UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
INSTITUTO DE BIOCIÊNCIAS – RIO CLARO

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS
(ZOOLOGIA)

Thermal regimes effects over stress indicators in rattlesnakes, *Crotalus durissus* (Serpentes: Viperidae)

AILTON FABRÍCIO NETO

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

Novembro - 2018

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Rio Claro
2018

Fabrício Neto, Ailton
F126t Thermal regimes effects over stress indicators in rattlesnakes, Crotalus durissus (Serpentes: Viperidae) / Ailton Fabrício Neto. -- Rio Claro, 2018
37 f. : il., tabs.

Dissertação (mestrado) - Universidade Estadual Paulista (Unesp), Instituto de Biociências, Rio Claro
Orientador: Denis Otávio Vieira Andrade
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1. Snakes. 2. Ectothermic. 3. Circadian Cycle. 4. Corticosterone. 5. Innate Immmunity. I. Título.

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CERTIFICADO DE APROVAÇÃO

TÍTULO DA DISSERTAÇÃO: Thermal regimes effects over stress indicators in rattlesnakes, Crotalus durissus (Serpentes: Viperidae)

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Rio Claro, 26 de outubro de 2018

AGRADECIMENTOS

Acima de tudo agradeço a ciência, que estimula o pensamento crítico e através do método científico tem desafiado o obscurantismo dogmático perpetuado por pseudociências perigosas como o movimento antivacina, além de outras potencialmente nocivas, como homeopatia e astrologia.

Agradeço a minha família, pelo apoio durante o mestrado e, previamente, durante toda a vida. Meu irmão, minha mãe e meu pai são responsáveis pelo tipo de pessoa que sou hoje, e por isso sou eternamente grato a todos.

Ao amor da minha vida, pelo incentivo para escrever esse texto quando a vontade faltava, pelas viagens e passeios gastronômicos (gordices), por ouvir minhas reclamações, enfim, por ser a pessoa maravilhosa que ela é.

Ao meu orientador, professor Denis, por todas as conversas relacionadas ao projeto, por ter me ajudado a treinar para coletar sangue das cascavéis, pelos serviços de marcenaria para construção de novos abrigos para as serpentes e pelas reuniões do laboratório na sua casa, sempre alegradas pelo Nick (saudades dele, *in memorian*), nas quais sempre pudemos conversar sobre os mais diversos temas.

Ao meu coorientador, professor Fernando, por ter acreditado no projeto nos passos iniciais e durante toda sua execução. Por todas as conversas sobre o design experimental do estudo, pela calma transmitida, pela recepção sempre acolhedora no laboratório de fisiologia da USP... Muito obrigado =D

Agradeço ao professor Pizo pela supervisão e orientação durante o processo de estágio docência, no qual tive a oportunidade de participar de uma experiência que julguei extremamente positiva... Acredito que o futuro do nosso país depende não só de maiores investimentos em educação, mas também de tornarmos as aulas mais práticas e interessantes, estimulando a curiosidade e o pensamento crítico dos estudantes, fugindo da tradicional “decoreba”. Agradeço também a turma de Ecologia de 2017, pelo interesse demonstrado no decorrer na disciplina e pela amizade desenvolvida.

Agradeço de maneira muito especial às alunas e alunos do laboratório do professor Fernando. Em especial, agradeço a Carla (Skol) por toda a ajuda com as análises estatísticas desenvolvidas nesse trabalho e o treinamento para as técnicas de capacidade bactericida do plasma, além das padronizações para este ensaio. A Bruna (Itubaína), pelo treinamento e ajuda para a execução dos primeiros kits de

corticosterona. Também agradeço a recepção dada por Ronyelle, Stefane, Stefanny, Aymam, Braz, Caroline, Faride, Diego, Adriana, Felipe e Débora, muito obrigado a todas e todos.

Agradeço ao Centro de Controle de Zoonoses de Itu (CCZ) pela doação de cascavéis para a realização desse estudo. Em especial, agradeço ao biólogo Diogo, que sempre nos recebeu muito bem no CCZ e se manteve em constante comunicação conosco, facilitando muito a fase de aquisição de animais para a execução dos experimentos.

Aos colegas de laboratório do jacarezário: Rafaela (Paquita), Amanda (Pink), Thiago (Pezinho), Juliana (Jujuba), Miguel, Luá, Rodolfo (Muzambinho) e Rodrigo (Penapolense), por todas as conversas, risadas, doces de carolina e saudações à mandioca realizadas durante esse período de mestrado.

Aos ajudantes durante as coletas de sangue, vitais para a execução desse trabalho e a quem espero ter transmitido uma visão mais realista sobre serpentes, abandonando preconceitos propagados através de alguns conhecimentos tradicionais e religiões: Sophia (Afilhadinha / Fuzuê), Augusto (Bad), Fernanda (Saúde), Vinícius (Bin Laden), Jade (Mística), Rafaela (Porteira), Nathália (Kiwi) e Maria Teresa (Vó), além das já citadas Paquita, Pink e Pezinho.

Agradeço ao Pedro (C3PO), Gabriel (Bebê) e Matheus pela ajuda com os materiais necessários para a montagem de esfregaços, além do treinamento para montar lâminas de qualidade.

Agradeço aos doutores Guilherme Gomes, Rafael Bovo e Célio Haddad, pelos comentários feitos na primeira avaliação desse projeto, os quais já foram incorporados durante a execução do mesmo, na medida do possível. Também agradeço aos membros da banca de qualificação, Ariovaldo Neto e Guilherme Gomes (novamente) por todas as sugestões oferecidas.

Agradeço aos técnicos do jacarezário Ayrton e Fernando pelas conversas e cafezinhos. Também agradeço aos técnicos da Zoologia, Fernando e Emygdio, por ceder alguns materiais importantes para a execução dos experimentos (heparina, por exemplo) e também pelos momentos de descontração.

Também agradeço ao pessoal da seção técnica de pós-graduação, em especial a Rosangela, Ivana e Felipe, por toda a ajuda nos tramites mais “burocráticos” e pela

calma e atenção transmitidas quando ficamos aflitos pelos prazos que estão se aproximando.

Agradeço aos queridos amigos e amigas de São Paulo: Roberto (Beto), Luiz, Tadashi, Lia e agora a Iolanda (Ioio), por todas as hospedagens solidárias quando precisei ir até lá para execução dos experimentos.

Um agradecimento muito especial às amizades mais verdadeiras que já tive: Sally, Meg (saudades de você pretinha), Nina, Pituca, Bóris, Tarzan, Groselha e em Rio Claro, Beta e Luke. E as que não estão mais em minha vida, mas nunca serão esquecidas: Miranda, Pixuinha, Tobias, Neguinha, Coca e Remela.

As pessoas que não estão mais conosco, porém sempre lembradas: Vó Laide, Tia Ana, Bisa Henriqueta, Michel, Tia Lígia, Maria Isabela, Tio Pedro, Tio Nego, Murilão... Quantas saudades deixaram.

Ao CNPq pela bolsa concedida (Processo 134635/2016-7) e a FAPESP, ao auxílio da técnica Raphaela e novamente ao professor Fernando pela compra dos kits de corticosterona utilizados nesse estudo (Processo 2014/16320-7).

Por fim, agradeço as serpentes... Sem as quais esse estudo não seria possível. Minha esperança é que um dia os seres humanos abandonem sua ignorância e aprendam mais sobre esses animais.

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ABSTRACT

Ectothermic animals have their physiological functions closely related to temperature. For example, metabolic and growth rates of these animals may be affected by body temperature. Studies focusing on the measurement of metabolic rates in snakes, for example, are usually performed by subjecting animals to constant temperature regimes. However, ectotherms (snakes included) are known to exhibit wide variations in their body temperature throughout the circadian cycle. This disagreement between thermal biology and experimental conditions could act as a potential stressor agent for ectotherms. The present study tested the effects of different thermal regimes, constant and fluctuating, throughout the circadian cycle on stress indicators in rattlesnakes (*Crotalus durissus*). A group of animals was exposed to the constant-to-fluctuating treatment, composed by a constant temperature regime (30°C) followed by a fluctuating regime (25°C, 1800 to 0600h, 35°C, 0600h to 1800h, mean 30°C); the second group was subjected to the fluctuating-to-constant treatment, composed of a fluctuating regime and, after, the constant. The animals were exposed to the treatments for a period of 24 days, and on the 12th day the change of thermal regime occurred. We collected blood samples on days 2, 10, 14 and 22 to measure heterophil:lymphocyte ratio (H:L), plasma bacteria killing ability (BKA) and corticosterone plasmatic levels (CORT). The shift between constant and fluctuating thermal regime acted as an acute stressor for snakes, promoting an increase in plasma CORT levels. Exposure to a fluctuating thermal regime at the onset of the experiment induced a decrease in the BKA of rattlesnakes that persists for the whole experiment, and males also present higher levels of BKA than females. H:L was not affected by any of the treatments, therefore it is possible to postulate that the shift between constant and fluctuating thermal regimes acted as a low intensity stressor. Our results highlight that acute shifts in thermal regimes can promote a stress response in snakes, even after long times of maintenance in captivity.

Keywords: Snakes. Ectothermic. Circadian cycle. Corticosterone. Innate Immunity.

RESUMO

Animais ectotérmicos têm suas funções fisiológicas fortemente influenciadas pela temperatura. Por exemplo, taxas metabólicas e de crescimento destes animais podem ser afetadas pela temperatura corpórea. Estudos com foco na medição de taxas metabólicas em serpentes, por exemplo, geralmente são realizados através da submissão dos animais a regimes constantes de temperatura. No entanto, ectotérmicos (serpentes inclusas) são conhecidos por apresentarem variações amplas de sua temperatura corpórea ao longo do ciclo circadiano. Esta discordância entre biologia termal e condições experimentais poderia funcionar como um potencial agente estressor para os ectotérmicos, o que poderia comprometer os resultados e interpretações de estudos, pois um aumento nos níveis de glucocorticóides pode afetar diversos atributos de vertebrados, como taxas metabólicas e resposta imune. O presente estudo testou os efeitos de diferentes regimes térmicos, constante e flutuante, ao longo do ciclo circadiano sobre indicadores de estresse em cascavéis (*Crotalus durissus*). Para tanto, um grupo de animais foi exposto ao tratamento constante-flutuante, composto por um regime constante de temperatura (30°C) seguido por um flutuante (25°C, 1800 às 0600h; 35°C, 0600h às 1800h, média de 30°C); o segundo grupo foi submetido ao tratamento flutuante-constante, composto por um regime flutuante e, após, o constante. Os animais foram expostos aos tratamentos por um período de 24 dias, sendo que no 12° dia ocorreu a mudança de regime termal. Efetuamos coletas de sangue nos dias 2, 10, 14 e 22 para medição da taxa heterófilo:linfócito (H:L), capacidade bactericida do plasma (CBP) e níveis plasmáticos de corticosterona (CORT). A mudança entre o regime termal constante e o flutuante agiu como um agente estressor para as serpentes, causando um aumento nos níveis plasmáticos de CORT. A exposição a um regime flutuante no início do experimento causou uma diminuição na CBP das cascavéis que persistiu por todo o experimento, e machos também apresentaram maior CBP do que fêmeas. H:L não foi afetada por nenhuma das variáveis independentes, portanto é possível que a mudança entre regimes termais agiu como um estressor de baixa intensidade. Nossos resultados demonstram que mudanças agudas em regimes termais podem causar uma resposta ao estresse em serpentes, mesmo após longos tempos de manutenção em cativeiro.

Palavras-chave: Serpentes. Ectotérmicos. Ciclo Circadiano. Corticosterona. Imunidade Inata.

INTRODUCTION

The environmental temperature is an important abiotic factor influencing living organisms, as it broadly affects behavioral and physiological aspects (Huey and Stevenson, 1979; Kingsolver and Woods, 1997; Angilletta et al., 2002). The relation between environmental temperature and behavioral or physiological attributes is particularly relevant for ectothermic animals, since their body temperatures generally are correlated with the environmental temperature and its fluctuations (Angilletta et al., 2002; Andrade, 2016). Thus, the effects of temperature on a wide variety of functions and physiological processes, such as metabolic (Dorcas et al., 2004) and growth (Niehaus et al., 2012) rates, for example, have been studied in ectothermic animals (Angilletta et al., 2002).

The influence of temperature on different physiological parameters in ectothermic animals is usually tested by submitting the animals to constant temperature regimes throughout the study, often for several consecutive days (Secor, 2009; Saxon et al., 2018). However, under natural conditions, the body temperature of ectotherms generally exhibits circadian variation (Niehaus et al., 2012; Kingsolver et al., 2015; Colinet et al., 2015), snakes included (Tozetti and Martins, 2008; Gomes and Almeida-Santos, 2012; Andrade, 2016; Brischoux et al., 2016). Thus, the maintenance of ectothermic animals under constant temperature regime during experimentation can be quite different from what is experienced under natural conditions. This discrepancy, we suspect, may lead to bias in data acquisition and mislead interpretations. Indeed, differences in thermal regime are known to affect metabolic (Gavira and Andrade, 2013; Stahlschmidt et al., 2015), growth rates (Niehaus et al., 2012; Kingsolver et al., 2015) and thermal tolerance (Zatsepina et al., 2000; Manenti et al., 2018) of ectothermic animals. Therefore, it is possible that the absence of temperature fluctuations by denying a thermally changing environment or the possibility for thermoregulation could be perceived as a stressor by these animals (Andrade, 2016).

The exposure of different vertebrates to stressful situations triggers the activation of the hypothalamic-hypophysis-adrenal axis (HHA), resulting in secretion of glucocorticoids (GC) into the bloodstream (Wingfield et al., 1998; Wingfield, 2013). The effects of this increase in GC levels can be adaptive or deleterious (Dickens et al., 2010; Lucas and French, 2012) depending on its temporal pattern (Martin, 2009;

Dickens et al., 2010). A short-term increase in GC may improve the immune response during the post-stress recovery (Dhabhar and McEwen, 1999; Sapolsky et al., 2000; Moore and Jessop, 2003) and may also increase energy recruitment trough an enhancement of the intermediary metabolism (Durant et al., 2008; Preest and Cree, 2008). However, long-term exposure to stressors can lead to a chronic elevation of GC levels in the bloodstream, resulting in deleterious effects on reproduction, growth rates, and the immune system (Guillette et al., 1995; Sapolsky et al. 2000; French et al., 2007; Dickens et al., 2010). In reptiles, changes in environmental temperature may also be associated with changes in GC levels (Dupoué et al., 2013; Jessop et al., 2016), which can in turn affect diverse physiological attributes, as metabolism (DuRant et al., 2008; Preest and Cree, 2008), digestive performance (Bonnet et al., 2013), reproductive behavior (Brischoux et al., 2016) and immune response (Graham et al., 2012). Therefore, the experimental exposure of ectotherms to constant thermal regime might represent a stressor factor, and this would potentially influence physiological parameters, but this possibility remains unverified.

The magnitude of the stress response in reptiles generally has a positive correlation with plasma levels of corticosterone (CORT), the main GC in these animals (Romero, 2004; Lind et al., 2018). Handling and restraint (Kreger and Mench, 1993; Schuett et al., 2004; Sykes and Klukowski, 2009), translocation (Heiken et al., 2016), water deprivation (Dupoué et al., 2014) and acute temperature changes (Dupoué et al., 2013) are considered stressors for snakes, by increasing CORT plasma levels, which can affect the immune response in a complex manner (Martin, 2009). For example, the maintenance of chronically elevated GC levels can lead to a decrease of plasma bacteria killing ability (BKA) (Neuman-Lee et al., 2015). Additionally, the release of CORT leads to an increase in the ratio between circulating heterophils to lymphocytes (H:L rate; Davis and Maerz, 2008; Davis et al., 2008). Given that changes in GC and leukocyte profile in the bloodstream show a different temporal dynamic in response to a stressor (Seddon and Klukowski, 2012; Sparkman et al., 2014), increase in H:L usually requires longer exposure to an stressor agent, and may also be associated to stressor agents with higher levels of intensity (Assis et al., 2015). Therefore, the determination of H:L ratio, plasma CORT levels, and BKA may help to evaluate the potential stress caused by exposure of ectotherms to constant temperature regimes.

Different thermal regimes can affect physiological attributes in ectothermic animals in divergent ways (Niehaus et al., 2012; Kingsolver et al., 2015; Saxon et al., 2018), including stress and immune response (Jessop et al., 2016; Stahlschmidt et al, 2017). More specifically, in snakes, metabolic rates (*Bothrops alternatus*, Gavira and Andrade, 2013; *Pantherophis guttatus*, Stahlschmidt et al., 2015; *Crotalus durissus*, Fabrício-Neto et al., unpublished data) and innate immunity (*P. guttatus*, Stahlschmidt et al, 2017) may present differences according to the exposure to a constant or fluctuating thermal regime. Therefore, the aim of the present study was to investigate how different thermal regimes affected stress indicators and parameters of the immune system in the South American rattlesnake (*C. durissus*). These indicators included the determination of plasma CORT levels, H:L ratio and also an indicator of innate immunity (BKA). The rattlesnakes (*C. durissus*) is relevant to this study since it presents a circadian variation of body temperature, of up to 10°C (Tozetti and Martins, 2008; Andrade, 2016), which can even affect the selection of microhabitats for this species (Tozetti and Martins, 2008; Gomes and Almeida-Santos, 2012). Our hypothesis is that the exposure of rattlesnakes to constant thermal regime will act as a stressor, promoting changes associated with acute and chronic effects on the immune response. To test our hypothesis, we divided the experimental animals into two groups: 1) Constant-to-fluctuating, in which snakes were exposed to a constant thermal regime (30°C), followed by a fluctuating thermal regime (25-35°C, in a 12:12h cycle). 2) Fluctuating-to-constant, in which the snakes were exposed to a fluctuating thermal regime, followed by a constant thermal regime. In both cases, treatments lasted for 24 days, with the change in thermal regimes happening at day 12. This experimental design was done to allow the assessment of a previous acclimation, to either constant or fluctuating conditions, over the exposure to a new thermal regime. We forecast that: 1) CORT and BKA will increase in the short term under the constant regime both at constant-to-fluctuating and fluctuating-to-constant treatments. 2) CORT and H:L will increase in the long term in the constant thermal regime at both treatments, and BKA will decrease. 3) CORT levels, BKA and H:L ratio will not change during the exposure to the fluctuating regime of the fluctuating-to-constant treatment and CORT and H:L will decrease in the constant-to-fluctuating treatment, whereas BKA will increase.

MATERIAL AND METHODS

Studied species and captivity

We used 24 adult individuals of *C. durissus* (15 males and 9 females) that were kept for at least 2 years in captivity, divided into two treatments: constant-to-fluctuating (8 males and 4 females) and fluctuating-to-constant (7 males and 5 females). The snakes were kept individually in transparent plastic boxes (480 x 270 x 133mm, 8.6L) with holes for ventilation, and lined with corrugated cardboard and a water bowl. Cages were kept in a room with controlled temperature (25 ± 2°C) and natural photoperiod, located in the Laboratory of Animal Physiology, Department of Zoology, in the Universidade Estadual Paulista from Rio Claro, SP. Snakes were fed with mice (*Mus musculus*) every 15 days, except when they were under experimentation, in which case they were fasted for 15 days prior to the trials.

The permissions for collection and maintenance of the animals were issued by ICMBIO (no. 22028-1), and all animals used in this study were collected in the state of São Paulo, Brazil. The experiments were conducted with the approval of the Ethics and Animal Use Committee (protocol no. 6613/2016) of the Instituto de Biociências, Universidade Estadual de São Paulo, Rio Claro, SP.

Experimental protocol

Snakes were subjected to one of the two experimental treatments: constant-to-fluctuating, composed by a 12 days period in which the temperature was kept constant at 30°C and, then, changed to a fluctuating thermal regime composed by 12:12h thermoperiods set to 25°C (1800h to 0600h) and 35°C (0600h to 1800h), which lasted for another 12 days. The second treatment, fluctuating-to-constant, replicated the duration, temperatures, and regime, however, the order of the exposure to the constant and fluctuating regimes were inverted. Snakes were randomly assorted between experimental treatments.

Three days before and after the end of the experimental period snakes had their body mass (Mars AS5500C, ± 0.01g) and snout-vent length (SVL, ± 0.1 cm) measured to calculate the body condition index (BCI), since BCI can affect GC levels in snakes (Lind and Beaupre, 2015). The index was represented by the standardized residuals of a linear regression between SVL and body mass (both data log10 transformed) (Brusch and DeNardo, 2017).

At the beginning of the experiments, snakes were transferred in their plastic boxes into climatic chambers (ELETROlab, model 122FC), in which the environmental temperature was controlled and photoperiod was set at 12:12 light (0600h to 1800h) and dark (1800h to 0600h) period. Blood samples (0.5 ml) were collected from the snakes at days 2, 10, 14, and 22 of the experimental treatments. To avoid the effect of the circadian variation in CORT levels, all snakes were sampled in the same time period (between 1200h and 1500h). On the days of blood collection, the daily increment in temperature of the fluctuating thermal regime, set from 25 to 35°C, was kept at 30°C for a period of 6 hours coincident with the sampling period, which ensured that the body temperatures of the snakes were the same between both experimental treatments (Fabrício-Neto et al., unpublished data). All blood samples were taken between January and March of 2018.

Blood sampling

Blood samples were collected through cardiocentesis of non-anesthetized immobilized animals, with heparinized 22G and 1.1/4" needles and 3 ml syringes (see Isaza et al., 2004; Dyer and Cervasio, 2008; Köbölkuti et al., 2009; Brown, 2010; Johnson, 2011; Bonnet et al., 2016). After restraint, snakes were placed in dorsal recumbency and the heart was located through visual inspection of the heartbeat. Snakes were immobilized between the fingers of the collector and, after cleaning the site with alcohol, the needle was inserted one or two ventral scales below the heart, aiming to puncture the ventricle. In all cases, blood samples were collected under 5.7 min (mean time= 3.36 ± 0.91min, min – max = 1.50 – 5.67) and by the same person (AFN).

Sample treatment

Soon after blood collection, two slides were prepared for analysis of the leukocyte profile (two drops of blood, 40-80 µl). The remaining blood was transferred to a test tube and centrifuged for 3 minutes at 4000 RPM. The plasma after centrifugation was separated into two aliquots, one for the CORT assay and one for the BKA assay. These samples were frozen at -80°C until transported (on dry ice) to the Department of Physiology of the University of São Paulo for analysis, and this stocking period did not exceed 20 days.

CORT assay

CORT samples were initially extracted with ether (Mendonça et al., 1996): 10 µl of plasma were placed into test tubes and 3 ml of ethyl ether were added to the plasma samples, and the resulting mixture was agitated for 30 seconds and then centrifuged at 4°C for 9 minutes at 1800 rpm. After, the tubes containing the samples were held at -80°C for 9 minutes and the liquid phase transferred to a new test tube, which were kept in a gas exhaustion chamber for evaporation of the ether for approximately 48 hours.

CORT was assessed through ELISA kits (Cayman Chemical, Cat. 501320). Prior to performing the assay, samples were resuspended in a "buffer" assay solution. After, 50 µl of calibrators and samples were added into each well of the respective 96 well plate in duplicate. Next, 50 µl of tracer and 50 µl of antiserum specific for CORT were added, and the plate was incubated overnight at 4°C. The plates were then washed 5 times with wash buffer (Wash Buffer, Cat No. 400062) and 200 µl of Ellman reagent (Cat No. 400050) were added to each well, and then incubated in an orbital shaker for one hour and 45 minutes. CORT concentrations were determined with a spectrophotometer (Molecular Devices - Spectra Max 250) with wavelength 412nm. The mean intra- and inter-assay coefficient of variation was 8.1 and 13.6%, respectively.

BKA assay

The BKA of *C. durissus* plasma was assessed *in vitro* by the exposure of the snake´s plasma to *Escherichia coli*, a known pathogen presenting ecological relevance for snakes (Brusch and DeNardo, 2017). This assay was adapted from French and Neuman-Lee (2011) and Assis et al. (2013). First, *E. coli* pellets (MicroBioLogics, # 24311 - ATCC 8739) were resuspended in 1 ml of sterile phosphate buffered saline (PBS). Then, 100 µl of this solution were added to 5 ml of sterile trypticasein broth (TSB broth), and this mixture was kept overnight at 37°C for growth of the bacteria. Next day, the concentration of bacteria was estimated by reading the optical density in a plate spectrophotometry apparatus (Molecular Devices - Spectra Max 250; wavelength: 600nm, 96-well ELISA plate) and serial dilutions with PBS were used to obtain the working concentration of microorganisms ($1x10^6$ microorganisms.ml^{-1}). Plasma samples were diluted in a sterile PBS solution (10 µl of plasma in 190 µl of PBS), and thereafter were added 10 µl of the *E. coli* working solution ($1x10^4$ microorganisms.ml^{-1}). The mixture was agitated, partitioned and separately incubated for 1 hour at each of

two temperatures: at 37°C, which is the optimal temperature for *E. coli* growth; and at 30°C which is the temperature of the constant thermal regime and also the average temperature of the fluctuating regime. To determine the positive control of each assay, 10 µl of the working solution of *E. coli* were added to 200 µl of sterile PBS, and the negative control was determined with 210 µl of sterile PBS, both incubated under the same conditions and in the same plate as the plasma samples. After incubation, 500 µl of TSB were added to all samples and the resulting solution agitated and transferred, in duplicate, to a 96-well culture plate (300 µl per well), which were then incubated at 30/37°C for two hours. At the end of the incubation period, we started a sequence of 4 readings of the optical density of the samples in spectrophotometer with intervals of 1 hour. The antimicrobial activity of the plasma was calculated as: *1 - (optical density of the sample / optical density of the positive control)*, representing the proportion of dead microorganisms in the plasma samples in relation to the positive control. For the calculations, we used the optical density data at the initial moment of the exponential growth phase of *E. coli* in the positive control, because at that moment it is evidenced the maximum growth of the bacteria and, consequently, the highest index of bactericidal capacity of the samples.

Leukocyte profile

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

Data treatment and statistical analysis

To test whether CORT, BKA and H:L levels (dependent variables) were affected by exposure to treatments, time of exposure or by the sex of animals, we submitted our

data to mixed linear models using the LMER function (package “lme4”; Bates et al., 2014; 2015), and a series of models was proposed to explain the results. Treatment to which the animals were exposed (categorical variable with two levels), the sex of the animals (categorical variable with two levels) and the time of exposure to the treatment (categorical variable with 4 levels) were defined as fixed factors. Some models also included interactions terms between the fixed factors (Table 1). As our experimental design involved repeated measures, in all the proposed models we included the animals ID as a random factor. The models were submitted to the AIC selection criterion, in which each competitive model receives a delta of AIC (dAIC) and a weight, and the model with lower dAIC is the best model to describe the data (Burham and Anderson, 2002). We also considered Akaike's weight in the explanatory power of the models with dAIC ≤ 2.0 (Burham and Anderson, 2002). Further, we examined the significance of the fixed effects in the selected models (using the “summary(model)” function in R), considering significant factors with t-values higher than 2 and smaller than -2.0 (Luke, 2017). Since CORT levels can affect BKA (Assis et al., 2015) and H:L ratio (Davis et al., 2008; Sparkman et al., 2014), we also performed another modeling stage, including the CORT concentration as a fixed factor, maintaining BKA and H:L ratio as dependent variables.

Repeated measures ANOVA were performed within treatment, time of exposure and sex to explore differences between BKA assays conducted at 30°C and 37°C. The ANOVA tests were followed by a Bonferroni adjustment for multiple comparisons. The significance level was set at $P < 0.05$.

Previous to any analyses, data was checked for normality and BKA data had to be SEN transformed to meet this assumption. All analyses were performed using the R software (version 3.5.0, R Core Team, 2018).

RESULTS

The descriptive analysis of all dependent variables is presented in Table 2. Time for blood collection and BMI did not influence any of the measured parameters and, therefore, these variables were not included in the final models. Models selected for each dependent variable are presented in Table 3.

Variation on plasma CORT levels were not explained by sex, time of exposure or treatment, but rather by an interaction between treatment and time of exposure (T = 2.11; Figure 1, Table 4). Rattlesnakes from the constant-to-fluctuating treatment exhibited increased CORT levels in comparison to those under the fluctuating-to-constant treatment on day 14, i. e., two days after thermal regimes were switched.

None of the independent variables affected the BKA at 30°C (Figure 2; Table 3). Males presented higher BKA at 37°C in comparison to females (sex: T = 2.24; Figure 3; Table 4). Snakes from the fluctuating-to-constant treatment presented a decrease in BKA at 37°C compared to snakes under the constant-to-fluctuating treatment (treatment: T = -3.64; Figure 3; Table 4), and time of exposure was not a significant factor.

Temperature tested (30°C vs 37°C) did not affect BKA for males ($F_{7,61}$ = 2.014; P = 0.07) and females ($F_{3,31}$ = 1.0; P = 0.46) in the constant-to-fluctuating treatment, neither for males ($F_{6,55}$ = 0.94; P = 0.49) and females ($F_{4,39}$ = 1.775; P = 0.132) in the fluctuating-to-constant treatment.

Variation in H:L ratio was not affected by treatment, time of exposure or sex (Table 3; Figure 4).

Plasma CORT levels did not influence variation in BKA at 30°C and 37°C and H:L ratio (null models selected in all comparisons, see Table 5).

DISCUSSION

Contrary to our expectation, our results show that maintenance of rattlesnakes under constant thermal regimes was not associated to chronic stress and immunosuppression. Instead, the change in thermal regime from constant to fluctuating was found to be the possible trigger of an acute stress response. Perhaps, a constant thermal regime, stable and predictable, facilitates acclimation by the snakes. Thus, the change to a more complex and less predictable thermal regime (i. e., fluctuating), could be perceived as an acute stressor agent. This would cause a compensatory response (Sapolsky et al., 2000; Martin, 2009), even though the change brings the animals to a condition closer to what is experienced in nature. Afterwards, as the fluctuating thermal regime extends, the stress response attenuates and vanish within 10 days.

The acute shift from constant to fluctuating thermal regimes resulted in increased plasma CORT levels in rattlesnakes, while such an effect was absent in snakes transitioning from fluctuating to constant regimes. In general, increased plasma CORT levels in ectotherms are associated with a stress response to a determined agent (Romero, 2004). We advocate that in our study the shift between constant and fluctuating thermal regime might have been a perturbation for rattlesnakes. Acute exposure to suboptimal temperatures can represent stressor agents for snakes, as already shown for *Antaresia childreni* (Dupoué et al., 2013). In this sense, the temperature of 30°C is close to *C. durissus* preferred temperature during activity (~32.4°C; Gavira and Andrade, unpublished data). Removing snakes from this optimal condition and inserting then into a new thermal regime (fluctuating) would be the trigger of a stress response. The acute increase in plasma CORT presented by rattlesnakes on the shift between constant and fluctuating thermal regime could have beneficial effects, allowing snakes to cope with this change in environmental temperature through (Sapolsky et al., 2000; Romero, 2004; Dickens et al., 2010). However, continuous elevated plasma CORT might be associated to deleterious effects over immune and reproductive systems (Moore and Mason, 2001). In this sense, the mitigation of the stress response within 10 days of exposure to thermal fluctuation might have attenuated any potential deleterious effects caused by chronically elevated plasma CORT. Temperature compensation has been studied in ectotherms after exposure to a new thermal environment (Angilletta, 2009). For instance, some snakes display a shift in metabolic rates (Gavira e Andrade, 2013; Stahlschmidt et al., 2015) and immune function (Stahlschmidt et al., 2017) in response to exposure to fluctuating regimes. Such changes might contribute to maintain a balanced organismal function in a new thermal environment (Dupoué et al., 2013), and plasma CORT levels may play a key role modulating these shifts.

Plasma CORT levels are related to physiological and biological attributes in ectotherms, such as metabolic rates in lizards (DuRant et al., 2008; Preest and Cree, 2008), reproductive cycle (Moore and Mason, 2001; Lind et al., 2018) and emergency state in snakes (Brischoux et al., 2016). In this sense, studies focusing on functional and ecological attributes are likely to be affected by alterations in plasma CORT levels caused by acute changes in environmental temperature, leading to results that may be affected by experimental bias. Our results highlight the importance of previous acclimation of ectotherms to the experimental thermal conditions in cases involving

acute changes in thermal regimes (e. g., acute temperature changes from what is presented at captivity), especially on studies focusing on the aforementioned biological and physiological attributes or in variation of plasma CORT concentrations itself (Dupoué et al., 2013).

Incubating temperature (30°C vs 37°C) did not affect BKA in *C. durissus*, which is contrary to the temperature-induced increment of the immune response usually seen in ectothermic organisms (Zimmerman et al., 2010; Graham et al., 2017; Stahlschmidt et al., 2017; Zimmerman et al., 2017; Ferguson et al., 2018). Although there are no studies focusing on the thermal performance of the immune response in *C. durissus*, it is a well-known feature of the vertebrate immune system that it can present optimal performance under a wide range of temperatures (Zimmerman et al., 2010; Butler et al., 2013). Indeed, some studies show that innate immune response become impaired only under cold or hot suboptimal temperatures (Zimmerman et al., 2010; Butler et al., 2013; Ferguson et al., 2018). In the specific case of *C. durissus*, its preferred temperature during activity averages 32.4°C (Gavira and Andrade, unpublished data). The test temperatures for BKA in the present study (30°C and 37°C) are just a few degrees below/above this optimal temperature, thus it seems plausible that such temperature variation would not be conducive to significant changes in BKA. On the other hand, some studies had found that ectotherms innate immunity increase almost linearly with temperature until thermal limits that can deactivate key proteins in the complement system (Graham et al., 2017; Zimmerman et al., 2017). Therefore, it seems that the immunosuppressive effect of temperature on the innate immunity performance of ectotherms can be variable among different organisms (Butler et al., 2013; Graham et al., 2017; Zimmerman et al., 2017).

When the incubating temperature was of 37°C, rattlesnakes exposed to the fluctuating-to-constant treatment presented decreased BKA, whereas snakes from the constant-to-fluctuating did not. As already discussed, 30°C represented a near-optimal temperature for *C. durissus*, and in this case, perhaps the BKA attribute can be maximized at this temperature. Therefore, it is plausible that rattlesnakes acclimated to constant temperature would present an increased BKA in comparison to snakes acclimated at fluctuating regime. In this sense, in the fluctuating regime the optimal temperature only occurs two times in a day, i. e., when the temperature is increasing from 25°C to 35°C and decreasing from 35°C to 25°C, and the remaining time was

composed of exposure to suboptimal temperatures (Niehaus et al., 2012). Studies focusing on the effect of fluctuating thermal regimes over snakes' immune function are almost non-existent, but in amphibians it is described that the exposure to fluctuating or novel thermal regimes causes a decrease in immune response (Raffel et al., 2006; Raffel et al., 2013), which are in accordance with our BKA results. Snakes initially subjected to the fluctuating thermal regime presented a decrease in BKA that lasted for the whole experiment. Different phenotypic variables may be characterized by different temporal courses of response to a stressor (Martin, 2009). Therefore, the exposure to a high amplitude thermal regime can produce negative effects over BKA that last for longer timer periods, as after 10 days of acclimation to a new constant thermal regime. In the context of our experiment, behavioral thermoregulation was denied, which could attenuate deleterious effects caused by temperature fluctuations (Kearney et al., 2009; Vasseur et al., 2014), whereas the animals exposed to constant thermal regime were exposed to optimal temperature throughout the experiment.

Regarding sex differences there was effect only for BKA, whereas males presented higher levels than females in both treatments throughout the experiment. This result partially diverges from those found for the corn snake (*Panterophis guttatus*), in which males presented higher levels of peak agglutination (another parameter used to assess the immune capability of the complement system) in comparison to females after exposure to a fluctuating thermal regime, but not prior to this treatment (Stahlschmidt et al., 2017). Sex differences in the innate immune response of snakes might be related to reproductive hormones. For ectotherms, in general, the immune response can be regulated by reproductive hormones, being that high levels of androgens or estrogens might be associated to immunosuppression (Titon et al., 2016; Szwejser et al., 2017). Indeed, the time in which our experiments were conducted coincided with the second stage (active phase) of vitellogenesis for *C. durissus* (Almeida-Santos and Orsi, 2002). Since vitellogenesis imposes a high energetic demand, a possible trade-off between reproductive costs and the activation of the innate immune activity in females may have been at play at the time of our experiments. Therefore, it is possible that high levels of sex-related hormones may have suppressed immune response in females of *C. durissus*.

We did not find any evidence that thermal regime, time of exposure or sex influenced H:L ratio. This result is contrary to our predictions and we did not find any correlation between plasma CORT levels and H:L ratio. In general, the absence of

correlation between H:L ratio and plasma CORT show that, although both are used as stress indices, the temporal response dynamics presented by each one can be quite distinct, thus making difficult to observe a correlation in samples collected at the same time, as previously reported for lizards (Seddon and Klukowski, 2012) and snakes (Sparkman et al., 2014). Besides, changes in H:L ratio is somehow a more conservative stress index, since it requires longer times of exposure to the stressor for a stress response to occur, whereas CORT levels can respond and even return to pre-stressor levels more quickly (Davies et al., 2008; Sparkman et al., 2014). Indeed, in the snake *Tamnophis elegans* H:L ratio can take from weeks to months to suffer changes in response to stressors (Sparkman et al., 2014). Also, it is possible that the intensity of the stressor may promote changes in the H:L ratio of ectotherms (Assis et al., 2015). The absence of effects on H:L ratio suggests that the shift from constant to fluctuating thermal regime represented an acute and low intensity stressor for these snakes, which is unlikely to cause heterophilia (increase in circulating heterophils numbers) and lymphopenia (decrease in circulating lymphocyte numbers) (Davies et al., 2008).

Changes from fluctuating to constant temperatures do not seem to cause stress in *C. durissus* under the experimental conditions used in this study. In this sense, it is possible that the constant temperature used (30°C) was somehow a mild condition, whereas exposure to very low (e. g., 10°C), or high (e.g., 38°C) constant temperatures could result in a stress response (Dupoué et al., 2013; Jessop et al., 2016) for rattlesnakes. We found that the transition from a constant thermal regime to a fluctuating thermal regime increased plasma CORT levels. We suggest that this response might be related to the transition between a constant and optimal condition to a more complex thermal regime. The initial exposure of snakes to a fluctuating regime promoted a reduced BKA, which was also influenced by sex, possibly through a differential modulation by sexual hormones, as females presented decreased BKA in comparison to males in both treatments. Our results present a caution perspective, since even after long times of captivity, ectotherms can show increased plasma CORT levels associated with changes in thermal regimes.

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TABLES

Table 1. Fitted models to assess the relation between the dependent variable (DV: corticosterone, bacteria killing ability and H:L ratio) and independent variables (treatment (TR), time of exposure (time) and sex). Animals ID was used as a random effect term.

Tested Models
1) DV ~ TR + (1 | ID)
2) DV ~ TR + Sex + (1 | ID)
3) DV ~ TR + Time + (1 | ID)
4) DV ~ TR + Time + Sex + (1 | ID)
5) DV ~ TR*Sex + (1 | ID)
6) DV ~ TR*Time + (1 | ID)
7) DV ~ TR*Sex + Time + (1 | ID)
8) DV ~ TR*Time + Sex + (1 | ID)
9) DV ~ TR + Time*Sex + (1 | ID)
10) DV ~ Null model

Table 2. Descriptive analysis of corticosterone plasmatic levels (CORT), bacteria killing ability at 30°C (BKA 30°C) and 37°C (BKA 37°C), and heterophil:lymphocyte ratio (H:L) of rattlesnakes (*Crotalus durissus*) in the Constant-to-fluctuating (CF) and Fluctuating-to-constant (FC) treatments. The thermal regime, constant (C) or fluctuating (F) is indicated in bold and duration of exposure to the treatment is described in days (d).

	CF (2d)		**C**F (10d)		C**F** (14d)		C**F** (22d)		**F**C (2d)		**F**C (10d)		F**C** (14d)		F**C** (22d)	
Variables	**N**	**Mean ± SE**	**N**	**Mean ± SE**	**N**	**Mean ± SE**	**N**	**Mean ± SE**	**N**	**Mean ± SE**	**N**	**Mean ± SE**	**N**	**Mean ± SE**	**N**	**Mean ± SE**
CORT (ng/ml) Males	8	19.01 ± 2.89	8	26.16 ± **7.**38	8	26.9 ± 7.95	7	28.82 ± 5.57	7	22.93 ± 3.97	7	39.14 ± 11.03	7	13.72 ± 2.39	7	18.95 ± 5.2
CORT (ng/ml) Females	4	28.95 ± 20.12	4	26.3 ± 11.75	4	37.31 ± 21.1	4	20.86 ± 12.97	5	29.42 ± 8.79	5	28.33 ± 5.51	5	15.81 ± 7.96	5	29.93 ± 4.06
BKA 30°C (%) Males	8	35.75 ± 10.04	8	37.79 ± 10.29	8	49.76 ± 11.95	7	22.87 ± 9.86	7	39.1 ± 9.55	7	44.29 ± 10.03	7	36.33 ± 12.45	7	32.45 ± 11.87
BKA 30°C (%) Females	4	48.38 ± 26.48	4	46.87 ± 26.48	4	27.17 ± 6.87	4	41.89 ± 24.21	5	41.22 ± 11.58	5	62.35 ± 18.45	5	29.17 ± 14.86	5	60.32 ± 15.55
BKA 37°C (%) Males	8	56.76 ± 11.97	8	41.51 ± 12.3	8	58.55 ± 10.49	7	36.23 ± 10.46	7	55.64 ± 14.68	7	61.23 ± 12.36	7	56.49 ± 8.41	7	45.12 ± 11.29
BKA 37°C (%) Females	4	48.79 ± 19.42	4	41.89 ± 23.47	4	44.66 ± 4.14	4	42.28 ± 23.97	5	44.97 ± 13.26	5	53.48 ± 21.36	5	47.65 ± 10.39	5	72.2 ± 12.8
H:L Males	8	0.06 ± 0.02	8	0.12 ± 0.04	8	0.16 ± 0.03	7	0.16 ± 0.05	7	0.09 ± 0.02	7	0.23 ± 0.06	7	0.18 ± 0.07	7	0.13 ± 0.03
H:L Females	4	0.06 ± 0.02	4	0.11 ± 0.06	4	0.09 ± 0.03	4	0.05 ± 0.01	5	0.05 ± 0.01	5	0.08 ± 0.03	5	0.05 ± 0.02	5	0.23 ± 0.18

N = valid N; Min = Minimun; Max = Maximum; SE = Standard Error.

Table 3. Models selected through the AIC criterion (models described in Table 1) for corticosterone (CORT), bacteria killing ability at 30°C (BKA30) and 37°C (BKA37) and heterophil:lymphocyte ratio (HL) in rattlesnakes (*Crotalus durissus*). AIC = Akaike information criterion; dAIC = delta AIC; df = degrees of freedom. Weight indicates the robustness of the model in the explanation of the data.

Dependent Variables	Selected Models	AIC	dAIC	Df	Weight
CORT	8	788.9	0.0	11	0.82
BKA30	10	180.1	0.0	3	0.84
BKA37	2	198.5	0.0	5	0.4
BKA 37	5	198.9	0.5	6	0.31
BKA37	1	199.2	0.8	4	0.27
HL	10	-101.2	0.0	3	0.95

Table 4. T-values of significant fixed effects from selected models explaining variance in corticosterone and immune function after exposure to different treatments: constant-to-fluctuating (CF) and fluctuating-to-constant (FC).

Selected model	Fixed effects	t-value
8 (CORT)	Intercept	3.36
	Treatment CF*Time of Exposure (14d)	2.11
2 (BKA37)	Intercept	0.19
	Treatment FC	-3.64
	Sex M	2.24
5 (BKA37)	Intercept	-0.75
	Sex M	2.66
1 (BKA 37)	Intercept	2.35
	Treatment FC	-3.75

CORT = corticosterone; BKA37 = bacterial killing ability determined at 37°C; CF = Constant-to-fluctuating; FC = Fluctuating-to-constante; M = Male.

Table 5. Models selected through the AIC criterion (models described in Table 1) for bacteria killing ability at 30°C (BKA30) and 37°C (BKA37) and heterophil:lymphocyte ratio (HL) in rattlesnakes (*Crotalus durissus*) when CORT was included as a fixed fator. AIC = Akaike information criterion; dAIC = delta AIC; df = degrees of freedom. Weight indicates the robustness of the model in the explanation of the data.

Dependent Variables	Selected Models	AIC	dAIC	Df	Weight
BKA30	10	180.1	0.0	3	0.99
BKA37	10	207.2	0.0	3	0.99
HL	10	-101.2	0.0	3	1.0

FIGURES

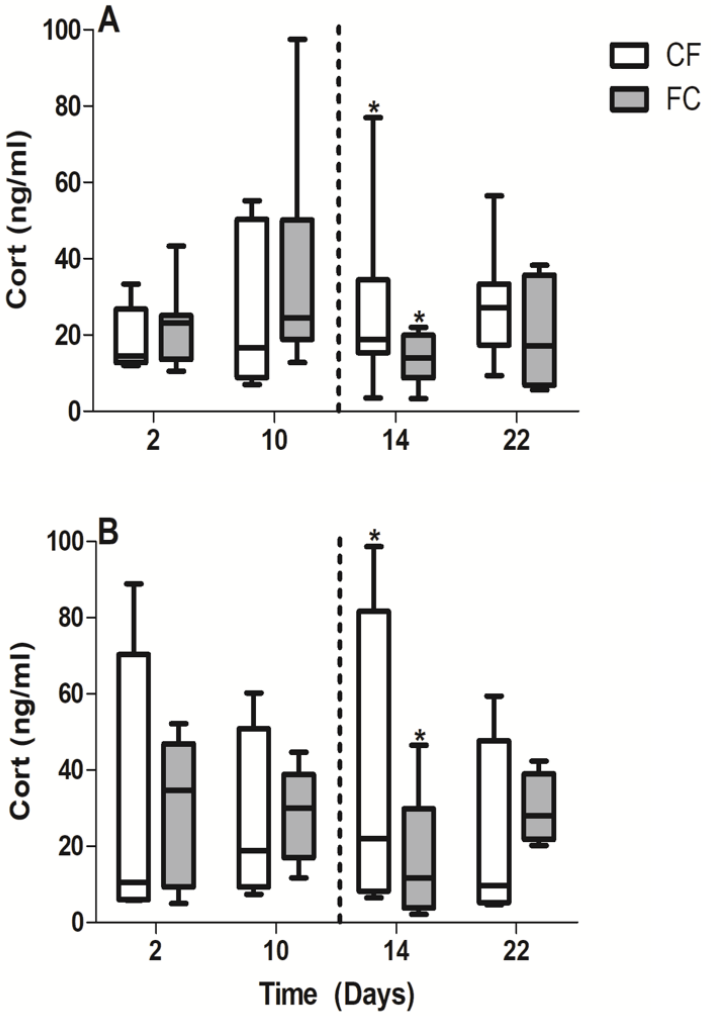

Figure 1 – Variation in corticosterone concentrations (Cort, in ng/ml) for males (A) and females (B) of *Crotalus durissus* (rattlesnakes). CF = Constant-to-fluctuating treatment; FC = Fluctuating-to-constant treatment. The vertical dotted line marks day 12, in which the shift between thermal regimes occurred. The horizontal lines inside the boxes indicates de median values, and the lower and upper whiskers represents respectively the 10th and the 90th percentile. The asterisks denote statistical difference between treatments.

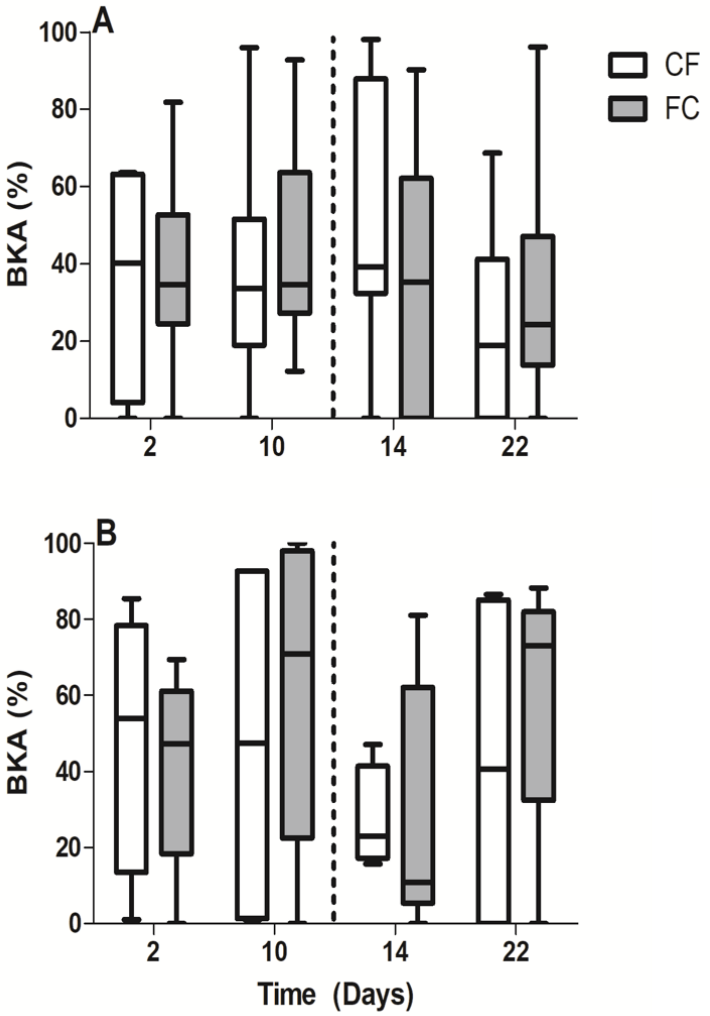

Figure 2 – Bacteria killing ability (BKA, %) at 30°C for males (A) and females (B) of *Crotalus durissus* (rattlesnakes). CF = Constant-to-fluctuating treatment; FC = Fluctuating-to-constant treatment. The vertical dotted line marks day 12, in which the shift between thermal regimes occurred. The horizontal lines inside the boxes indicates de median values, and the lower and upper whiskers represents respectively the 10^{th} and the 90^{th} percentile.

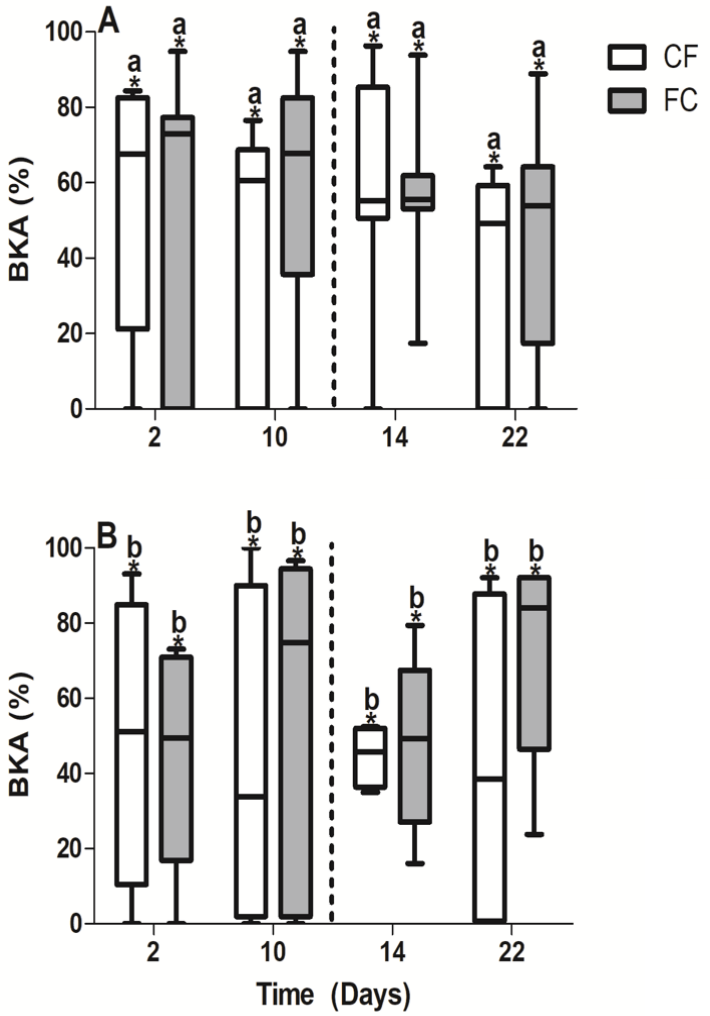

Figure 3 – Bacteria killing ability (BKA, %) at 37°C for males (A) and females (B) of *Crotalus durissus* (rattlesnakes). CF = Constant-to-fluctuating treatment; FC = Fluctuating-to-constant treatment. The vertical dotted line marks day 12, in which the shift between thermal regimes occurred. The horizontal lines inside the boxes indicates de median values, and the lower and upper whiskers represents respectively the 10th and the 90th percentile. The asterisks denote statistical difference between treatments, and different letters indicates significant differences between sexes.

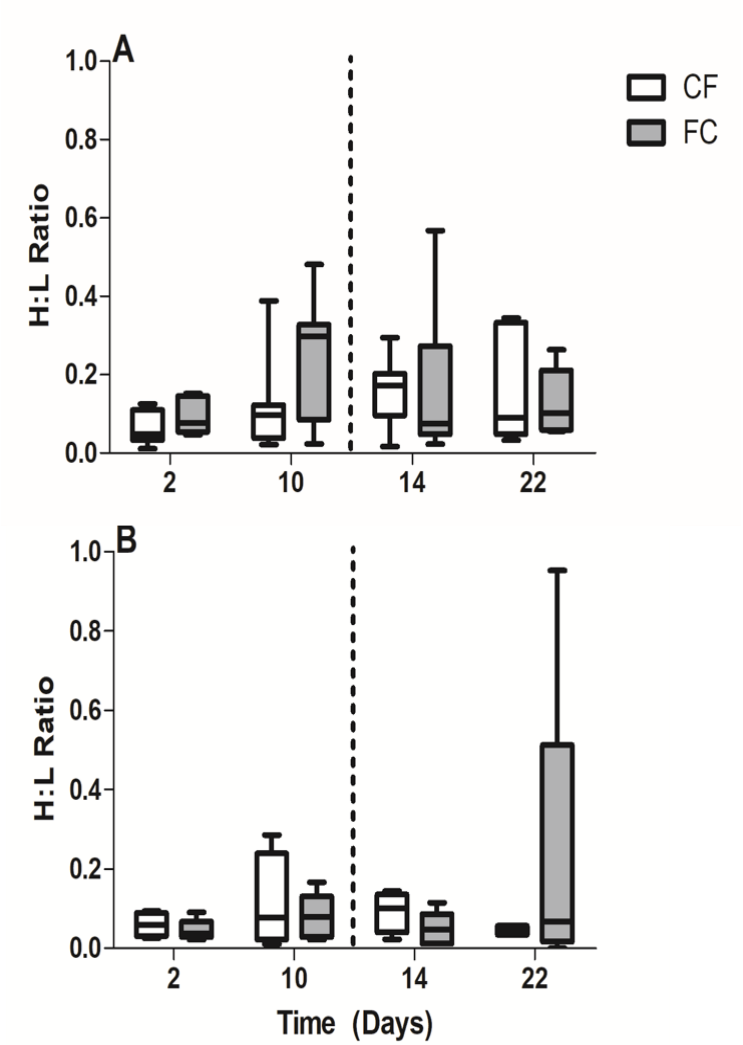

Figure 4 – Heterophil to lymphocyte ration (H:L ratio) for males (A) and females (B) of *Crotalus durissus* (rattlesnakes). CF = Constant-to-fluctuating treatment; FC = Fluctuating-to-constant treatment. The vertical dotted line marks day 12, in which the shift between thermal regimes occurred. The horizontal lines inside the boxes indicates de median values, and the lower and upper whiskers represents respectively the 10th and the 90th percentile.