FEDERAL UNIVERSITY OF SÃO CARLOS
CENTER OF BIOLOGICAL SCIENCES AND HEALTH
INTERINSTITUTIONAL PROGRAM OF POST-GRADUATION IN PHYSIOLOGICAL SCIENCES UFSCar/UNESP

SAMANTA APARECIDA CASTRO

THE ROLE OF NITRIC OXIDE AND ADRENERGIC STIMULATION ON THE CARDIOVASCULAR ADJUSTMENTS IN SOUTH AMERICAN RATTLESNAKES (*Crotalus durissus*)

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Dissertation presented to the Interinstitutional Postgraduate Program in Physiological Sciences of Federal University of São Carlos, as part of the requirements to obtain the Master's degree in Physiological Sciences.

Advisor: Dr. Cléo Alcantara Costa Leite

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To my parents, Maria and José, for the love, encouragement, dedication, teachings and for offering me the opportunity to study. Thank you for allowing my dream become true!

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I dedicate

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EPIGRAPH

“The Knowledge serves to enchant people,
not to humiliate them.”

Mário Sérgio Cortella

“Humility differs from subservience. A humble person knows that it does not know everything. It's the person who knows it is not the only one who knows. It's the one who knows that together with another person they will know a lot more. It's the person who knows that even together, they will never know everything to be known.”

Mário Sérgio Cortella

RESUMO

Entender a base da regulação do tônus vascular é importante para compreender a fisiologia cardiovascular de qualquer vertebrado. Investigações recentes em répteis tentaram essa descrição trabalhando em animais anestesiados. Infelizmente, essa abordagem pode ser ineficaz para acessar mecanismos que dependem de algum nível de modulação autonômica. O objetivo deste estudo foi investigar o papel do óxido nítrico (NO) e da estimulação adrenérgica no controle do tônus vascular em répteis. Este estudo utilizou o modelo de cascavel descerebrada desenvolvido para evitar o efeito deletério dos anestésicos na modulação cardiovascular. Descobrimos que o NO é um vasorelaxante eficaz em cascavéis e existe um nível de repouso na produção de NO. O nitroprussiato de sódio (SNP) causou vasodilatação sistêmica, seguida por aumento da condutância sistêmica (G_{sys}) e fluxo sistêmico ($\dot{Q}_{sys}$), e esse resultado foi associado à redução da pressão arterial média sistêmica (PAM_{sys}). Como resposta, o débito cardíaco (DC) foi elevado. Na circulação pulmonar, o SNP aumentou a condutância pulmonar (G_{pul}) e reduziu a pressão arterial média pulmonar (PAM_{pul}), enquanto o fluxo pulmonar ($\dot{Q}_{pul}$) permaneceu inalterado. A vasodilatação sistêmica após a L-arginina (L-Arg) foi semelhante ao efeito da injeção de SNP. Embora não houvesse efeitos da L-Arg na circulação pulmonar. Portanto, em cascavéis, o NO é sintetizado via L-Arg e tem papel parcial na regulação local do tônus vascular sistêmico. Em contraste, a vasculatura pulmonar é menos reativa ao SNP e a produção de NO parece não depender da L-Arg. Adrenalina e fenilefrina causaram vasoconstrição sistêmica que foi abolida pela fentolamina, demonstrando que esta resposta foi mediada por receptores α-adrenérgicos. A injeção de fentolamina causou uma acentuada vasodilatação, seguida de aumento da G_{sys} e $\dot{Q}_{sys}$, e esteve efeito foi associado à redução da PAM_{sys}. A injeção de propranolol promoveu bradicardia, com consequente diminuição do DC e $\dot{Q}_{sys}$, e aumento do tônus vascular sistêmico, sem alteração da G_{sys} e PAM_{sys}. Esses resultados indicam que a modulação adrenérgica, via α-receptores, é quantitativamente mais relevante do que o ramo β-receptor do barorreflexo, para modular a PAM em *Crotalus*. A vasculatura pulmonar é menos responsiva à estimulação adrenérgica. Além disso, a fentolamina foi mais eficaz do que propranolol em alterar a G_{pul}. Portanto, sugerimos que a estimulação adrenérgica, pelo ramo simpático do sistema nervoso autônomo, é mais eficaz do que a atividade cardíaca, em modular o tônus vascular em cascavéis.

Palavras-chave: sistema nervoso autônomo, descerebração, óxido nítrico, répteis, ativação simpática, tônus vascular.

ABSTRACT

Understanding the basis of vascular tone regulation is important to comprehend the cardiovascular physiology of any vertebrate. Recent investigations on reptiles have tried that description working on anesthetized animals. Unfortunatelly, such approach can be ineffective to access mechanisms that rely on some level of autonomic modulation. The aim of this study was to investigate the role of nitric oxide (NO) and of adrenergic stimulation on the control of vascular tone in reptiles. This study used the developed decerebrate rattlesnake model in order to avoid the deleterious effect of anesthetics on cardiovascular modulation. We found that NO is effective vasorelaxing in rattlesnakes and there is a resting level of NO production. Sodium nitroprusside (SNP) caused systemic vasodilation followed by systemic conductance (G_{sys}) and systemic flow ($\dot{Q}_{sys}$) increasing, and it was associated with systemic mean arterial pressure (MAP_{sys}) reduction. As a response, cardiac output (CO) was elevated. In the pulmonary circulation, SNP increased pulmonary conductance (G_{pul}) and reduced pulmonary mean arterial pressure (MAP_{pul}), while pulmonary flow ($\dot{Q}_{pul}$) remained unaffected. The systemic vasodilation after L-arginine (L-Arg) was similar to the effect of SNP injection. Although, there were no effects of L-Arg on the pulmonary circulation. Therefore, in rattlesnakes, NO is synthetized via L-Arg and it has partial role on the local regulation of systemic vascular tone. In contrast, the pulmonary vasculature is less reactive to SNP and also, NO production seems not to be dependent on the L-Arg. Adrenaline and phenylephrine caused systemic vasoconstriction that was abolished by phentolamine, demonstrating this response was mediated by α-adrenergic receptors. Injection of phentolamine caused a marked vasodilatation followed by G_{sys} and $\dot{Q}_{sys}$ increasing, and it was associated with MAP_{sys} reduction. Injection of propranolol promoted bradycardia, with consequent decreasing of CO and $\dot{Q}_{sys}$ and increased of systemic vascular tone, without changing the G_{sys} and MAP_{sys}. These results indicate adrenergic modulation via α-receptors is quantitatively more relevant to modulate MAP than the β-receptors branch of barorreflex in *Crotalus*. Pulmonary vasculature is less responsive to adrenergic stimulation. Also, phentolamine was more effective in alter G_{pul} than propranolol. Therefore, we suggest that the adrenergic stimulation by the sympathetic branch of the autonomic nervous system is more effective in modulate vascular tone than cardiac activity in rattlesnakes.

Key words: autonomic nervous system, decerebration, nitric oxide, reptiles, sympathetic activation, vascular tone.

LIST OF SYMBOLS AND ABBREVIATIONS OF THE CHAPTER I

SNA: sistema nervoso autônomo

NO: óxido nítrico

L-Arg: L-arginina

SNP: nitroprussiato de sódio

NOS: enzima NO sintase

eNOS: NOS endotelial

L-NAME: éster metílico da N-nitro-l-arginina

i.v: intravenoso

CO_2: dióxido de carbono

SDA: ação dinâmica específica

P_{sys}: pressão arterial sistêmica

P_{pul}: pressão arterial pulmonar

$\dot{Q}_{sys}$: fluxo sanguíneo sistêmico

$\dot{Q}_{pul}$: fluxo sanguíneo pulmonar

$\dot{Q}_{LAo}$: fluxo sanguíneo no arco sistêmico esquerdo

PAM_{sys}: pressão arterial média sistêmica

PAM_{pul}: pressão arterial média pulmonar

f_H: frequência cardíaca

DC: débito cardíaco

V_{stot}: volume sistólico total

R_{sys}: resistência sistêmica

R_{pul}: resistência pulmonar

G_{sys}: condutância sistêmica

G_{pul}: condutância pulmonar

Net-Shunt D-E: desvio líquido da direita para esquerda

ACh: acetilcolina

EDHF: fator hiperpolarizante derivado do endotélio

H_2O_2: peróxido de hidrogênio

Cu, Zn-SOD: enzima Cu, Zn-superóxido dismutase

P_{VC}: pressão venosa central

MCFP: pressão média de enchimento circulatório

LIST OF SYMBOLS AND ABBREVIATIONS OF CHAPTERS II AND III

NO: nitric oxide

NO^{-2}: nitrite

NO^{-3}: nitrate

L-Arg: L-arginine

SNP: sodium nitroprusside

NOS: enzyme NO synthase

eNOS: endothelial NOS

nNOS: neuronal NOS

iNOS: inducible NOS

ANS: autonomic nervous system

CNS: central nervous system

L-NAME: N(ω)-nitro-L-arginine methyl ester

SDA: specific dynamic action

PCO_2: partial pressure of carbon dioxide

$\dot{Q}_{sys}$: systemic blood flow

$\dot{Q}_{pul}$: pulmonary blood flow

$\dot{Q}_{LAo}$: left systemic arch blood flow

MAP_{sys}: systemic mean arterial pressure

MAP_{pul}: pulmonary mean arterial pressure

f_H: heart rate

CO: cardiac output

V_{stot}: total stroke volume

R_{sys}: systemic resistance

R_{pul}: pulmonary resistance

G_{sys}: systemic conductance

G_{pul}: pulmonary conductance

Net R-L shunt: right-to-left net blood shunt

Net L-R shunt: left-to-right net blood shunt

ACh: acetycholine

EDHF: endothelium-derived hyperpolarizing factor

H_2O_2: hydrogen peroxide

Cu, Zn-SOD: enzyme Cu, Zn-superoxide dismutase

NADPH: nicotinamide adenine dinucleotide phosphate

FAD: flavin adenosine dinucleotide

FMN: flavin mononucleotide

BH_4: tetrahydrobiopterin

K_{Ca}: calcium-activated potassium channels

Phent: phentolamine

Propr: propranolol

P_{CV}: central venous pressure

MCFP: mean circulatory filling pressure

SUMMARY

CHAPTER I

Síntese em português

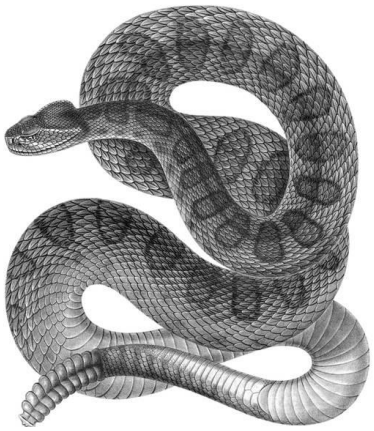

Image source: https://bit.ly/2KpzvVe

1. INTRODUÇÃO

O sistema cardiovascular de vertebrados é modulado pelo SNA. Assim, coração e vasos parecem estar sob ação tônica do SNA em todos os grupos analisados até o momento (Taylor et al., 1999; 2014). Em répteis, além do efeito de catecolaminas circulantes, o coração e vasos recebem inervação direta de fibras simpáticas adrenérgicas excitatórias e parassimpáticas colinérgicas inibitórias (Morris e Nilsson, 1994). O sistema vascular de répteis pode receber inervação excitatórias de fibras simpáticas tanto na vasculatura central quanto na periférica, além de inervação excitatórias parassimpáticas na vasculatura central, principalmente no circuito pulmonar (Taylor et al., 1999; 2009; Leite et al., 2013; 2014). Apesar dessa descrição geral estar disponível, há pouca informação sobre formas e mecanismos de ajustes cardiovasculares em répteis. Informações provenientes desse grupo são de fundamental importância para basear qualquer teoria sobre a evolução de mecanismos e processos no grupo dos vertebrados. Deste modo, o estudo da regulação do tônus vascular dos circuitos sistêmico e pulmonar têm implicações importantes para a compreensão da fisiologia de répteis e possibilita um melhor entendimento do funcionamento de mecanismos para ajustes cardiovasculares nos vertebrados.

A anatomia cardíaca de répteis é especialmente interessante, pois seu ventrículo é apenas parcialmente dividido, permitindo a distribuição seletiva do sangue entre as circulações sistêmica e pulmonar (desvio sanguíneo intracardíaco) (Hicks, 1998). A possibilidade da mistura de fluxos de sangue rico e pobre em oxigênio foi vista, durante muito tempo, como um mecanismo cardiovascular ineficiente, uma vez que era diferente do encontrado em aves e mamíferos (Foxon, 1955). No entanto, esse pensamento mudou quando estudos demonstraram que o grau de mistura do sangue dentro do coração de répteis é controlado pelo SNA e que o mesmo varia de acordo com o estado fisiológico do animal, em resposta a variações do meio interno ou externo (Hicks e Malvin 1992; Comeau e Hicks, 1994; Hicks e Comeau, 1994; Hicks, 1994).

Atualmente, está bem estabelecido que o controle vagal colinérgico da artéria pulmonar determina variações nos padrões de desvio sanguíneo intracardíaco, por afetar a condutância do circuito pulmonar (Johansen and Burggren, 1980; Hicks, 1998; Taylor et al, 2009). Além disso, há modulação da condutância vascular, através da estimulação adrenérgica na circulação sistêmica, contudo, não se sabe se essa estimulação contribuiu para o controle de desvio sanguíneo intracardíaco ou quanto pode afetar a condutância pulmonar nas diferentes espécies de répteis (Lillywhite e Donald, 1994). Do mesmo modo, vários estudos se dedicaram a caracterizar o papel do NO no sistema cardiovascular de répteis (Crossley et al., 2000; Axelsson

et al., 2001; Galli et al., 2005b; Donald and Broughton, 2005; Skovgaard et al., 2005b; Broughton and Donald, 2007). Em alguns desses trabalhos, foi relatado que a inibição da síntese de NO aumentou o tônus vascular sistêmico, demonstrando que a liberação tônica de NO contribui para a regulação basal da condutancia sistêmica (Knight e Burnstock, 1993; Axelsson et al. 2001). Contudo, esses dados mostram apenas uma análise qualitativa da presença desse mecanismo.

A ocorrência de influências adrenérgicas e do NO já foi observada experimentalmente em cascavéis anestesiadas (Galli et al., 2005b; Galli et al., 2007). O uso da anestesia para permitir instrumentações mais invasivas, possibilitando múltiplas medidas concomitantes, é relevante do ponto de vista qualitativo. Entretanto, inferências quantitativas, limites de ajustes nos sistemas investigados, ou qualquer integração em resposta a desafios proporcionados por alterações dos meios internos e externos é muito limitada, uma vez que o SNA está em disfunção, decorrente do anestésico utilizado.

Dentro da temática abordada, a utilização da cascavel como modelo de estudo deve-se a facilidade de obtenção do animal, ao montante de informações disponíveis sobre o sistema cardiovascular dessa espécie e pela representatividade que a mesma apresenta na ordem Squamata. Ela torna-se relevante, uma vez que as conclusões provenientes das investigações com essa espécie podem ser extrapoladas para o grupo. Além disso, as serpentes demonstraram ser importantes modelos para estudos dos mecanismos envolvidos na evolução de adaptações cardiovasculares devido, principalmente, a diversidade na biologia, ecologia e características comportamentais desses animais. Deste modo, o modelo de estudo com o uso de cascavel descerebrada pode trazer implicações importantes para a pesquisa nessa área. Ele permite que sejam realizadas investigações da função autonômica do animal, com instrumentação para múltiplas medidas simultâneas, sem o uso de anestesia. Tal modelo abre um largo campo de investigações para o entendimento de padrões mecanísticos e funções autonômicas em répteis.

Portanto, diante dos argumentos apresentados, trabalhamos com uma série de hipóteses nesta investigação: (1) que o modelo de animal, com o uso de cascavel descerebrada é uma técnica eficiente para obtenção de dados relacionados aos processos regulatórios do SNA, visto que em tal modelo ele está operante. (2) sugerimos também que a ação do NO nos ajustes vasculares é acentuada e relevante em ambos os circuitos, sistêmico e pulmonar. (3) além disso, acreditamos que a estimulação adrenérgica tem efeito em ambos os circuitos e é capaz de afetar o desvio sanguíneo intracardíaco, por propiciar maior redução relativa da condutância sistêmica, em comparação com a pulmonar. (4) acreditamos ainda, que a relevância do controle autonômico vascular é subestimada, devido ao uso de modelos animais anestesiados e, portanto,

desprovidos de tônus autonômico e de suporte funcional para apresentação de ajustes regulatórios. Essa investigação tem relevância por inserir um novo modo de analisar a presença e eficácia de ajustes cardiovasculares e questiona alguns dos dados anteriormente produzidos com animais anestesiados.

2. OBJETIVOS

O objetivo geral do estudo foi investigar o papel do NO e da estimulação adrenérgica na regulação dos ajustes cardiovasculares em Squamata.

Tais objetivos podem, ainda, ser segmentados em dois principais:

- Avaliar o papel e quantificar a relevância funcional do efeito do NO nos ajustes vasculares em cascavéis (*Crotalus durissus*);
- Investigar o papel e quantificar a relevância funcional do efeito da estimulação α e β - adrenérgica na regulação dos ajustes vasculares em cascavéis (*Crotalus durissus*).

3. MATERIAL E MÉTODOS

3.1 Obtenção e manutenção dos animais

Para esse estudo, foram utilizadas 21 cascavéis sul-americanas (*Crotalus durissus*), de ambos os sexos (829 ± 47g). As serpentes foram doadas pelo Instituto Butantan em São Paulo e pela UNESP em Rio Claro, e então, transportadas para o biotério do Departamento de Ciências Fisiológicas da UFSCar, São Carlos. Os animais foram mantidos em compartimentos individuais, em temperatura controlada (28 ± 2^{o}C) e ciclo claro:escuro, 12:12h. As serpentes tinham livre acesso à água e eram alimentadas a cada duas semanas. A alimentação era interrompida duas semanas antes de qualquer procedimento experimental, para evitar o efeito do incremento metabólico pós-prandial nos animais de estudo (Andrade et al., 1997). Todos os procedimentos utilizados foram analisados e aprovados pela CEUA/UFSCar.

3.2 Anestesia e eutanásia

Primeiramente, os animais foram sedados por inalação de CO_2, até a perda total de reflexos de ajuste corporal e insensibilidade ao toque (Forslid et al., 1986; Wang et al., 1993; Leite et al., 2013; 2014). Esse procedimento permitiu manuseio seguro para inserção de tubo endotraqueal para ventilação mecânica e anestesia com isoflurano (5% para indução e 2-3% para manutenção). Após anestesia completa, o animal foi mantido com ventilação artificial (4-

5 ventilações por minuto e volume de 30 ml·kg^{-1}). Anestésico local, lidocaína (2% - Pearson, Brasil) foi aplicado no local de cada incisão realizada. Todos os animais passaram por cuidados pós-operatórios com injeções de antibiótico (Chemitril, 11 mg·kg^{-1} - Chemitec) e analgésico/anti-inflamatório (Flunixin, 1,1 mg·kg^{-1} – Chemitec), ainda sob anestesia. O processo de descerebração, como remete a um procedimento sobre perda de processamento central e integridade sensorial e cognitiva irreversível, é considerado o momento da eutanásia do animal (Silverman et al., 2005). Assim, após a descerebração e implantação de equipamentos de registro dos parâmetros cardiovasculares, os animais foram recuperados dos efeitos do isoflurano e mantidos por 24h, para então, iniciar os protocolos experimentais. Uma vez finalizado os protocolos experimentais, os animais receberam injeção intravenosa de barbitúrico (Tiopental, 100 mg·kg^{-1} - Pearson, Brasil), seguida por solução hipersaturada de K^+. Após parada cardíaca, as carcaças foram apropriadamente acondicionadas para posterior descarte pelo serviço de coleta especializado da UFSCar.

3.3 Descerebração

No processo de descerebração, o córtex cerebral e tálamo foram separados do restante do sistema nervoso central, o que eliminou o processamento central de nocicepção e qualquer processamento relacionado à percepção e/ou sofrimento (Silverman et al., 2005). A transecção ocorreu ao nível do *Tectum*, e todas as estruturas rostrais a secção foram cauterizadas e retiradas, eliminando o efeito de ativação do córtex, o que é necessário para qualquer nível de consciência (Pickering and Paton, 2006). O propósito desse procedimento foi ter um animal passível de instrumentação para registro concomitante de múltiplos parâmetros fisiológicos, sem a percepção ou qualquer processamento de sofrimento ou dor, e sem o efeito deletério da anestesia sobre as funções autonômicas.

3.4 Instrumentação

Após o processo de descerebração e ainda sob o efeito do isoflurano, foi realizada uma incisão ventrolateral (3 - 4cm), imediatamente rostral à posição do coração, para expor a área com os vasos centrais. Após essa etapa, foi realizada a canulação sistêmica (canulação oclusiva da arterial vertebral para inserção do cateter no arco sistêmico direito) e pulmonar (canulação oclusiva de um ramo proximal da artéria pulmonar para inserção do cateter na artéria pulmonar) (PE20). Além disso, inserimos sondas de fluxo no arco sistêmico esquerdo e artéria pulmonar. Ao final do procedimento, as cânulas e sondas foram exteriorizadas e fixadas com pontos de sutura no dorso do animal, para evitar que se deslocassem durante o experimento. O animal

teve a incisão suturada e foi posicionado em decúbito ventral para as medições. Tais procedimentos permitiram o registro contínuo e concomitante de P_{sys}, P_{pul}, $\dot{Q}_{LAo}$ e $\dot{Q}_{pul}$ (sistema de transdução, amplificação e registro de Transonic Systems e Powerlab – ADInstruments). Com esses dados foi possível calcular PAM_{sys} e PAM_{pul}, f_H, $\dot{Q}_{sys}$, DC, V_{stot}, Net-Shunt, R_{sys} e R_{pul}, além da G_{sys} e G_{pul}.

3.5 Protocolos experimentais

3.5.1 Curva dose-resposta para o L-NAME

Para obtenção da curva dose-resposta para o L-NAME (n = 6, 597 ± 86g), foi utilizada uma série de injeções i.v.: 0,5 ml·kg^{-1} de solução salina (0,9%), seguida por doses crescentes de L-NAME (0,1 mg·kg^{-1}; 1 mg·kg^{-1}; 10 mg·kg^{-1} e 100 mg·kg^{-1}). Os parâmetros cardiovasculares foram analisados de acordo com o efeito de doses crescentes do fármaco, verificando o ponto de maior resposta. Após análise da curva dose-resposta resultante, foi escolhida a menor dose, que claramente apresentava resposta cardiovascular para ser utilizada no protocolo seguinte.

3.5.2 Avaliação do papel do NO nos ajustes vasculares

Neste protocolo (n = 7, 983 ± 50g), cada animal recebeu uma injeção i.v. do doador de NO (SNP – 2,5 µg·kg^{-1}) para determinar a existência de resposta a esse tipo de sinalizador. Em seguida, um substrato para a produção endógena de NO (L-Arg – 50 mg·kg^{-1}) foi injetado para verificação da presença de vias de produção de NO. Finalmente, a síntese de NO foi bloqueada por administração do inibidor de NOS (L-NAME – 10 mg·kg^{-1}) para análise da presença de produção tônica de NO. Para verificação da inibição, foi administrado L-Arg e, uma segunda perfusão de SNP. Tal protocolo nos permitiu avaliar se a capacidade de resposta ao NO ainda estava intacta. Cada fármaco teve seu efeito analisado através de alterações das variáveis cardiovasculares, avaliadas ao longo do tempo pós-injeção.

3.5.3 Avaliação do papel da estimulação adrenérgica nos ajustes vasculares

Para este protocolo (n = 8, 852 ± 40g), cada animal recebeu injeções i.v. de fenilefrina (5 µg·kg^{-1}) e adrenalina (2 µg·kg^{-1}) antes e depois da administração da droga bloqueadora dos receptores α-adrenérgicos, fentolamina (2 mg·kg^{-1}). Após essa etapa, foi administrado propranolol (2 mg·kg^{-1}) para o bloqueio β-adrenérgico e, novamente, adrenalina (2 µg·kg^{-1}) e

fenilefrina (5 μg·kg^{-1}). Cada fármaco teve seu efeito analisado através de alterações das variáveis cardiovasculares, avaliadas ao longo do tempo pós-injeção.

Todas as injeções foram realizadas através do cateter da artéria vertebral. A injeção inicial de salina foi utilizada para determinação do efeito cardiovascular de injeção i.v. em si. A administração de qualquer droga somente foi realizada após tempo adequado para retorno dos parâmetros à condição basal ou ao estado estacionário normal. Tais procedimentos são idênticos e retratam o que foi realizado em todos os protocolos experimentais. Todos os fármacos utilizados foram adquiridos na Sigma-Aldrich Brasil Ltda.

3.6 Análises estatísticas

Cada parâmetro cardiovascular na curva dose-resposta para o L-NAME foi analisado com o teste de Friedman e teste post-hoc de Student-Newman-Keuls. A presença dos efeitos da L-Arg e SNP, antes e após a injeção de L-NAME, foram analisados com o teste de Wilcoxon. Os efeitos da adrenalina e fenilefrina, antes e após o bloqueio dos receptores α e β-adrenérgicos, foram analisados com os testes de Friedman ou ANOVA One-way, de acordo com a distribuição dos dados. O teste t pareado ou teste de Wilcoxon foram usados para comparar os efeitos da adrenalina e fenilefrina em relação à sua pré-injeção, assim como os efeitos da fentolamina e propranolol. Todos os dados foram apresentados como média ± EPM e as diferenças foram determinadas seguindo o nível de confiança de 95% (valor $P < 0,05$).

4. PRINCIPAIS RESULTADOS ENCONTRADOS

4.1 Efeito do NO nos ajustes cardiovasculares

Considerando as alterações obtidas na condutância vascular sistêmica, a dose de L-NAME escolhida para investigar o papel do NO nos ajustes cardiovasculares da cascavel foi de 10 mg·kg^{-1}. Quando o L-NAME inibiu a síntese de NO, houve aumento do tônus vascular, causando redução na G_{sys}, no entanto, a f_H e PAM_{sys} permaneceram inalteradas. Essa resposta demonstra que para compensar o efeito da elevação da vasoconstrição sistêmica, a cascavel foi capaz de diminuir o DC, causando redução compensatória do $\dot{Q}_{sys.}$ Não houve efeitos do L-NAME na circulação pulmonar (Tabela 1), de forma que tal aumento de G_{sys} não acarretou alteração do desvio sanguíneo intracardiaco.

O SNP induziu relaxamento na vasculatura sistêmica e os efeitos não foram inibidos após L-NAME. Sua injeção promoveu vasodilatação sistêmica (R_{sys}: 0,15 ± 0,03 para 0,06 ±

0,01 kPa·ml^{-1}·min^{-1}·kg^{-1}) e diminuição da PAM_{sys} (5,48 ± 0,50 para 3,26 ± 0,31 kPa). Como resposta compensatória à hipotensão, houve aumento do DC (57,50 ± 4,60 para 76,36 ± 6,30 ml·min^{-1}·kg^{-1}), devido ao aumento da f_H (34,95 ± 2,48 para 38,90 ± 2,23 b.p.m), com consequente aumento do $\dot{Q}_{sys}$ (40,94 ± 4,82 para 61,28 ± 7,55 ml·min^{-1}·kg^{-1}). Na circulação pulmonar, observamos redução da R_{pul} (0,24 ± 0,06 para 0,18 ± 0,04 kPa·ml^{-1}·min^{-1}·kg^{-1}) e diminuição da PAM_{pul} (2,98 ± 0,31 para 2,18 ± 0,20 kPa). No entanto, $\dot{Q}_{pul}$ permaneceu inalterado após injeção de SNP (16,56 ± 4,74 para 15,09 ± 3,27 ml·min^{-1}·kg^{-1}). O Net-Shunt D-E não foi alterado pela injeção de SNP (0,49 ± 0,16 para 0,30 ± 0,08).

Os efeitos das injeções de L-Arg foram semelhantes aos do SNP e causaram vasodilatação sistêmica, devido à diminuição da R_{sys} (0,17 ± 0,04 para 0,08 ± 0,01 kPa·ml^{-1}·min^{-1}·kg^{-1}). A vasodilatação foi associada ao aumento da G_{sys} (7,74 ± 1,44 para 16,12 ± 3,60 ml·KPa^{-1}·min^{-1}·kg^{-1}) e consequente redução da PAM_{sys} (5,57 ± 0,50 para 4,20 ± 0,44 KPa). Como respostas compensatórias, a f_H (33,43 ± 2,83 para 36,38 ± 2,22 b.p.m) e V_{stot} (1,63 ± 0,16 para 2,06 ± 0,21 ml·kg^{-1}) foram elevados após L-Arg, aumentando o DC (53,83 ± 4,96 para 73,65 ± 5,82 ml·min^{-1}·kg^{-1}) e $\dot{Q}_{sys.}$ (39,81 ± 4,70 a 59,60 ± 6,72 ml·min^{-1}·kg^{-1}). Todas as respostas das variáveis fisiológicas cardiovasculares sistêmicas foram significativamente atenuadas após o tratamento com L-NAME. O único parâmetro pulmonar alterado após injeção de L-Arg foi a PAM_{pul} (3,02 ± 0,37 para 2,60 ± 0,24 KPa), sendo esta queda associada à redução do Net-Shunt (0,42 ± 0,12 a 0,27 ± 0,07). Não houve efeitos da injeção de L-Arg nas demais variáveis pulmonares (R_{pul}: 0,27 ± 0,06 para 0,21 ± 0,05 kPa·ml^{-1}·min^{-1}·kg^{-1}; $\dot{Q}_{pul}$: 14,03 ± 3,80 para 14,06 ± 3,17 ml·min^{-1}·kg^{-1}; G_{pul}: 5,03 ± 1,23 para 5,70 ± 1,00 ml·KPa^{-1}·min^{-1}·kg^{-1}).

4.2 Efeio da estimulação adrenérgica nos ajustes cardiovasculares

A injeção de adrenalina causou vasoconstrição sistêmica, devido ao aumento da R_{sys} (1,33 ± 0,27 para 2,22 ± 0,45 kPa·ml^{-1}·min^{-1}·kg^{-1}). Essa resposta vasoconstritora foi associada à diminuição da G_{sys} (0,72 ± 0,10 para 0,43 ± 0,06 ml·KPa^{-1}·min^{-1}·kg^{-1}) e, consequente, aumento da PAM_{sys} (5,07 ± 0,27 para 7,66 ± 0,33 KPa). No entanto, a f_H (26,41 ± 2,48 para 30,50 ± 2,08 b.p.m), V_{stot} (1,55 ± 0,14 para 1,24 ± 0,20 ml·kg^{-1}), DC (40,31 ± 8,06 para 36,83 ± 8,08 ml·min^{-1}·kg^{-1}) e $\dot{Q}_{sys}$ (27,72 ± 2,85 para 24,28 ± 3,02 ml·min^{-1}·kg^{-1}) permaneceram constantes. A PAM_{pul} foi a única variável alterada após injeção de adrenalina (2,50 ± 0,24 para 3,31 ± 0,30 KPa). Os demais parâmetros pulmonares não foram afetados ($\dot{Q}_{pul}$: 15,03 ± 6,21 para 16,72 ± 6,47 ml·min^{-1}·kg^{-1}; R_{pul}: 2,30 ± 0,90 para 2,25 ± 0,84 kPa·ml^{-1}·min^{-1}·kg^{-1}; G_{pul}:

1,68 ± 1,15 para 1,32 ± 0,72 ml·KPa^{-1}·min^{-1}·kg^{-1}). O Net-Shunt D-E não foi alterado pela injeção de adrenalina (0,15 ± 0,05 para 0,22 ± 0,07).

O efeito da injeção de fenilefrina foi semelhante ao da adrenalina e causou vasoconstrição sistêmica. A vasoconstrição ocorreu devido ao aumento da R_{sys} (1,52 ± 0,20 para 1,98 ± 0,26 kPa·ml^{-1}·min^{-1}·kg^{-1}), e essa resposta foi associada à diminuição da G_{sys} (0,71 ± 0,08 para 0,54 ± 0,05 ml·KPa^{-1}·min^{-1}·kg^{-1}) e aumento da PAM_{sys} (5,28 ± 0,21 para 6,10 ± 0,30 KPa). De forma semelhante ao tratamento com adrenalina, a f_H (27,80 ± 2,57 para 27,22 ± 2,50 b.p.m), V_{stot} (1,46 ± 0,17 para 1,34 ± 0,16 ml·kg^{-1}), DC (42,67 ± 8,35 para 39,01 ± 8,84 ml·min^{-1}·kg^{-1}) e $\dot{Q}_{sys}$ (27,70 ± 2,78 para 24,50 ± 2,74 ml·min^{-1}·kg^{-1}) permaneceram inalterados. Não houve efeitos da fenilefrina na circulação pulmonar (PAM_{pul}: 2,34 ± 0,32 para 2,40 ± 0,34 KPa; $\dot{Q}_{pul}$: 19,00 ± 8,18 para 18,84 ± 8,37 ml·min^{-1}·kg^{-1}; R_{pul}: 1,22 ± 0,73 para 1,26 ± 0,71 kPa·ml^{-1}·min^{-1}·kg^{-1}; G_{pul}: 5,40 ± 3,12 para 5,07 ± 3,03 ml·KPa^{-1}·min^{-1}·kg^{-1}). O Net-Shunt D-E não foi alterado pela injeção de fenilefrina (0,55 ± 0,37 para 0,60 ± 0,38).

A injeção de fentolamina reduziu a R_{sys} (1,41 ± 0,30 para 0,41 ± 0,10 kPa·ml^{-1}·min^{-1}·kg^{-1}) e causou uma vasodilatação sistêmica acentuada, que foi associada ao aumento da G_{sys} (0,70 ± 0,12 para 2,46 ± 0,40 ml·KPa^{-1}·min^{-1}·kg^{-1}) e diminuição da PAM_{sys} (5,10 ± 0,35 para 2,73 ± 0,25 KPa). Como resposta compensatória à hipotensão, houve aumento da f_H (26,10 ± 2,72 para 42,10 ± 1,15 b.p.m), enquanto o V_{stot} (1,58 ± 0,15 para 1,54 ± 0,13 ml·kg^{-1}) e DC (42,33 ± 10,53 para 65,94 ± 4,62 ml·min^{-1}·kg^{-1}) permaneceram inalterados. No entanto, houve aumento significativo do $\dot{Q}_{sys}$ após o bloqueio dos receptores α-adrenérgicos (26,32 ± 2,94 para 48,58 ± 4,15 ml·min^{-1}·kg^{-1}). O único parâmetro alterado após a injeção de fentolamina foi a G_{pul}, na qual aumentou (4,28 ± 2,66 para 6,38 ± 2,78 ml·KPa^{-1}·min^{-1}·kg^{-1}). Não houve efeitos da fentolamina nos demais parâmetros pulmonares (PAM_{pul}: 2,53 ± 0,33 para 2,00 ± 0,13 KPa; $\dot{Q}_{pul}$: 15,70 ± 7,12 para 21,14 ± 4,68 ml·min^{-1}·kg^{-1}; R_{pul}: 2,15 ± 1,00 para 0,66 ± 0,30 kPa·ml^{-1}·min^{-1}·kg^{-1}). O Net-Shunt D-E foi mantido (0,13 ± 0,06 para 0,21 ± 0,05), apesar das alterações sistêmicas causadas pela injeção de fentolamina.

Após o tratamento com fentolamina, houve inibição do tônus simpático vascular, causando vasodilatação sistêmica. A injeção de adrenalina não foi capaz de elevar a R_{sys} (0,80 ± 0,22 para 0,44 ± 0,08 kPa·ml^{-1}·min^{-1}·kg^{-1}), enquanto a G_{sys} foi mantida elevada (1,41 ± 0,30 para 2,21 ± 0,30 ml·KPa^{-1}·min^{-1}·kg^{-1}) e a PAM_{sys} foi reduzida (4,03 ± 0,43 para 3,08 ± 0,23 KPa). A queda da R_{sys} foi proporcionamente maior que a PAM_{sys} e assim, houve aumento do $\dot{Q}_{sys}$ (40,74 ± 5,75 para 46,88 ± 4,25 ml·min^{-1}·kg^{-1}). O aumento do $\dot{Q}_{sys}$ pode ter ocorrido devido a manutenção compensatória de f_H (40,46 ± 1,90 para 42,42 ± 1,21 b.p.m), V_{stot} (1,33 ± 0,18

para 1,40 ± 0,10 ml·kg^{-1}) e DC (55,25 ± 6,25 para 61,03 ± 4,00 ml·min^{-1}·kg^{-1}). Não houve efeitos da adrenalina na circulação pulmonar (PAM_{pul}: 2,03 ± 0,16 para 2,08 ± 0,23 KPa; $\dot{Q}_{pul}$: 18,43 ± 6,68 para 18,20 ± 5,01 ml·min^{-1}·kg^{-1}; R_{pul}: 2,32 ± 1,33 para 2,36 ± 1,43 kPa·ml^{-1}·min^{-1}·kg^{-1}; G_{pul}: 6,04 ± 3,00 para 5,90 ± 2,73 ml·KPa^{-1}·min^{-1}·kg^{-1}). O Net-Shunt D-E foi mantido (0,17 ± 0,06 para 0,16 ± 0,06), mesmo com as alterações sistêmicas exercidas pela fentolamina e injeção de adrenalina.

Após o bloqueio dos receptores α-adrenérgicos com fentolamina, o efeito da fenilefrina também foi abolido. Não houve efeitos nos parâmetros cardiovasculares analisados (PAM_{sys}: 4,10 ± 0,43 para 3,87 ± 0,34 KPa; R_{sys}: 0,88 ± 0,18 para 0,74 ± 0,14 kPa·ml^{-1}·min^{-1}·kg^{-1}; G_{sys}: 1,41 ± 0,30 para 1,58 ± 0,25 ml·KPa^{-1}·min^{-1}·kg^{-1}; $\dot{Q}_{sys}$: 41,95 ± 5,92 para 44,92 ± 4,94 ml·min^{-1}·kg^{-1}; PAM_{pul}: 2,00 ± 0,20 para 2,05 ± 0,20 KPa; R_{pul}: 1,34 ± 1,10 para 1,25 ± 1,00 kPa·ml^{-1}·min^{-1}·kg^{-1}; G_{pul}: 6,78 ± 3,35 para 6,88 ± 3,34 ml·KPa^{-1}·min^{-1}·kg^{-1}; $\dot{Q}_{pul}$: 19,66 ± 7,20 para 20,83 ± 6,80 ml·min^{-1}·kg^{-1}; f_H: 40,40 ± 1,76 para 40,78 ± 1,60 b.p.m; V_{stot}:1,41 ± 0,17 para 1,52 ± 0,14 ml·kg^{-1}; DC:57,06 ± 7,10 para 61,86 ± 4,64 ml·min^{-1}·kg^{-1}). O Net-Shunt D-E não foi alterado pela injeção de fenilefrina após o bloqueio dos receptores α-adrenérgicos (0,56 ± 0,38 para 0,54 ± 0,34).

O bloqueio β-adrenérgico com propranolol causou uma bradicardia acentuada (38,20 ± 2,23 para 23,75 ± 1,55 b.p.m), com consequente diminuição do DC (52,67 ± 6,41 para 32,77 ± 4,71 ml·min^{-1}·kg^{-1}) e do $\dot{Q}_{sys}$ (39,65 ± 5,83 para 25,65 ± 4,58 ml·min^{-1}·kg^{-1}). No entanto, V_{stot} (1,44 ± 0,18 para 1,46 ± 0,24 ml·kg^{-1}) permaneceu inalterado. O controle periférico compensou as alterações de DC e manteve a G_{sys} (1,42 ± 0,30 para 0,88 ± 0,20 ml·KPa^{-1}·min^{-1}·kg^{-1}) e PAM_{sys} (3,80 ± 0,30 para 4,01 ± 0,36 KPa) inalterados. Na circulação pulmonar, observamos respostas semelhantes. Houve aumento da R_{pul} (2,53 ± 1,43 para 3,86 ± 1,80 kPa·ml^{-1}·min^{-1}·kg^{-1}), contudo a G_{pul} (5,37 ± 2,88 para 2,33 ± 1,14 ml·KPa^{-1}·min^{-1}·kg^{-1}) e a PAM_{pul} (2,10 ± 0,21 para 2,36 ± 0,30 KPa) não foram alteradas. A redução de DC foi associada à queda do $\dot{Q}_{pul}$ após a injeção de propranolol (16,92 ± 6,17 para 7,95 ± 2,90 ml·min^{-1}·kg^{-1}). O Net-Shunt D-E não foi alterado pela injeção de propranolol (0,15 ± 0,06 para 0,13 ± 0,05).

Após o bloqueio concomitante α e β-adrenérgico, a adrenalina causou vasoconstrição sistêmica semelhante à observada com a injeção controle de adrenalina. Essa vasoconstrição ocorreu devido ao aumento da R_{sys} (1,78 ± 0,36 para 2,43 ± 0,40 kPa·ml^{-1}·min^{-1}·kg^{-1}). Este efeito foi associado ao aumento da PAM_{sys} (4,20 ± 0,37 para 5,18 ± 0,40 KPa) e redução do $\dot{Q}_{sys}$ (17,58 ± 1,58 para 14,52 ± 1,05 ml·min^{-1}·kg^{-1}), porém a G_{sys} permaneceu inalterada (0,53 ± 0,08 para 0,37 ± 0,02 ml·KPa^{-1}·min^{-1}·kg^{-1}). A f_H (19,51 ± 1,84 para 18,92 ± 1,92 b.p.m), V_{stot}

(1,42 ± 0,20 para 1,30 ±0,17 ml·kg^{-1}) e o DC (26,91 ± 3,94 para 23,46 ± 3,90 ml·min^{-1}·kg^{-1}) não foram alterados com a administração de adrenalina após fentolamina e propranolol. Não houve efeitos da adrenalina na circulação pulmonar (PAM_{pul}: 2,02 ± 0,17 para 2,47 ± 0,27 KPa; $\dot{Q}_{pul}$: 6,08 ± 1,62 para 5,78 ± 1,17 ml·min^{-1}·kg^{-1}; R_{pul}: 2,23 ± 1,07 para 3,10 ± 1,45 kPa·ml^{-1}·min^{-1}·kg^{-1}; G_{pul}: 1,31 ± 0,67 para 1,28 ± 0,70 ml·KPa^{-1}·min^{-1}·kg^{-1}). O Net-Shunt D-E não foi alterado pela injeção de adrenalina após o bloqueio dos receptores α e β-adrenérgicos (0,17 ± 0,06 para 0,24 ± 0,08).

Após o tratamento com fentolamina e propranolol, o efeito vasoconstritor da fenilefrina foi semelhante ao da injeção controle. A fenilefrina causou aumento da R_{sys} (2,46 ± 0,24 para 3,15 ± 0,30 kPa·ml^{-1}·min^{-1}·kg^{-1}) e este efeito foi associado a diminuição na G_{sys} (0,42 ± 0,04 para 0,34 ± 0,03 ml·KPa^{-1}·min^{-1}·kg^{-1}) e aumento da PAM_{sys} (4,47 ± 0,27 para 5,03 ± 0,27 KPa). Como resposta compensatória à hipertensão, houve diminuição da f_H (17,85 ± 2,33 para 17,37 ± 2,33 b.p.m). O V_{stot} (1,45 ± 0,23 para 1,37 ± 0,22 ml·kg^{-1}) e DC (24,37 ± 3,43 para 22,87 ± 3,83 ml·min^{-1}·kg^{-1}) permaneceram inalterados, enquanto $\dot{Q}_{sys}$ reduziu (14,71 ± 1,25 para 12,80 ± 1,18 ml·min^{-1}·kg^{-1}). O único parâmetro alterado foi a PAM_{pul} (2,22 ± 0,31 para 2,44 ± 0, 35 KPa), na qual aumentou com a administração de fenilefrina após fentolamina e propranolol. Não houve efeitos da fenilefrina nos demais parâmetros pulmonares ($\dot{Q}_{pul}$: 5 18 ± 1,00 para 5,36 ± 1,00 ml·min^{-1}·kg^{-1}; R_{pul}: 2,30 ± 1,26 para 2,50 ± 1,42 kPa·ml^{-1}·min^{-1}·kg^{-1}; G_{pul}: 3,42 ± 1,94 para 3,45 ± 2,00 ml·KPa^{-1}·min^{-1}·kg^{-1}). O Net-Shunt D-E não foi alterado pela injeção de fenilefrina após o bloqueio dos receptores α e β-adrenérgicos (0,81 ± 0,53 para 0,98 ± 0,63).

5. DISCUSSÃO GERAL

5.1 Efeito do NO no circuito sistêmico

Em nosso estudo, o L-NAME reduziu a G_{sys} e o $\dot{Q}_{sys}$, sugerindo que há uma liberação contínua de NO na circulação sistêmica, responsável por manter o tônus vascular sistêmico basal na condição de repouso. Esse resultado difere do que foi relatado em estudos anteriores que utilizaram cascavéis anestesiadas (Skovgaard et al., 2005b; Galli et al., 2005b), na qual concluíram que essa espécie parece não precisar do NO para manutenção do tônus vascular sistêmico basal. Assim como demonstrado em mamíferos, houve vasoconstrição sistêmica após a inibição da NOS em tartarugas, lagartos varanídeos e pítons (Crossley et al., 2000; Skovgaard et al., 2005b). Esse achado é consistente com estudos *in vitro* realizados em crocodilos estuarinos. Nesta espécie, a vasoconstrição aórtica em resposta à adrenalina levou a uma

vasodilatação vascular reflexa, e essa resposta foi reduzida após o tratamento com L-NAME (Axelsson et al., 2001). Resultados semelhantes também foram observados em vasos de *Thamnophis sirtalis parietalis*, na qual a adição de L-NAME promoveu aumento no tônus vascular (Knight e Burnstock, 1993).

O doador exógeno de NO, SNP, causou uma acentuada vasodilatação sistêmica na cascavel, demonstrando a presença de receptores de NO na vasculatura sistêmica dessa espécie. Esse efeito é consistente com dados anteriores sobre a ação do SNP em répteis (Crossley et al. 2000; Skovgaard et al. 2005b, Galli et al., 2005b). Resultados semelhantes também foram observados em vasos sistêmicos isolados de *Thamnophis sirtalis parietalis*, na qual relaxaram em resposta a ACh e SNP (Knight e Burnstock, 1993). Entretanto, o uso de ACh e SNP não fornece informações sobre qual mecanismo endógeno é responsável pela produção cardiovascular *in vivo* de NO (Skovgaard et al., 2005b). Portanto, para abordar o mecanismo fisiológico por trás da síntese de NO em cascavel, administramos L-Arg, um substrato para produção endógena de NO.

A L-Arg causou efeitos semelhantes à injeção de SNP. As respostas a L-Arg foram significativamente atenuadas após injeção de L-NAME, sugerindo que o efeito foi dependente da produção endógena de NO. Esse resultado foi semelhante ao relatado por Galli et al. (2005b). No entanto, embora possamos concluir que a dose utilizada de L-NAME foi capaz de inibir a síntese de NO, a inibição pode não ter sido completa, uma vez que observamos uma vasodilatação remanescente. Além disso, não podemos descartar a possibilidade de que a vasodilatação após a inibição da NOS possa ter ocorrido devido ao efeito vasodilatador causado por outros moduladores endógenos.

Em mamíferos, as células endoteliais podem controlar o tônus vascular pela liberação contínua de NO ou EDHF (Félétou e Vanhoutte, 2004). Em grandes artérias, o NO é responsável pelo relaxamento vascular, enquanto nas artérias de resistência, o EDHF tem um papel mais pronunciado (Shimokawa et al., 1996; Takaki et al., 2008). O mecanismo endógeno responsável pela vasodilatação dos microvasos pela liberação de EDHF tem participação da enzima NOS. As isoformas da NOS, especialmente a eNOS, são capazes de produzir NO e ânions superóxido, e este último pode ser dismutado pela enzima Cu, Zn-SOD, sintetizar EDHF/H_2O_2 e promover vasodilatação (Takaki et al., 2008). Caso o L-NAME apresente efeitos diferentes, de acordo com o calibre dos vasos, isso explicaria os resultados obtidos em nosso estudo. Em grandes artérias, o L-NAME impediu a síntese e liberação do NO, porém, nas artérias de resistência, seu efeito foi mínimo. Assim, nos microvasos, houve maior produção de EDHF e, portanto, um relaxamento residual foi observado, mesmo após a inibição da NOS. O

papel do EDHF no tônus vascular desse grupo é uma possibilidade intrigante e estudos *in vitro* são importantes para testar a presença e o efeito do EDHF nos diferentes leitos vasculares em Squamata.

5.2 Efeito da estimulação adrenérgica no circuito sistêmico

Assim como as substâncias vasoativas apresentadas anteriormente, a adrenalina e fenilefrina também demonstraram a importância da estimulação adrenérgica para o controle do tônus vascular em *C. durissus*. A vasoconstrição sistêmica causada pela adrenalina e fenilefrina foi revertida após o tratamento com fentolamina, o que demonstra que esta resposta é mediada pelos receptores α-adrenérgicos. Além disso, a fentolamina causou expressiva vasodilatação sistêmica e aumento da G_{sys}, indicando a importância dos receptores α-adrenérgicos no controle do tônus vascular sistêmico basal e, consequentemente, da P_{sys} na cascavel em repouso. Esses resultados foram semelhantes aos relatados em cascavéis anestesiadas (Skals et al., 2005; Galli et al., 2007) e tartarugas (Overgaard et al., 2002). A fenilefrina causou respostas semelhantes à adrenalina, embora menos acentuadas. Tal aspecto demonstra que ambos os ramos para ajustes barorreflexos são relevantes para manutenção da PAM no animal não anestesiado. Quando o SNA está atuante, mesmo sob estímulo adrenérgico, a f_H não é alterada. Da mesma forma, quando há redução da condutância por estímulo α-adrenérgico, a f_H é capaz de manter a PAM. A presença de inervação adrenérgica em vasos de Squamata, como *Elaphe obsoleta* e *Crotalus viridis* já foi demonstrada em estudos histoquímicos (Donald e Lillywhite, 1988; Lillywhite e Donald, 1994). Além disso, estudos realizados em *Trachemys scripta* e *Python regius* observaram constrição da vasculatura sistêmica em resposta a ativação dos receptores α-adrenérgicos e vasodilatação quando os receptores β-adrenérgicos foram estimulados (Wang et al., 2000; Hicks e Farrell, 2000; Overgaard et al., 2002), dando maior suporte à importância da estimulação adrenérgica para os ajustes vasculares em répteis.

Em cascavéis anestesiadas, a injeção de propranolol causou queda da G_{sys} e PAM_{sys} (Galli et al., 2007). Os autores sugeriram que tal fato indicaria a presença de um tônus β-adrenérgico basal na vasculatura sistêmica. No entanto, em nosso estudo, não houve efeitos na G_{sys} e PAM_{sys}, portanto, podemos sugerir que os receptores α-adrenérgicos são preponderantes no controle do tônus vascular sistêmico em *Crotalus*. Nossos resultados não dão suporte a hipótese da presença e/ou relevância de receptores β-adrenérgicos para manutenção das condições hemonidâmicas durante o repouso. Em outras espécies de serpentes, como *Bitis arietans*, *Python regius* e *Boa constrictor*, o efeito cronotrópico positivo da adrenalina foi abolido pelo propranolol (Hedberg e Nilsson, 1975; Wang et al., 2000, 2001a). Galli e

colaboradores observaram que a fentolamina também aboliu a taquicardia causada pela adrenalina e esse efeito foi atribuído pela frequência máxima, em resposta à queda da pressão arterial, promovida pelo antagonista do receptor α-adrenérgico (Galli et al., 2007). No entanto, em nosso estudo, a injeção de adrenalina não alterou, significantemente, a f_H, nem mesmo após os tratamentos com fentolamina ou propranolol. Assim, sugerimos que, as compensações autonômicas causadas por retirada simpática ou elevação parassimpática foram rápidas o suficiente para compensar o cronotropismo adrenérgico. E que, principalmente, o ramo vascular afetado pela estimulação adrenérgica é preponderante para o controle de PAM e G_{sys}, se comparado à ação cardíaca.

Em relação ao controle do volume sanguíneo, nem o V_{stot} nem o $\dot{Q}_{sys}$ foram alterados após a adrenalina e fenilefrina, semelhante ao encontrado por Galli et al. (2007) e Skals et al. (2005). Como observado em mamíferos (Guyton, 1955) e répteis (Enok et al., 2016), existe uma relação entre MCFP, enchimento cardíaco e V_{stot} em cascavéis (Skals et al., 2005). A falta de efeito da adrenalina no V_{stot} na cascavel também foi observada em peixes (Zhang et al., 1998) e, uma possível explicação para essa resposta seriam as alterações na pós-carga e resistência venosa, uma vez que esse efeito pode estar relacionado com o equilíbrio entre constrição venosa e maior pós-carga (Skals et al., 2005). Galli et al. (2007) atribuíram a manutenção do V_{stot} com o aumento da contratilidade, uma vez que o propranolol foi capaz de reduzir o V_{stot}. No entanto, em nosso estudo, não observamos alteração do V_{stot} após o tratamento com propranolol, portanto, parece mais plausível que a manutenção do V_{stot} tenha ocorrido devido à constrição venosa e pós-carga aumentada. Skals et al. (2005) também demonstraram que a resistência venosa e a Pcv aumentaram em resposta à estimulação α-adrenérgica na cascavel, e esse resultado poderia explicar por que a adrenalina e a fenilefrina não alteraram o V_{stot} após a fentolamina. Deste modo, em repouso, o aumento do tônus venoso é incapaz de alterar V_{stot} e, portanto, o aumento da atividade simpática pode exercer uma influência mais significativa no retorno venoso em situações em que o aumento do metabolismo é necessário, como exercício, digestão ou aumento da temperatura (Skals et al., 2005).

5.3 Efeito do NO no circuito pulmonar

A vasculatura pulmonar da cascavel não foi afetada pelo L-NAME, em contraste com o papel óbvio do NO na circulação sistêmica. A injeção de L-NAME não teve efeito sobre a G_{pul}, sugerindo que o NO não contribui para o controle do tônus vascular basal na circulação pulmonar em cascavéis em repouso, a 25ºC. Esse resultado está de acordo com o que foi previamente sugerido em estudos com tartarugas e outras serpentes (Crossley et al., 2000;

Skovgaard et al., 2005b). A falta de resposta do circuito pulmonar ao L-NAME não exclui a importância do NO para o controle do tônus vascular pulmonar. Talvez, o NO possa afetar a circulação pulmonar e ter um papel em outras condições fisiológicas, como no aumento da temperatura ou SDA, que denotam um aumento no metabolismo. Essa condição é apoiada pelo aumento do DC, que precisa ser seguido por ajustes sistêmicos e pulmonares para manter o equilíbrio do desvio sanguíneo intracardíaco.

Apesar de não haver tônus relevante em repouso, a vasculatura pulmonar é capaz de responder ao NO, já que o SNP reduziu a R_{pul} e PAM_{pul}. Após a injeção do L-NAME, o SNP causou maior queda da R_{pul} e aumento mais acentuado da G_{pul}, demonstrando que a administração do SNP teve efeito no tônus vascular da circulação pulmonar e que a inibição da NOS aumenta a capacidade de relaxamento desse circuito. Esses achados discordam de relatos anteriores baseados em estudos com tartarugas (Crossley et al. 2000) e cascavéis anestesiadas (Skovgaard et al. 2005b; Galli et al., 2005b). Uma possível explicação para a maior vasodilatação observada na vasculatura pulmonar após o L-NAME pode ser, novamente, atribuída a presença de EDHF. A inibição da NOS leva a um aumento na atividade da enzima envolvida com a síntese de H_2O_2, com consequente potencialização do relaxamento da musculatura lisa vascular (Takaki et al., 2008). No entanto, por que isso ocorre no circuito pulmonar e não no circuito sistêmico, ainda não é totalmente compreendido. Talvez, a vasculatura pulmonar seja mais sensível ao EDHF do que a vasculatura sistêmica e por isso, esse padrão de resposta tenha sido observado somente na circulação pulmonar.

A injeção de L-Arg não causou vasodilatação pulmonar em *Crotalus*. Isso é semelhante às respostas relatadas por Galli et al. (2005b). Deste modo, nosso estudo indica que a vasodilatação dos vasos pulmonares, mediada pela liberação de NO, não é um mecanismo dependente de L-Arg. Sugerimos ainda, que a ausência de resposta clara no circuito pulmonar pode ser devido a síntese de NO, *in vivo,* ter sido limitada pela falta de substâncias ou cofatores importantes para a produção endógena de NO (Umans e Levi, 1995). Ou, que a redução do fluxo sanguíneo na circulação pulmonar pode ter influenciado a magnitude de resposta à L-Arg, afetando seu efeito sobre a vasculatura pulmonar. Acreditamos ainda que, a falta de resposta pode estar relacionada com a produção não enzimática do NO, embora essa reação pareça improvável que ocorra em tecidos biológicos, como já mencionado por Ignarro (1990). Estes resultados mostram a necessidade de estudos que elucidem quais são os mecanismos evolutivos responsáveis pela produção endógena de NO em ambos os circuitos nos vertebrados basais.

5.4 Efeito da estimulação adrenérgica no circuito pulmonar

Semelhante ao que ocorreu com o NO, a vasculatura pulmonar também foi menos responsiva aos agonistas adrenérgicos. Esses dados estão de acordo com o que foi observado em cascavéis anestesiadas (Galli et al., 2007) e tartarugas (Overgaard et al., 2002). Por outro lado, a injeção de fentolamina levou a um aumento da G_{pul}, demonstrando que os receptores α-adrenérgicos são importantes para o controle do tônus vascular pulmonar na cascavel. Esses achados discordam de relatos anteriores de Galli et al. (2007), onde não houve alterações na G_{pul}. O tratamento com propranolol, no entanto, não afetou a G_{pul}, indicando que os receptores β-adrenérgicos são necessários apenas para manutenção do relaxamento tônico, semelhante ao que também foi relatado por Galli et al., (2007) e Overgaard et al. (2002). Segundo Overgaard e colaboradores (2002), não houve constrição significativa da vasculatura pulmonar, em *Trachemys,* quando os receptores α-adrenérgicos foram estimulados, e esse efeito foi semelhante em *Crotalus*, uma vez que a adrenalina não causou alteração da R_{pul}. Em *Trachydosaurus rugosus* e *Trachemys scripta*, a estimulação dos receptores β-adrenérgicos causaram vasodilatação pulmonar (Berger, 1972; Overgaard et al., 2002), e este efeito foi confirmado na cascavel, uma vez que houve uma resposta vasoconstritora após o tratamento com propranolol.

A fenilefrina causou respostas semelhantes à adrenalina, mas não afetou a PAM_{pul}. Da mesma forma, em *Chrysemys scripta* e *Chelodina longicollis*, a injeção de fenilefrina não promoveu alterações na circulação pulmonar (Milsom et al., 1977; Berger, 1972). A falta de resposta da circulação pulmonar em Squamata por agonistas adrenérgicos é conflitante com a extensa inervação adrenérgica presente na vasculatura pulmonar desses animais (McLean e Burnstock, 1967; Donald e Lillywhite, 1988; Donald et al., 1990b). No entanto, um ponto importante a ser mencionado é que a presença de receptores e capacidade de responder à ação de uma determinada substância não significa necessariamente que o animal sempre responda com a mesma intensidade. Um exemplo claro desse fenômeno é que a cascavel responde de maneira diferente aos efeitos de numerosos mediadores químicos. Vários estudos demonstraram que a circulação pulmonar é menos responsiva a diversas substâncias, como o neuropeptídeo gama, a bradicinina, o NO e o inibidor da NOS (Crossley et al., 2000; Galli et al., 2005a, b; Skovgaard et al., 2005a, b). Essas evidências mostram que, para garantir a regulação local e manter uma adequada ventilação e perfusão, a ativação simpática não é tão eficiente e, portanto, o controle parassimpático da artéria pulmonar pode ser uma das principais fontes de regulação da perfusão pulmonar (Galli et al., 2007).

Sempre que for necessário aumentar a demanda metabólica, o controle vascular do tecido abre leitos vasculares aumentando a G_{sys}. Em répteis, a maior demanda de oxigênio

arterial pode ser efetuada através do aumento do $\dot{Q}_{sys}$ ou pela redução do Net-Shunt D-E (Wang et al., 2001b; Wang e Hicks, 1996). Em cascavéis anestesiadas, a injeção de adrenalina elevou a razão $\dot{Q}_{pul}/\dot{Q}_{sys}$. Embora, em nosso experimento, G_{pul} e o Net-Shunt tenham permanecido estáveis, demonstrando que o controle do desvio intracardíaco pode ser dinâmico o suficiente para anular as alterações na hemodinâmica da circulação sistêmica. Em nossos achados, observamos ainda que a injeção de fenilefrina não afetou o Net-Shunt D-E, e esse resultado foi semelhante ao relatado por Skals e colaboradores (2005). A vasculatura pulmonar pode ser tonicamente estimulada por agonistas adrenérgicos, no entanto, mudanças na condutância e, portanto, padrões de desvio intracardíaco são, principalmente, controlados pelo controle vagal da artéria pulmonar, como havia sido mencionado por Galli et al. (2007). Assim, parece que a regulação parassimpática do sistema cardiovascular é mais efetiva que a regulação simpática para o controle do desvio intracardíaco e manutenção do conteúdo arterial de oxigênio no circuito pulmonar de *C. durissus* (Wang et al., 2001a, b; Taylor et al., 2009; Leite et al., 2013, 2014).

6. CONCLUSÕES

Diante dos resultados obtidos, sugerimos que, em *Crotalus durissus*, o NO é sintetizado via mecanismo L-Arg e tem papel parcial na regulação local do tônus vascular na circulação sistêmica. Em contraste, a vasculatura pulmonar é menos reativa ao SNP e não responde a L-Arg, demonstrando que, neste circuito, a produção de NO não é um mecanismo dependente de L-Arg. Assim, sugerimos que a vasodilatação mediada por NO tem um papel importante nos ajustes cardiovasculares sistêmicos na condição de repouso em Squamata. E que, os ajustes vasculares do circuito pulmonar, promovidos pelo NO, poderiam ser relevantes em outras condições fisiológicas, com maior demanda energética.

A estimulação adrenérgica, por sua vez, tem papel fundamental na regulação local do tônus vascular sistêmico. Enquanto, a vasculatura pulmonar é menos responsiva aos agonistas adrenérgicos. Alem disso, podemos indicar que os receptores α-adrenérgicos são mais eficientes do que os receptores β-adrenérgicos no controle da pressão arterial em *Crotalus*. A falta de efeitos da estimulação adrenérgica nos parâmetros cardíacos indica ainda que, os agonistas adrenérgicos têm mais ação vascular do que ação cardíaca no controle do tônus vascular sistêmico e pulmonar nessa espécie. A falta de mudança no G_{pul} e no Net-Shunt demonstra que a regulação parassimpática do sistema cardiovascular é mais efetiva que a regulação simpática para o controle do desvio sanguíneo intracardíaco em *Crotalus*. Os dados apresentados até o momento permitem discussões seguras acerca da importância do SNA para o controle do tônus

vascular em répteis e demonstra que o modelo da cascavel descerebrada possibilitou a obtenção de dados cardiovasculares consistentes. Deste modo, a descerebração mostrou ser uma ótima alternativa para estudos que envolvem mecanismos autonômicos, enfatizando sua importância e possíveis implicações na área da fisiologia evolutiva.

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8. TABELA E LEGENDA

Tabela 1: Alteração máxima dos parâmetros cardiovasculares na curva dose-resposta para o L-NAME em *C. durissus*

L-NAME	PAM_{sys}	$\dot{Q}_{sys}$	G_{sys}	PAM_{pul}	$\dot{Q}_{pul}$	G_{pul}	DC
Pré-injeção	5,50 ± 0,25	55,16 ± 15,46	10,32 ± 3,00	4,23 ± 0,54	11,60 ± 4,25	3,40 ± 1,50	66,74 ± 18,35
0,1 mg·kg^{-1}	5,75 ± 0,30	46,05 ± 15,00*	8,20 ± 2,60*	4,10 ± 0,56	8,37 ± 2,71	2,50 ± 0,92	54,40 ±16,65*
Pré-injeção	5,58 ± 0,41	46,67 ± 14,16	9,35 ± 3,24	4,81 ± 0,44	6,17 ± 1,43	1,44 ± 0,40	52,84 ± 14,83
1 mg·kg^{-1}	5,95 ± 0,35	40,01 ± 12,58*	7,36 ± 2,56*	4,45 ± 0,60	5,43 ± 1,40	1,50 ± 0,45	45,44 ± 13,11*
Pré-injeção	5,74 ± 0,37	41,40 ± 13,80	7,83 ± 2,80	4,47 ± 0,51	6,40 ± 1,55	1,66 ± 0,50	47,80 ± 15,10
10 mg·kg^{-1}	5,90 ± 0,40	35,80 ± 12,90*	6,61 ± 2,51*	4,40 ± 0,54	5,60 ± 1,61	1,40 ± 0,36	41,36 ± 14,30
Pré-injeção	5,71 ± 0,35	38,60 ± 12,73	7,21 ± 2,51	4,40 ± 0,57	5,60 ± 1,46	1,56 ± 0,53	44,20 ± 13,70
100 mg·kg^{-1}	6,00 ± 0,33	23,60 ± 9,10*	4,14 ± 1,64*	4,17 ± 0,55	3,73 ± 1,43	0,94 ± 0,36	27,31 ± 10,08*

Valores médios dos parâmetros cardiovasculares antes e após (no momento de maior efeito) diferentes doses de L-NAME. PAM_{sys} - pressão arterial média sistêmica (KPa); $\dot{Q}_{sys}$ - fluxo sistêmico (ml·min^{-1}·kg^{-1}); G_{sys} - condutância sistêmica (ml·KPa^{-1}·min^{-1}·kg^{-1}); PAM_{pul} - pressão arterial média pulmonar (KPa); $\dot{Q}_{pul}$ - fluxo pulmonar (ml·min^{-1}·kg^{-1}); G_{pul} - condutância pulmonar (ml·KPa^{-1}·min^{-1}·kg^{-1}); DC – débito cardíaco (ml·min^{-1}·kg^{-1}). *N = 6*. Os valores foram apresentados como média ± EPM. * Indica diferença significativa dos valores pré-injeção (P <0,05) - teste de Friedman.

CHAPTER II

Role of nitric oxide on the cardiovascular adjustments in South American rattlesnakes (*Crotalus durissus)*

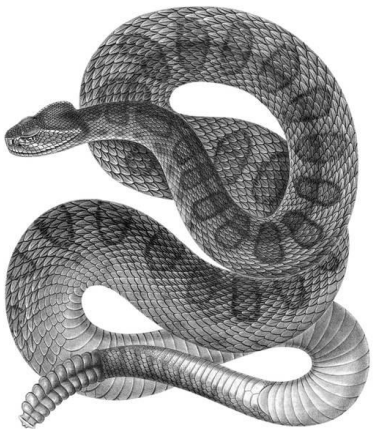

Image source: https://bit.ly/2KpzvVe

1. INTRODUCTION

1.1 The relevance of NO signaling

The evolutionary history of NO is long and its origin may relate to changes on the chemical composition of the atmosphere. A possible hypothesis that explains the emergence of this gas shows that NO appeared as a form of chemical protection against cytotoxic ozone, which increased as molecular oxygen arised on the palaeoatmosphere, due respiratory process of cyanobacteria. It is possible that such environment favoured the use of the earliest form of NO signaling (Feelisch and Martin, 1995). The importance of NO as one of the first signaling molecules is observed in all eukaryotes (González-Domenech and Muñoz-Chápuli, 2010). Currently, is well established that NO has an important role in multiple levels of organism function, like local blood flow regulaton, arterial pressure adjustment, neurotransmission, and inflammatory responses in mammals (Umans and Levi, 1995; Mungrue et al. 2003). NO is a highly reactive gas that has a half-life of 5 to 8 seconds due to its rapid oxidation and consequent formation of NO^{-2} and NO^{-3} (Ignarro, 1990; Conger, 1994). It is well known that endogenous synthesis of NO can result from the oxidation of L-arginine, which is converted to L-citrulline by NOS enzyme (Ignarro, 1990; Marletta, 1993) (Fig. 1). Three NOS isoforms are responsible for NO synthesis: eNOS, present in vascular endothelial cells (Moncada et al., 1991) and platelets (Radomski et al., 1990); nNOS, present in neurons (Knowles et al., 1989), and inducible NOS (iNOS) produced in inflammatory cells (Moncada et al., 1991).

In fish, only the nNOS and iNOS isoforms were found, while the eNOS isoform was observed in all tetrapods (González-Domenech and Muñoz-Chápuli, 2010; Olson et al., 2012). So, we can infer that NO-mediated vasodilation could be related with the evolutionary process of water-air transition of vertebrate group (Miller and vanhoutte, 2000; Toda and Ayajiki, 2006), regarding the required vascular adjustments to deal with the gravity effects on the cardiovascular function. Such hypothesis emphasizes the importance of investigations in reptiles as a polyfiletic group that was first really terrestrial group of vertebrates. In this context, Squamata is a key group to study the role of vasoactive substances and so to provide insites of the evolution of NO system for the vertebrate group development.

1.2 NO signaling on reptiles

Due to it strategic phylogenetic position for evolutionary views about a number of mechanism, a number of reptile species have been studied to understand the role of NO in their cardiovascular system: one testudine, *Trachemys scripta*; one crocodilian, *Crocodylus porosus*;

and six squamates, *Thamnophis sirtalis parietalis, Crotalus durissus, Python regius, Tiliqua scincoides, Varanus exanthematicus,* and *Salvator merianae* (Knight and Burnstock, 1993; Hylland et al.,1996; Söderström et al., 1997; Crossley et al., 2000; Axelsson et al., 2001; Galli et al., 2005b; Donald and Broughton, 2005; Skovgaard et al., 2005b; Broughton and Donald, 2007). The information presented to this date comes from *in vitro* or studies with anesthetized animals. Anesthesia depress the ANS and so its use can cause underestimation of cardiovascular responses and it could even obliterate important mechanisms. So, its use delimits a qualitative value to those studies. According to Mosley and collaborators, the use of increasing doses of the inhaling anesthetic isoflurane, for example, leads a decrease of blood pressure and f_H in *Iguana iguana* (Mosley et al., 2004). Also, one study perfomed with *Crotalus durissus* demonstrated that pentobarbital highly increase of f_H and $\dot{Q}_{pul}$ in resting animals (Taylor et al., 2009). These results show that anesthetesia affects the autonomic mechanism modulating cardiovascular responses and such effects can confuse quantitative and qualitative interpretation of results during the study of regulatory mechanisms.

In studies performed in reptiles, NOS inhibition via L-NAME injection increased systemic vascular tone, demonstrating that NO contributes to resting regulation of the systemic vasculature (Knight and Burnstock, 1993; Söderström et al., 1997; Crossley et al., 2000; Axelsson et al., 2001; Skovgaard et al., 2005b). However, in rattlesnake, the L-NAME injection did not significantly change systemic vascular resistance (Galli et al., 2005b), contradicting what had been observed in other reptile species and pointing for multiple function or variable relevance of NO in that group. Furthemore, all studied species showed a low influence of NO production in pulmonary circulation (Crossley et al. 2000; Skovgaard et al. 2005b; Galli et al., 2005b). The reason why the pulmonary and systemic circuits would have different responses is still unknown. Therefore, one hypothesis that could explain the diversity of results in systemic circulation and the low responsiveness of pulmonary circulation to NO is that the use of anesthetized animals can change the vascular tone, thus preventing NO release (Barnes and Liu, 1995; Skovgaard et al., 2005b). Anesthesia has been appointed as the responsible for increases in pulmonary circuit perfusion (Taylor et al., 2009). That would affect local regulation of the pulmonary vasculature. Thus, data in anesthetized animals are different from fully recovered or non-anaesthetised animals (Galli et al., 2005b).

Moreover, in contrast to other snakes, the rattlesnake seems to have elevated pulmonary blood pressures (Galli et al., 2005b), allowing a better understanding of the regulation of pulmonary vascular resistance in this species. In addition, recent *in vitro* research has demonstrated that the pulmonary artery of rattlesnakes is reactive to NO (Filogonio, 2016).

Such study evidence the importance of NO for overall vascular control of this species, as well as the need for an alternative model for such studies, free of the detrimental effects of anesthesia in function modulation. Thus, in view of the arguments presented, we work with the hypothesis that NO action on the vascular adjustments is accentuated and relevant in both circuits, systemic and pulmonary.Therefore, the aim of the present study was to investigate the role and functional relevance of NO for regulation of resting vascular tone in systemic and pulmonary circuits of rattlesnakes. In this study, we use a recently validated decerebrated animal model that, safely, permits the multiple cardiovascular measurements without anesthesia and maintain the integrity of ANS and local modulatory mechanisms.

2. MATERIALS AND METHODS

2.1 Experimental animals

For this study, we used 13 South American rattlesnakes, *Crotalus durissus* (Linnaeus, 1758), of both sexes (805 ± 72g) (Fig. 2). The snakes were obtained from Butantan Institute in São Paulo and UNESP in Rio Claro, and transported to animal care facility on the Department of Physiological Sciences at UFSCar, São Carlos. The animals were kept in individual compartments (44 x 50 x 43 cm), in a temperature-controlled environment (28 ± 2ºC), 12:12 light: dark cycle. Snakes were fed on mice to saciety every two weeks and *ad libitum* supply of water. Feeding was interrupted 15 days prior to any experimental procedure to avoid the effect of SDA (Andrade et al., 1997). All experiments were performed at room temperature, 25°C. All procedures were approved by the committee on animal use CEUA/UFSCar.

2.2 Anesthesia and euthanasia

For the instrumental procedure, snakes were previously sedated in an increased PCO_2 environment until the loss of righting reflexes and touch sensitivity (Forslid et al., 1986; Wang et al., 1993; Leite et al., 2013; 2014). That allowed safe manipulation for traqueal intubation to mechanical ventilation (5 breaths·min^{-1}; volume: 30 ml·kg^{-1}; SAR-830/P Ventilator) with isoflurane, 5% for induction and 3-2% for anesthesia maintenance. Loss of muscle tone was used to verify anesthetic plane. Each snake received lidocaine injection (2% - Pearson, Brazil) at each incision site and injections of antibiotic (Chemitril, 11 mg·kg^{-1} - Chemitec) and anti-inflammatory with analgesic action (Flunixin, 1.1 mg·kg^{-1} - Chemitec). Experimental protocol was performed 24h after surgery for decerebration and instrumentation. This made it possible to obtain data after anesthetic effects were dissipated. Decerebration procedure is inreversible,

so, it was considered the euthanasia moment of the animal (Silverman et al., 2005). Anestesia recovery and animal viability after the decerebration procedure applied have been validated previously (Rocha et al., 2019 – *submitted;* Tavares et al., 2019 – *submitted*). After completion of the experimental protocols, each snake received i.v. injections of barbiturate (Tiopental, 100 mg/kg - Pearson, Brazil) followed by hipersaturated K^+ solution. After cardiac arrest, the carcases were appropriately conditioned for later disposal by the UFSCar specialized collection service.

2.3 Decerebration

Decerebration process was accomplished by dorsoventral cauterization at the level of the *Tectum* and removal of all nervous structures rostral to it. Removal of these structures, including cerebral cortex and thalamus, eliminates the central processing of any nociception and processing high level of perception (Silverman et al., 2005). For the decerebration procedure, an incision was made on the medial region of the head, allowing access to the skull (Fig. 3A). Using a mini drill bit (AWT/MR-115), a small aperture (approximately 4 mm in diameter) was made on the cranial casing, allowing access to the menings just over the *Tectum*. The menings were opened, superficial arteries were cauterized. That prepared for a transverse section crossing the first third portion of *Tectum* structure to the base of the skull (Fig. 3B-C). All the nervous tissue, rostral to the incision was cauterized and removed (Fig. 3D). Absorbable gelatin sponge (gelfoam) was placed on the remain cavity to stabilize the remaining nervous tissue and to avoid subsequent bleeding. The purpose of decerebration was to eliminate cortex activation, which is necessary for any normal information integration that leads to perception or any considered kind of awareness (Pickering and Paton, 2006, Dobson and Harris, 2012). The remaining structures allowed for a euthanized decerebrated animal preparation with functional autonomic modulation (Rocha et al., 2019 – *submitted;* Tavares et al., 2019 – *submitted*).

2.4 Cardiovascular data recording

During the time course of the same anesthetic plane, a ventrolateral incision of approximately 3 - 4 cm, just rostral to the heart position, exposed systemic and pulmonary central vessels. Occlusive cannulation allowed catheters insetion into pulmonary artery and right systemic arch (PE20) through a branch of the unique pulmonary artery and the vertebral artery, respectively. This allowed P_{pul} and P_{sys} recording and i.v. drug injection. Pressure transducers were calibrated against a mercury column.

In addition, flow probes were placed over the left aortic arch and pulmonary artery, in order to record $\dot{Q}_{LAo}$ and $\dot{Q}_{pul}$, respectively (1.5PR – 1.5PR, Transonic Systems). The probes were fixed around the vessels and filled with gel to allow signal transmission. At the end of the procedure, cannulas and probes were externalized through the access incision and fixed with stitches to the snakes' dorsum in order to prevent signal artifacts due to probe movement. After instrumentation, snakes were positioned in ventral decubitus and the anesthesia terminated. These procedures allowed continuous recording of P_{sys}, P_{pul}, $\dot{Q}_{LAo}$ and $\dot{Q}_{pul}$ using the respective amplifiers and AD recording system (Powerlab, ADInstruments; Transonic). The recording signals provided calculation of MAP_{sys}, MAP_{pul}, f_H, $\dot{Q}_{sys}$, CO, V_{stot}, Net-Shunt, R_{sys}, R_{pul}, besides G_{sys} and G_{pul}, and so the model allowed for access the overall cardiovascular responses to pharmacological protocols.

2.5 Experimental protocols

2.5.1 L-NAME dose-response curve

The L-NAME dose used for the following protocols was the dose which produced evident alteration in a designed dose-response curve (n = 6, 597 ± 86g). The dose response protocol for L-NAME had serial i.v. injections: saline (0.5 ml·kg^{-1}, 0.9%) and L-NAME doses (0.1 mg·kg^{-1} - 0.37 mM; 1 mg·kg^{-1} - 3.7 mM; 10 mg·kg^{-1} - 37 mM and 100 mg·kg^{-1} - 370 mM). All doses were administered as i.v. bolus injections through the vertebral artery catheter. All parameters were verified to attest basal steady state recovery before following dose was injected.

2.5.2 The role of NO on the cardiovascular adjustments

To access the role of NO for cardiovascular adjustments, rattlesnakes (n = 7, 983 ± 50g) were submitted to serial i.v. injection of 0.9% saline solution (control injection); followed by SNP injection, a NO donor (2.5 μg·kg^{-1}; 0.008mM); L-Arg, a substrate for the endogenous production of NO (50 mg·kg^{-1}; 287 mM); and also L-NAME, the NOS inhibitor (10 mg·kg^{-1} - 37mM). After that, in order to verify whether NOS was inhibited and to verify that the response to NO was intact, L-Arg and SNP were injected again. Hemodynamic variables were continuous recorded. The doses of L-NAME used in this study were defined in previous dose-response protocol. SNP and L-Arg doses were based on previous investigation in rattlesnakes (Galli et al., 2005b).

Each decerebrated rattlesnake was considered recovered from anesthesia. The snakes presented muscle tone, spontaneous ventilation, and touch reflex movements. The animals were capable of rising tail and head, and also to perform non-directional locomotor movements after being repetitively touched. No alarm response, tail rattling or hissing was observed. So, in order to avoid disrupting implanted catheters and probes due the movements of animal, a non-depolarizing neuromuscular inhibitor was injected (i.v. injection of gallamine triethiodide – 0.5 $mg \cdot kg^{-1}$). Such injection did not change the cardiovascular parameters recorded. All drugs were administered as bolus injections through the sistemic catheter. All parameters were back to normal stable values before following injection. Previous test with slow i.v. saline injection summing the total volume of the protocol did not change cardiovascular parameters recorded. All drugs were purchased from Sigma-Aldrich Brasil Ltda.

2.6 Calculations and statistics

Recordings were analysed for data collection with the Labchart software (ADInstrumets). Systemic and pulmonary mean arterial pressures were calculated from the equation:

$$MAP = 1/3\ (Psystolic) + 2/3\ (Pdiastolic) \quad (1)$$

Heart rate was derived from the P_{sys} signal. $\dot{Q}_{sys}$ was calculated from the $\dot{Q}_{LAo}$, using the correction factor provided by Filogonio (2014):

$$\dot{Q}_{sys} = 2.6 \times \dot{Q}_{LAo}\ \text{(Filogonio et al., 2014)} \quad (2)$$

Cardiac output was calculated as the sum of $\dot{Q}_{sys}$ and $\dot{Q}_{pul}$ per time, and total stroke volume (pulmonary + systemic) was calculated as:

$$V_{stot} = \dot{Q}_{tot} \cdot f_H^{-1}\ \text{(Crossley et al., 1998; Galli et al. 2005b)} \quad (3)$$

Systemic and pulmonary vascular resistance were calculated as:

$$R = P \cdot \dot{Q}^{-1}\ \text{(Lautt, 1989)} \quad (4)$$

Systemic and pulmonary vascular conductance were calculated as:

$$G = \dot{Q} \cdot P^{-1} \text{ (Lautt, 1989; O'Leary, 1991)} \quad (5)$$

Intracardiac shunting was calculated by the ratio:

$$\dot{Q}_{pul} \cdot \dot{Q}_{sys}^{-1} \text{ (Hicks, 1994)} \quad (6)$$

After parameters calculations, Grubbs' test was used to detect outlier values on the analyzed groups. Data normality was analised using D'Agostino & Pearson omnibus test. The dose response curve for L-NAME was analysed using Friedman and Student-Newman-Keuls post-hoc test. The effects of L-Arg and SNP regarding to its pre-injection, as well as the effects of L-Arg and SNP before and after L-NAME were analised using Wilcoxon test. Statistical analyzes were performed with specific designed statistical softwares, GraphPad Prism 5 or SigmaPlot 11. All data were presented as mean ± SEM and differences were denoted as significant according 95% confidence level ($P < 0.05$).

3. RESULTS

L-NAME increased vascular tone and its effect is clear 10s after administration. G_{sys} decreased after all doses of L-NAME (Fig. 4), while f_H and MAP_{sys} remained unchanged (Figs. 4-5). As a compensatory result, to avoid the effect of systemic vasoconstriction, the rattlesnake decrease CO, mainly, by V_{stot} reduction. That is present as a tendency after the dose of 0.1 $mg \cdot kg^{-1}$, but it is significant only in doses of 1 $mg \cdot kg^{-1}$ and 100 $mg \cdot kg^{-1}$ (Fig. 5). CO reduction decreased $\dot{Q}_{sys}$ in all doses of L-NAME (Fig. 4). There were no effects of L-NAME on pulmonary circulation. Net-Shunt demonstrated by $\dot{Q}_{pul} \cdot \dot{Q}_{sys}^{-1}$ ratio (6) was not altered and so, resting R-L shunt was stable over the time course of the response (Fig. 5). Considering the clear effect on cardiovascular variables, mainly on the vascular conductance reduction, we chose the dose of 10 $mg \cdot kg^{-1}$ to investigate the role of NO on the cardiovascular system.

SNP induced relaxation on systemic vasculature and the effects was not inhibited after L-NAME (Figs. 6, 7, 8 and Table 1). SNP injection reduced R_{sys} (0.15 ± 0.03 to 0.06 ± 0.01 $kPa \cdot ml^{-1} \cdot min^{-1} \cdot kg^{-1}$) causing systemic vasodilation and decrease of MAP_{sys} (5.48 ± 0.50 to 3.26 ± 0.31 KPa). As a compensatory response to hypotension, there were significant rise of f_H (34.95 ± 2.48 to 38.90 ± 2.23 $beats \cdot min^{-1}$) and so, CO increased (57.50 ± 4.60 to 76.36 ± 6.30 $ml \cdot min^{-}$

^{1}·kg^{-1}), with a consequent increased of $\dot{Q}_{sys}$ (40.94 ± 4.82 to 61.28 ± 7.55 ml·min^{-1}·kg^{-1}). V_{stot} was not changed after SNP injection (1.70 ± 0.19 to 2.03 ± 0.25 ml·kg^{-1}).

There were similar responses on pulmonary circulation. We observed R_{pul} reduction (0.24 ± 0.06 to 0.18 ± 0.04 kPa·ml^{-1}·min^{-1}·kg^{-1}), and MAP_{pul} decreasing (2.98 ± 0.31 to 2.18 ± 0.20 KPa). Although, $\dot{Q}_{pul}$ remained unchanged following SNP injection (16.56 ± 4.74 to 15.09 ± 3.27 ml·min^{-1}·kg^{-1}). Net R-L shunt was not changed after SNP injection (0.49 ± 0.16 to 0.30 ± 0.08). Differently from what was observed on systemic vasculature, the NOS inhibition after L-NAME injection affected pulmonary circulation, causing expressive fall on R_{pul} (-21.01 ± 6.73 to -34.31 ± 5.61%) and higher G_{pul} increase (32.41 ± 13.85 to 58.31 ± 14.48%) (Fig. 7F-H and Table 1). Although the Net-Shunt was reduced with SNP injection after L-NAME (Table 1), when we compared SNP effects before and after L-NAME, there were not significant change in this parameter (Fig. 8D). The maximum vasodilation caused by SNP started after one min (1.21 ± 0.17 min) and lasted one min (1.0 ± 0.17 min).

The effects of L-Arg injections were similar to those of SNP and caused systemic vasodilation and so, R_{sys} decreased (0.17 ± 0.04 to 0.08 ± 0.01 kPa·ml^{-1}·min^{-1}·kg^{-1}). The vasodilation was associated with G_{sys} increase (7.74 ± 1.44 to 16.12 ± 3.60 ml·KPa^{-1}·min^{-1}·kg^{-1}), and consequent MAP_{sys} reduction (5.57 ± 0.50 to 4.20 ± 0.44 KPa). As compensatory responses, f_H (33.43 ± 2.83 to 36.38 ± 2.22 beats·min^{-1}) and V_{stot} (1.63 ± 0.16 to 2.06 ± 0.21 ml·kg^{-1}) were significantly elevated following L-Arg, increasing CO (53.83 ± 4.96 to 73.65 ± 5.82 ml·min^{-1}·kg^{-1}) and $\dot{Q}_{sys}$ (39.81 ± 4.70 to 59.60 ± 6.72 ml·min^{-1}·kg^{-1}). All responses of cardiovascular physiological variables were significantly attenuated after treatment with L-NAME (Figs. 6, 9, 10 and Table 1).

L-Arg injection did not affected the most pulmonary parameters (R_{pul}: 0.27 ± 0.06 to 0.21 ± 0.05 kPa·ml^{-1}·min^{-1}·kg^{-1}; $\dot{Q}_{pul}$: 14. 03 ± 3.80 to 14.06 ± 3.17 ml·min^{-1}·kg^{-1}; G_{pul}: 5.03 ± 1.23 to 5.70 ± 1.00 ml·KPa^{-1}·min^{-1}·kg^{-1}). However, MAP_{pul} was reduced after L-Arg injection (3.02 ± 0.37 to 2.60 ± 0.24 KPa), and this response was attenuated after NOS inhibition (Fig. 9B). The MAP_{pul} decrease was associated to a Net-Shunt reduction (0.42 ± 0.12 to 0.27 ± 0.07). The maximum vasodilatory effect following L-Arg started about 40 s (40 ± 12 s) and lasted less than 60 s (45 ± 6 s).

4. DISCUSSION

4.1 The role of NO for resting cardiovascular adjustments

L-NAME injection reduced G_{sys} and $\dot{Q}_{sys}$, suggesting there is a continuous release of NO on the systemic circulation that maintains basal systemic vascular tone in resting condition. That result differs from what has been reported in previous studies on rattlesnakes (Skovgaard et al., 2005b; Galli et al., 2005b) and agree with previous studies on other reptiles. We suggest that the detrimental effect of anesthesia on autonomic modulation can mask the presence of some mechanism, mainly when that is working on a basal level. In mammals, the production of NO, as a potent endothelium-dependent vasodilator, occurs when receptors of endothelial cells are stimulated by bradykinin, adenosine diphosphate, serotonina, substance P, ACh or according to shear stress produced by blood flow increasing (Ignarro, 1990; Moncada et al., 1991; Conger, 1994). These stimulus lead to eNOS activation and increase NO production. Under basal conditions, there is a tonic release of NO and the NOS inhibition via L-NAME cause significant vasoconstriction (Vallance et al., 1989; Wennmalm, 1994).

As well as demonstrated in mammals, there was a significant systemic vasoconstriction following NOS inhibition in turtles, varanid lizards and pythons (Crossley et al. 2000; Skovgaard et al. 2005b). This finding is consistent with *in vitro* studies performed in estuarine crocodile. The vasoconstrition of the aortic anastomosis in response to adrenaline leaded a reflex vascular vasodilatation, and this response was reduced after treatment with L-NAME (Axelsson et al., 2001). Similar results were also observed on the garter snake aorta, *Thamnophis sirtalis parietalis*, in which, addition of L-NAME caused an increase in vascular tone (Knight and Burnstock, 1993).

Despite the obvious role of NO on the systemic vasculature in mammals, the contribution of it for the basal vascular tone in the pulmonary circuit remains controversial (Hampl and Herget, 2000). Some studies evidenced the absence of changes in pulmonary pressure or resistance after NOS inhibition (Barer et al., 1993; Cremona et al., 1994). As observed in mammals, the pulmonary vasculature of rattlesnakes was not affected by L-NAME, in contrast to the obvious role of NO on the systemic circulation. Injection of L-NAME had no effect on G_{pul}, suggesting that NO do not contribute to control of basal vascular tone on the pulmonary circulation in resting snakes, at 25^{o}C. That is according to what has been previously suggested in studies with turtles and ratllesnakes, which did not find changes in P_{pul}, R_{pul} and G_{pul} after L-NAME injection (Crossley et al., 2000; Skovgaard et al., 2005b). However, Galli and collaborators showed there was a small, but significant rise on the P_{pul} after L-NAME (Galli et al., 2005b).

We point out for the efficiency of a compensatory response involving V_{stot} reduction in order to decrease CO and consequently to reduce $\dot{Q}_{sys}$ and stabilize MAP_{sys}. These results differs

of data observed by Galli et al., (2005b). The authors did not observe changes on the V_{stot} and $\dot{Q}_{sys}$, but found an increased of f_H following L-NAME. Besides that, no L-NAME dose was able to affect G_{pul} and $\dot{Q}_{pul}$. So, we suggest that the pulmonary vascular tone is nonexistent at the study condition. This conclusion does not reject the existence of NO modulation on the pulmonary circulation under other physiological conditions. Temperature increase or SDA specially increase metabolism and so, the pulmonary circuit demand. Such condition is supported by CO increase and that need to be followed by conductance adjustments of the systemic and pulmonary circuits in order to provide the appropriate shunt balance. The role of NO on the vascular tone of pulmonary circuit could be related to those conditions. Such quantitative inference is possible since our experimental model has functional ANS with appropriate cardiovascular adjustments. Despite that, since L-Arg injection did not affected pulmonary circulation, its presence probably would require another production pathway.

4.2 The role of NO on systemic circulation

The exogenous NO-donor SNP caused large systemic vasodilatation in rattlesnakes, demonstrating the presence of NO receptors on the systemic vasculature. This vasodilation was enough to reduce P_{sys} as a result of R_{sys} fall. Such alteration is followed by compensatory effort to maintain P_{sys} with consequentily rise on the f_H and CO. These effects are consistent with previous data about the SNP effect in reptiles (Crossley et al. 2000; Skovgaard et al. 2005b, Galli et al., 2005b). Similar results have been also observed in isolated vessels, which sections of systemic arch was relaxed in response to ACh and SNP in *Thamnophis sirtalis parietalis* (Knight and Burnstock, 1993). In freshwater turtle, ACh led an increase in cerebral blood flow velocity and this response was inhibited after L-NAME injection. Based on that, the authors suggested that NO has an important role in relaxation of turtle brain vessels (Hylland et al. 1996; Söderström et al., 1997). Despite that, the use of ACh and SNP gives no information about which endogenous mechanism is the responsible for *in vivo* cardiovascular production of NO (Skovgaard et al. 2005b; Toda and Ayajiki, 2006). Therefore, to address the physiological mechanism behind NO synthesis in rattlesnake we administered L-Arg, a substrate for NO endogenous production.

L-Arg caused effects similar to SNP. The responses to L-Arg were significantly attenuated after L-NAME injection, suggesting that the effect was dependent of endogenous production of NO. This result indicates systemic effect has occurred, and that L-Arg acts at the site of administration. That result was similar to the one reported by Galli et al. (2005b). Although we can conclude that the used dose of L-NAME was able to inhibit NO synthesis, the

inhibition may not have been complete. *In vitro* studies demonstrated that 100 µM of L-NAME is able to completely abolish the ability of ACh to relax the garter snake aorta (Knight and Burnstock, 1993). However, despite we injected 1000-fold higher concentration of L-NAME, our *in vivo* experimental protocol may not have been enough to NOS inhibition. In addition, we can not discharge the possibility that vasodilation after NOS inhibition may have occurred due the vasorelaxing effect caused by other endogenous modulators. The validation of such inference is beyond our protocol.

In mammals, endothelial cells can control the vascular tone by continuous NO releasing. However, the existence of another alternative pathway has been demonstrated. EDHF has been suggested to have the same vasodilator role (Félétou and Vanhoutte, 2004). It is already well established that some vasoactive substances act differently according to blood vessels caliber. In large arteries, NO is reponsible by vascular relaxation, while in resistance arteries, EDHF have pronounced role on vascular tone modulation (Shimokawa et al., 1996; Takaki et al., 2008). The endogenous mechanism responsible for microvessels vasodilation by EDHF releasing have participation of NOS enzime. The NOS isoforms, especially eNOS, is able to produce NO and superoxide anions, and the latter can be dismutated by Cu, Zn-SOD enzyme synthesizing EDHF/H_2O_2 (Takaki et al., 2008). So, EDHF hyperpolarizes the vascular smooth muscle cells opening K_{Ca} channels and so, promote vasodilation. Such different L-NAME effect in large and small vessels could explain the results obtained in our study. In large arteries, L-NAME prevented the synthesis and release of NO, however, in resistance arteries, its effect was minimal. Thereby, in microvessels, there was higher production of EDHF and therefore, a residual relaxation was observed even after NOS inhibition.

The role of EDHF on the vascular tone of this group is an intriguing possibility. *In vitro* studies are important to test the presence and effect of EDHF on the different vascular beds. Besides that, the quantification of the effect and relevance of endogenous modulators, NO and EDHF, would provide better understanding of these signaling molecules on cardiovascular adjustments in Squamata. So, we can suggest that, on the rattlesnake, there is an important, but partial role for NO in regulating systemic vascular relaxation for the final tone and other mediators seems to be involved on the vascular adjusments. This is a new possibility that contradicts other studies that reported that rattlesnake does not seem to need of NO for maintenance of basal systemic vascular tone (Skovgaard et al. 2005b, Galli et al., 2005b). The use of unanesthetised animal, provided by the decerebrated snake was critical to observe the possibility of this mechanism.

4.3 The role of NO on pulmonary circulation

SNP reduced R_{pul} and despite MAP_{pul} reduction, $\dot{Q}_{pul}$ was maintained constant. These effects indicated that pulmonary vasculature is able to respond the NO. Following injection L-NAME, SNP caused higher effect descreasing R_{pul} and increasing of $G_{pul.}$ So, SNP had effect on the vascular tone of the pulmonary circulation and NOS inhibition increases the capacity of relaxation of this circuit. These findings disagree of previous reports studies on turtles (Crossley et al. 2000) and on rattlesnakes (Skovgaard et al. 2005b; Galli et al., 2005b). A possible reason to explain the lack changes on the R_{pul} in rattlesnakes and turtles after SNP injection is that reptiles with an undivided circulation, and highter pulmonary blood pressure are less dependent on NO regulation on the pulmonary circulation (Crossley et al. 2000; Galli et al., 2005b; Skovgaard et al. 2005). So, the stimulation of pulmonary artery by the vagus nerve is the main mechanisms to regulate pulmonary resistance and consequently, to affect the intracardiac shunting (Galli et al., 2005b, Berger 1972; Burggren 1977, Hicks 1994). In such experiments, SNP injection did not cause vasodilation in pulmonary vasculature. One possible explanation for a higher vasodilation in pulmonary vasculature after L-NAME in our study can be, again, attributed to EDHF. NOS inhibition leads to an increase on the activity of the enzyme involved on H_2O_2 synthesis, with consequent potencialization of vascular smooth muscle relaxation (Takaki et al., 2008). However, the presence of such mechanim in the pulmonary circuit is still not understood. The pulmonary vasculature may be more sensitive to EDHF than the systemic vasculature and therefore, this pattern of response has been observed only on the pulmonary circulation.

L-Arg injection did not cause pulmonary vasodilation in *Crotalus*. That is similar to the responses reported by Galli et al. (2005b). Another interesting data is that, in our study, we observed a MAP_{pul} reduction following L-Arg injection, and this effect can be related to control of blood shunting out of pulmonary circulation. This explanation is consistent, since that Net-Shunt was reducted after L-Arg, demonstrating the blood flow was redirected to systemic circulation. So, our finding shows the basal production of NO affects the R_{sys}/R_{pul} balance in a way that it can influence, but does not to control, the intracardiac shunt in rattlesnakes.

Thus, our study demonstrate that pulmonary circuit vasodilation is not a L-Arg-dependent mechanism. The absence of clear response on the pulmonary circuit can still be addressed by three hypotheses. First, the reduction of blood flow in the pulmonary circulation may have influenced the magnitude of response to L-Arg, affecting its effect on the pulmonary vasculature. Second, NO synthesis by pulmonary vasculature (*in vivo*) may have been limited by other substances such as co-substrate NADPH or cofactors like as FAD, FMN, heme,

calmodulin and BH_4, that are important factors for NO production (Umans and Levi, 1995). Another explanation for the lack of response can be related with the nonenzymatic producion of NO. Though this reaction seems improbable that occurs in biological tissues, as already mentioned by Ignarro (1990). The present results indicate the need of further studies regarding the evolutionary mechanism responsible to endogenous production of NO on both circuits in vertebrates.

5. CONCLUSIONS

We suggesting that, in rattlesnakes, NO is synthetized via L-Arg mechanism and it has partial role on basal relaxation to provide the final vascular tone of systemic circulation. In contrast, pulmonary vasculature is less reactive to SNP and it is unresponsive to L-Arg. Hence, we suggest that NO-mediated vasodilation has an important role for systemic cardiovascular adjustments in resting condition in reptiles. And, pulmonary vascular adjustments promoted by NO could be relevant in other physiological conditions like as temperature increase or SDA. Besides, the obtaining data without the use of anesthesia made it possible to observe precise and different responses than those presented in studies with anesthetized animals. The main changes were observed on the control of vascular tone promoted by SNP on the pulmonary vasculature. Thus, decerebrated rattlesnake model allowed the getting of cardiovascular data consistent and safe, demonstrating to be great alternative for studies, which involve mechanisms that rely some level of autonomic activation.

6. REFERENCES

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7. FIGURES AND LEGENDS

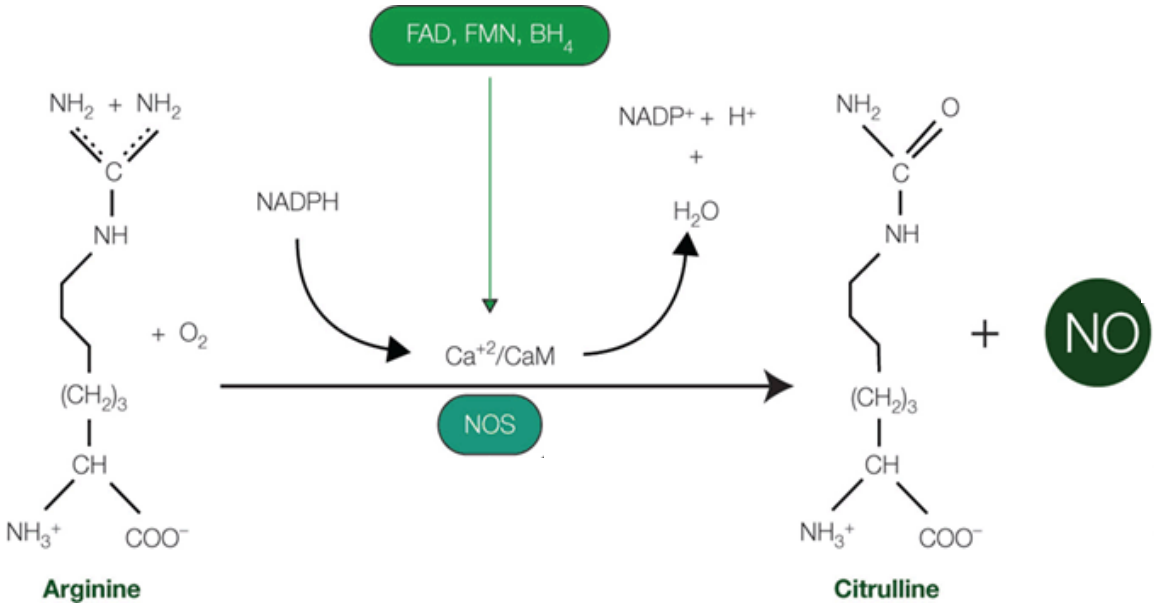

Fig. 1. Nitric oxide synthesis. Endogenous synthesis of NO from the oxidation of arginine, which is converted to citrulline by NOS enzyme. Co-substrate nicotinamide adenine dinucleotide phosphate (NADPH) or cofactors like as tetrahydrobiopterin (BH4), flavin mononucleotide (FMN), calmodulin (CaM) and flavin adenine dinucleotide (FAD) are important factors for NO production. Adapted from Freire et al. (2009). Pain modulation by nitric oxide in the spinal cord - *Frontiers in Neuroscience*, p. 176.

Fig. 2. Experimental animal. South American rattlesnakes, *Crotalus durissus* (Linnaeus, 1758)**.** (Image: Oda, G. M.).

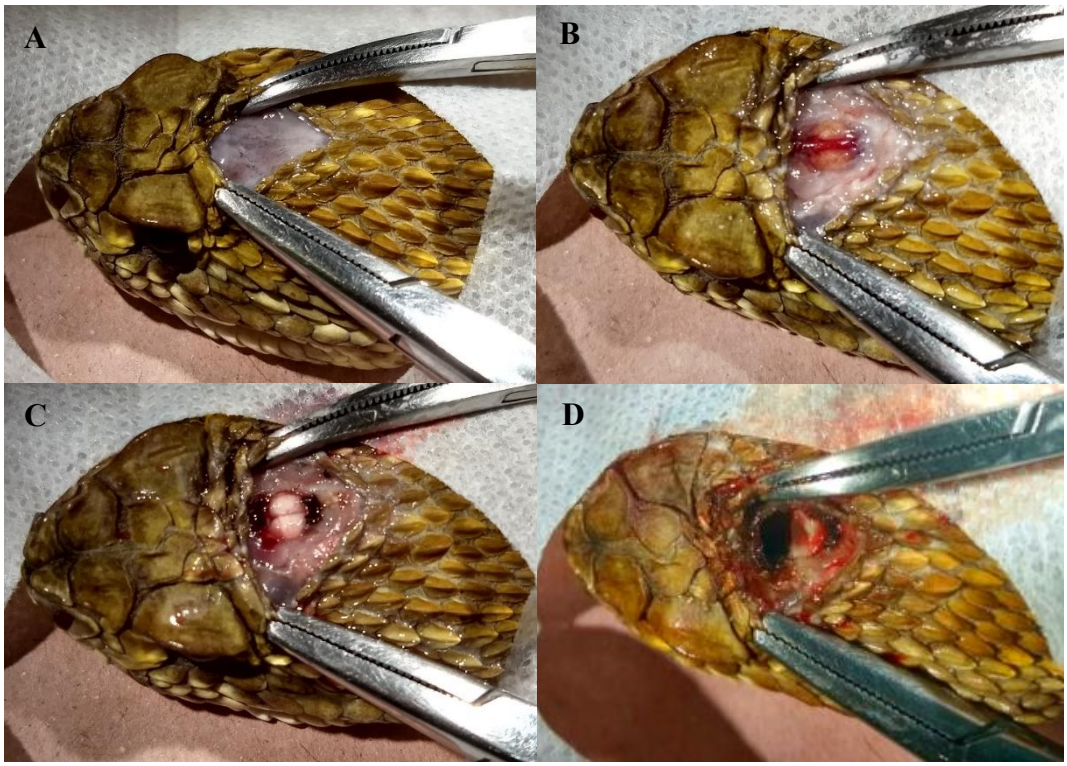

Fig. 3. Progress of decerebration procedure in *C. durissus*. (A) opening in the medial region of the head, allowing access to skull. (B) aperture in the cranial casing, allowing access to superficial arterie. (C) cauterization of the superficial arterie and visualization of *Tectum* structure. (D) transverse section at the first third of *Tectum* structure, cauterization and removal of the the nervous tissue rostral to incision. (Images: Castro, S. A.).

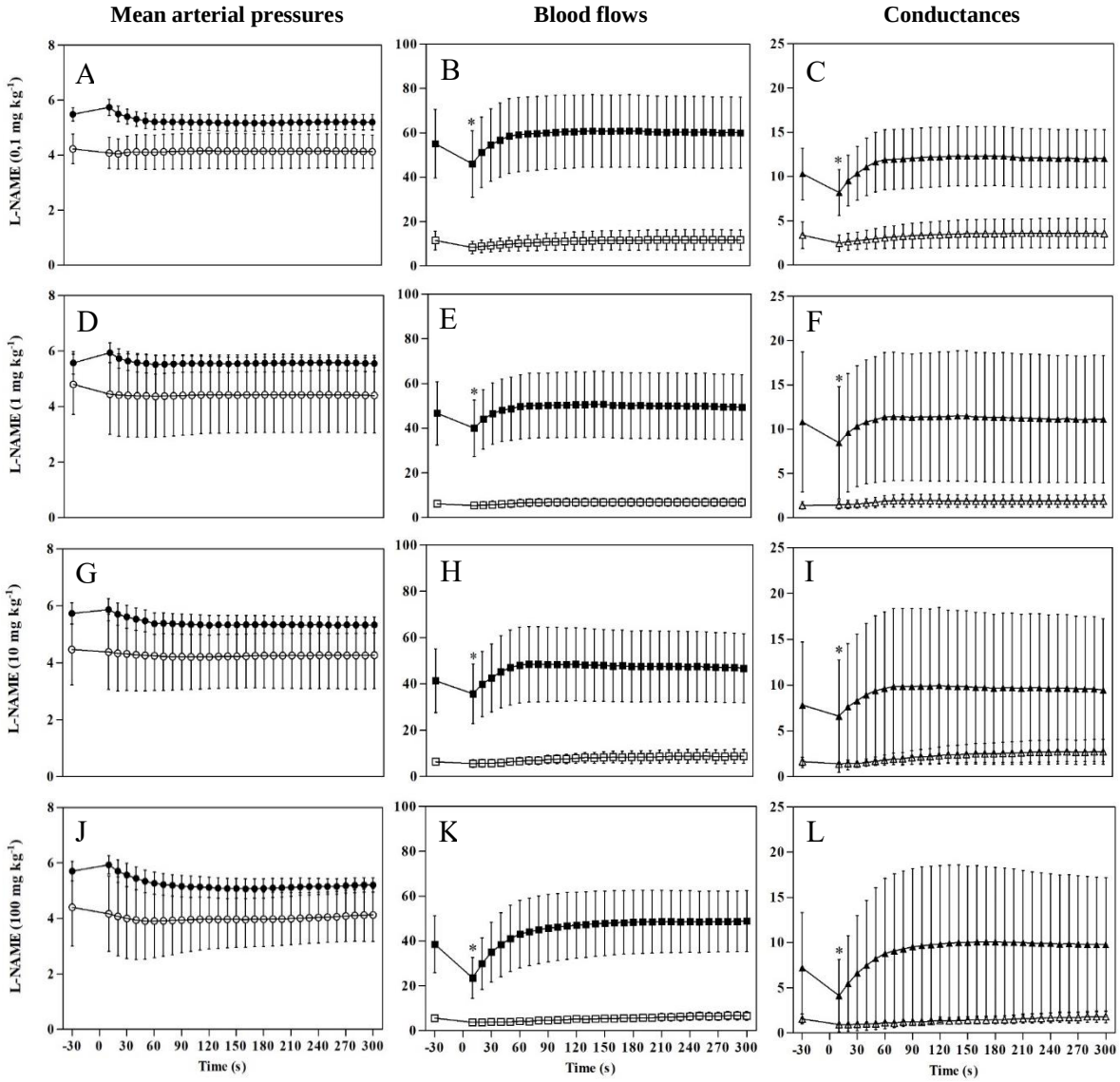

Fig. 4. Effects of different doses of L-NAME over time on pressure, blood flow, and conductance on the systemic and pulmonary circulations. A, B and C: L-NAME (0,1 mg·kg^{-1}); D, E and F: L-NAME (1 mg·kg^{-1}); G, H and I: L-NAME (10 mg·kg^{-1}); J, K and L: L-NAME (100 mg·kg^{-1}). Systemic mean arterial pressure - MAP_{sys} (filled circle - KPa); pulmonary mean arterial pressure - MAP_{pul} (unfilled circle - KPa); systemic blood flow - $\dot{Q}_{sys}$ (filled square - ml min^{-1} kg^{-1}); pulmonary blood flow - $\dot{Q}_{pul}$ (unfilled square - ml min^{-1} kg^{-1}); systemic conductance – G_{sys} (filled triangle - ml min^{-1} kg^{-1} KPa^{-1}); pulmonary conductance – G_{pul} (unfilled triangle - ml min^{-1} kg^{-1} KPa^{-1}). *N = 6.* Values are mean with S.E.M. * Denotes significant difference mean against pre-injection values (-30 s) ($P < 0.05$) - Friedman repeated measures analysis of variance on ranks.

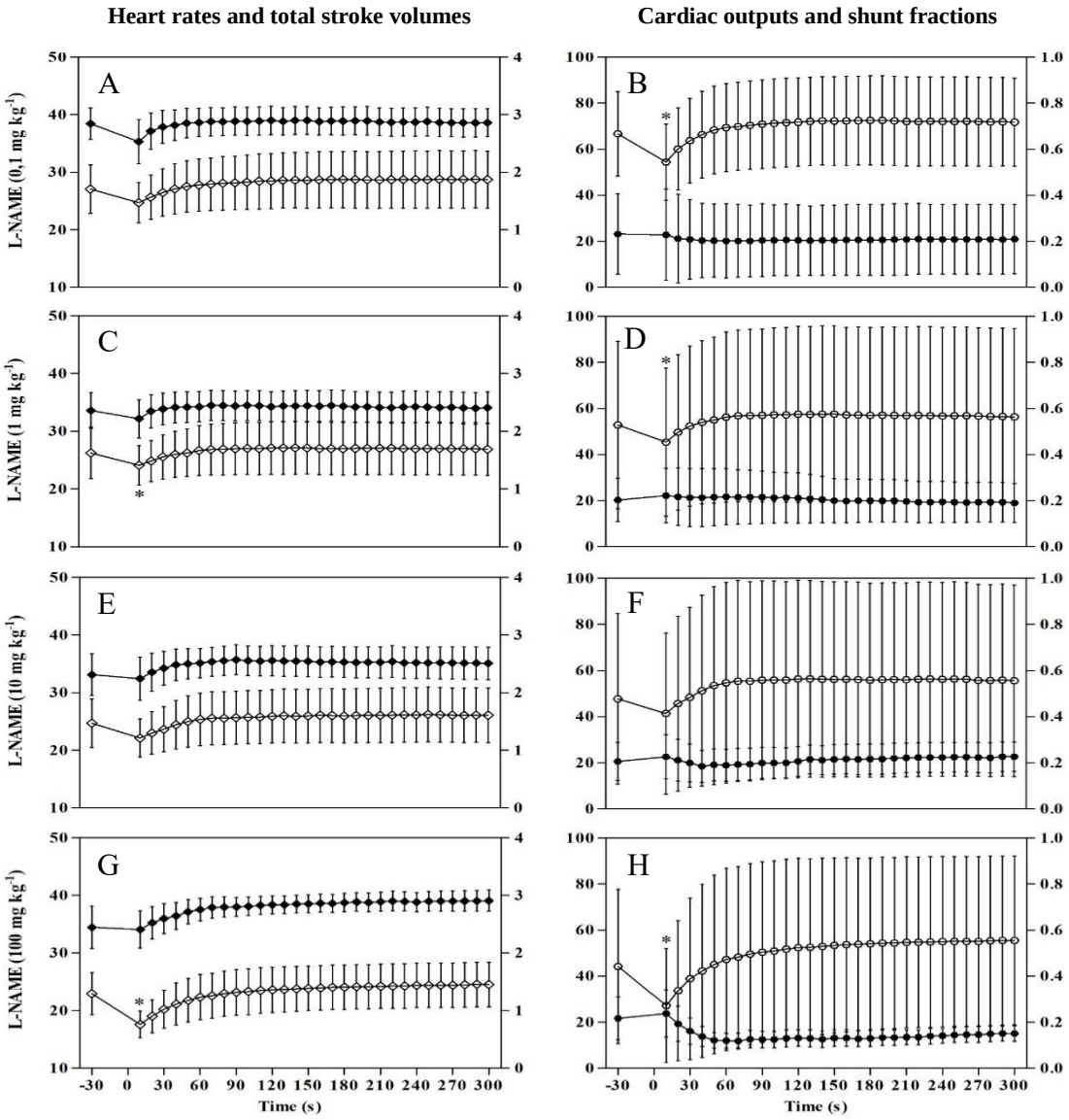

Fig. 5. Effects of different doses of L-NAME over time on heart rate, total stroke volume, cardiac output, and intracardiac shunting on the systemic and pulmonary circulations. A and B: L-NAME (0,1 mg·kg^{-1}); C and D: L-NAME (1 mg·kg^{-1}); E and F: L-NAME (10 mg·kg^{-1}); G and H: L-NAME (100 mg·kg^{-1}). Heart rate - f_H (filled diamond - beats min^{-1}); total stroke volume - V_{stot} (unfilled diamond - ml·kg^{-1}); cardiac output – CO (unfilled hexagon - ml min^{-1} kg^{-1}); Net-Shunt - $\dot{Q}_{pul}\cdot\dot{Q}_{sys}^{-1}$ (filled hexagon). *N = 6*. Values are mean with S.E.M. * Denotes significant difference mean against pre-injection values (-30 s) ($P < 0.05$) - Friedman repeated measures analysis of variance on ranks.

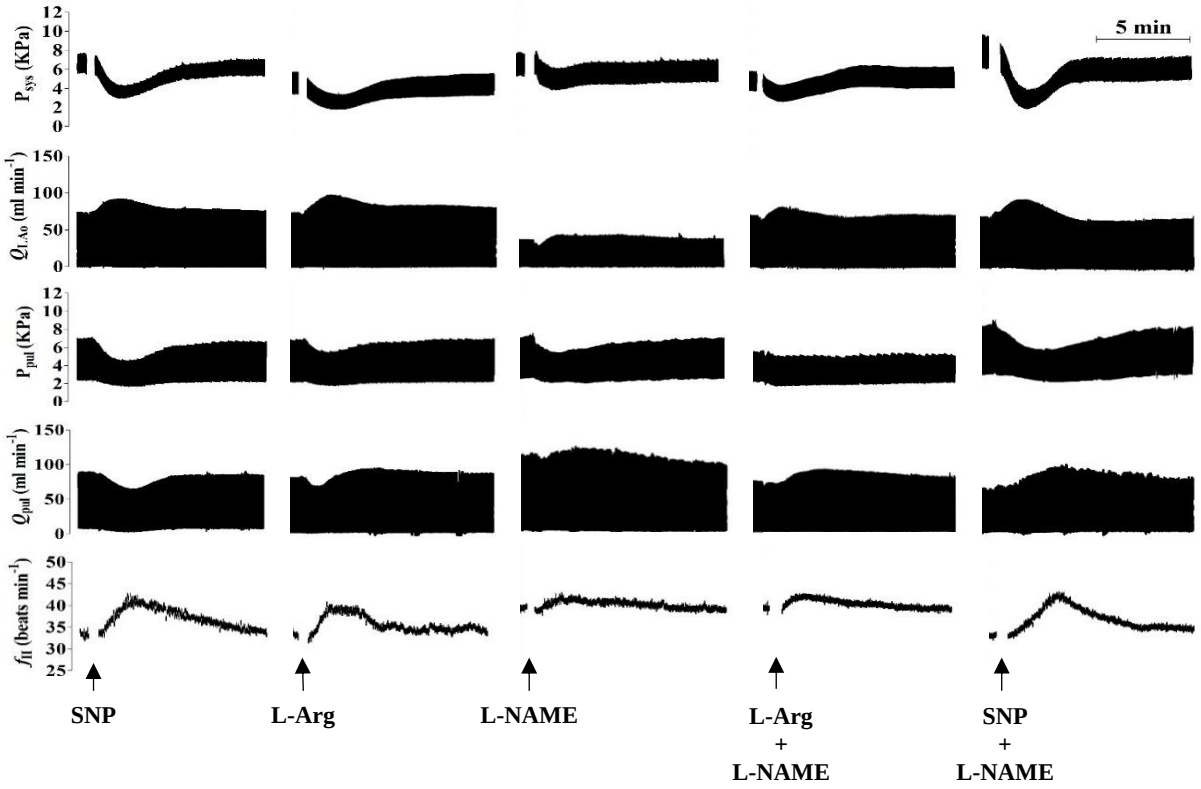

Fig. 6. Original traces demonstrating the effect of SNP, L-Arg and L-NAME i.v. injections on cardiovascular parameters in *Crotalus durissus*. SNP, sodium nitroprusside (2.5 μg·kg^{-1}); L-Arg, L-arginine (50 mg·kg^{-1}) and L-NAME, N(ω)-nitro-L-arginine methyl ester (10 mg·kg^{-1}). Systemic arterial pressure - P_{sys} (KPa); left aortic arch blood flow - $\dot{Q}_{LAo}$ (ml·min^{-1}); pulmonary arterial pressure – P_{pul} (KPa); pulmonary blood flow - $\dot{Q}_{pul}$ (ml·min^{-1}); heart rate - f_H (beats·min^{-1}). Arrows indicate the moment of injections.

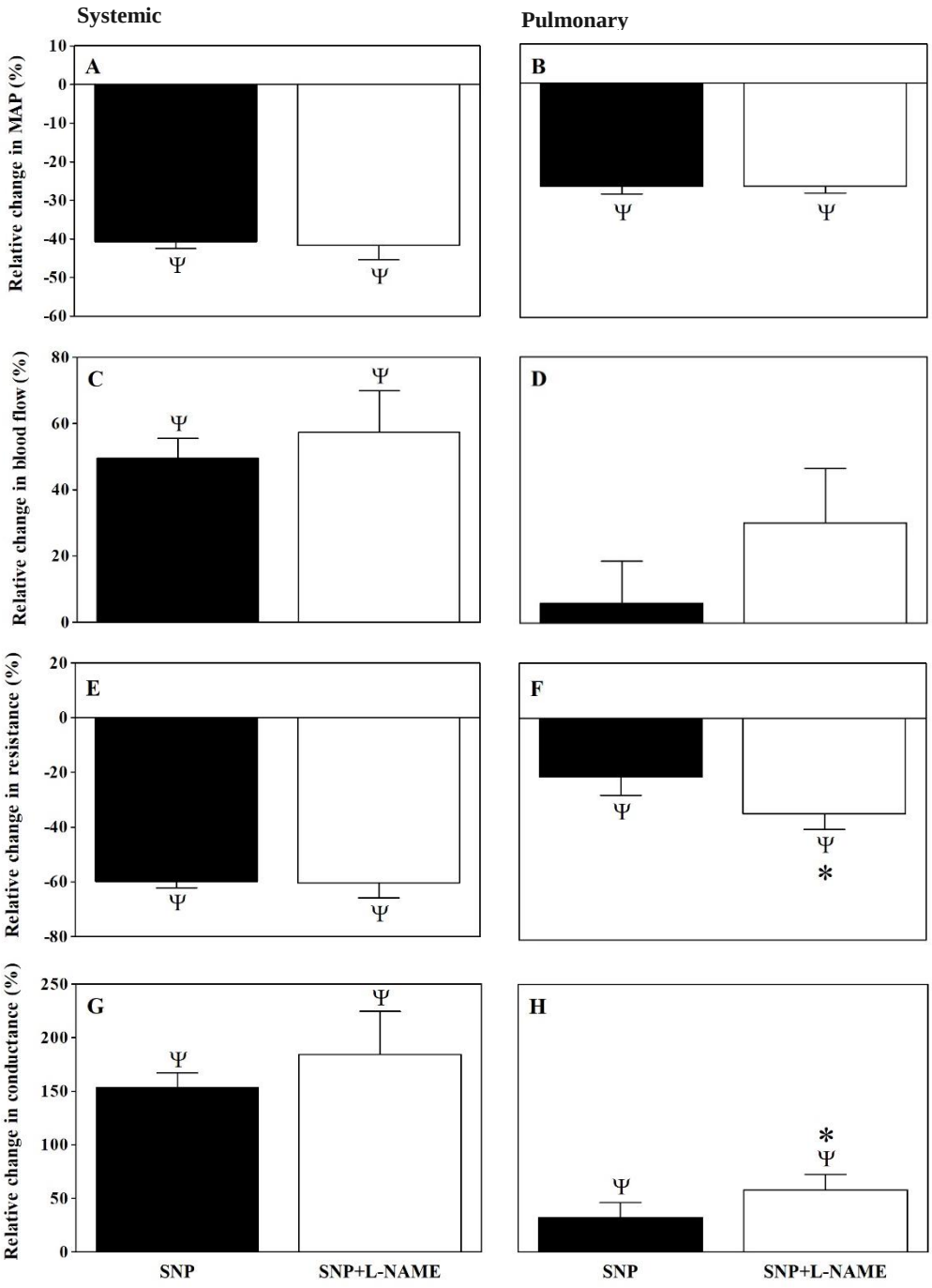

Fig. 7. Relative change (%) on haemodynamic parameters of *C. durissus* after injection of SNP (2.5 µg·kg^{-1}), before and after L-NAME (10 mg·kg^{-1}) injection. Systemic (A) and pulmonary (B) mean arterial pressures, MAP; systemic (C) and pulmonary blood flow (D); systemic (E) and pulmonary (F) vascular resistance; systemic (G) and pulmonary (H) vascular conductance. In the systemic circulation: n = 7. In the pulmonary circulation: $\dot{Q}_{pul}$, n = 7; MAP_{pul}, R_{pul} and G_{pul}, n = 6. Values are mean ± S.E.M. $^{\Psi}$ Denotes difference against value pre-injection value ($P < 0.05$) and * denotes difference against values after L-NAME injection ($P < 0.05$) - Wilcoxon matched-pairs signed rank test.

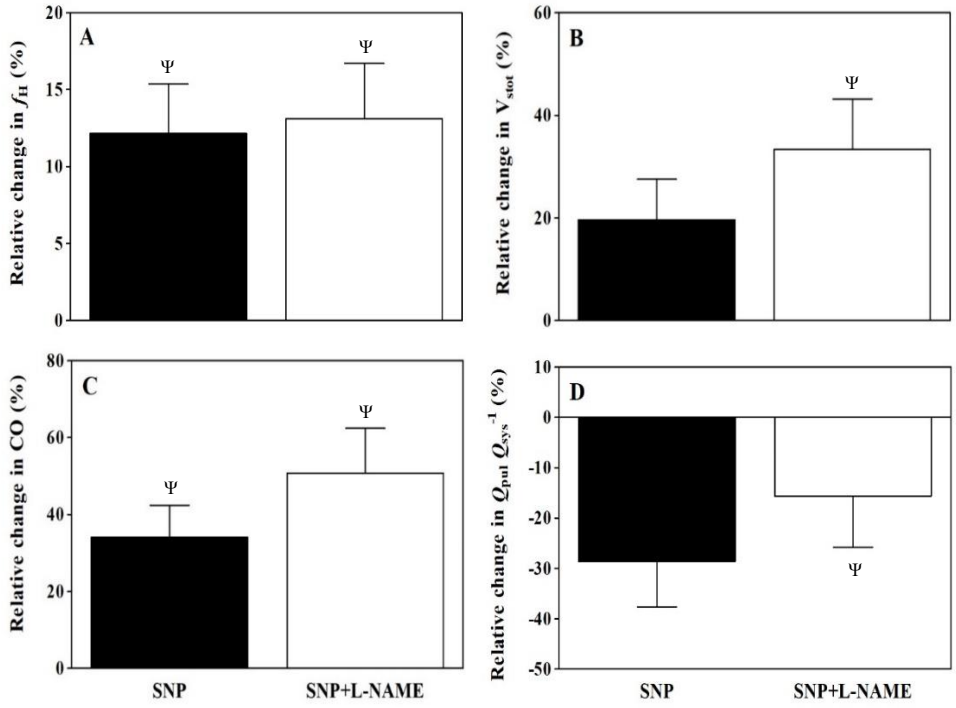

Fig. 8. Relative change (%) on haemodynamic parameters of *C. durissus* after SNP injection (2.5 μg·kg^{-1}), before and after L-NAME (10 mg·kg^{-1}) injection. Heart rate, f_H (A); total stroke volume, V_{stot} (B); cardiac output, CO (C); Net-Shunt, $\dot{Q}_{pul}\cdot\dot{Q}_{sys}^{-1}$ (D). *N* = 7. Values are mean ± S.E.M. $^{\Psi}$ Denotes difference against value pre-injection value ($P < 0.05$) - Wilcoxon matched-pairs signed rank test.

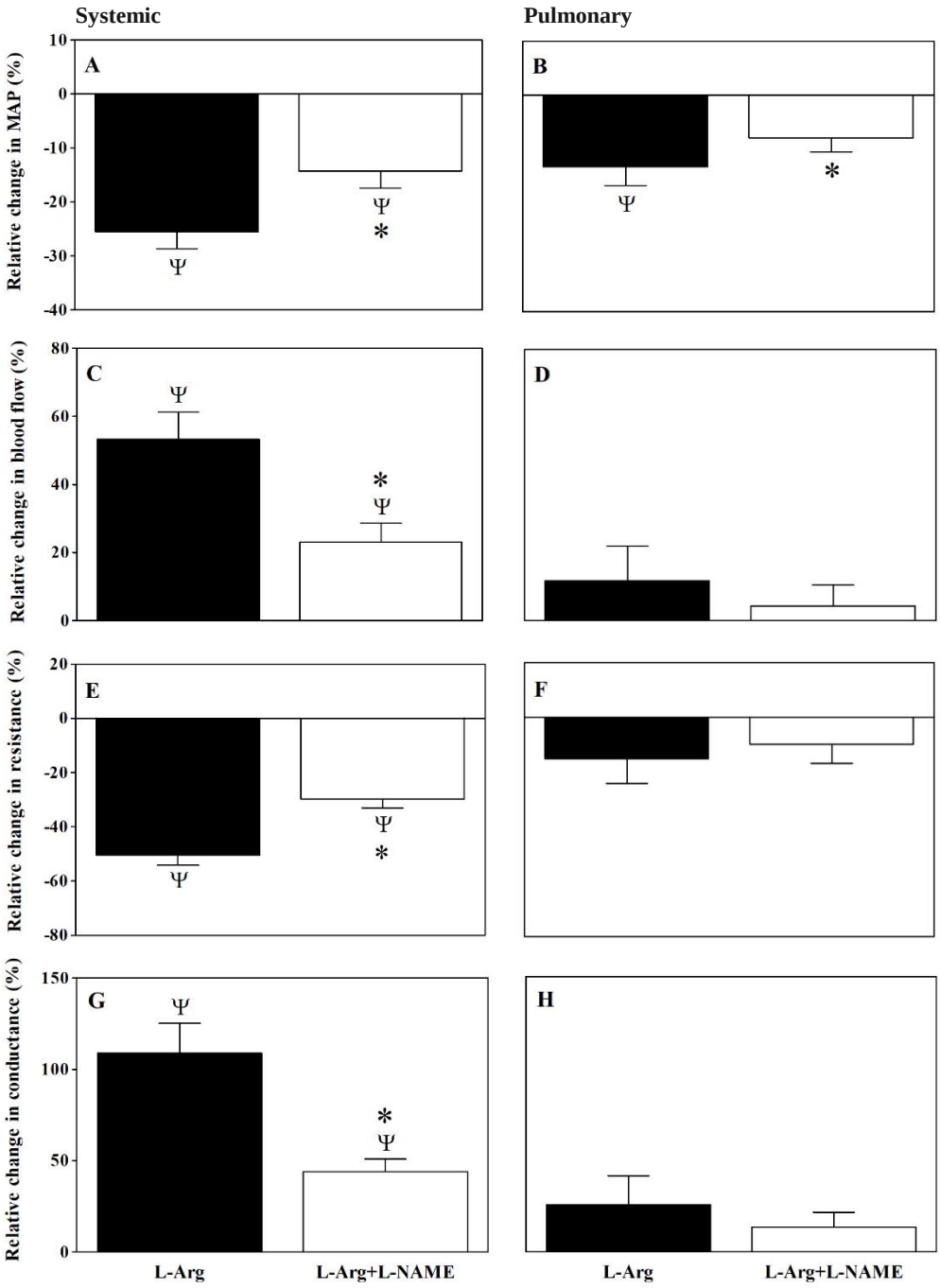

Fig. 9. Relative change (%) on haemodynamic parameters of *C. durissus* after L-Arg injection (50 mg·kg^{-1}), before and after L-NAME (10 mg·kg^{-1}) injection. Systemic (A) and pulmonary (B) mean arterial pressures, MAP; systemic (C) and pulmonary blood flow (D); systemic (E) and pulmonary (F) vascular resistance; systemic (G) and pulmonary (H) vascular conductance. In the systemic circulation: n = 7. In the pulmonary circulation: $\dot{Q}_{pul}$, n = 7; MAP_{pul}, R_{pul} and G_{pul}, n = 6. Values are mean ± S.E.M. $^{\Psi}$ Denotes difference against value pre-injection value ($P < 0.05$) and * denotes difference against values after L-NAME injection ($P < 0.05$) - Wilcoxon matched-pairs signed rank test.

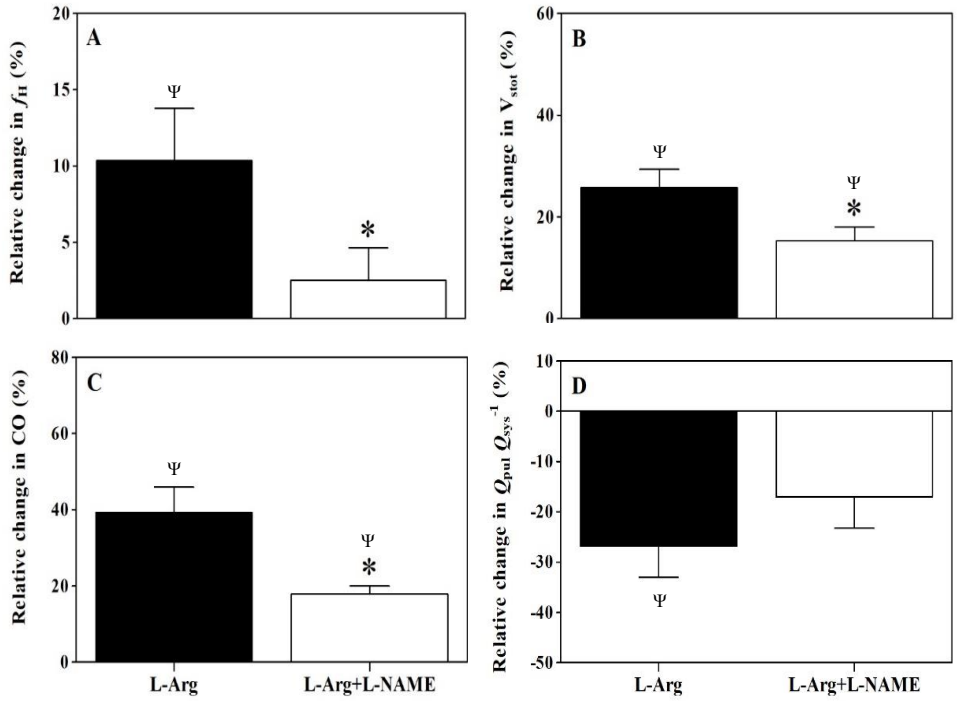

Fig. 10. Relative change (%) on haemodynamic parameters of *C. durissus* after L-Arg injection (50 mg·kg^{-1}), before and after L-NAME (10 mg·kg^{-1}) injection. Heart rate, f_H (A); total stroke volume, V_{stot} (B); cardiac output, CO (C); Net-Shunt, $\dot{Q}_{pul}\cdot\dot{Q}_{sys}^{-1}$ (D). N = 7. Values are mean ± S.E.M. $^{\Psi}$ Denotes difference against value pre-injection value ($P < 0.05$) and * denotes difference against values after L-NAME injection ($P < 0.05$) - Wilcoxon matched-pairs signed rank test.

8. TABLE AND LEGEND

Table 1: Effects of saline, SNP, L-arg and L-NAME injections on haemodynamic variables of *C. durissus*

Variables	Pre-injection	Saline	Pre-injection	SNP + L-NAME	Pre-injection	L-Arg + L-NAME
MAP_{sys} (KPa)	5.40 ± 0.51	5.46 ± 0.50	6.23 ± 0.46	3.65 ± 0.38 *	5.65 ± 0.37	4.86 ± 0.38 *
$\dot{Q}_{sys}$ ($ml \cdot min^{-1} \cdot kg^{-1}$)	43.76 ± 5.00	44.64 ± 5.00	34.53 ± 3.72	55.60 ± 8.00 *	35.61 ± 4.04	42.93 ± 4.27 *
R_{sys} ($kPa \cdot ml^{-1} \cdot min^{-1} \cdot kg^{-1}$)	0.14 ± 0.02	0.14 ± 0.02	0.20 ± 0.04	0.09 ± 0.03 *	0.18 ± 0.04	0.13 ± 0.02 *
G_{sys} ($ml \cdot min^{-1} \cdot kg^{-1} \cdot KPa^{-1}$)	8.83 ± 1.62	8.74 ± 1.50	5.90 ± 0.96	17.40 ± 3.60 *	6.70 ± 1.07	9.43 ± 1.50 *
MAP_{pul} (KPa)	2.91 ± 0.30	2.92 ± 0.25	3.23 ± 0.40	2.40 ± 0.33 *	3.11 ± 0.40	2.86 ± 0.33
$\dot{Q}_{pul}$ ($ml \cdot min^{-1} \cdot kg^{-1}$)	17.57 ± 5.04	17.40 ± 5.03	11.50 ± 4.00	12.94 ± 3.80	13.10 ± 5.31	14.46 ± 5.03
R_{pul} ($kPa \cdot ml^{-1} \cdot min^{-1} \cdot kg^{-1}$)	0.23 ± 0.07	0.25 ± 0.08	0.35 ± 0.07	0.22 ± 0.04 *	0.36 ± 0.10	0.34 ± 0.11
G_{pul} ($ml \cdot min^{-1} \cdot kg^{-1} \cdot KPa^{-1}$)	6.40 ± 1.51	6.36 ± 1.60	3.89 ± 1.23	5.65 ± 1.35 *	4.30 ± 1.50	4.75 ± 1.41
f_H ($beats \cdot min^{-1}$)	35.73 ± 2.53	35.92 ± 2.30	32.76 ± 2.86	36.62 ± 2.53 *	33.56 ± 3.00	34.33 ± 3.04
V_{stot} ($ml \cdot kg^{-1}$)	1.78 ± 0.14	1.77 ± 0.12	1.44 ± 0.10	1.95 ± 0.24 *	1.50 ± 0.10	1.70 ± 0.08 *
CO ($ml \cdot min^{-1} \cdot kg^{-1}$)	61.33 ± 3.00	62.03 ± 3.21	46.02 ± 3.41	68.51 ± 5.39 *	48.71 ± 4.01	57.38 ± 4.67 *
$\dot{Q}_{pul} \cdot \dot{Q}_{sys}^{-1}$	0.51 ± 0.17	0.49 ± 0.16	0.42 ± 0.17	0.36 ± 0.15 *	0.50 ± 0.22	0.40 ± 0.15

Values are mean ± S.E.M. before (pre-injection) and after saline, SNP and L-Arg injections, both after L-NAME inhibition. MAP_{sys} - systemic mean arterial pressure; $\dot{Q}_{sys}$ - systemic flow; R_{sys} - systemic resistance; G_{sys} - systemic conductance; MAP_{pul} - pulmonary mean arterial pressure; $\dot{Q}_{pul}$ - pulmonary flow; R_{pul} - pulmonary resistance; G_{pul} - pulmonary conductance; f_H - heart rate; V_{stot} - total stroke volume; CO - cardiac output and $\dot{Q}_{pul} \cdot \dot{Q}_{sys}^{-1}$ - Net-Shunt. In the systemic circulation: n = 7. In the pulmonary circulation: $\dot{Q}_{pul}$, n = 7; MAP_{pul}, R_{pul} and G_{pul}, n = 6. f_H, V_{stot}, CO and $\dot{Q}_{pul} \cdot \dot{Q}_{sys}^{-1}$, n = 7. * Denotes difference against pre-injection values ($P < 0.05$) - Wilcoxon matched-pairs signed rank test.

CHAPTER III

Role of α and β-adrenergic regulation for cardiovascular adjustments in South American rattlesnakes (*Crotalus durissus)*

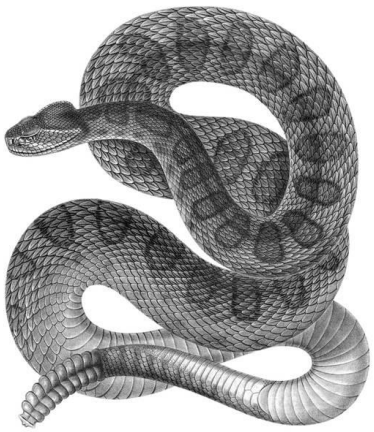

Image source: https://bit.ly/2KpzvVe

1. INTRODUCTION

The circulatory system of vertebrates is under continuous autonomic regulation. Heart and pulmonary central vessels are inervated by, respectively, inhibitory and excitatory parasympathetic vagal fibers. Moreover, heart and systemic vascular system are directly inervated by excitatory sympathetic adrenergic fibers (Morris and Nilsson, 1994; Taylor et al., 2009; Leite et al., 2013, 1014). The regulation of systemic and pulmonary vascular tone has important implications for Testudines and Squamata, since the ventricle of these animals is partially divided and so, it is able to allow different proportions of blood recirculation in systemic and/or pulmonary systems (Hicks et al., 1996; Hicks, 1998). Despite cardiac shunt function is still under debate, the presence of nervous regulation of systemic and pulmonary vasculature affecting cardiac shunt has been experimentaly demonstrated (Eme et al., 2009; Leite et al., 2013, 2014).

Different factors influencing intracardiac shunting have been described in *Trachemys scripta* (Hicks and Malvin, 1992; Hicks and Comeau, 1994; Hicks, 1994; Hicks et al., 1996) and *Crotalus durissus* (Galli et al., 2005b; Taylor et al., 2009; Leite et al., 2013, 2014; Filogonio et al., 2016). In these animals, intracardiac shunt is influenced by alterations in pulmonary and systemic vascular conductances (Hicks and Krosniunas, 1996; Crossley et al., 1998). Histochemical studies have demonstrated that pulmonary and systemic vasculature are innervated by adrenergic fibers (Smith and Macintyre, 1979; Donald and Lillywhite, 1988; Donald et al., 1990a). Hence, adrenergic excitatory stimuli can alters the balance of vascular resistance between systemic and pulmonary circulations, affecting the Net-Shunt (Lillywhite and Donald, 1989). Intravenous administration of adrenaline was able to eliminate systemic venous admixture in aortic arches on *Pseudemys scripta*, abolishing the R-L shunt (Hicks and Malvin 1992). According Comeau and Hicks (1994), adrenaline injection may promote large net L-R shunt in turtles. Increased sympathetic stimulation is often associated with exercise and stressful conditions in snakes, and it is also related with reduction of vagal tone (Wang et al., 2001a). Those alterations would lead to relative decrease in G_{sys} and G_{pul} increase, reducing net R-L shunt and so, directing blood to pulmonary circulation. That would resout in increasing systemic oxygen delivery to sustain the increased metabolic demands (Wang and Hicks, 1996; Wang et al., 2001b).

As previously described, adrenergic stimulation seems to have great influence on the control of cardiac shunt and so, a number of reptile species have been studied to understand the distribution of sympathetic inervation and their role for hemodynamic adjstments: five testudines species, *Chrysemys* scripta, *Trachemys scripta*, *Chelodina longicollis*, *Pseudemys*

scripta and *Pseudemys elegans*; one crocodilian, *Crocodylus porosus*; and seven squamates, *Crotalus durissus*, *Tiliqua rugosa, Elaphe obsoleta, Bitis arietans, Ophidae colubridae, Acrochordus granulatus and Trachysaurus rugosus* (Luckhardt and Carlson 1921; Milsom et al., 1977, Overgaard et al., 2002; Berger, 1972; Hicks and Malvin, 1992; Comeau and Hicks, 1994; Van Harn et al, 1973; Franklin and Axelsson, 1994; Galli et al., 2007; Skals et al., 2005b; Donald and Lillywhite, 1988; Donald et al., 1990a,b; Hedberg and Nilsson, 1975; Smith and MacIntyre 1979; Lillywhite and Donald, 1989; McLean and Burnstock, 1967; Furness and Moore, 1970).

On the other hand, it is well established that cholinergic control of pulmonary vasculature exerts an important role on intracardiac shunt (Johansen and Burggren, 1980; Hicks, 1998; Taylor et al, 2009). Studies demonstrate that electrical stimulation of the vagus nerve resulted in R_{pul} increase and consequent decrease in $G_{pul,}$ allowing R-L shunt increasing. And, the effect of vagal stimulation on testudines and squamates has been abolished by atropine injection (Luckhardt and Carlson 1921; Berger 1972, 1973; Burggren 1977; Milsom et al., 1977; Smith and MacIntyre 1979; Hicks and Comeau 1994). Hence, the control of pulmonary artery constriction has been demonstrated important for cardiac shunt for noncrocodilians reptiles (White, 1976; Leite et al., 2013). Rattlesnakes, when deprived of the vagal inervation in pulmonary artery can not control cardiac shunt (Taylor et al., 2009; Leite et al, 2013, 2014). Such lack of control remains despite sympathetic influences are intact, questioning the relevance of sympathetic system for shunt adjustments.

However, despite the undeniable relevance of the mentioned studies for description of autonomic mechanism influencing cardiac shunt adjustments and their roles, the conclusions presented so, in majority, far are based on evidences rised by analyses in anesthetized animals or deeply instrumented animals. Such conditions have questenable relevance when used to investigate the relative relevance of sympathetic and parasympathetic systems, since the experimental designe unevitably have deleteriour effect on autonomic balance and so, on the hability to provide autonomic adjustments on the cardiovascular system. Such conditions can lead us to inaccurate assumptions regarding fine adjustments of cardiovascular responses. One clear exemplo of deleteriour effect of anesthesia on hemodynamic variables of reptiles was demonstrated by Taylor et al. (2009) and Skals et al. (2005), in which the f_H and $\dot{Q}_{pul}$ of fully recovered rattlesnakes were lower than those observed in anesthetized rattlesnakes. In addition, studies with snakes equipped with ECG electrodes showed progressive recovery of f_H only after 120 h of anesthetic induction (Campbell et al., 2006). Then, as suggested by Taylor et al. (2009), experimental studies with invasive and/or complex instrumentation such as flow-tube implants

and vagal snares seem to impair the postoperative recovery of cardiac vagal tone, and this effect could affect interpretations and the adequate functioning of the autonomic responses on cardiovascular system. So, alternatives for autonomic studies are necessary and fundamental to find out which are the main mechanisms responsible for the control of vascular tone and blood pressure in reptiles. Thus, the decerebrate animal model is a method used to remove possible undesirable effects of anesthesia in the experimental protocols, reducing to the maximum the number of variables that may affect the conclusions about regulatory mechanisms.

Therefore, due to the importance of the adrenergic agonists for the regulation of the cardiovascular system in reptiles, we hypothesized that adrenergic stimulation will be able to affect vascular resistance on the systemic and pulmonary circuits and so, increase relative G_{pul} in rattlesnakes. However, when capable to provide proper autonomic responses, such tendence of increasing L-R shunt will be compensated by pulmonary circulatory control, in order to maintain shunt balance. Observing that compensation would provide interesting information on the relative capacity of both systems to influence cardiac shunt, on the compensatory time of hemodynamic alterations. So, we suppose that sympathetic control of cardiac shunt can change Net-Shunt after alterations on metabolic. When any disturbance changes the balance between systemic and pulmonary resistances without demanding alterations in tissue perfusion, autonomic adjustments will be able to compensate the alteration and maintain Net-Shunt stable despite the systemic resistance. Thus, the aim of the present study was to investigate the role and functional relevance of adrenergic stimulation on the regulation of resting vascular tone on the systemic and pulmonary circuits of rattlesnakes. In this study, we use a decerebrated animal model that permits to obtaining several hemodynamic parameters without the deletery effect of anesthesia over autonomic mechanisms.

2. MATERIALS AND METHODS

2.1 Experimental animals

For this study, we used eight South American rattlesnakes, *Crotalus durissus* (Linnaeus, 1758), of both sexes (852 ± 40g) (Fig. 2 of CHAPTER II). Each animal was kept in individual compartments (44 x 50 x 43 cm), in a temperature-controlled environment (28 ± 2^{o}C), 12:12 light: dark cycle. Snakes were fed on mice to saciety every two weeks and *ad libitum* supply of water. Feeding was interrupted 15 days prior to any experimental procedure to avoid the effect of SDA (Andrade et al., 1997). All experiments were performed at room temperature, 25°C. All procedures were approved by the committee on animal use CEUA/UFSCar.

2.2 Anesthesia and euthanasia

For the instrumental procedure, snakes were previously sedated in an increased PCO_2 environment until the loss of righting reflexes and touch sensitivity (Forslid et al., 1986; Wang et al., 1993; Leite et al., 2013; 2014). That allowed safe manipulation for traqueal intubation to mechanical ventilation (5 breaths·min^{-1}; volume: 30 ml·kg^{-1}; SAR-830/P Ventilator) with isoflurane, 5% for induction and 3-2% for anesthesia maintenance. Each snake received lidocaine injection (2% - Pearson, Brazil) at each incision site and injections of antibiotic (Chemitril, 11 mg·kg^{-1} - Chemitec) and anti-inflammatory with analgesic action (Flunixin, 1.1 mg·kg^{-1} - Chemitec). Experimental protocol was performed 24h after surgery for decerebration and instrumentation. This made it possible to obtain data after anesthetic effects were dissipated. Decerebration procedure is inreversible, so, it was considered the euthanasia moment of the animal (Silverman et al., 2005). Anestesia recovery and animal viability after the decerebration procedure applied have been validated previously (Rocha et al., 2019 – *submitted;* Tavares et al., 2019 – *submitted*). After completion of the experimental protocols, each snake received i.v. injections of barbiturate (Tiopental, 100 mg/kg - Pearson, Brazil) followed by hipersaturated K^+ solution until cardiac arrest. The carcases were appropriately conditioned for later disposal by the UFSCar specialized collection service.

2.3 Decerebration

Decerebration process was accomplished by dorsoventral cauterization at the level of the *Tectum* and removal of all nervous structures rostral to it. Removal of these structures, including cerebral cortex and thalamus, eliminates the central processing of any nociception and processing high level of perception (Silverman et al., 2005). For the decerebration procedure, an incision was made in the medial region of the head, allowing access to the skull (subtopic 2.3 and Fig. 3A of CHAPTER II). Using a mini drill bit, a small aperture (approximately 4 mm in diameter) was made in the cranial casing, allowing access to the menings just over the *Tectum*. The menings were opened, superficial arteries were cauterized. That prepared for a transverse section crossing the first third portion of *Tectum* structure to the base of the skull (Fig. 3B-C of CHAPTER II). All the nervous tissue, rostral to the incision was cauterized and removed (Fig. 3D of CHAPTER II). Absorbable gelatin sponge (gelfoam) was placed in the remain cavity to stabilize the remaining nervous tissue and to avoid subsequent bleeding. The purpose of decerebration was to eliminate cortex activation, which is necessary for any normal information integration that leads to perception or any considered kind of awareness (Pickering and Paton, 2006, Dobson and Harris, 2012). The remaining structures

allowed for a euthanized decerebrated animal preparation with functional autonomic modulation (Rocha et al., 2019 – *submitted;* Tavares et al., 2019 – *submitted*).

2.4 Cardiovascular data recording

During the time course of the same anesthetic plane, a ventrolateral incision of approximately 3 - 4 cm, just rostral to the heart position, exposed systemic and pulmonary central vessels. Occlusive cannulation allowed catheters insetion into pulmonary artery and right systemic arch (PE20) through a branch of the unique pulmonary artery and the vertebral artery, respectively. This allowed P_{pul} and P_{sys} recording and i.v. drug injection. Pressure transducers were calibrated against a mercury column.

In addition, flow probes were placed over the left aortic arch and pulmonary artery, in order to record $\dot{Q}_{LAo}$ and $\dot{Q}_{pul}$, respectively (1.5PR – 1.5PR, Transonic Systems). The probes were fixed around the vessels and filled with gel to allow signal transmission. After instrumentation, snakes were positioned in ventral decubitus and the anesthesia terminated. These procedures allowed continuous recording of P_{sys}, P_{pul}, $\dot{Q}_{LAo}$ and $\dot{Q}_{pul}$ using the respective amplifiers and AD recording system (Powerlab, ADInstruments; Transonic). The recording signals provided calculation of MAP_{sys}, MAP_{pul}, f_H, $\dot{Q}_{sys}$, CO, V_{stot}, Net-Shunt, R_{sys}, R_{pul}, besides G_{sys} and G_{pul}.

2.5 Experimental protocol

2.5.1 The role of adrenergic stimulation for hemodynamic adjustments

Injections of α- and β-adrenergic agonists and antagonists were used to investigate how adrenergic stimulation affects cardiovascular parameters. First, the animal received an i.v. injection of 0.9% saline solution. Then, phenylephrine (5 $\mu g \cdot kg^{-1}$) and adrenaline (2 $\mu g \cdot kg^{-1}$) were administered before and after α-adrenergic blockade with a non-selective antagonist, phentolamine (2 $mg \cdot kg^{-1}$). Prior to subsequent injections, the parameters were expected to be stabilized. Subsequently, propranolol (2 $mg \cdot kg^{-1}$), a non-selective antagonist, was administered for β-adrenergic blockade, again, followed by injections of adrenaline (2 $\mu g \cdot kg^{-1}$) and phenylephrine (5 $\mu g \cdot kg^{-1}$). The doses used in the protocol were based on previous dose response tests. We consider appropriate, the minimum dose that elicit higher evidente cardiovascular response.

As observed in previous study, decerebrated rattlesnake presents body movement in response to touch stimulus and to avoid disrupting and/or catheters and probes displacement a

non-depolarizing neuromuscular inhibitor was injected (i.v. injection of pancuronium bromide – 0.01 $mg \cdot kg^{-1}$). All drugs were administered as bolus injections through the vertebral artery catheter. The drugs used in the study were administered in volumes varing between 0.2 – 1.0 $mL \cdot kg^{-1}$, according to the drug. All drugs were purchased from Sigma-Aldrich Brasil Ltda.

2.6 Calculations and statistics

Recordings were analysed for data collection with the Labchart software (ADInstrumets). Systemic and pulmonary mean arterial pressures were calculated from the equation:

$$MAP = 1/3\ (Psystolic) + 2/3\ (Pdiastolic) \quad (1)$$

Heart rate was derived from the P_{sys} signal. $\dot{Q}_{sys}$ was calculated from the $\dot{Q}_{LAo}$, using the correction factor provided by Filogonio (2014):

$$\dot{Q}_{sys} = 2.6 \times \dot{Q}_{LAo}\ \text{(Filogonio et al., 2014)} \quad (2)$$

Cardiac output was calculated as the sum of $\dot{Q}_{sys}$ and $\dot{Q}_{pul}$ per time, and total stroke volume (pulmonary + systemic) was calculated as:

$$V_{stot} = \dot{Q}_{tot} \cdot f_H^{-1}\ \text{(Crossley et al., 1998; Galli et al. 2005b)} \quad (3)$$

Systemic and pulmonary vascular resistance were calculated as:

$$R = P \cdot \dot{Q}^{-1}\ \text{(Lautt, 1989)} \quad (4)$$

Systemic and pulmonary vascular conductances were calculated as:

$$G = \dot{Q} \cdot P^{-1}\ \text{(Lautt, 1989; O'Leary, 1991)} \quad (5)$$

Intracardiac shunting was calculated by the ratio:

$$\dot{Q}_{pul} \cdot \dot{Q}_{sys}^{-1}\ \text{(Hicks, 1994)} \quad (6)$$

After parameters calculations, Grubbs' test was used to detect outlier values in the analyzed groups. Data normality was analised using D'Agostino & Pearson omnibus test. Repeated Measures ANOVA or Friedman test was used to compare the effects of adrenaline and phenylephrine before and after phentolamine and propranolol injection. The Paired t Test or the Wilcoxon matched-pairs signed rank test was used to compare the effects of adrenaline and phenylephrine regarding to its pre-injection, as well as the effects of phentolamine and propranolol. Statistical analyzes were performed with specific designed statistical software, GraphPad Prism 5. All data were presented as mean ± SEM and differences were denoted as significant according 95% confidence level ($P < 0.05$).

3. RESULTS

Adrenaline injection caused a systemic vasoconstriction and so, R_{sys} increases (Fig. 2E). The vasoconstriction was associated with G_{sys} decrease and a consequent increase in MAP_{sys} (Figs. 1-2A-2G and Table 1). f_H, V_{stot}, CO and $\dot{Q}_{sys}$ remained stable (Figs. 2C-3 and Table 1). The only parameter altered after adrenaline injection was MAP_{pul} (Fig. 2B and Table 1), however, other parameters remained constant (Fig. 1 and Table 1). Net R-L shunt demonstrated by $\dot{Q}_{pul} \cdot \dot{Q}_{sys}^{-1}$ ratio (6) was not changed after adrenaline (Fig.3D and Table 1). The maximum vasoconstriction effect of adrenaline begun after one min (1.17 ± 0.13 min) and lasted about two min (2.08 ± 0.45 min).

The effects of phenylephrine injections were similar to those of adrenaline and caused systemic vasoconstriction (Fig. 4E). The vasoconstriction caused R_{sys} increase and this response was associated with G_{sys} reduction and MAP_{sys} increase (Figs. 1-4A-4G and Table 2). f_H, V_{stot}, CO and $\dot{Q}_{sys}$ remained stable (Figs. 4C-5 and Table 2). There were no effects of phenylephrine on the pulmonary circulation (Figs. 1-4 and Table 2). Resting mean net R-L shunt was maintened after phenylephrine injection (Fig. 5D and Table 2). The maximum vasoconstriction effect of phenylephrine begun after 30 s (38 ± 13 s) and lasted less than two min (1.30 ± 0.36 min).

Phentolamine injection reduced R_{sys} and caused a marked systemic vasodilation that was associated with G_{sys} increase and MAP_{sys} decreased (Fig. 6A). As a compensatory response, there were significant rise on f_H (Fig. 6B). Although, V_{stot} and CO remained unchanged (Fig. 6B-6C). Nevertheless, $\dot{Q}_{sys}$ rised after α-adrenergic blockade (Figs. 6B). The only parameter altered after phentolamine was G_{pul}, which was increased (Fig. 6A). There were no observable

effects of phentolamine on the other pulmonary parameters analyzed. Net R-L shunt was not altered after α-adrenergic blockade (Fig. 6C). The maximum vasodilatory effect of phentolamine begun after four min (4.46 ± 0.51 min) and lasted about three min (3.2 ± 0.55 min).

Following treatment with phentolamine, the stimulatory effect of adrenaline was abolished and a systemic vasodilation was observed (Fig. 1). Adrenaline injection reduced the R_{sys} causing G_{sys} increase and MAP_{sys} decrease (Fig. 2A-2E-2G and Table 1). As a compensatory response, $\dot{Q}_{sys}$ rised (Fig. 2C and Table 1). f_H, V_{stot} and CO remained unchanged (Fig. 3 and table 1). However, V_{stot} was increase when compared to the adrenaline control injection (Fig. 3B). There were no effects of adrenaline on the pulmonary circulation (Figs 1-2 and Table 1). Net R-L shunt was not changed after blockade of α-adrenergic receptors (Fig. 3D and Table 1).

After α-adrenergic blockade with phentolamine, the effect of phenylephrine was also abolished. There were no effects on the analyzed cardiovascular parameters (Table 2). When we compare the injection of phenylephrine after phentolamine with the results of control injection, we observe that R_{sys} decreased, G_{sys} increased, and we identify a rise in V_{stot} and so, the CO and $\dot{Q}_{sys}$ increased (Figs. 4E-4G-5B-5C). Net R-L shunt was not altered following injection of phenylephrine after phentolamine (Fig. 5D and Table 2).

β-adrenergic blockade with propranolol caused marked bradycardia, with consequent CO and $\dot{Q}_{sys}$ reduction (Figs. 1-6E). However, V_{stot}, G_{sys} and MAP_{sys} remained unchanged (Fig. 6D-6F). To avoid a systemic hypotension and to maintain the G_{sys}, there were R_{sys} increase, causing increase of peripheral vascular resistance and consequent systemic vasoconstriction (Fig. 6D). There were similar responses on the pulmonary circulation. We observed R_{pul} increase, but G_{pul} and MAP_{pul} was not altered (Fig. 6D). CO decreased, causing $\dot{Q}_{pul}$ reduction after propranolol injection (Figs. 1-6E). Net R-L shunt was not altered after propranolol (Fig. 6F). The maximum bradycardia occurred after ten minute of propranolol injection (10.7 ± 2.73 min) and this effect remained until the next injection (33.25 ± 4.32 min).

Following concomitant α and β-adrenergic blockade, adrenaline caused systemic vasoconstriction similar to that the one observed after adrenaline control injection. This vasoconstriction was due R_{sys} increase (Fig. 2E and Table 1). This effect was associated with MAP_{sys} increased and $\dot{Q}_{sys}$ reduction. However, G_{sys} remained unchanged (Figs. 1-2A-2C-2G and Table 1). f_H, CO and V_{stot} was not altered (Table 1). When we compared the control injection of adrenaline with injection of adrenaline after phentolamine, we observed a significant

decreased of f_H (Fig. 3A). And, when we compared the injection of adrenaline after phentolamine with injection of adrenaline after phentolamine and propranolol, we found that there was decrease of CO, with consequent reduction of $\dot{Q}_{sys}$ (Figs. 2C-3C). There were no effects of adrenaline on pulmonary circulation (Fig. 1 and Table 1). As well as observed with control injection of adrenaline, the net R-L shunt was not changed after α and β-adrenergic blockade (Fig. 3D and Table 1).

After treatment with phentolamine and propranolol, the vasoconstrictive effect of phenylephrine was similar to phenylephrine control injection. Phenylephrine increased R_{sys} and this effect was associated with G_{sys} decrease and MAP_{sys} increase (Figs. 1-4A-4E-4G and Table 2). As a compensatory response, there f_H decreased, although this response was not different of the one after phenylephrine control injection (Figs. 1-5A and table 2). V_{stot} and CO remained unchanged, and was $\dot{Q}_{sys}$ reduced (Figs. 1-4C-5B-5C and Table 2). MAP_{pul} was the only parameter altered (2.22 ± 0.31 to 2.44 ± 0.35 KPa), which increased following phenylephrine injection after α and β-adrenergic blockade (Fig. 4B). However, this response was not different from the one following phenylephrine control injection. There were no effects of phenylephrine on the other pulmonary parameters analyzed. (Figs. 1-4 and Table 2). Net R-L shunt was not modified (Fig. 5D and Table 2) following phenylephrine injection after treatment with phentolamine and propranolol.

4. DISCUSSION

4.1 Limitations of the study

Pancuroniun bromide is a non-depolarising muscle relaxant and it has been shown to be a potent neuromuscular blocker with minimal ganglion-blocking and histamine releasing properties (Buckett et al., 1968). *In situ* studies on cat preparations showed that pancuronium have some effect on reducing the cardiac effect of electrical stimulation of the vagus nerve. But, it did not block the response to ACh injection. This effect suggested that pancuronium acted stabilising the post-synaptic membrane (Bonta et al., 1968). Studies on dogs and guinea-pigs demonstrated that the carbachol and ACh depressor effect on blood pressure was reduced, while their negative chronotropic and inotropic effects were completely blocked by pancuronium. These results indicate that pancuronium specifically block cardiac cholinergic receptors (Saxena and Bonta, 1970). So, we would expect f_H and CO were less affected after atropine injection. In fact, Kelman and Kennedy (1971) demonstrated pancuronium alters the cardiac cholinergic receptor. According to Booij and coworkers (1980), the pancuronium increased f_H,

P_{pul}, CO and decreased systemic vascular response. In our study, f_H increased in 34% indicating some level of cholinergic blockade. Our findings are consistent with the description of pancuronio effect in humans (Kelman and Kennedy, 1971) and dogs (Booij et al., 1980). Although, we found no effects on blood pressure. Besides, we observed that f_H retornted to baseline before our experimental protocol, around 27 beats·min^{-1}. Also, we also observed clear cardiovascular responses after adrenergic agonists and antagonists injections. Hence, it is safe to assume effective autonomic compensation after pancuronium injection and maintenance of capacity for autonomic regulation of the hemodynamic parameters studied.

4.2 The role of adrenergic stimulation on systemic circulation

Systemic vasoconstriction caused by adrenaline and phenylephrine was reversed after phentolamine treatment, what demonstrate that this response is mediated by α-adrenergic receptors. Furthermore, the phentolamine caused an expressive systemic vasodilatation and consequent rise of 252% on G_{sys}, indicating the presence an important α-adrenergic vascular tone for R_{sys} and P_{sys} control in resting rattlesnake. These results were similar to those the reported in anesthetized rattlesnakes (Skals et al., 2005; Galli et al., 2007) and turtles (Overgaard et al., 2002), although the increase on G_{sys} in anesthetized rattlesnakes was of lower magnitude, with about 129% change. The α-agonist phenylephrine caused similar responses to adrenaline, but less pronounced and without affecting MAP_{sys}. The less pronounced effects of phenylephrine on systemic vasculature in *Crotalus* could be related to a suposed lower α-adrenergic receptor affinity, as suggested by Skals and coworkers (2005). This aspect demonstrates that both branches for baroreflex adjustments are relevant for maintenance of MAP in the non-anesthetized animal.

Another important point is the expectation that there would be no change in MAP with the injection of adrenaline after blockade of α-adrenergic receptors. However, a reduction in blood pressure was observed and this response was associated with the interaction of adrenaline with β-adrenergic receptors. The vasodilatory effect promoted by the injection of adrenaline after phentolamine may be attributed by the activation of vascular β_2-receptors, responsible for triggering reduction in peripheral resistance and consequent P_{sys} reduction, similar to founded in studies with dogs, in which was observed significant decrease in MAP after β_2-adrenergic-mediated effect of adrenaline (Vaknin et al., 2001). The presence of adrenergic innervation in vessels of Squamata, such as *Elaphe obsoleta* and *Crotalus viridis* have also already been demonstrated by histochemical studies (Donald and Lillywhite, 1988; Lillywhite and Donald, 1994). In addition, studies on *Trachemys scripta* and *Python regius* observed constriction of

systemic vasculature in response to α-adrenergic receptors activation, and vasodilation when the β-adrenergic receptors were stimulated (Wang et al., 2000; Hicks and Farrell, 2000; Overgaard et al., 2002), giving greater support to the importance of adrenergic stimulation for the vascular adjustments in reptiles.

In anesthetized rattlesnakes, propranolol injection caused G_{sys} and MAP_{sys} reduction, demonstrating a basal β-adrenergic tone on systemic circulation (Galli et al., 2007). However, in our study, there were no effect on G_{sys} or MAP_{sys}, and so, we can suggest that α-receptors are more efficiently than β-receptors to control systemic vascular tone in *C. durissus*. Such differences between the two studies can be explained by the effects of anesthesia on vascular tone control in this specie. In the study with anesthetized rattlesnakes, the anesthetic may have affected autonomic mechanisms, and therefore, the sympathetic regulation may not have been sufficient to maintain blood pressure. Another explanation for these effects may be related to baroreceptors. According to Seagard et al. (1983), the baroreflex response of anesthetized animals is affected by isoflurane, making it a mechanism unable to compensate for changes in blood pressure. Similarly, anesthesia may increase perfusion of the pulmonary circuit (Taylor et al., 2009) and this effect could reduce the blood volume of the systemic circuit and change the conductance in this circuit. According Galli and collaborators (2007), the injection of propranolol promoted bradycardia, triggering a decrease in CO and, consequently, $\dot{Q}_{sys}$ reduction. However, in our study, this change in CO was off set by increased vascular resistance, maintaining vascular conductance and blood pressure constant. This response further emphasizes the importance of α-adrenergic receptors on controlling blood pressure in *Crotalus*, demonstrating that the sympathetic tone of the peripheral vasculature was determinant to maintain the conductance and to ensure the tissue perfusion, even when the cardiac control was absent.

In other snake species, such as *Bitis arietans*, *Python regius* and *Boa constrictor*, the chronotropic effect of adrenaline was abolished by propranolol (Hedberg and Nilsson, 1975; Wang et al., 2000, 2001a). Galli and collaborators observed that phentolamine also abolished the tachycardia caused by adrenaline, and this effect was attributed by maximal rate in response to fall on the blood pressure promoted by α-adrenergic receptor antagonist (Galli et al., 2007). Nevertheless, in our study, adrenaline injection did not evoke significantly alteration in $f_{H,}$ not even after treatments with phentolamine or propranolol. However, following concomitant α and β-adrenergic blockade there was a small but significant bradycardia with the injection of phenylephrine. And, this effect may be associated with the indirect activation of the α_2-receptors

responsible for reducing the presynaptic release of noradrenaline in the synaptic cleft, leading to a decrease in f_H (Murrell and Hellebrekers, 2005).

The heart of reptiles is innervated by adrenergic fibres that promote rise in contraction force and f_H through of the activation of β-receptors present in the pace-maker region, atria and ventricle (Hedberg and Nilsson, 1975, Van Harn et al., 1973; Morris and Nilsson, 1994). However, the sympathetic adrenergic innervation seems less efficiently to control blood pressure via β-receptors. Thus, we suggest that, in *Crotalus*, adrenergic stimulation cause higher vascular effect than cardiac alterations. And so, the vascular branch of the baroreflex mechanism was functional and it ables to compensate the MAP reduction promoted by phentolamine, increasing the f_H. This evidence shows that the decerebrate rattlesnake model is an interesting alternative to demonstrate the role of autonomic functions in cardiovascular control by preserving higher capacity for autonomic regulation. In addition, the blockade of α-receptors with phentolamine leads to increase the release of noradrenaline (Starke et al., 1971), and it potentiates the reflex tachycardia promoted by the decrease of MAP, emphasizing the compensatory response capacity of the resting rattlesnake.

Similar to Galli et al., (2007) and Skals et al. (2005) reported, we observed no V_{stot} or $\dot{Q}_{sys}$ changes after adrenaline and phenylephrine. V_{stot} is determined by difference between end-diastolic and end-systolic volumes, as well as by cardiac filling, contractility and afterload (Skals et al., 2005). As observed in mammals (Guyton, 1955) and reptiles (Enok et al., 2016), there is a relationship between MCFP, cardiac filling and V_{stot} in rattlesnakes (Skals et al., 2005). Lack of adrenaline effect on the V_{stot} in rattlesnake was also observed in fish (Zhang et al., 1998), and one possible explanation for this response would be the alterations in afterload and venous resistance, since this result can be related with balance between venous constriction and higher afterload (Skals et al., 2005). Galli et al. (2007) attributed the maintenance of V_{stot} with increased of contractility, once propranolol was able to reduce V_{stot}. However, in our study, we did not observed V_{stot} alteration after treatment with propranolol, and so, it seems more plausible that V_{stot} maintenance after adrenergic stimulation may be related to venous constriction and afterload increased. Skals et al. (2005) also demonstrated that venous resistance and P_{cv} increased in response to α-adrenergic stimulation in rattlesnake, and this result could explain why adrenaline and phenylephrine did not alter V_{stot} after phentolamine. The effects of adrenergic stimulation on MCFP and venous resistance in *Crotalus* show that this specie has a pronounced adrenergic regulation of the venous system and, so, it is able to control the V_{stot} (Skals et al., 2005). This find is consistent with previous studies with isolated central veins from the ratsnake, *Elaphe obsoleta quadrivittata*, which was contracted in response to adrenaline

(Conklin et al., 1996). Thus, at rest, increased venous tone is unable to alter V_{stot} and, therefore, the increase of sympathetic activity maybe exert a more significant influence on venous return, in situations where increased metabolism is required, such as exercise, digestion, or temperature increase (Skals et al., 2005).

4.3 The role of adrenergic stimulation on pulmonary circulation

In contrast to consistent effect of adrenergic stimulation on systemic circulation, we found that pulmonary vasculature is less responsive to adrenaline or phynephrine. That is similar to what was observed in anesthetized rattlesnakes (Galli et al., 2007) and turtles (Overgaard et al., 2002). On the other hand, the phentolamine injection leaded to G_{pul} increase, demonstrating that α-adrenergic receptors are also important to control of pulmonary vascular tone in rattlesnakes. These findings oposes what was previously reported by Galli et al. (2007). Propranolol treatment, however, did not altered G_{pul}, indicating β-adrenergic receptors are required only to maintain tonic relaxation, similar to previous studies (Galli et al., 2007; Overgaard et al. 2002). In turtles, there was no significant constriction of pulmonary vasculature when α-adrenergic receptors were stimulated (Overgaard et al., 2002), and this effect was similar in *Crotalus*, since adrenaline did not cause R_{pul} alteration. β-adrenergic receptors caused pulmonary vasodilatation when it was stimulated in *Trachydosaurus rugosus* and *Trachemys scripta* (Berger, 1972; Overgaard et al., 2002). This effect was confirmed on the rattlesnake, in which there was a vasoconstrictor response following propranolol injection.

The phenylephrine caused responses similar to adrenaline injection, but no effect on MAP_{pul}. Similarly, in *Chrysemys scripta* and *Chelodina longicollis*, the injection of phenylephrine did not promote changes on pulmonary circulation (Milsom et al., 1977; Berger, 1972). Squamates' lack of adrenergic agonists response on pulmonary circulation is conflictant with the extensive adrenergic innervation present on that vasculature (McLean and Burnstock, 1967; Donald and Lillywhite, 1988; Donald et al., 1990b). Nevertheless, an important point to be mentioned is that the presence of receptors and ability to respond to the action of a particular substance does not necessarily mean that the animal will always respond with the same intensity. A clear example of this phenomenon is that the rattlesnake responds differently to the effects of numerous chemical mediators. Several studies demonstrated the pulmonary circulation is less responsive to several substances, such as neuropeptide gamma, bradykinin, NO, and NOS inhibitor (Crossley et al., 2000; Galli et al., 2005a,b; Skovgaard et al., 2005a,b). These reports show that to ensure local regulation and so, in order to maintain adequate ventilation and perfusion, sympathetic activation is not enough efficient to provide the proper

hemodynamic adjustments and, therefore, that parasympathetic control of pulmonary artery might be the main source of pulmonary hemodynamic regulation (Galli et al., 2007).

Whenever is necessary to increase metabolic demand, the tissue vascular control open vascular beds increasing G_{sys}. The rise in $\dot{Q}_{sys}$ is provided by autonomic regulation of heart function to maintain homeostasia. However, in reptiles, arterial oxygen deliver may also be increased through a reduction in net R-L shunt without alteration in ventilation and/or cardiac work (Wang et al., 2001b; Wang and Hicks, 1996). In anesthetized rattlesnakes, adrenaline injection rised $\dot{Q}_{pul} \cdot \dot{Q}_{sys}^{-1}$ ratio. Although, in our experiment, G_{pul} and Net-Shunt remained stable, demonstrating that shunt control can be dynamic enough to override alterations in systemic circulation hemodynamics. The role of blood flows control in maintaining of Net-Shunt is an interesting matter and deserve dedicated experiments. In our findings, we observed still that phenylephrine injection did not affect the net R-L shunt, and this result was similar to the one reported by Skals and coworkers (2005). The pulmonary vasculature may be tonically stimulated by adrenergic agonists, nevertheless, changes in conductance and, therefore, shunt patterns, are mainly controlated by vagal control of the pulmonary artery (Galli et al., 2007; Taylor et al.; 2009; Leite et al., 2013, 2014; Filogonio et al., 2016). Thus, it seems that the parasympathetic regulation of the cardiovascular system of *Crotalus* is more effective than the sympathetic regulation for the control of intracardiac shunting and maintenance of arterial oxygen content on pulmonary circuit (Wang et al., 2001a,b; Taylor et al., 2009; Leite et al., 2013, 2014).

5. CONCLUSIONS

We concluded that, in rattlesnakes, the adrenergic stimulation has an important role for local regulation of vascular tone on the systemic circulation. Pulmonary vasculature is less reactive to adrenaline and phenylephrine, demonstrating that systemic circulation is the main target to peripheral conductance adjustments at rest. Resting tone of systemic circuit is maintained rather by α-adrenergic stimulation and have higher influence in maintain MAP_{sys} than adjustments of cardiac work. Also, β-receptors have no role for maintenance of resting hemodynamics. In addition, the lack of change in G_{pul} and Net-Shunt demonstrate that parasympathetic regulation of the cardiovascular system is more effective than the sympathetic regulation for intracardiac shunting control in *Crotalus*. Besides, as previously observed, the decerebrated rattlesnake model is an usefull alternative for studies that rely on ANS functionality. The use of decerebrate animals confer greater safety and advantage compared to the use of anesthetized animals, since several responses were different or of magnitudes greater

than those observed in studies with anesthetized rattlesnakes. Thus, decerebration has shown to be a great alternative for studies involving autonomic mechanisms, emphasizing its importance and possible implications on area of evolutionary physiology.

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8. FIGURES AND LEGENDS

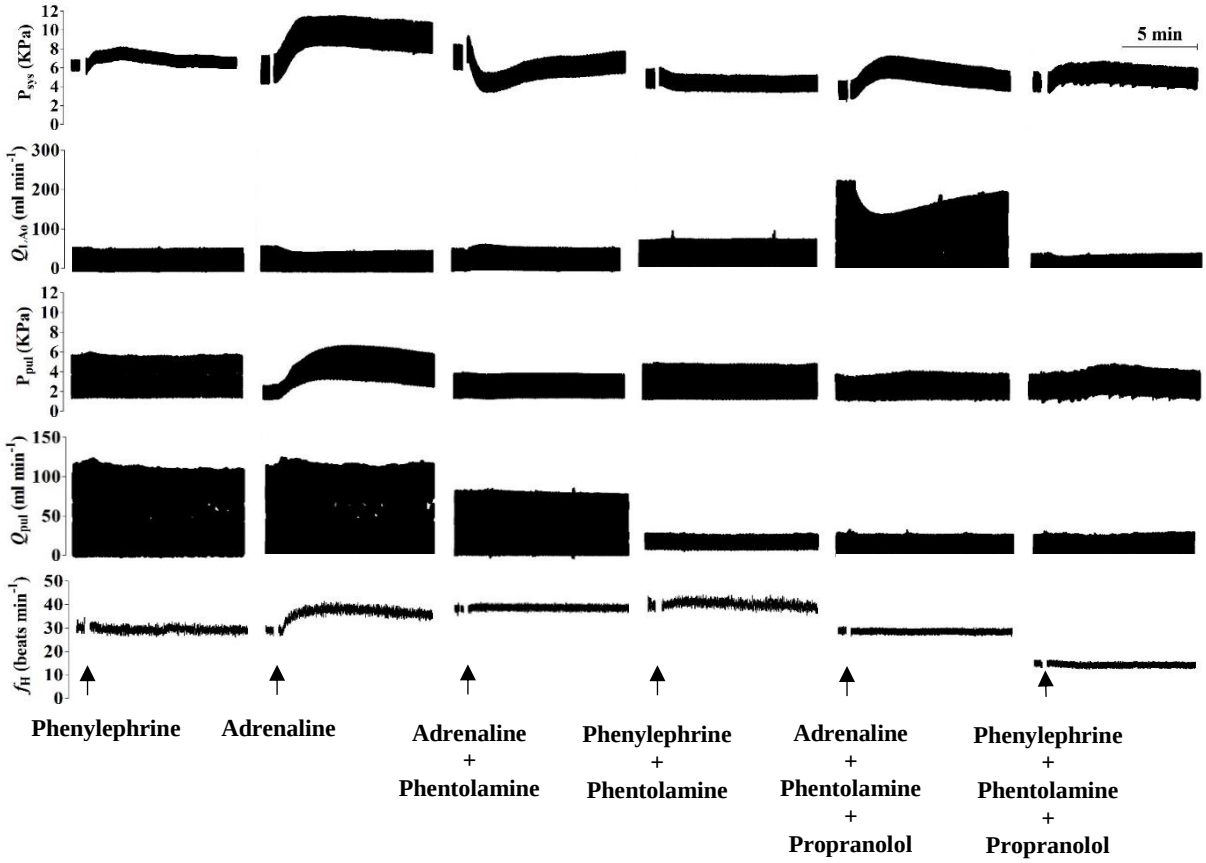

Fig. 1. Original traces demonstrating the cardiovascular effects of adrenergic agonists and antagonists injections in *Crotalus durissus*. Phenylephrine (5 μg·kg^{-1}); adrenaline (2 μg·kg^{-1}); phentolamine (2 mg·kg^{-1}) and propranolol (2 mg·kg^{-1}). Systemic arterial pressure - P_{sys} (KPa); left aortic arch blood flow - $\dot{Q}_{LAo}$ (ml·min^{-1}); pulmonary arterial pressure – P_{pul} (KPa); pulmonary blood flow - $\dot{Q}_{pul}$ (ml·min^{-1}); heart rate - f_H (beats·min^{-1}). Arrows indicate time of injection.

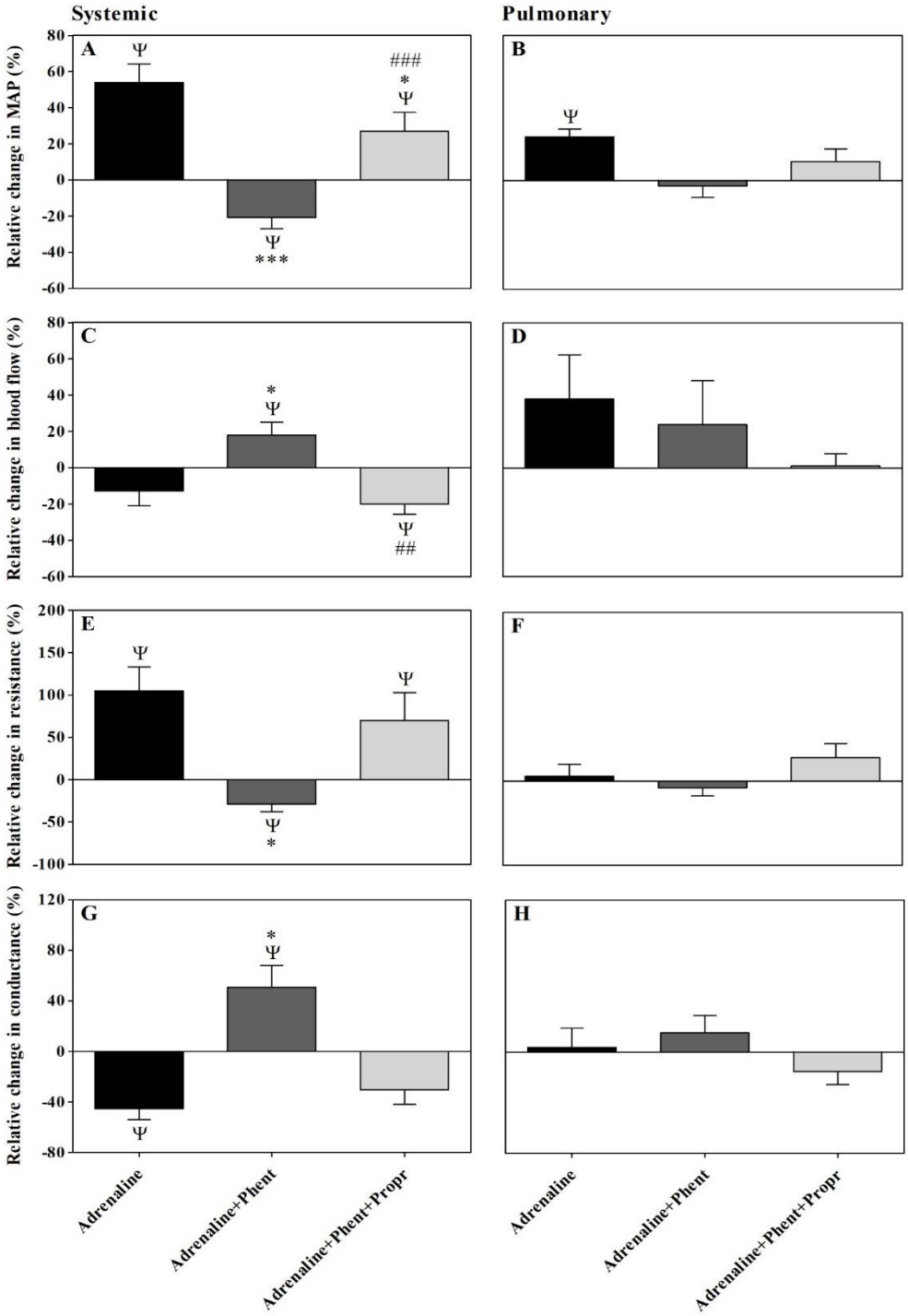

Fig. 2. Relative change (%) on haemodynamic variables following injection of adrenaline (2 µg·kg^{-1}); adrenaline after phentolamine (2 mg·kg^{-1}); adrenaline after phentolamine and propranolol, (2 mg·kg^{-1}). Systemic (A) and pulmonary (B) mean arterial pressures, MAP; systemic (C) and pulmonary blood flow (D); systemic (E) and pulmonary (F) vascular resistance; systemic (G) and pulmonary (H) vascular conductance. In the systemic circulation: MAP_{sys}, n = 8, $\dot{Q}_{sys}$, n = 7; R_{sys} and G_{sys}, n = 6. In the pulmonary circulation: MAP_{pul}, R_{pul} and G_{pul}, n = 6; $\dot{Q}_{pul}$, n = 7. Values are mean with S.E.M. $^{\Psi}$ Denotes difference against pre-injection value ($P < 0.05$); * denotes difference against adrenaline control injection and $^{\#}$ denotes difference against adrenaline after phentolamine and propranolol injection. Repeated Measures ANOVA or Friedman test ($P < 0.05$)

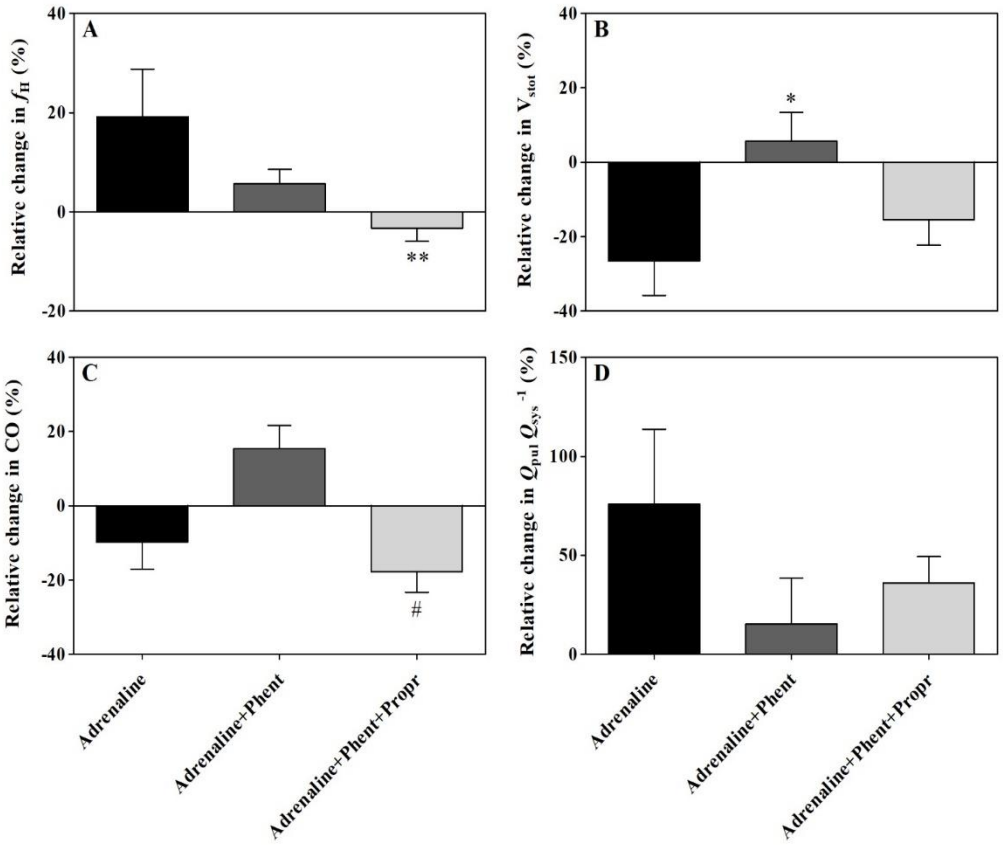

Fig. 3. Relative changes (%) on cardiac pumpimg following injection of adrenaline (2 µg·kg^{-1}); adrenaline after phentolamine (2 mg·kg^{-1}); adrenaline after phentolamine and propranolol, (2 mg·kg^{-1}). Heart rate, f_H (A); total stroke volume, V_{stot} (B); cardiac output, CO (C); Net-Shunt, $\dot{Q}_{pul}\cdot\dot{Q}_{sys}^{-1}$ (D). f_H, n = 8; CO, n = 7; V_{stot} and $\dot{Q}_{pul}\cdot\dot{Q}_{sys}^{-1}$, n = 6. Values are mean with S.E.M. * Denotes difference against adrenaline control injection and # denotes difference against adrenaline injection after phentolamine - Friedman test. ($P < 0.05$)

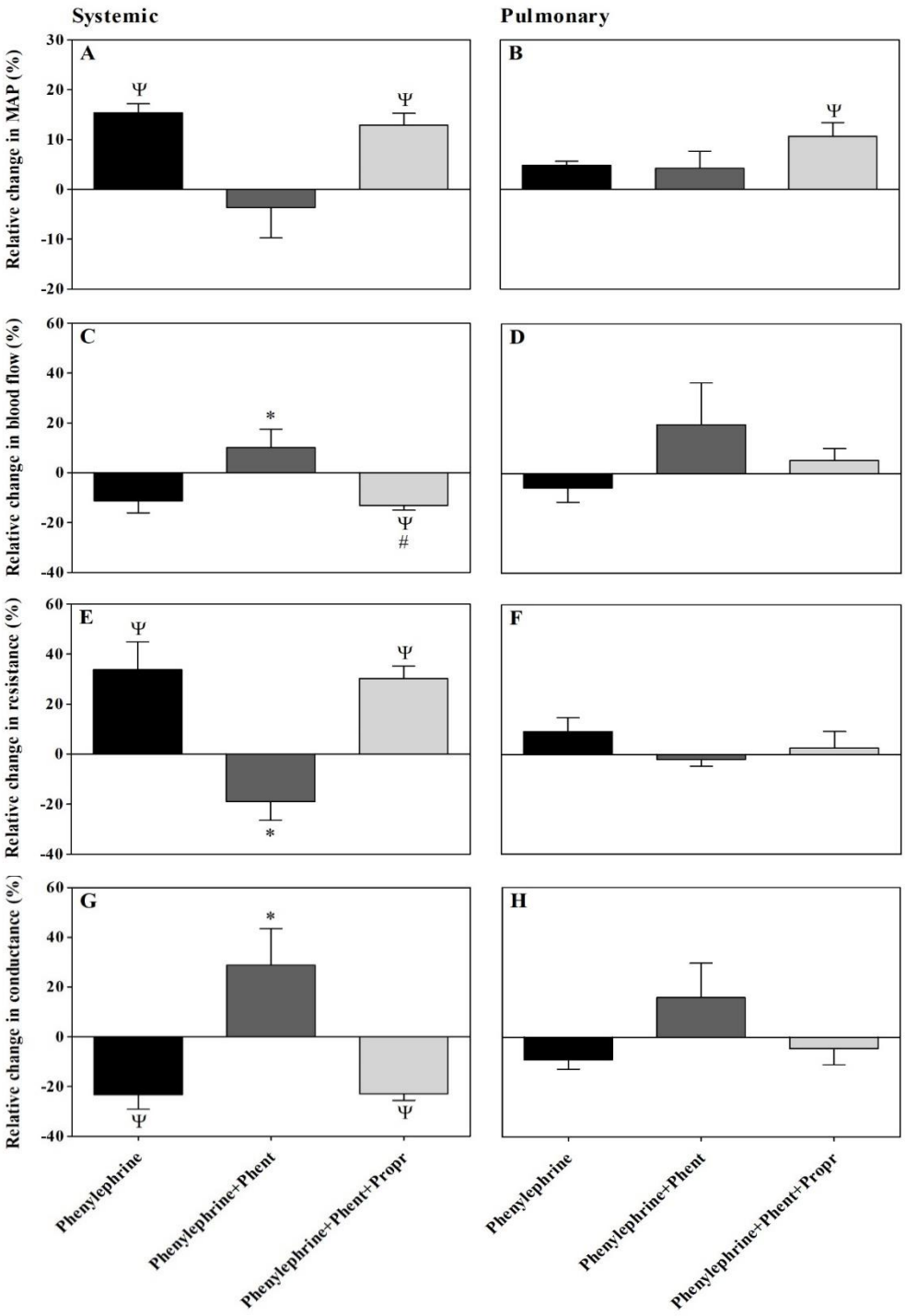

Fig. 4. Relative changes (%) on haemodynamic variables following injection of phenylephrine (5 µg·kg^{-1}); phenylephrine after phentolamine (2 mg·kg^{-1}); phenylephrine after phentolamine and propranolol, (2 mg·kg^{-1}). Systemic (A) and pulmonary (B) mean arterial pressures, MAP; systemic (C) and pulmonary blood flow (D); systemic (E) and pulmonary (F) vascular resistance; systemic (G) and pulmonary (H) vascular conductance. In the systemic circulation: MAP_{sys}, n = 7, $\dot{Q}_{sys}$, n = 6; R_{sys} and G_{sys}, n = 5. In the pulmonary circulation: MAP_{pul} and R_{pul}, n = 5; $\dot{Q}_{pul}$ and G_{pul}, n = 6. Values are mean with S.E.M. $^{\Psi}$ Denotes difference against pre-injection value; * denotes difference against phenylephrine control injection; and $^{\#}$ denotes difference against phenylephrine injection after phentolamine - Friedman test ($P < 0.05$).

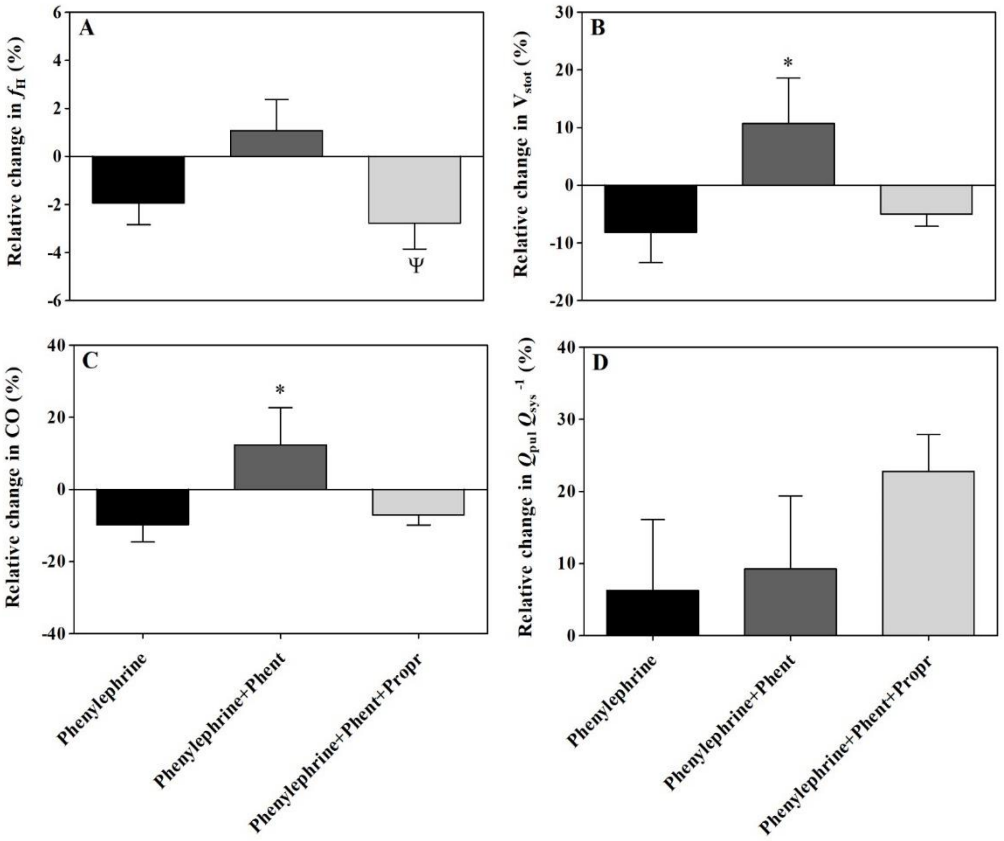

Fig. 5. Relative changes (%) on cardiac pumping following injection of phenylephrine (5 µg·kg^{-1}); phenylephrine after phentolamine (2 mg·kg^{-1}); phenylephrine after phentolamine and propranolol, (2 mg·kg^{-1}). Heart rate, f_H (A); total stroke volume, V_{stot} (B); cardiac output, CO (C); Net-Shunt, $\dot{Q}_{pul}\cdot\dot{Q}_{sys}^{-1}$ (D). f_H, n = 7; V_{stot}, CO and $\dot{Q}_{pul}\cdot\dot{Q}_{sys}^{-1}$, n = 5. Values are mean with S.E.M. $^{\Psi}$ Denotes difference against pre-injection value ($P < 0.05$) and * denotes difference against phenylephrine control injection - Friedman test ($P < 0.05$).

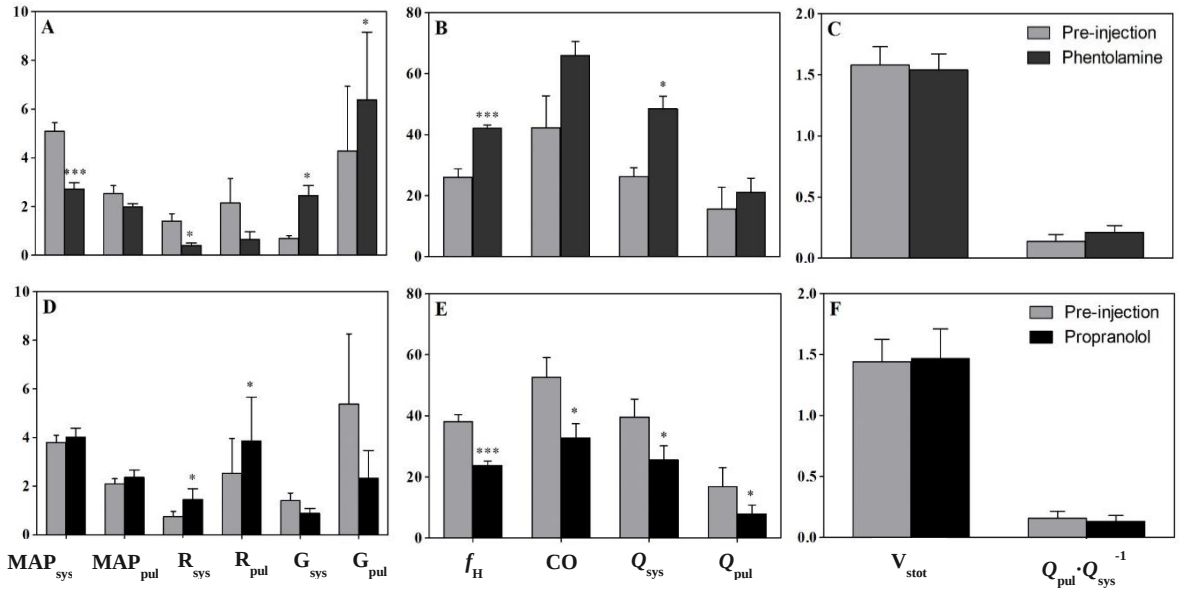

Fig. 6. Effects of bolus injections of phentolamine (A, B and C: 2 mg·kg^{-1}) and propranolol (D, E and F: 2 mg·kg^{-1}) in South American rattlesnakes, *Crotalus durissus*. MAP_{sys} - systemic mean arterial pressure (KPa); MAP_{pul} - pulmonary mean arterial pressure (KPa); R_{sys} - systemic resistance (kPa·ml^{-1}·min^{-1}·kg^{-1}); R_{pul} - pulmonary resistance (kPa·ml^{-1}·min^{-1}·kg^{-1}); G_{sys} - systemic conductance (ml·min^{-1}·kg^{-1}·KPa^{-1}); G_{pul} - pulmonary conductance (ml·min^{-1}·kg^{-1}·KPa^{-1}); f_H - heart rate (beats·min^{-1}); CO - cardiac output (ml·min^{-1}·kg^{-1}); $\dot{Q}_{sys}$ - systemic flow (ml·min^{-1}·kg^{-1}); $\dot{Q}_{pul}$ - pulmonary flow (ml·min^{-1}·kg^{-1}); V_{stot} - total stroke volume (ml·kg^{-1}) and $\dot{Q}_{pul}\cdot\dot{Q}_{sys}^{-1}$ - Net-Shunt. Light grey bars represent control values (pre-injection); dark grey bars represent values after injection of phentolamine; black bars represent values after propranolol. Phentolamine - in the systemic circulation: MAP_{sys}, n = 8; R_{sys}, n = 7; $\dot{Q}_{sys}$ and G_{sys}, n = 6. In the pulmonary circulation: MAP_{pul} and $\dot{Q}_{pul}$, n = 7; R_{pul} and G_{pul}, n = 6. f_H, n = 8; V_{stot}, CO and $\dot{Q}_{pul}\cdot\dot{Q}_{sys}^{-1}$, n = 5. Propranolol - in the systemic circulation: MAP_{sys}, n = 8; $\dot{Q}_{sys}$, R_{sys} and G_{sys}, n = 6. In the pulmonary circulation: MAP_{pul} and $\dot{Q}_{pul}$, n = 7; R_{pul} and G_{pul}, n = 6. f_H, n = 8; CO, n = 6; V_{stot} and $\dot{Q}_{pul}\cdot\dot{Q}_{sys}^{-1}$, n = 5. * Denotes differences against pre-injection values - Paired t test or Wilcoxon matched-pairs signed rank test ($P < 0.05$).

9. TABLES AND LEGENDS

Table 1: Haemodynamic variables in rattlesnakes, *C. durissus*, following adrenaline injection, before and after phentolamine and propranolol

Variables	Pre-injection	Adrenaline	Pre-injection	Adrenaline + Phentolamine	Pre-injection	Adrenaline + Phentolamine + Propranolol
MAP_{sys} (KPa)	5.07 ± 0.27	7.66 ± 0.33**	4.03 ± 0.43	3.08 ± 0.23*	4.20 ± 0.37	5.18 ± 0.40*
$\dot{Q}_{sys}$ ($ml \cdot min^{-1} \cdot kg^{-1}$)	27.72 ± 2.85	24.28 ± 3.02	40.74 ± 5.75	46.88 ± 4.25*	17.58 ± 1.58	14.52 ± 1.05*
R_{sys} ($kPa \cdot ml^{-1} \cdot min^{-1} \cdot kg^{-1}$)	1.33 ± 0.27	2.22 ± 0.45*	0.80 ± 0.22	0.44 ± 0.08*	1.78 ± 0.36	2.43 ± 0.40*
G_{sys} ($ml \cdot min^{-1} \cdot kg^{-1} \cdot KPa^{-1}$)	0.72 ± 0.10	0.43 ± 0.06*	1.41 ± 0.30	2.21 ± 0.30*	0.53 ± 0.08	0.37 ± 0.02
MAP_{pul} (KPa)	2.50 ± 0.24	3.31 ± 0.30*	2.03 ± 0.16	2.08 ± 0.23	2.02 ± 0.17	2.47 ± 0.27
$\dot{Q}_{pul}$ ($ml \cdot min^{-1} \cdot kg^{-1}$)	15.03 ± 6.21	16.72 ± 6.47	18.43 ± 6.68	18.20 ± 5.01	6.08 ± 1.62	5.78 ± 1.17
R_{pul} ($kPa \cdot ml^{-1} \cdot min^{-1} \cdot kg^{-1}$)	2.30 ± 0.90	2.25 ± 0.84	2.32 ± 1.33	2.36 ± 1.43	2.23 ± 1.07	3.10 ± 1.45
G_{pul} ($ml \cdot min^{-1} \cdot kg^{-1} \cdot KPa^{-1}$)	1.68 ± 1.15	1.32 ± 0.72	6.04 ± 3.00	5.90 ± 2.73	1.31 ± 0.67	1.28 ± 0.70
f_H ($beats \cdot min^{-1}$)	26.41 ± 2.48	30.50 ± 2.08	40.46 ± 1.90	42.42 ± 1.21	19.51 ± 1.84	18.92 ± 1.92
V_{stot} ($ml \cdot kg^{-1}$)	1.55 ± 0.14	1.24 ± 0.20	1.33 ± 0.18	1.40 ± 0.10	1.42 ± 0.20	1.30 ± 0.17
CO ($ml \cdot min^{-1} \cdot kg^{-1}$)	40.31 ± 8.06	36.83 ± 8.08	55.25 ± 6.25	61.03 ± 4.00	26.91 ± 3.94	23.46 ± 3.90
$\dot{Q}_{pul} \cdot \dot{Q}_{sys}^{-1}$	0,15 ± 0.05	0.22 ± 0.07	0.17 ± 0.06	0.16 ± 0.06	0.17 ± 0.06	0.24 ± 0.08

Absolute values are mean ± S.E.M. of adrenaline injection before and after injection of phentolamine and propranolol. MAP_{sys} - systemic mean arterial pressure; $\dot{Q}_{sys}$ - systemic flow; R_{sys} - systemic resistance; G_{sys} - systemic conductance; MAP_{pul} - pulmonary mean arterial pressure; $\dot{Q}_{pul}$ - pulmonary flow; R_{pul} - pulmonary resistance; G_{pul} - pulmonary conductance; f_H - heart rate; V_{stot} - total stroke volume; CO - cardiac output and $\dot{Q}_{pul} \cdot \dot{Q}_{sys}^{-1}$ - Net-Shunt. In the systemic circulation: MAP_{sys}, n = 8, $\dot{Q}_{sys}$, n = 7; R_{sys} and G_{sys}, n = 6. In the pulmonary circulation: MAP_{pul}, R_{pul} and G_{pul}, n = 6; $\dot{Q}_{pul}$, n = 7. * Denotes differences against pre-injection values ($P < 0.05$) - Paired t test or Wilcoxon matched-pairs signed rank test ($P < 0.05$).

Table 2: Cardiovascular parameters in rattlesnakes, *C. durissus*, following phenylephrine injection, before and after phentolamine and propranolol

Parameters	Pre-injection	Phenylephrine	Pre-injection	Phenylephrine + Phentolamine	Pre-injection	Phenylephrine + Phentolamine + Propranolol
MAP_{sys} (KPa)	5.28 ± 0.21	6.10 ± 0.30*	4.10 ± 0.43	3.87 ± 0.34	4.47 ± 0.27	5.03 ± 0.27*
$\dot{Q}_{sys}$ ($ml \cdot min^{-1} \cdot kg^{-1}$)	27.70 ± 2.78	24.50 ± 2.74	41.95 ± 5.92	44.92 ± 4.94	14.71 ± 1.25	12.80 ± 1.18*
R_{sys} ($kPa \cdot ml^{-1} \cdot min^{-1} \cdot kg^{-1}$)	1.52 ± 0.20	1.98 ± 0.26*	0.88 ± 0.18	0.74 ± 0.14	2.46 ± 0.24	3.15 ± 0.30*
G_{sys} ($ml \cdot min^{-1} \cdot kg^{-1} \cdot KPa^{-1}$)	0.71 ± 0.08	0.54 ± 0.05*	1.41 ± 0.30	1.58 ± 0.25	0.42 ± 0.04	0.34 ± 0.03*
MAP_{pul} (KPa)	2.34 ± 0.32	2.40 ± 0.34	2.00 ± 0.20	2.05 ± 0.20	2.22 ± 0.31	2.44 ± 0.35*
$\dot{Q}_{pul}$ ($ml \cdot min^{-1} \cdot kg^{-1}$)	19.00 ± 8.18	18.84 ± 8.37	19.66 ± 7.20	20.83 ± 6.80	5.18 ± 1.00	5.36 ± 1.00
R_{pul} ($kPa \cdot ml^{-1} \cdot min^{-1} \cdot kg^{-1}$)	1.22 ± 0.73	1.26 ± 0.71	1.34 ± 1.10	1.25 ± 1.00	2.30 ± 1.26	2.50 ± 1.42
G_{pul} ($ml \cdot min^{-1} \cdot kg^{-1} \cdot KPa^{-1}$)	5.40 ± 3.12	5.07 ± 3.03	6.78 ± 3.35	6.88 ± 3.34	3.42 ± 1.94	3.45 ± 2.00
f_H ($beats \cdot min^{-1}$)	27.80 ± 2.57	27.22 ± 2.50	40.40 ± 1.76	40.78 ± 1.60	17.85 ± 2.33	17.37 ± 2.33*
V_{stot} ($ml \cdot kg^{-1}$)	1.46 ± 0.17	1.34 ± 0.16	1.41 ± 0.17	1.52 ± 0.14	1.45 ± 0.23	1.37 ± 0.22
CO ($ml \cdot min^{-1} \cdot kg^{-1}$)	42.67 ± 8.35	39.01 ± 8.84	57.06 ± 7.10	61.86 ± 4.64	24.37 ± 3.43	22.87 ± 3.83
$\dot{Q}_{pul} \cdot \dot{Q}_{sys}^{-1}$	0.55 ± 0.37	0.60 ± 0.38	0.56 ± 0.38	0.54 ± 0.34	0.81 ± 0.53	0.98 ± 0.63

Absolute values are mean ± S.E.M. of phenylephrine injection before and after injection of phentolamine and propranolol. MAP_{sys} - systemic mean arterial pressure; $\dot{Q}_{sys}$ - systemic flow; R_{sys} - systemic resistance; G_{sys} - systemic conductance; MAP_{pul} - pulmonary mean arterial pressure; $\dot{Q}_{pul}$ - pulmonary flow; R_{pul} - pulmonary resistance; G_{pul} - pulmonary conductance; f_H - heart rate; V_{stot} - total stroke volume; CO - cardiac output and $\dot{Q}_{pul} \cdot \dot{Q}_{sys}^{-1}$ - Net-Shunt. In the systemic circulation: MAP_{sys}, n = 7, $\dot{Q}_{sys}$, n = 6; R_{sys} and G_{sys}, n = 5. In the pulmonary circulation: MAP_{pul} and R_{pul}, n = 5; $\dot{Q}_{pul}$ and G_{pul}, n = 6. * Denotes differences against pre-injection values - Wilcoxon test ($P < 0.05$).