy mainly forested areas, whereas *B. alternatus* and *C. durissus* are open area dwellers. Our results showed that T_{pref} at night was higher than during daytime, in any species. Interespecifically, diurnal T_{pref} did not differ among species, indicating a potential energy economy to the snakes during inactivity period. At nighttime *B. jararaca* and *C. durissus* selected higher temperatures than *B. alternatus* and *B. moojeni*, a pattern that still remains to be understood. The southernmost species, *Bothrops alternatus*, exhibited the lowest CT_{min} among all species. The open area dwellers, *B. alternatus* and *C. durissus*, exhibited similar and also higher CT_{max} than the forested species, *B. jararaca* and *B. moojeni*. A higher CT_{max} indicate that these open area dweller species would be less vulnerable to a potential thermal stress caused by climate warming in comparison to the forested species. In conclusion, our outcomes highlight that both T_{pref} and critical limits were congruent with the type of environment occupied by the snakes, from the point of view of phytophysiognomy.

KEYWORDS: Thermoregulation; Crotalinae snakes; Thermal preference; Thermal tolerance; Habitat occupancy.

1. INTRODUCTION

Temperature affects dramatically the physiological processes of animals and, therefore, body temperature (T_b) regulation is pivotal for the adequate functioning of physiological systems (Cossins and Bowler, 1987). Ectotherms rely primarily on external heat sources and behavioral adjustments (e.g., shifts in posture, microhabitat selection, basking) to thermoregulate (Reinert, 1984; Huey, 1991; Blouin-Demers and Weatherhead, 2001a; 2001b; 2002). As a consequence, the accuracy of T_b regulation may be constrained by the availability of thermal niches in the environment (Huey and Stevenson, 1979). Additionally, because physiological functions of ectotherms are temperature-dependent (Angilletta, 2009), behavioral thermoregulation is largely accepted to influence performance and fitness (Huey, 1982; Grant, 1990).

Thermoregulation in ectotherms is determined by a complex balance between costs and benefits (Huey and Slatkin, 1976; Blouin-Demers and Nadeau, 2005; Herczeg et al., 2008; Lelièvre et al., 2010). In this sense, adjustments in thermoregulatory behavior to compensate adverse conditions may increase the energy and time animals invest in thermoregulation (Huey and Slatkin, 1976; Lelièvre et al., 2011). On the other hand, body temperature regulation may result in benefits associated to the optimization of various physiological processes at a given temperature or thermal interval (Dawson, 1975; Huey and Bennett, 1987; Hertz et al., 1993; Angilletta et al., 2002; Blouin-Demers and Nadeau, 2005; but see Fitzgerald et al., 2003). Central to this concept, particularly for squamate reptiles, is the fact that animals, under permissible conditions, will exhibit a preferred body temperature (T_{pref}), sometimes equated as a thermal range. This thermal preference has been shown to differ among species and be influenced by a number of biotic and abiotic factors (Dawson, 1975; Huey and Slatkin, 1976; Rosen, 1991; Du et al., 2006; Lelièvre et al., 2010; Dupoué et al., 2015). Usually, T_{pref} is determined in the laboratory by placing the animals in a thermal gradient (Angilletta et al., 2002 and references therein), where thermoregulatory costs are zeroed, and animals are allowed to freely choose their preferred body temperature while active (Huey, 1982). Besides this T_{pref} for activity, as expected, thermal preference may also be exerted during the inactivity period and, therefore, thermal preference may vary along the different periods of the day associated with the fact of the animal being active or not (Al-Johany and Al-Sadoon, 1996; Angilletta et al., 1999; Bontrager et al., 2006; Lin et al., 2007).

Extending below and above the range of thermal preference, ectotherms are usually still able to engage in different behaviors, even at decreased levels of performance (Huey and Slatkin, 1976; Herczeg et al., 2003; Blouin-Demers and Nadeau, 2005). However, they eventually will reach a lower or upper thermal limit in which performance, and consequently survival, will be inviable. These extremes, referred as the critical thermal maximum (CT_{max}) and critical thermal minimum (CT_{min}), are generally determined by experiments of thermal tolerance, in which animals are subjected to cooling or heating, under pre-defined rates, until they reach an "end point" in which behavioral performance becomes critically compromised (Terblanche et al., 2007). Although the ultimate “end point” indicator of thermal tolerance is the animal’s death, it is generally accepted that "ecologically and physiology lethal" temperatures are those in which animals lost their ability of coordinated movement and would fail to escape from the critical temperatures (Cowles and Bogert, 1944; Doughty, 1994; Lutterschmidt and Hutchison, 1997). In this regard, a commonly adopted indicator of this “ecological death” is the loss of the righting reflex, when the animal fail, within a pre-established latency period, to right its body after being overturned on its back (Lutterschmidt and Hutchison, 1997).

In snakes, T_{pref} and critical thermal limits (CTL) vary intra and interspecifically, being influenced by a number of organismal and environmental variables. For example, T_{pref} and CTL are known to be influenced by feeding (Tsai and Tu, 2005; Sievert at al., 2013), pregnancy (Dupoué et al., 2015), hydration (Ladyman and Bradshaw, 2003), and pathological conditions (Burns et al., 1996) in snakes. Also, acclimation (Jacobson and Whitford, 1970; Lelièvre et al., 2011; Dupoué et al., 2015) and seasonal acclimatization (Graham et al., 1971; Joy and Crews, 1987; Row and Blouin-Demers, 2006; Lelièvre et al., 2011; Bovo et al., 2012) are known to influence thermal preference and tolerance in snakes. In general, differences in the thermal tolerance and preference among populations of a single species (Clarke, 2003; Hoekstra, 2015; Llewelyn et al., 2016; 2017) or among different species (Jacobson and Whitford, 1970; Huang et al., 2007; Li et al., 2009; Lelièvre et al., 2010; 2011; Moreno-Azócar et al., 2012) are often associated to concurrent differences in environmental and climatic parameters of the habitats occupied (Huey, 1991; Blouin-Demers and Weatherhead, 2001a). In conclusion, thermal preference and tolerance are central attributes of the thermal biology of ectothermic organisms, including snakes, probably reflecting historical and/or adaptive differences. In this regard, the exam of thermal preference and tolerance in different snake species of known degree of relatedness and differing in habitat occupancy and some other biological traits, might be particularly enlightening.

Herein, we select a few representative snake species from the Neotropical Crotalinae subfamily to determine possible differences in thermal preference and tolerance in an attempt to approach the questions previously commented. More specifically, we elected three species of the *Bothrops* genus (*B. alternatus*, *B. jararaca*, and *B. moojeni*) and the assigned outgroup for them, the South-American rattlesnake *Crotalus durissus*. The Neotropical pitvipers from the *Bothrops* genus is an apparently monophyletic clade of about 50 species, occurring throughout diverse landscapes in Central and South America (Hoge and Romano-Hoge, 1981; Campbell and Lamar, 2004; Greene, 1992; Alencar et al., 2016). Within the *Bothrops* genus, approximately seven lineages are recognized and the species herein studied, *B. alternatus*, *B. jararaca*, and *B. moojeni*, are representative of the '*alternatus*', '*jararaca*', and '*atrox*' groups, respectively (Carrasco et al., 2012). These species possess wide geographic distribution with considerable overlapping. *Bothrops jararaca* occurs mainly in eastern Brazil, as well as northeast Paraguay and northern Argentina, whereas *B. moojeni* is found in from central-western to the south of Brazil, extending to northern Argentina and east of Bolivia and Paraguay. *Bothrops alternatus* is the southernmost snake species, occupying the regions central-west, south-eastern and southern of Brazil, being also found in Uruguay, Paraguay and northern Argentina (Campbell and Lamar, 2004). In relation to the microhabitat, both *B. jararaca* and *B. moojeni* are primarily found in forested areas, the latter being often associated with gallery forests (Sazima, 1992; Nogueira et al., 2003; Sawaya et al., 2008; Barbo et al., 2011; Pires et al., 2012). In addition, while *B. moojeni* may also be found in open areas of Brazilian Cerrado (Martins et al., 2002; Nogueira et al., 2003), *B. jararaca* is mostly common in areas of Atlantic Forest, seldom found in open areas (Sazima, 1992; Hartmann, et al., 2009; Sousa et al., 2012; Rocha et al., 2014). *Bothrops alternatus* inhabits regions of open savanna ("campo sujo") close to riparian areas (Sazima, 1992; Martins et al., 2001; Lema, 2002; Campbell and Lamar, 2004; Sawaya et al., 2008). *Crotalus durissus* occurs mainly in open formations (Colli et al., 2002; Marques et al., 2004; Bastos et al., 2005; Tozzetti and Martins, 2008) and its geographic distribution overlaps those of all three *Bothrops* species (Campbell and Lamar, 2004). In general, *C. durissus* is largely acknowledged as a more generalist and tolerant species in comparison to *Bothrops*, with some indications that deforestation may indeed benefit its populations (Bastos et al., 2005; Duarte and Menezes, 2013).

Thus, on the basis of the differences in macro habitat preference and use among the different species herein investigated, we predict that thermal preference and tolerance will vary concurrently. More specifically, we expect that thermal tolerance will be greater in *B. alternatus* and *C. durissus*, particularly in the latter, compared to *B. jararaca* and *B. moojeni*. Similarly, we expect *C. durissus* and *B. alternatus* exhibit a thermal preference for higher temperatures than *B. jararaca* and *B. moojeni*.

2. MATERIAL AND METHODS

2.1. Study animals

We used thirty-three both sexes snakes (*Bothrops alternatus* = 7; *B. jararaca* = 9; *B. moojeni* = 8; and *Crotalus durissus* = 9). Snakes were collected in several locations of São Paulo state, southern Brazil and also received from donations by other São Paulo state research institutes.

Animals were housed at the Laboratório de Fisiologia Animal Comparada, at Universidade Estadual Paulista (UNESP), Rio Claro municipality, São Paulo state, Brazil. They were placed individually in wooden cages with glass front and side holes for ventilation (30 x 29 x 27 cm), lined with corrugated cardboard kept in room with temperature controlled at approximately 26°C (± 2°C). Animals were fed monthly with a single mouse (*Mus musculus*) and had free access to water. Only apparently healthy animals and not undergoing ecdysis were used in the experiments.

All snakes were fasted for a period of 15 to 20 days before the experiments to avoid any influence of digestion on the experiments (see Tattersall et al., 2004; Tsai and Tu, 2005; Bontrager et al., 2006; Wall and Shine, 2008; Sievert et al., 2013). All animals were obtained under a license for capture and transport issue by the "Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis" (IBAMA, process number and 22028-1). Procedures were approved by the "Animal Use Ethic Committee" (CEUA) from Instituto de Biociências, Universidade Estadual Paulista (protocol number 1655, March 12th 2014).

2.2. Temperature data recording

Body temperature (T_b) was recorded using thermal data loggers (iButton Thermochron, Maxim Integrated Products, Sunnyvale, CA, USA; model: DS1922L-F50; precision: 0.0625°C), computer-programmed via a contact reader for ibuttons (Blue dots, Maxim Integrated Products, Sunnyvale, CA, USA; model: DS1402D-DR8) and specific software (OneWireViewer, Maxim Integrated Products, Sunnyvale, CA, USA; version: 4.05). Sampling interval was set at 10 minutes for thermal preference determination and at 1 minute for the thermal tolerance trials. After being programmed, loggers were waterproofed by dipping them in liquid synthetic rubber coating (Plasti Dip International, Performix Brand, Blaine, MN, USA) (Fig. 6), and left for drying for approximately 12 hours (see Robert and Thompson, 2003; Roznik and Alford, 2012). Before coating, thermal loggers were all calibrated against a mercury-in-glass thermometer.

2.3. Surgical process

Each snake was implanted with two temperature data loggers (total mass ~ 7.5 g, 1.1–4.6% of snake body mass), which were programmed differently in order to record T_b variation during T_{pref} and CTL determinations (see above). Thus, snakes underwent only one surgical process for data logger implantation minimizing the potential stress associated with this procedure.

For logger implantation, snakes were previously anesthetized with isoflurane gas inhalation, and then partially placed in restraining tubes (i.e., with their first third inside the tubes) for a safe handling. Non-responsiveness and pupil dilation were used to indicate unconsciousness. Local asepsis was done with a 10% solution of povidone-iodine and, about ten minutes before the coeliotomy, lidocaine hydrochloride (2 $mg.kg^{-1}$) was injected subcutaneously into the region of the cut for local anesthesia. Skin incision (2 to 4 cm) was made with a scalpel in the left side of medial portion of snakes' body, just above the area dividing dorsal and ventral scales, and the sterilized ibuttons (alcohol dipping) were inserted into intraperitoneal cavity (Fig. 6). Next, incision was closed by using non-absorbable nylon surgical suture and broad-spectrum antibiotic (enrofloxacin, 10 $mg.kg^{-1}$) injected intramuscularly. Total duration of each operation was about 20 min and all snakes recovered from anesthesia within 5–15 min after the end of surgery, when they were touch responsive and started to move. Doses for all drugs were adjusted according to the snakes' body mass, obeying the proportions of body mass and concentration (Mosley, 2006). All surgical procedures were conducted by following the instructions and techniques of Alworth et al. (2011) for snakes' surgery. Animals were allowed to recover from the surgical procedure for at least 5 days before experimentation. Upon the completion of the experiments, the data loggers were retrieved following the same surgical procedures as for implantation.

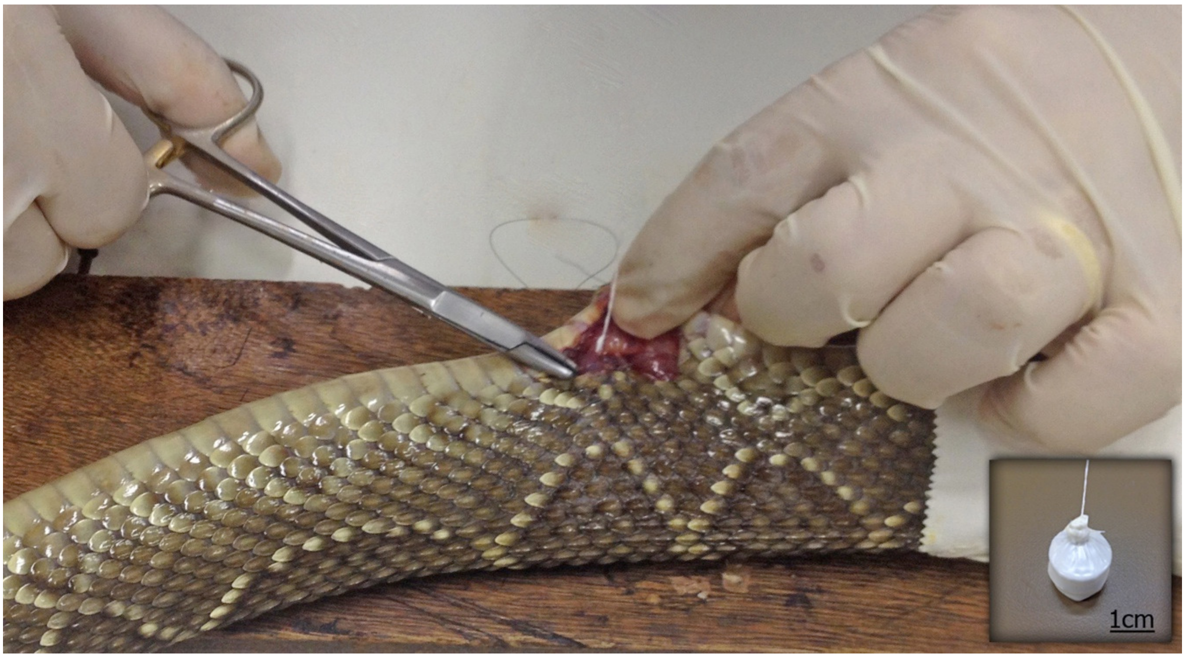

Figure 6. Implanting of previously encapsulated and waterproofed ibuttons (highlighted in the lower right corner), in the intraperitoneal cavity of an anesthetized rattlesnake (*C. durissus*).

2.4. Thermal preference

We determined the thermal preference (T_{pref}) of snakes by measuring their selected body temperatures in a rectangular thermal gradient (4 m x 0.5 m) fenced by 90 cm high plywood walls (Figs. 7 e 8). This gradient was arranged inside a room with controlled air temperature at 25ºC ± 2°C under a photoperiod of 12L:12D, with photophase starting at 06:00. Substratum was composed by a copper plate rested on top of two groups of opposing heat exchangers made of galvanized steel (Fig. 7). Copper substratum was subdivided into eight sections with adhesive markers (black electrical tape), which provided greater clarity in identifying the selected site by the animals (Fig. 8). Two thermostatic water baths (Tecnal, Tecnal Equipamentos Científicos, Piracicaba, SP, BRA; model: TE-184; precision: 0.1°C) were used to control the temperature of the heat exchangers by pumping hot (45ºC) or cold (3ºC) water into each opposing group of them.

Heat transfer from the heat exchangers and the copper plate substratum was modulated by interposing a polyester sheet between them. By varying the thickness of this sheet, we were able to implement a gradual and linear temperature variation from 11 to 35°C (Fig. 9), along the major axis of the gradient, whose accuracy was constantly checked with a thermal (infrared) imaging camera (FLIR Systems, ThermaCAM, Wilsonville, OR, USA; model: SC640; sensitivity: <0.1°C; Fig. 10).

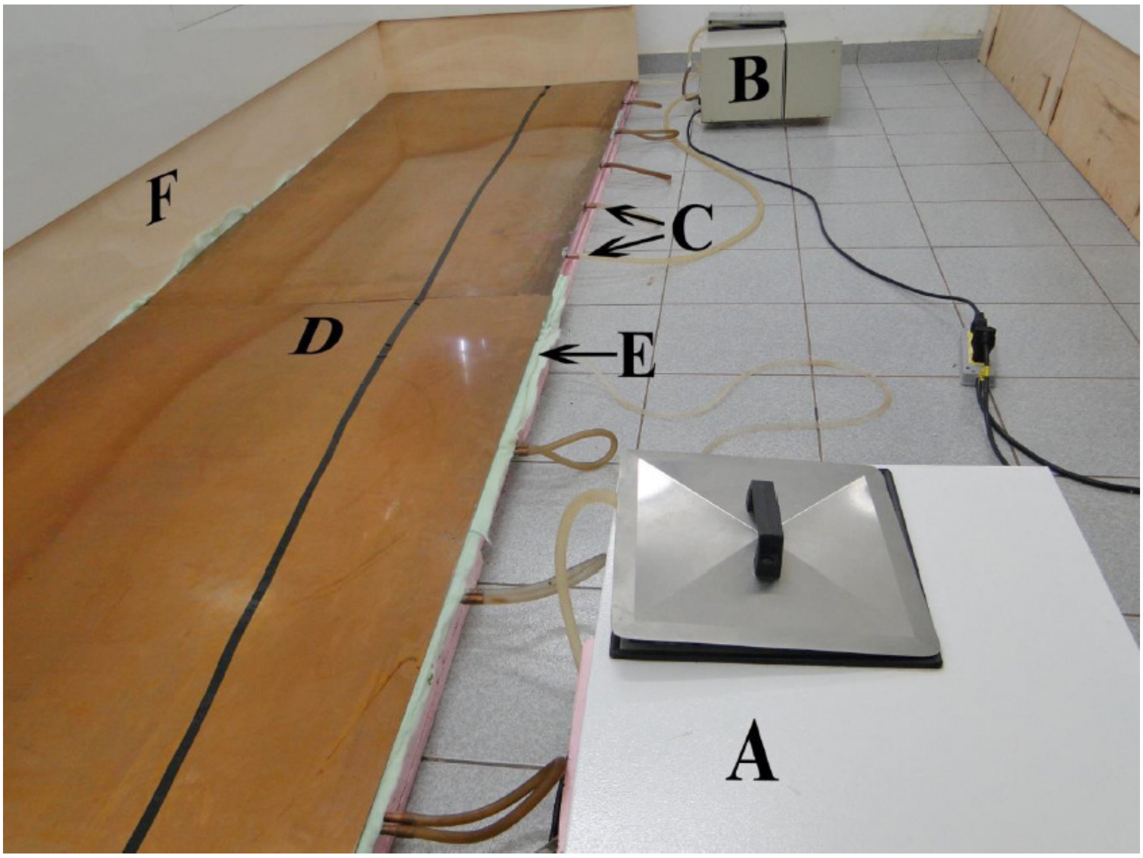

Figure 7. Thermal gradient and its components: Thermostatic baths with hot (A) and cold (B) water; Hose plugs of heat exchangers (C); Copper substratum (D); Sheets of fiber glass wool (E); Plywood walls that delimit the gradient (F). Just for the photo, one of the sidewalls was removed for a better perception of the system structures (courtesy of Adriana Fuga).

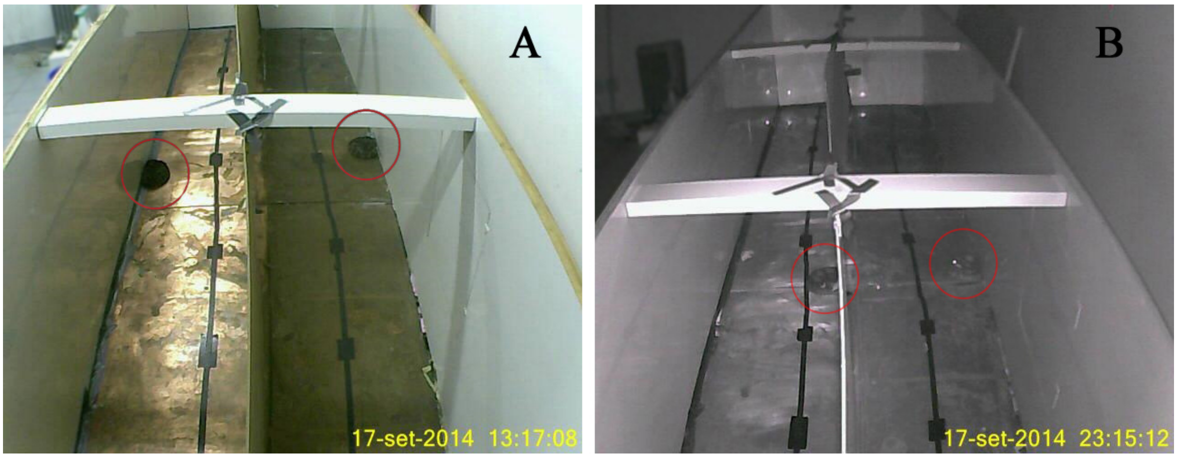

Figure 8. Image of the thermal gradient taken at T_{pref} experiment during daytime (A) and nighttime (B). Red circles show the snakes' position in the gradient. Arena was divided into two equal parts for the simultaneous measurement of two individuals.

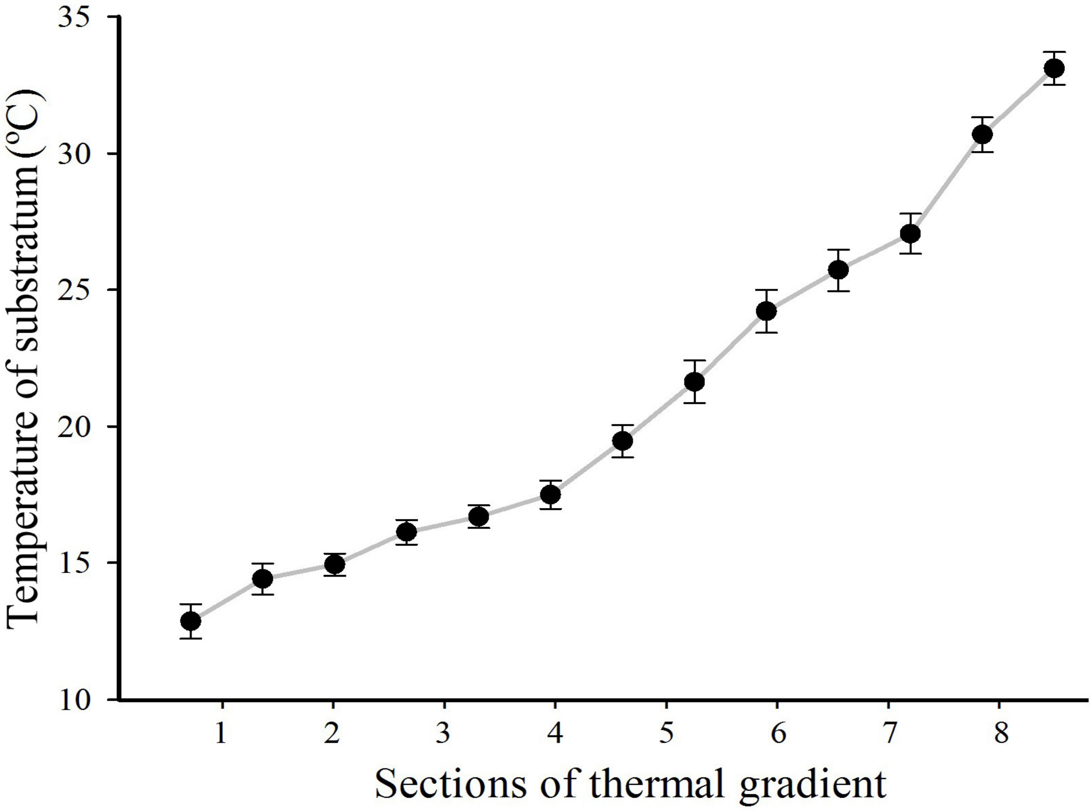

Figure 9. Temperatures registered along the eight sections (50 cm length each) of the copper plate substratum during thermal gradient validation. Circles represent the temperature recorded in each one of thirteen thermal loggers (distant about 30 cm apart) arranged on the plate. Temperature variation pattern shows that the thermal gradient was gradual and linear. Data are displayed as mean ± SE over 24 hours.

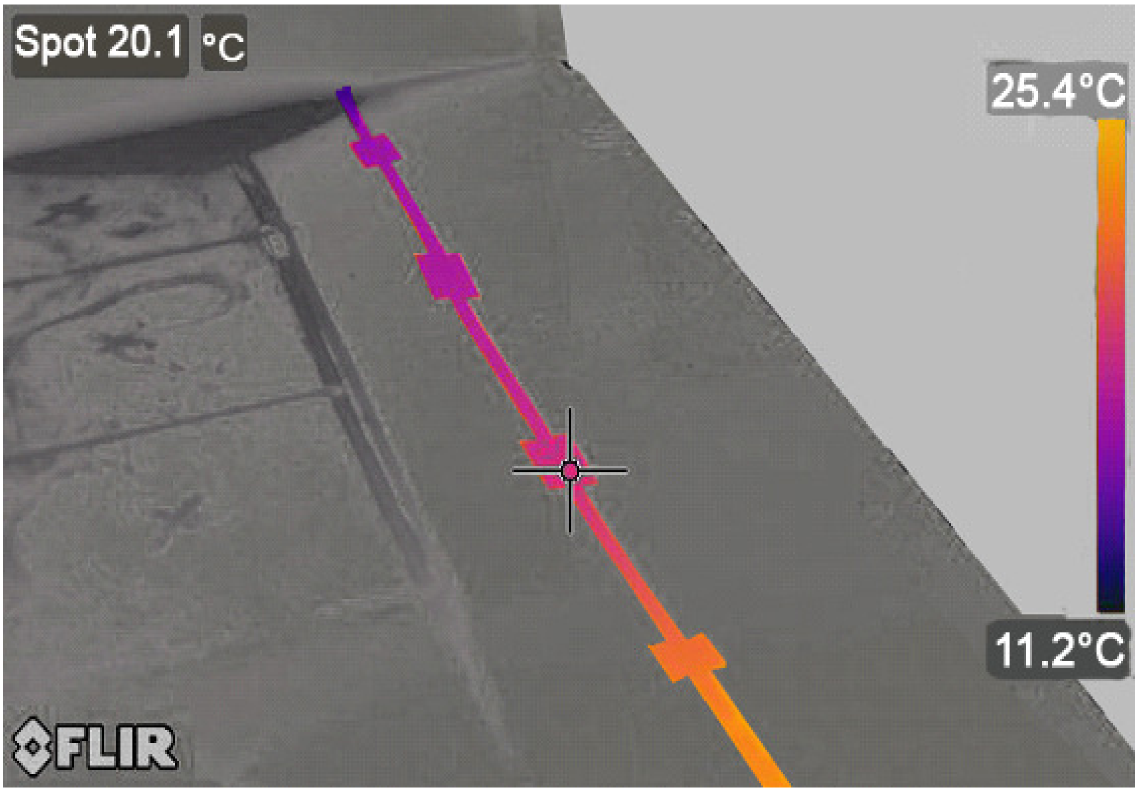

Figure 10. Thermal image showing one half (the cold part, ~ 11 to 25°C) of the copper plate covering the thermal gradient. Note the gradual temperature change in the electrical tape (colored center strip) attached to the substratum.

Thermal preference was determined by releasing the snakes into the thermal gradient, where they were left undisturbed for a period of four days. From these measurements, the first 12 hours were allowed for acclimation and data obtained during this period was not included in the estimation of T_{pref}. Since there was a clear circadian pattern in activity for all snake species investigated (Fig. 11), we analyzed thermal preference separately for the activity and inactivity periods (see also Angilletta et al., 1999). For the snakes we investigated, activity was concentrated during nighttime, with animals remaining inactive throughout daytime. Thus, from the three days of body temperature recordings, we selected up to 3 periods lasting from 1 to 3 hours each, for the daytime or nighttime periods. In all cases, we tried to select periods in which snakes kept a relatively steady-state body temperature. Lastly, we calculated a mean value for each of the selected periods and averaged them in a grand-mean corresponding to the nighttime and daytime periods, which were accepted to represent the T_{pref} for the active and inactive periods, respectively.

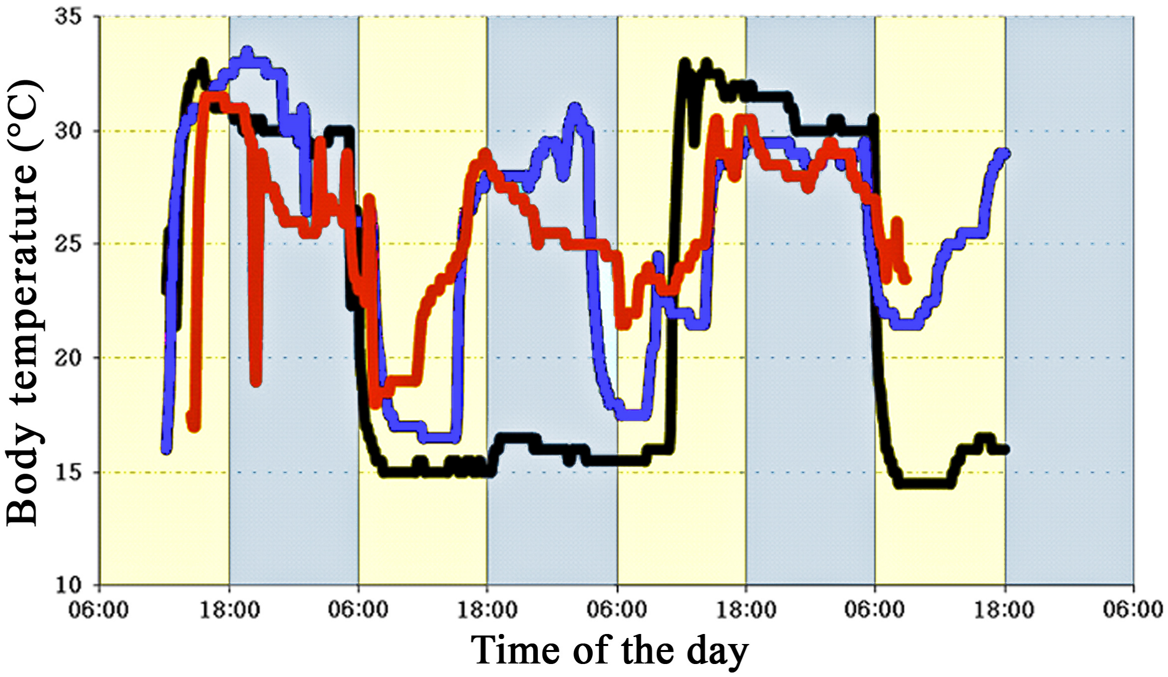

Figure 11. Pattern of thermal preference of three individuals of *B. alternatus* within the thermal gradient (~ 11 to 35°C) over four consecutive days. Note how snakes typically select colder temperatures during daytime (lights on, yellow stripes) and warmer temperatures at night (lights off, blue stripes).

2.5. Thermal tolerance

Snakes were individually placed in a wired mesh container (20 x 9 cm and 30 x 10 cm, diameter x height, mesh size 1 x 1 cm; Fig. 12), which allowed for rapid temperature equilibration. Thereafter, they were placed inside a climatic chamber (122FC, Eletrolab), where they remained at 25°C for one hour before the beginning of the experiments. Immediately after this acclimation period, the temperature of the chamber was either decreased or increased at the rate of 1°C every 6 minutes, in both cases starting at the initial temperature of 25°C. At every five minutes, the snake's chamber was gently turned in 180°, which cause the animals to fall back on their dorsum. When snakes took more than ten seconds to return to their normal position (Doughty, 1994; Navas et al., 2007; Belasen et al., 2017), the experiment was ended. Trials were conducted between 08:30 and 13:00 and CT_{min} always preceded CT_{max} determinations, allowing a minimum recovery period of 48 hours between both measurements. At the end of experiments, animals were placed in a bowl with water at room temperature (~ 26°C) to accelerate the recovery process from thermal stress caused by CTL.

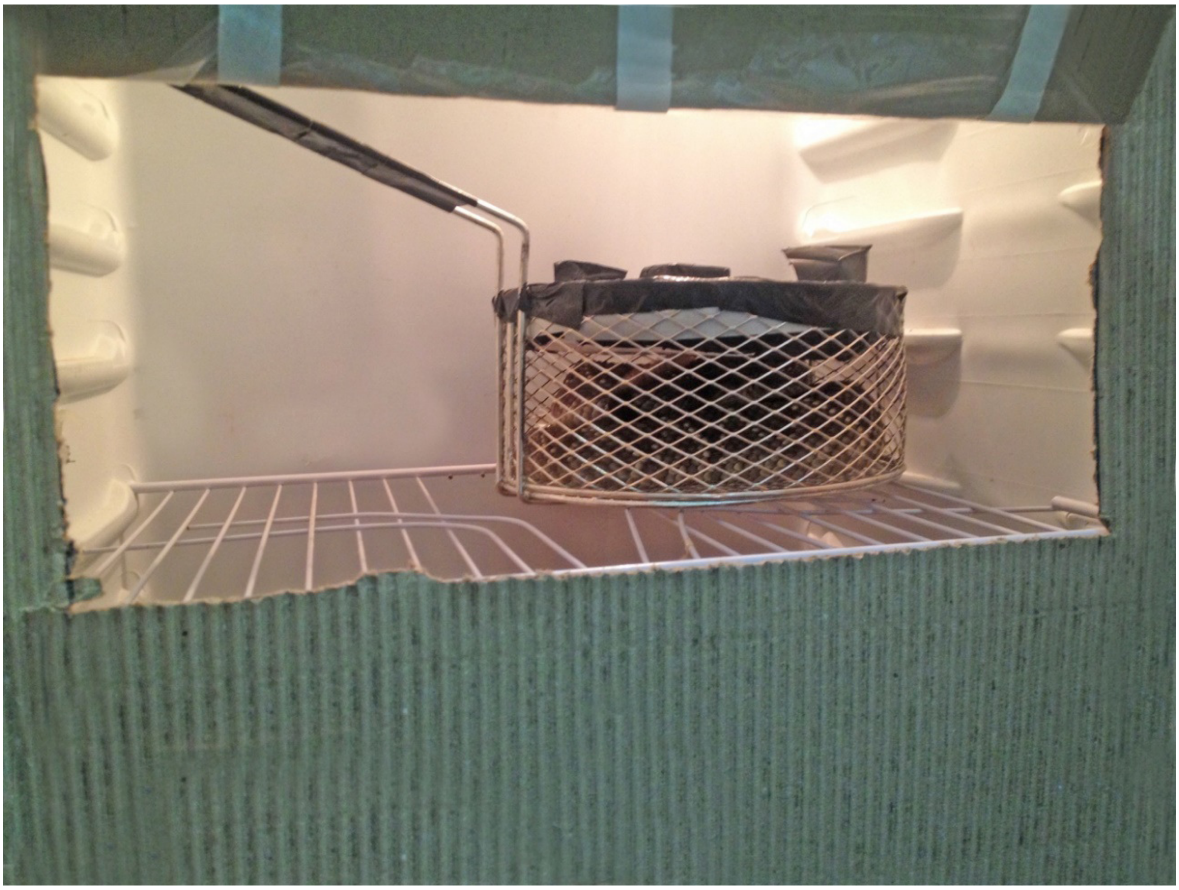

Figure 12. A rattlesnake inside the wired mesh container used to verify the critical thermal limits (i.e., to turn snakes on their dorsum; see text for details) arranged inside a BOD climatic chamber.

2.6. Data analysis

Although we are aware that conventional statistical approach disregards the effect of common ancestry on data independence among different species (Harvey and Pagel, 1991), we did not perform a phylogenetically informed analysis of our results due to the low number of species investigated (see also Li et al., 2009; Qu et al., 2011; Guillon et al., 2014; Watson and Burggren, 2016; Gienger et al., 2017). Our approach was based on the fact that little or no phylogenetic signal can be detected in sample sizes with *n* less than twenty species (Blomberg et al., 2003; Revell, 2010; Münkemüller et al., 2012).

We used Student's t-test to compare T_{pref} between nighttime and daylight for each species as well as to compare the time taken by the snakes to reach CT_{min} and CT_{max}. All data were first analyzed via a one-way analysis of covariance (ANCOVA), using species as factor and body mass as the covariate. Once it was determined that mass was not a significant covariate, this variable was dropped from the model and the analyses were run for the temperature and species factors using one or two-way ANOVA, followed by Holm-Sidak test for *post hoc* multiple comparisons. To compare T_{pref} among species we used a two-way ANOVA with period (night or day) and species as the factors. We performed one-way ANOVA for comparisons of CT_{min} and CT_{max} among species. Before the statistical tests, we evaluated the parametric premises of normality (Shapiro-Wilk) and equality of variances (Levene's test). When we observed lack of normality and/or homoscedasticity of variance, the data were logarithmized and the test repeated. All tests followed Zar (1999), and the results are presented as mean ± standard error. The significance level was set at $P < 0.05$.

3. RESULTS

3.1. Thermal preference

Snake's body masses did not differ among species ($F_{3,25} = 1.12$; $P = 0.359$; Table 3). Analyses of covariance indicated that T_{pref} was not affected by body mass among species in any period (daylight: $F_{3,25} = 0.199$, $P = 0.895$; nighttime: $F_{3,25} = 0.083$; $P = 0.968$). Snakes selected temperatures at night higher than daylight in all species studied (*B. alternatus*, $t_8 = 6.30$; *B. jararaca*, $t_{14} = 6.47$; *B. moojeni*, $t_{12} = 13.38$; *C. durissus*, $t_{12} = 7.25$; $P < 0.001$ in all species; Fig. 13; Table 3). Interespecifically, T_{pref} during daylight was not different among species ($P \geq 0.205$), but at nighttime *B. jararaca* and *C. durissus* ($P = 0.766$) selected higher temperatures than *B. alternatus* and *B. moojeni* ($F_{3,50} = 0.276$; $P < 0.05$ in both cases). Nocturnal T_{pref} for *B. alternatus* was higher than for *B. moojeni* ($P < 0.05$; Fig. 13; Table 3).

Table 3. Body mass and prefered body temperature of four snake species measured in a thermal gradient.

	n	Body mass (g)	Thermal preference (°C)	
			Daytime	Nighttime
B. alternatus	7	423.54 ± 59.50 (216.90–643.20)	18.35 ± 1.76 (14.79–27.90)	30.02 ± 0.59 (28.22–31.92)
B. jararaca	8	294.44 ± 70.63 (95.50–658.10)	18.76 ± 1.95 (10.74–24.99)	31.62 ± 0.81 (28.70–33.79)
B. moojeni	7	356.99 ± 42.64 (227.53–550.00)	14.82 ± 0.91 (10.53–18.07)	27.37 ± 0.23 (26.70–28.63)
C. durissus	7	417.97 ± 51.55 (161.20–549.30)	18.52 ± 1.78 (13.80–25.00)	32.42 ± 0.71 (28.66–34.19)

Data are shown as mean ± SE (minimum and maximum values).

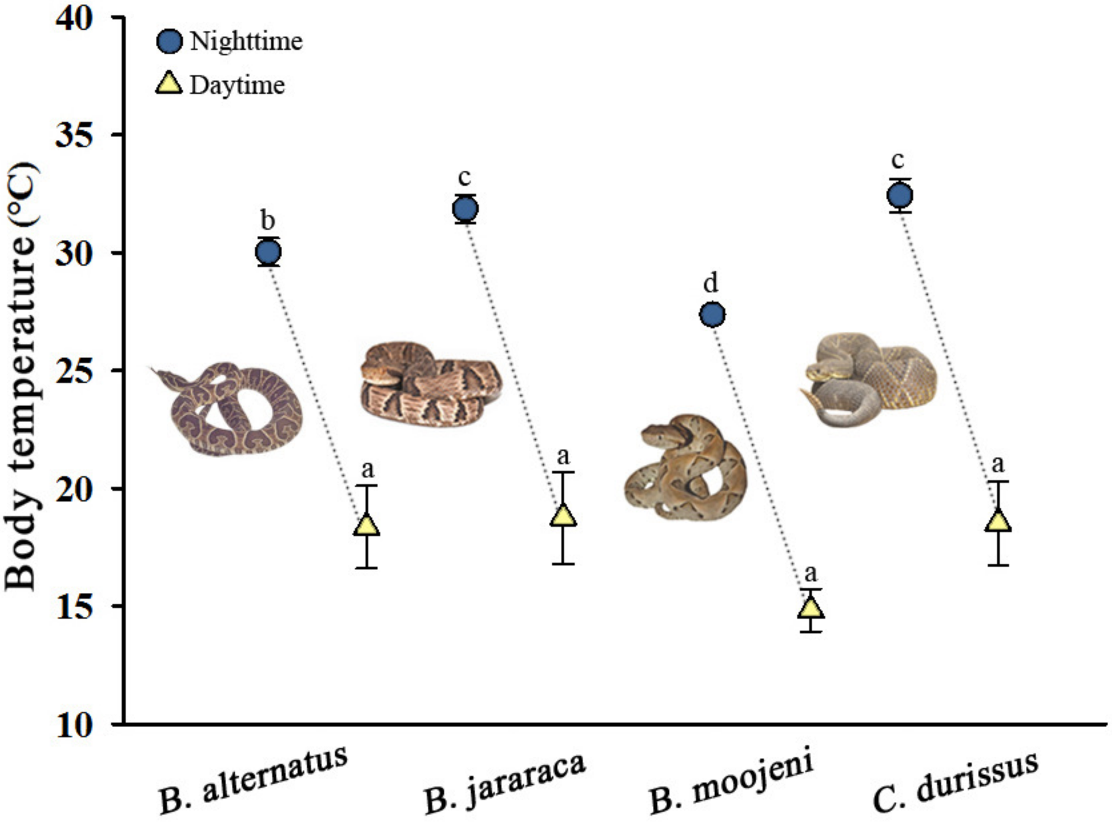

Figure 13. Thermal preference of four Viperidae snakes during day and nighttime periods. Dotted lines refer to the thermal amplitude selected by the snakes. Different letters indicate significant differences among species and periods. Symbols and bars indicate mean ± SE, respectively.

3.2. Thermal tolerance

Snake's body masses did not differ among species ($F_{3,29}$ = 1.83; P = 0.164; Table 4). Snakes took more time to reach CT_{min} (~ 167 min) than CT_{max} (~ 128 min) (*B. alternatus*, t_{12} = 8.96; *B. jararaca*, t_{16} = 7.11; *B. moojeni*, t_{14} = 6.25; $P < 0.001$ in all cases; Fig. 14), except for *C. durissus* (t_{16} = 1.88; P = 0.078). Analyses of covariance indicated that CTL were not affected by body mass among species (CT_{min}: $F_{3,29}$ = 2.51, P = 0.081; CT_{max}: $F_{3,29}$ = 1.21; P = 0.326).

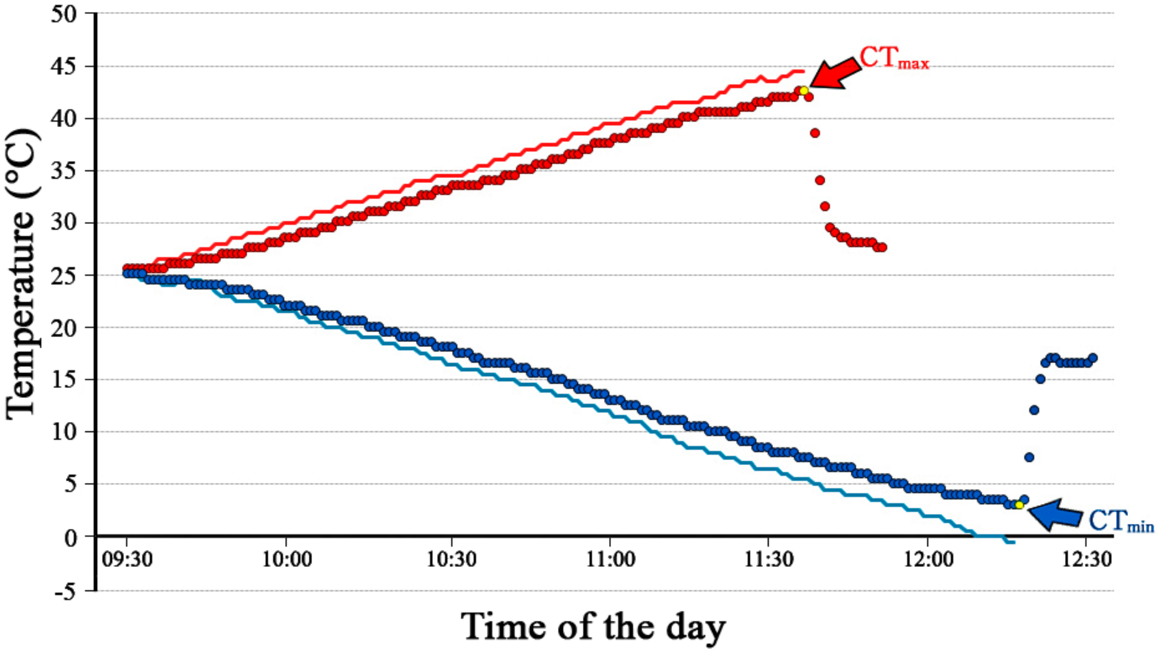

Figure 14. Representative body (circles) and climatic chamber air (lines) temperatures variation during the measurement of thermal tolerance in an individual of *B. alternatus*. Cooling and heating trials are represented in blue and red, respectively. Arrows indicate the moment when snake lost the righting reflex and was removed from the climatic chamber (yellow point).

Thermal stress responses exhibited by the snakes basically followed the same sequence. For CT_{min}: I) decrease followed by an interruption of the tail beat; II) decrease followed by an interruption of the number of attacks (strikes); III) decrease followed by an interruption of tongue flicking; IV) lethargy; V) decrease in speed to right and coil themselves in a coordinated manner when placed on their dorsum; and finally, VI) loss of the righting response (LRR). For measurements of CT_{max}: I) restlessness and escape attempt; II) decrease followed by an interruption of the tail beat and number of attacks; III) decrease followed by an interruption of tongue flicking; IV) lethargy; V) cloacal discharge (about 60% of cases; VI) slowing of the righting reflex; VII) gaping (observed for all species, in about 95% of cases; Fig. 15) and; VIII) loss of the righting reflex.

Bothrops alternatus exhibited the lowest CT_{min} among all species ($P < 0.05$ in all cases), whereas *B. jararaca* had lower CT_{min} values than *B. moojeni* ($F_{3,29} = 15.61$; $P < 0.005$; Fig. 16; Table 4). CT_{max} values for *Bothrops alternatus* and *C. durissus* were similar ($P = 0.307$) and higher than for *B. jararaca* and *B. moojeni* ($F_{3,29} = 18.35$; $P < 0.005$ in both cases; Fig. 16; Table 4).

Figure 15. *Crotalus durissus* (above) and *Bothrops alternatus* (below) exhibiting thermal gaping behavior, at the end of a critical thermal maxima experiment.

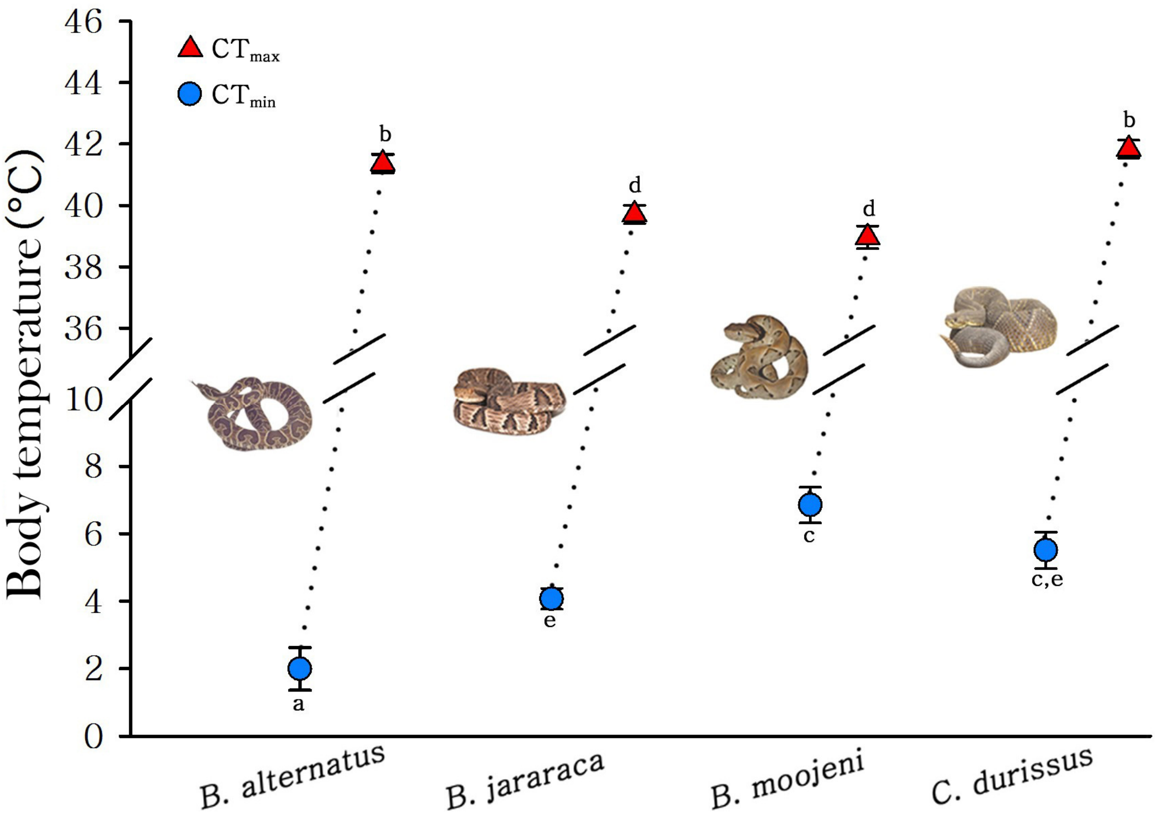

Figure 16. Minimum and maximum critical temperatures in four Viperidae snakes. Different letters indicate significant differences among species. Symbols and bars indicate mean ± SE, respectively.

Table 4. Body mass and minimum and maximum thermal limits of four snake species.

	N	**Body mass (g)**	**CT_{min} (°C)**	**CT_{max} (°C)**
B. alternatus	7	423.54 ± 59.49 (216.90–643.20)	1.99 ± 0.63 (-0.50–4.00)	41.36 ± 0.31 (40.50–42.65)
B. jararaca	9	294.90 ± 62.29 (95.50–658.10)	4.07 ± 0.31 (3.00–5.61)	39.71 ± 0.30 (38.50–41.16)
B. moojeni	8	351.61 ± 37.32 (227.53–550.00)	6.85 ± 0.53 (5.08–8.81)	38.96 ± 0.37 (37.69–40.41)
C. durissus	9	451.70 ± 51.03 (161.20–711.20)	5.51 ± 0.54 (3.50–8.50)	41.84 ± 0.29 (40.50–42.92)

Data are shown as mean ± SE (minimum and maximum values).

4. DISCUSSION

We found significant differences in thermal preference and tolerance among the four Viperidae snakes studied. However, the pattern of variation in thermal preference along the circadian cycle was very similar in all snake species studied. Snakes selected higher temperatures during the nighttime period, which coincide with the period that they are most active in nature (Greene, 1992; Sazima, 1992; Nogueira et al., 2003; Sawaya et al., 2008; Tozetti and Martins, 2013) and in the laboratory (personal observation). This pattern of concurrent variation in T_{pref} and activity along the circadian cycle is consistent with previous studies in squamates (e.g., Huey and Bennett, 1987; Schlesinger and Shine, 1994; Angilletta et al., 1999; Kearney and Predavec, 2000; see also Angilletta et al., 2002) including snakes (Gibbons and Semlitsch, 1987; Al-Johany and Al-Sadoon, 1996; Slip and Shine, 1998; Webb et al., 2004; Anderson et al., 2005; Tsai and Tu, 2005; Bontrager et al., 2006; Lin et al., 2007; Lelièvre et al., 2010). The preference for higher temperatures during the active period, nighttime in this case, may be related to the fact that behavioral performance in several ecologically relevant activities, such as foraging, prey capture, locomotion, and mating are importantly influenced by temperature and, therefore, can be optimized at physiologically adequate higher body temperatures (Gibbons and Semlitsch, 1987; Huey, 1982; Angilletta, 2009). On the other hand, as metabolic expenditure decreases with temperature in ectothermic organisms, including snakes (see Capítulo I), the selection of lower body temperatures during the inactive period is certainly beneficial in terms of minimizes energy expenditure (Greene, 1992; 1997; Secor and Nagy, 1994; Shine et al., 2003). Indeed, the estimated RMR reduction (see Capítulo I), as a function of change in thermal preference between day and night, may account for a decrease from 67 up to 80% in daily energetic expenditure (*B. alternatus* = 67.5%; *B. jararaca* = 80.5%; *B. moojeni* = 76.6; *C. durissus* = 78.5%).

Interestingly, we did not found differences in thermal preference for the inactive period among the snakes studied. On the other hand, during nighttime, *B. jararaca* and *C. durissus* selected higher temperatures than *B. moojeni* and *B. alternatus*. The explanation of these results is probably multi-factorial and hard to isolate. Perhaps, differences in thermoregulatory effort between day and night may relax body temperature regulation during the inactive daytime period compared to a more precise thermoregulation during the active period. As a result, intrinsic interspecific differences in preferred body temperature would be more likely detected during the nighttime. Also, it could be that selective forces acting on the T_{pref} during inactivity, for example associated to energy economy (commented above), might have a more general influence than those acting during the period in which the snakes are active. These explanations, however, still requires experimental validation. Whatever the case, the interspecific variation in T_{pref} during the active period seems to correlate well with broad differences in habitat occupancy among different snake species. Indeed, a species known to occur in open areas of the savanah-like Cerrado formation, which are generally warmer than forested habitats within the same geographical range (see Colli et al., 2002; Tozetti and Martins, 2008; 2013), *C. durissus* was found to have the highest T_{pref} during activity. Apparently contradictory with this idea, *B. moojeni*, also associated with the Cerrado formation, exhibited the lowest T_{pref} during activity. This disparate association, however, may be related to differences in microhabitat use. In fact, while *C. durissus* is usually found on drier patches within the Cerrado (Tozetti and Martins, 2008), *B. moojeni* inhabits mostly riparian areas (Nogueira et al., 2003). Somewhat unexpectedly, we found that the T_{pref} during activity in the almost strictly forest dweller *B. jararaca* (Sazima, 1988; Rocha et al., 2014) was higher in comparison to the other *Bothrops* species. This result does not agree with the general idea that open landscapes would be associated with higher T_{pref} and, therefore, currently unidentified differences in microhabitat use and thermal biology might be at play. A better assessment of microhabitat use and the quantitative characterization of thermal niches among distinct snake species are pivotal to understand the potential association between thermal preference and habitat occupancy.

Upper thermal limits for terrestrial ectotherms are generally viewed as less labile than lower thermal limits (Hertz et al., 1983; Moreno-Azócar et al., 2012; Araújo et al., 2013; Hoffmann et al., 2013; Sandblom et al., 2016; von May et al., 2017). In fact, thermal tolerance data indicate that CT_{min} of snakes is more variable intra- and interspecifically than CT_{max}, and our results agree with this pattern. Among the snake species investigated, we found that CT_{max} varied by 2.9ºC compared to the 4.9ºC variation found in CT_{min} (see Fig. 17). According to Araújo et al. (2013), divergence in conservatism of lower and upper thermal limits is likely to be the consequence of different physiological processes operating near CTL. Larger variation of lower thermal limits seems to be a consequence of differences in thermodynamic thermal effects on maintenance rates of ion homeostasis (e.g. MacMillan et al., 2012). On the other hand, the relatively low fluctuation in organisms' CT_{max} is partially attributed to their limited ability to tolerate the denaturating effects of extreme temperatures on membranes and proteins (Bartholomew, 1977; Somero, 1995; Tattersall et al., 2012; Araújo et al., 2013; Lillywhite, 2014), which thereby constrains their functional state (Hochachka and Somero, 2002; Sandblom et al., 2016; see also Angilletta, 2009). In regard to CT_{max}, we observed during its determination that all snake species herein investigated exhibited the gaping behavior as they approached CT_{max}. Although commonly reported for lizards and crocodilians (Heatwole et al., 1973), this interesting behavior had only been observed in other three snake species (Jacobson and Whitford, 1971; Johnson, 1973; Huang et al., 2007). Under situations of heat stress, gaping is largely accepted as a way to increase evaporation from the buccal cavity and upper airways surfaces and, therefore, assist in cooling (see Tattersall et al., 2006). However, if the referred mechanism is broadly widespread in other snake species, and also to what extent it may help in increasing CT_{max}, these questions remain to be investigated.

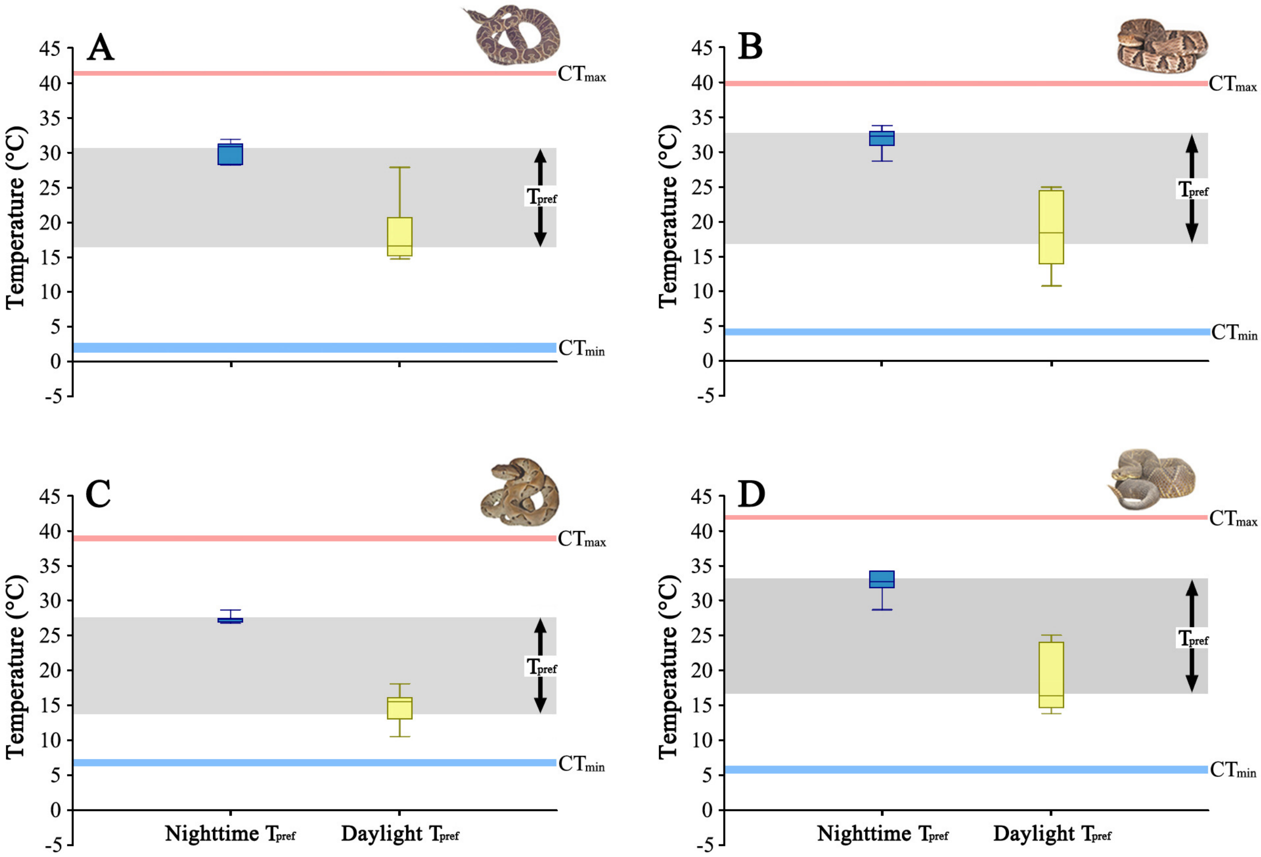

Figure 17. Upper (blue lines) and lower (red lines) thermal limits of *B. alternatus* (A), *B. jararaca* (B), *B. moojeni* (C), and *C. durissus* (D). The shaded gray zones delimit the ranges of diel preferred temperature, composed by nocturnal (dark blue boxes) and diurnal (yellow boxes) snakes' T_{pref}. Values are shown as means ± SE. Horizontal lines inside the boxes represent the median, boxes represent lower and upper quartiles, and whiskers the minimum and maximum values.

Discussions on interespecific differences in CTL are likely to be confounded by the same factors previously commented for differences in T_{pref}. However, considering the current available information, our results indicate that the interespecif pattern of CT_{max} variation among the snake species herein investigated may be consistent with broad differences in habitat occupancy. Indeed, in those species that thrive mostly in open formations, *C. durissus* and *B. alternatus* (see Mesquita and Brites, 2003; Tozetti and Martins, 2008; 2013), CT_{max} was higher in those species than in *B. jararaca* and *B. moojeni*, which are primarily found in forested and riparian areas (Sazima, 1992; Nogueira et al., 2003). Also, partially following this dichotomy between species inhabiting open and covered landscapes, CT_{min} was found to be lower in *B. alternatus* compared to *B. jararaca* and *B. moojeni*. However, the CT_{min} of C. *durissus* was similar to *B. jararaca* and *B. moojeni* and considerably higher than that of *B. alternatus*. Perhaps, these results may encompass differences in other aspects of the thermal biology and microhabitat use between *B. alternatus* and *C. durissus*. Indeed, *C. durissus* seems to be active at higher body temperatures (Nogueira et al., 2003; Bastos et al., 2005; Tozetti and Martins, 2008) besides having a T_{pref} for activity considerably higher (present study) than *B. alternatus*. Similar as for T_{pref}, the validity of the association between CTL and habitat occupancy will depend on a solid assessment of microhabitat use and the quantitative characterization of the thermal niches of the different snake species.

An informative way to conjugate thermal tolerance and preference is the calculation of the thermal safety margins (CT_{max} - T_{pref}; Bennett and John-Alder, 1986), which informs on how close to upper thermal limit animals select their preferred body temperatures. In our study, all snake species selected temperatures between 8 to 10°C below their CT_{max}, whereas during inactivity T_{pref} was 9 to 14°C above CT_{min} (Fig. 17). At nighttime, snakes selected areas relatively close to their CT_{max} (i.e., low thermal safety margins), a usual pattern for most Squamata, which generally select temperatures closer to CT_{max} when active (Brattstrom, 1965; Huey, 1982; but see Bennett and John-Alder, 1986). Perhaps, the low thermal safety margins in all snake species here are related to the fact that the majority of terrestrial ectotherms, due to their high thermal requirements (Araújo et al., 2013), are currently living at or beyond the limits of their physiological heat tolerance (Sunday et al., 2014). In this sense, if in one hand the exposure of snakes to low T_b promotes energy economy (discussed above), on the other hand, the preference for higher temperatures at night would improve their fitness (Araújo et al., 2013).

As previously acknowledged, some caveats may apply to our outcomes. Firstly, the potential influence of phylogeny on the results that, presently, we were unable to isolate. However, significant differences were apparent and seem to be associated with differences in snakes' ecological attributes and habitat occupied by our focal species. Secondly, differences in body size among experimental groups could be at play. However, the potential influence of this variable was incorporated in our analysis and, therefore, we could weigh its relevance while discussing our results, whenever was the case. Anyway, we are confident that our conclusions reflect a well grounded pattern, at least for the species herein studied, which highlight that both T_{pref} and critical limits are congruent with the type of environment occupied by the snakes, from the point of view of phytophysiognomy. If this apply to other viperid species, it remains to be examined.

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CONCLUSÕES GERAIS

1) A temperatura afetou a TMR e a PEA em todas as espécies de serpente, de modo que o consumo de oxigênio e a taxa de perda de água aumentaram juntamente com a temperatura;

2) A TMR dos animais pareceu se refletir com o tipo de ambiente das espécies: A 15°C, *Bothrops alternatus* (espécie com distribuição mais ao sul dentre as quatro estudadas) e *B. moojeni* (que apresentou a menor T_{pref}) tiveram as taxas mais elevadas. A 35°C, as espécies de área aberta (*B. alternatus* e *Crotalus durissus*), sujeitas a maiores variações de temperatura no ambiente natural, apresentaram TMR menores que as serpentes de área florestada (*B. jararaca* e *B. moojeni*), onde a temperatura ambiente é tipicamente mais constante;

3) *Crotalus durissus*, frequentemente encontrada em ambientes mais áridos, apresentou as menores taxas de PEA em todas as temperaturas. As serpentes do gênero *Bothrops* não diferiram quanto à PEA a 15 e 25°C. A 35°C, entretanto, *B. alternatus* apresentou PEA menor que as espécies congêneres. Desta forma, particularmentes em temperaturas mais elevadas, as espécies de área aberta tiveram taxas de PEA menores que as de área florestadas, onde a umidade relativa do ar é sempre elevada e temperatura raramente se extende acimda dos 30°C;

4) Todas as espécies de serpentes, nas quais predominam hábitos noturnos, apresentaram T_{pref} durante a noite maiores que a T_{pref} durante o dia;

5) Durante o período de inatividade das serpentes (i.e., durno) a T_{pref} não variou entre as espécies. À noite, *B. jararaca* e *C. durissus* apresentrarm os maiores valores de T_{pref}, seguidos por *B. alternatus* e *B. moojeni*.

6) Para todas as espécies, o CT_{min} foi mais lábil que o CT_{max}. O CT_{min} foi diferente nas três espécies de *Bothrops*, sendo que *B. alternatus* é a espécie mais tolerante ao frio, seguida por *B. jararaca* e *B. moojeni*. *Crotalus durissus* apresentou CT_{min} similar aos de *B. jararaca* e *B. moojeni*.

7) Os valores de CT_{max} foram relacionados com o tipo de hábitat das serpentes. Espécies de áreas abertas (*B. alternatus* e *C. durissus*) foram mais tolerantes ao calor do que as espécies de hábitats florestados (*B. jararaca* e *B. moojeni*).

8) Os resultados dos parâmetros fisiológicos analisados foram congruentes com o tipo de hábitat ocupado pelas serpentes, do ponto de vista da fitofisionomia. *Bothrops alternatus* e, principalmente, *C. durissus*, espécies de áreas abertas, demonstraram ser menos susceptíveis a condições ambientais adversas do que espécies que habitam áreas florestadas (*B. jararaca* e *B. moojeni*), visto que sob temperaturas elevadas (35°C), apresentaram menor TMR, PEA e maior tolerância ao calor.