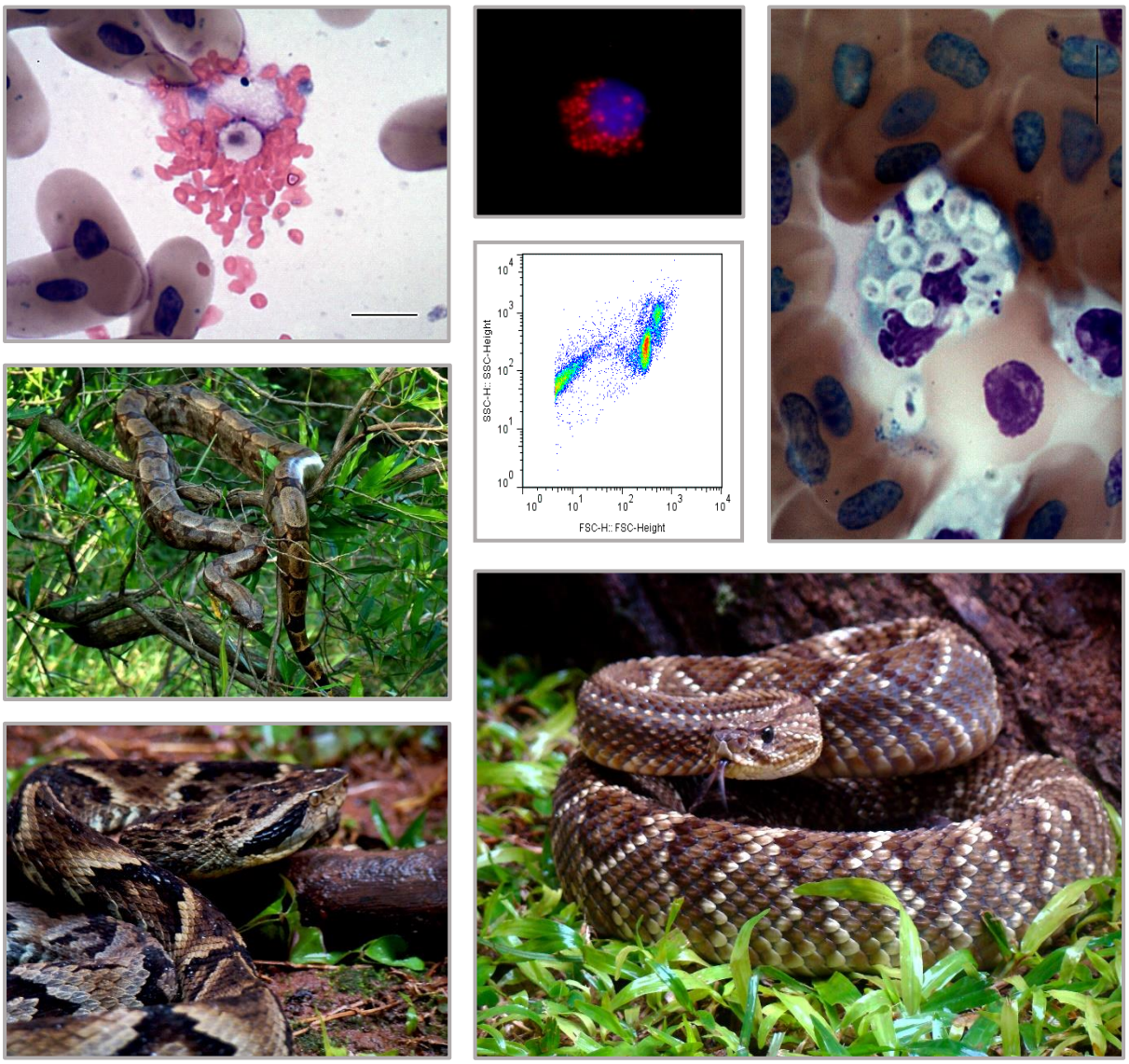
UNIVERSIDADE DE SÃO PAULO
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

MARCELO PIRES NOGUEIRA DE CARVALHO

Functional analysis of peripheral blood leucocytes of snakes (Boidae and Viperidae) during the process of adaptation to captivity



São Paulo
2018

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Functional analysis of peripheral blood leucocytes of snakes (Boidae and Viperidae) during the process of adaptation to captivity

Thesis submitted to the Postgraduate Program in Comparative and Experimental Pathology of the School of Veterinary Medicine and Animal Science of the University of São Paulo to obtain the Doctor's degree in Sciences

Department:
Pathology

Area:
Comparative and Experimental Pathology

Advisor:
Prof. José Luiz Catão-Dias, Ph.D.

São Paulo
2018

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Área de concentração: Patologia Experimental e Comparada.
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São Paulo, 16 de outubro de 2014

CERTIFICADO

Certificamos que o Projeto intitulado "Análises funcional e imunofenotípica de leucócitos de sangue periférico de serpentes neotropicais durante o processo de adaptação ao cativeiro", protocolado sob o CEUA nº 8322070714, utilizando 90 Répteis (45 machos e 45 femeas), sob a responsabilidade de José Luiz Catão Dias, foi aprovado na reunião de 15/10/2014, e está de acordo com os princípios éticos de experimentação animal da Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo.

We certify that the Research "Phenotypic and functional analysis of neotropical snakes peripheral blood leukocytes during the process of adaptation to captivity", protocol number CEUA 8322070714, utilizing 90 Reptiles (45 males and 45 females), under the responsibility José Luiz Catão Dias, was approved in the meeting of day 10/15/2014, and agree with Ethical Principles in Animal Research adopted by Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science of the University of São Paulo.

Atenciosamente,

Profa. Dra. Denise Tabacchi Fantoni
Presidente da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia
Universidade de São Paulo

São Paulo, 14 de março de 2018
CEUA N 8322070714

Ilmo(a). Sr(a).
Responsável: José Luiz Catão Dias
Área: 0
José Luiz Catão Dias (orientador)

Título da proposta: "Análise funcional de leucócitos de sangue periférico de serpentes (Boidae, Elapidae e Viperidae) durante o processo de adaptação ao cativeiro".

Parecer Consubstanciado da Comissão de Ética no Uso de Animais FMVZ/USP

A Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, no cumprimento das suas atribuições, analisou e **APROVOU** a Notificação (versão de 20/fevereiro/2018) da proposta acima referenciada.

Resumo apresentado pelo pesquisador: "Segundo a secretaria da CEUA, não consegui e não conseguirei atualizar os dados do meu projeto de pesquisa pelo sistema Orion devido a uma atualização programada pelo programador do sistema. Estou no aguardo desta atualização do sistema Orion desde o dia 11 de dezembro de 2017. Visto que até o momento a atualização não foi realizada, faço minha solicitação por meio desta notificação. O motivo da solicitação se refere a formação de um grupo controle necessário para validação dos dados já coletados. Os próximos experimentos são simples e facilmente exequíveis dentro do prazo proposto pelo cronograma já aprovado. Ainda, mando em anexo autorização sisbio, vinculada ao projeto de pesquisa, contemplando os indivíduos e espécies abaixo citados. Solicito inclusão de 15 machos da espécie Bothrops jararaca Solicito inclusão de 15 fêmeas da espécie Bothrops jararaca Solicito inclusão de 15 machos da espécie Crotalus durissus Solicito inclusão de 15 fêmeas da espécie Crotalus durissus Solicito inclusão de 15 machos da espécie Naja kaouthia Solicito inclusão de 15 fêmeas da espécie Naja kaouthia".

Comentário da CEUA: "Inclusão de grupo controle devidamente justificada.".

Profa. Dra. Anneliese de Souza Traldi
Presidente da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Profa. Dra. Claudia Madalena Cabrera Mori
Vice-Presidente da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

São Paulo, 02 de maio de 2018
CEUA N 8322070714

Ilmo(a). Sr(a).
Responsável: José Luiz Catão Dias
Área: 0
José Luiz Catão Dias (orientador)

Título da proposta: "Análise funcional de leucócitos de sangue periférico de serpentes (Boidae e Viperidae) durante o processo de adaptação ao cativeiro".

Parecer Consubstanciado da Comissão de Ética no Uso de Animais FMVZ/USP

A Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, no cumprimento das suas atribuições, analisou e **APROVOU** a Alteração do cadastro (versão de 02/maio/2018) da proposta acima referenciada.

Resumo apresentado pelo pesquisador: "Solicito alteração de título por não incluir na tese nenhuma espécie de serpente pertencente à família Elapidae.".

Comentário da CEUA: "Alteração de título aprovada.".

Profa. Dra. Anneliese de Souza Traldi
Presidente da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Profa. Dra. Claudia Madalena Cabrera Mori
Vice-Presidente da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Ministério do Meio Ambiente - MMA
Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio
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Autorização para atividades com finalidade científica

Número: 44990-10	Data da Emissão: 02/05/2018 14:15	Data para Revalidação*: 01/06/2019

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Dados do titular

Nome: MARCELO PIRES NOGUEIRA DE CARVALHO	CPF: 333.781.038-17
Título do Projeto: ANÁLISE FUNCIONAL DE LEUCÓCITOS DE SANGUE PERIFÉRICO DE SERPENTES (Boidae e Viperidae) DURANTE O PROCESSO DE ADAPTAÇÃO AO CATIVEIRO	
Nome da Instituição : Faculdade de Medicina Veterinária e Zootecnia USP	CNPJ: 63.025.530/0019-33

Cronograma de atividades

#	Descrição da atividade	Início (mês/ano)	Fim (mês/ano)
1	Coleta de sangue de serpentes em cativeiro, mantidas junto ao biotério de serpentes do Butantan.	07/2014	07/2018

Observações e ressalvas

1	As atividades de campo exercidas por pessoa natural ou jurídica estrangeira, em todo o território nacional, que impliquem o deslocamento de recursos humanos e materiais, tendo por objeto coletar dados, materiais, espécimes biológicos e minerais, peças integrantes da cultura nativa e cultura popular, presente e passada, obtidos por meio de recursos e técnicas que se destinem ao estudo, à difusão ou à pesquisa, estão sujeitas a autorização do Ministério de Ciência e Tecnologia.
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Outras ressalvas

1	Todas as serpentes a serem utilizadas na pesquisa pertencem ao plantel do Laboratório de Herpetologia do Instituto Butantan.

Equipe

#	Nome	Função	CPF	Doc. Identidade	Nacionalidade
1	KATHLEEN FERNANDES GREGO	colaboradora	851.044.347-53	067170985 IFP-RJ	Brasileira
2	José Luiz Catão Dias	colaborador	029.597.888-00	8914397-8 SSP-SP-SP	Brasileira
3	SÁVIO STEFANINI SANT'ANNA	colaborador	130.023.398-22	9813932-0 SSP-SP	Brasileira

Locais onde as atividades de campo serão executadas

#	Município	UF	Descrição do local	Tipo
1	SAO PAULO	SP	Instituto Butantan	Fora de UC Federal

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Nome da Instituição : Faculdade de Medicina Veterinária e Zootecnia USP	CNPJ: 63.025.530/0019-33

Atividades X Táxons

#	Atividade	Táxons
1	Coleta/transporte de amostras biológicas ex situ	Crotalus durissus, Bothrops jararaca, Naja kaouthia, Boa constrictor
2	Manutenção temporária (até 24 meses) de vertebrados silvestres em cativeiro	Boa constrictor

Material e métodos

1	Amostras biológicas (Répteis)	Sangue
2	Método de captura/coleta (Répteis)	Outros métodos de captura/coleta(NÃ£o haverÃ¡ coleta de animais em natureza)
3	Método de marcação (Répteis)	Outros métodos de marcação(NÃ£o haverÃ¡ nenhum tipo de marcaÃ§Ã£o)

Destino do material biológico coletado

#	Nome local destino	Tipo Destino
1	INSTITUTO BUTANTAN	
2	Faculdade de Medicina Veterinária e Zootecnia USP	

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Registro de coleta imprevista de material biológico

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Táxon*	Qtde.	Tipo de amostra	Qtde.	Data

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* Identificar o espécime no nível taxonômico possível.

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EVALUATION FORM

Author: CARVALHO, Marcelo Pires Nogueira de

Title: **Functional analysis of peripheral blood leucocytes of snakes (Boidae and Viperidae) during the process of adaptation to captivity**

Thesis submitted to the Postgraduate Program in Comparative and Experimental Pathology of the School of Veterinary Medicine and Animal Science of the University of São Paulo to obtain the Doctor's degree in Sciences

Date:_____/_____/_____

Committee Members

Prof._____

Institution: _____Decision: _____

Prof._____

Institution: _____Decision: _____

Prof._____

Institution: _____Decision: _____

Prof._____

Institution: _____Decision: _____

Prof._____

Institution: _____Decision: _____

To my family and friends.

ACKNOWLEDGEMENTS

To my advisor, Professor José Luiz Catão Dias, for whom I have great admiration and respect, for allowing this work to be carried out, for your constant encouragement in the research field, for the exceptional orientation, for your generosity, and for your example as a professional and as a person.

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To my dear ones, Pedro and Priscilla, for understanding my absence sometimes, and for your essential support. You will always be my greatest incentive in life.

To my awesome family, parents, sister, uncles, cousins, and grandparents, for the family structure that you gave me.

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This work was jointly developed with the Laboratory of Wildlife Comparative Pathology (LAPCOM), Department of Pathology - FMZ - USP and with the Laboratory of Herpetology, Butantan Institute.

RESUMO

CARVALHO, M. P. N. **Análise funcional de leucócitos de sangue periférico de serpentes (Boidae e Viperidae) durante o processo de adaptação ao cativeiro.** [Functional analysis of peripheral blood leucocytes of snakes (Boidae and Viperidae) during the process of adaptation to captivity]. 2018. 122 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2018.

O processo inflamatório é conhecido por proteger os vertebrados de lesões e infecções. No entanto, sob uma perspectiva imunológica, as funções dos leucócitos no processo inflamatório das serpentes são pouco compreendidas. Neste contexto, a caracterização leucocitária nestes animais ainda não está definida, havendo discordância na literatura sobre os tipos celulares existentes e suas classificações. Devido à grande variação citoquímica apresentada por estas células, uma análise focada apenas na morfologia óptica é insuficiente para determinar os diferentes tipos celulares. Assim, o empego de métodos mais avançados, como a citometria de fluxo, são importantes para o melhor entendimento da função e da origem de cada tipo celular. Em particular, estudos sobre o estresse crônico induzido pelo cativeiro em répteis demonstram que níveis séricos de corticosterona aumentam durante o período de adaptação, e estão associados à imunossupressão; no entanto, ainda não está claro como as funções leucocitárias são alteradas nestas condições. Neste cenário, o objetivo do presente estudo foi pesquisar gradientes de densidade (ficoll e percoll) para a recuperação de leucócitos provenientes do sangue periférico de serpentes, e caracterizar, por meio da citometria de fluxo, as células recuperadas com base no tamanho, presença de grânulos e complexidade interna (organelas), e em suas funções celulares inatas (*burst* oxidativo e fagocitose) frente a desafios bacterianos, fúngicos e químicos, possibilitando, desta forma, a avaliação qualitativa e quantitativa das funções apresentadas pelos leucócitos constituintes do sistema imunológico de três serpentes neotropicais, *Boa constrictor*, *Bothrops jararaca* e *Crotalus durissus*. Ainda, o estudo propôs correlacionar o desempenho destas funções com as concentrações séricas de corticosterona em viperídeos (*B. jararaca* e *C. durissus*) recém capturados da natureza, em dois momentos de adaptação ao cativeiro (após cinco e 60 dias de cativeiro). A comparação entre os dois métodos de recuperação leucocitária (ficoll e percoll) não apresentou diferença estatística em relação aos tipos e proporção dos leucócitos recuperados do sangue periférico de serpentes de mesma espécie. Nas três espécies, após análise por citometria de fluxo, as populações leucocitárias

obtidas nos citogramas foram identificadas mediante procedimento de *sorting* celular, com posterior análise por microscopia óptica, sendo compostas por azurófilos, heterófilos, linfócitos grandes e linfócitos pequenos. Em relação às funções leucocitárias, heterófilos, linfócitos e azurófilos apresentaram capacidade de fagocitose nas três espécies. Com relação ao *burst* oxidativo, apenas os azurófilos apresentaram uma atividade significativa em relação aos respectivos valores basais para as espécies investigadas. Na fase de adaptação ao cativeiro, a atividade fagocítica dos heterófilos de *B. jararaca* mostrou correlação positiva com os níveis séricos de corticosterona. Entretanto, a porcentagem de fagocitose apresentada pelos azurófilos e a quantidade de partículas ingeridas pelos linfócitos apresentaram correlações negativas. Para *C. durissus*, a porcentagem de fagocitose dos linfócitos também apresentou correlação negativa com os níveis de corticosterona. Em ambas as espécies, não houve correlação entre a atividade de *burst* oxidativo e as concentrações séricas de corticosterona. Acreditamos que os dados gerados nesta pesquisa contribuirão para o desenvolvimento de novos testes diagnósticos, análises filogenéticas, estudos ecotoxicológicos e ecoimunológicos, além de auxiliar no manejo clínico-sanitário de serpentes em cativeiro.

Palavras-chave: *Burst* oxidativo. Citometria de fluxo. Corticosterona. Fagocitose. Serpentes.

ABSTRACT

CARVALHO, M. P. N. **Functional analysis of peripheral blood leucocytes of snakes (Boidae and Viperidae) during the process of adaptation to captivity.** [Análise funcional de leucócitos de sangue periférico de serpentes (Boidae e Viperidae) durante o processo de adaptação ao cativeiro]. 2018. 122 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2018.

Inflammatory processes are known to protect vertebrates from injuries and infections. However, from an immunological perspective the role of leukocytes in snakes' inflammatory process is poorly understood. Within this context, leukocyte classification in these animals is not clearly defined, with authors disagreeing on existent cell types and their classification. Due to the great variation of snakes' leukocytes on cytochemistry, an analysis focused exclusively on optical morphology is insufficient to determine different cell types. Thus, additional methods, as flow cytometry, are important to better understand the function and origin of each cell type. In particular, studies of chronic stress induced by captivity in reptiles demonstrate that serum corticosterone levels rise during the adaptation period and are associated with immune suppression. However, it is not known how the leukocyte functions of reptiles are altered under these conditions. The objective of this research was to adapt leukocyte density gradients (ficoll and percoll) for snake blood samples, and characterize recovered cells based on size, presence of granules and internal complexity (organelle), and on their cellular innate functions (oxidative burst and phagocytosis), in front of bacterial, fungal and chemical challenges, by flow cytometry; enabling the qualitative and quantitative assessment of innate activities presented by cells constituting the immune system of *Boa constrictor*, *Bothrops jararaca* and *Crotalus durissus*. Additionally, the study proposes to correlate innate leukocyte functions performances with serum corticosterone concentrations, in recent wild-caught viperids *(B. jararaca* and *C. durissus*), at two moments of adaptation to captivity (5 and 60 days of captivity). Comparison between gradient methods for leukocyte isolation did not show any statistical difference in types and proportion of leukocytes' populations between snakes of the same species. When verified by means of flow cytometric cell sorting and confirmed by optical microscopy, populations were mainly composed of azurophils, heterophils, large lymphocytes and small lymphocytes. Concerning innate leukocyte functions, heterophils, lymphocytes and azurophils were involved in the phagocytosis response. Regarding oxidative burst activity, only azurophils presented a

significative and strong oxidative burst when compared to their respective baseline. During adaptation to captivity process, *B. jararaca* heterophils phagocytic activity showed a positive correlation with serum corticosterone levels. However, the percentage of phagocytosis presented by azurophils and the quantity of particles ingested by lymphocytes, presented a negative correlation with serum corticosterone concentrations. For *C. durissus*, phagocytosis displayed by lymphocytes also presented a negative correlation with serum corticosterone levels. In both viper's species, oxidative burst activity showed no correlation with serum corticosterone concentrations. We believe that the data generated in this research will contribute to the development of new diagnostic tests, phylogenetic analyzes, ecotoxicological and ecoimmunological studies, as well as assisting in the clinic-sanitary management of snakes in captivity.

Keywords: Corticosterone. Flow cytometry. Oxidative burst. Phagocytosis. Snakes.

SUMMARY

1 GENERAL INTRODUCTION

Inflammation is fundamentally an organic defense mechanism, whose final benefit is the elimination of the initial cause of cellular lesion and the consequences of this lesion (KUMAR et al., 2005). The inflammatory response consists of two main components: a vascular and a cellular reaction (KUMAR et al., 2005); and a critical function of the cellular response is the leukocytes migration to the lesion and their subsequent activation to perform the host defense functions (KUMAR et al., 2005).

The available literature currently describes hematological studies in some species of reptiles, but the classification and function of leukocyte subpopulations is controversial (TUCUNDUVA; BORELLI; SILVA, 2001). In addition, most of the available data is based on results from a small number of individuals (TUCUNDUVA; BORELLI; SILVA, 2001). In this context, the phenotypic and functional classification of leukocytes in snakes is still not clearly defined, and there is no consensus among authors regarding existing cell types, their nomenclature and functions (SAINT GIRONS, 1970; MATEO et al., 1984; WOOD; EBANKS, 1984; SYPIK; BORYSENKO, 1988; KNOTKOVA et al., 2002; CLAVER; QUAGLIA, 2009; PARIDA; DUTTA; PAL, 2014).

Some authors differentiate snakes' granulocytes into three cell groups - eosinophils, azurophils and neutrophils - while others in only two - eosinophils and heterophils or eosinophils and neutrophils (SAINT GIRONS, 1970; MATEO et al., 1984; WOOD; EBANKS, 1984; SYPEK; BORYSENKO, 1988; KNOTKOVA et al., 2002). Also, despite the general classification, the presence of eosinophils in the peripheral blood of snakes is questionable (ROSSKOPF Jr., 2000), mainly because this cell type is not evidenced in most species and has been described only in *Ophiophagus hannah* and *Naja naja* (SALAKIJ et al., 2002; PARIDA; DUTTA; PAL, 2014). Azurophils also present divergences in their classification, for some authors these cells represent only immature forms of monocytes (CAMPBELL, ELLIS, 2007), while for others they comprise a cell with unique characteristics and functions, present in high numbers in the peripheral blood of snakes (HAWKEY; DENNET, 1989; ALLEMAN et al., 1992; RASKIN, 2000).

In addition to non-consensual leukocyte classification, the study of the immune response in reptile species has become important for understanding the evolution of B cells throughout vertebrate classes, since evidence for a developmental relationship between B cells and macrophages has led to the hypothesis that B cells evolved from a phagocytic predecessor

(ZIMMERMAN et al., 2010). The recent identification of phagocytic IgM+ cells in fishes and amphibians supports this hypothesis, but raises the question of when, evolutionarily, was phagocytic capacity lost in B cells. Since reptiles are the only amniotic ectothermic vertebrates and represent the evolutionary link between non-amniotic (fish and amphibians) and homeothermic amniotic vertebrates (birds and mammals) reptiles possibly comprehend the last vertebrate class to present phagocytic B cells (ZIMMERMAN et al., 2009). In this context, a better understanding of reptile species immunology may provide important insights into the evolution and functions of the vertebrate immune system (ZIMMERMAN et al., 2010).

Due to the large morphological and cytochemical variation of snakes' leukocytes, an analysis focused only at the morphology becomes insufficient to determine the different cell types. Therefore, the need for complementary studies with improved methodologies, such as flow cytometry, is required for a better understanding of the function and characterization of each leukocyte type (ZANOTTI, 2007).

Flow cytometry is a cell analysis methodology that allows the simultaneous evaluation of several cellular characteristics. By means of this technique the cells are individually evaluated as they pass, in liquid medium, by one or more bundles of incident lights (lasers). The main cellular aspects that influence the frontal and lateral dispersions of the laser are: shape, membrane characteristics, size, presence of granules and internal complexity (organelles). Through the use of antibodies and fluorochromes, characteristics such as the identification of membrane and intracellular proteins, functional aspects and the cycle of cell proliferation can also be evaluated. These characteristics make flow cytometry a unique method and a valuable work tool in research and diagnosis areas (JANEWAY et al., 2000; AFFONSO, 2006).

In particular, wild animals kept in artificial environments are confronted with a range of potentially stressful changes, including abiotic environmental sources such as artificial lights, exposure to sound disturbances, repellent odors, and inappropriate temperatures and substrates (MORGAN; TROMBORG, 2007). In conclusion, studies of chronic stress induced by captivity in reptiles demonstrate that the process of adaptation to captive conditions promotes an elevation of serum corticosterone levels, which is associated with reproductive failure and immune suppression (GUILLET; CREE; ROONEY, 1995; GREGO; 2006). In chronically stressed individuals, the risk of developing infectious diseases increases due to immune suppression (GUILLET; CREE; ROONEY, 1995; GREGO, 2006;), but it is not known how leukocytes functions are affected under these conditions.

In this sense, the present study purposes were to optimize density gradient leukocyte isolation protocols for snake blood, and to establish flow cytometry parameters defining size

and complexity to gate and characterize different leukocyte series in *Boa constrictor*, *Bothrops jararaca*, and *Crotalus durissus*. From there on, access qualitative and quantitatively the innate cellular activities (phagocytosis and oxidative burst) presented by the leukocytes' subsets isolated from the peripheral blood of these snakes. Furthermore, since reptile glucocorticoids have been shown to increase in front of environmental changes with consequent alteration of innate leukocyte functions, as phagocytosis and oxidative burst (RODRÍGUEZ et al., 2001), and to promote variations in the number and proportion of circulating leukocytes (MCKAY; CIDLOWSKI 2003; GOESSLING et al., 2015), the study also aimed to report innate cellular functions (phagocytosis and oxidative burst) and haematological profiles (differential leukocyte count and heterophil: lymphocyte ratios) interactions with serum corticosterone levels in recent wild-caught viperids, at two moments of adaption to captivity (at 5 and 60 days of captivity).

The results of this thesis are expected to contribute to the morphological and functional classification, as well as to the evolutionary analysis of the leukocytes of vertebrates. Nevertheless, the correlation of serum corticosterone levels with leukocyte's subpopulations functional performances and distribution in the peripheral blood, may contribute to better comprehend how environmental change stress can affects leukocyte functions and haematological parameters in snakes. The present study results may also assist in the sanitary management of snakes in captivity, providing another tool in the clinical evaluation of these animals and collaborating with researches in the ecoimmunology and ecotoxicology fields.

2 FLOW CYTOMETRIC CHARACTERIZATION OF PERIPHERAL BLOOD LEUKOCYTE POPULATIONS OF 3 NEOTROPICAL SNAKE SPECIES: *Boa constrictor, Bothrops jararaca,* AND *Crotalus durissus*

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2.1 ABSTRACT

Background: The reptilian immune system is represented by innate, humoral, and cell-mediated mechanisms, involving different types of blood leukocytes. The development of optimized methods for the advanced study of origin and function of reptilian blood leukocytes is needed.

Objectives: The purpose of the study was to optimize leukocyte density gradient isolation protocols from snake peripheral blood samples and characterize recovered cells by flow cytometry based on size and internal complexity for a qualitative and semi-quantitative assessment of leukocyte populations in one boa (*Boa constrictor*), and 2 viper species (*Bothrops jararaca, Crotalus durissus*).

Methods: Blood samples from 30 snakes (10 from each species, 5 males and 5 females) were collected in tubes with sodium heparin. Fresh blood was centrifuged with either ficoll-paque PLUS or percoll density gradients for leukocyte isolation. Flow cytometric leukocyte gates were defined based on size (forward scatter [FSC]) and internal complexity (side scatter [SSC]). Relative leukocyte differential counts after sorting the cells in these gates in one snake for each species were compared to conventional light microscopic differential counts on unsorted isolated leukocytes.

Results: There was no statistical difference in the relative leukocyte populations, including heterophils, azurophils, and small and large lymphocytes between samples isolated by ficoll or percoll. Four leukocyte gates were identified based on their location in FSC/SSC cytograms. The relative leukocyte differential counts after sorting in single animals showed some agreement with the light microscopy differential count on unsorted cells.
Conclusions: Based on FSC and SSC, 4 distinct leukocyte populations were found in ficoll or percoll density gradient isolated leukocytes from peripheral blood from boa and viper species. Further optimization of the technique should allow the performance of functional assays.

Keywords: Ficoll. Forward scatter. Side Scatter. Hematology. Immune System. Percoll.

2.2 INTRODUCTION

Inflammatory processes are known to protect vertebrates from injuries and infections (TUCUNDUVA; BORELLI; SILVA, 2001); however, from an immunologic perspective, the role of granulocytic and mononuclear leukocytes in snakes' inflammatory processes is poorly understood. In particular, leukocyte classification in these species is not yet clearly defined, with authors disagreeing on existing cell types and their classification (KNOTKOVA et al., 2002; JACOBSON, 2007). Published data on reptilian leukocyte classification are proposing 6 cell series - azurophilic, basophilic, and eosinophilic granulocytes, monocytes, and small and large lymphocytes (KNOTKOVA et al., 2002; JACOBSON, 2007). However, the presence of eosinophils in the peripheral blood of snakes has been debated (ROSSKOPF, 2000), especially because their existence has not been convincingly demonstrated in most species, except in king cobras (*Ophiophagus hannah*) (SALAKIJ et al., 2002). Likewise, azurophils represent another leukocyte type that has been differentiated as an immature monocyte by some authors (CAMPBELL; ELLIS, 2007), while others consider azurophils as cells with unique characteristics and roles, present only in reptiles, and found in lesser numbers in the blood of lizards, chelonians, and crocodilians, but in greater numbers in snakes (HAWKEY; DENNET, 1989; ALLEMAN; JACOBSON; RASKIN, 1992; RASKIN, 2000).

Despite of wide morphologic and cytochemical leukocyte variations in snakes, analysis based on light microscopic morphology on conventionally stained blood smears is generally

sufficient to determine different cell types. However, advanced studies are required to understand cellular functions and thus better characterize each leukocyte population (ZANOTTI, 2007). The present study purpose was to optimize density gradient leukocyte isolation protocols from snake blood, and to establish flow cytometry parameters defining size and complexity to gate and characterize different leukocyte series in *Boa constrictor*, *Bothrops jararaca*, and *Crotalus durissus*.

2.3 MATERIAL AND METHODS

2.3.1 Animals

The present study was approved by the Butantan Institute Ethics Committee for Animal Use, registry number 1105/13, by the School of Veterinary Medicine and Animal Science, University of São Paulo, Ethics Committee on Animals Use (Registry Number 8322070714), and by SISBIO (Authorization System and Information in Biodiversity) license 44990. Adult male and female snakes of the Herpetology Laboratory, Butantan Institute, Brazil, were included in the study. They were housed in acclimatized standard rooms with defined lighting cycles, at temperatures between approximately 25°C and 27°C, and 60% relative humidity. The snakes were kept in individual plastic containers (60 x 40 x 15 cm) with a corrugated fiberboard substrate and tap water *ad libitum* supplied by a plastic water fountain drinker.

Blood samples from 30 snakes assumed clinically healthy based on feeding routine, behavior, successful shedding, and body score were collected on 3 consecutive days (10 individuals per day) and analyzed immediately on the same day. From each species, 5 males and 5 females were sampled.

2.3.2 Blood collection and differential leukocyte count

Blood samples were collected from the ventral coccigeal vein, using a heparinized disposable hypodermic needle (Becton Dickinson, Curitiba, Brazil) attached to a plastic syringe (Becton Dickinson). Samples were immediately transferred into tubes containing sodium heparin (Becton Dickinson, Franklin Lakes, NJ, USA) and transported at room temperature, within a maximum of 20 minutes, to the laboratory for processing and analysis.

A differential leukocyte count on a total of 100 cells, including heterophils, basophils, lymphocytes, and azurophils, following the classification previously described (ALLEMAN; JACOBSON; RASKIN, 1999) was performed by an expert in reptile hematology on individual blood smears made shortly after blood collection and stained according to an adapted May–Grünwald–Giemsa/Rosenfeld technique (ROSENFELD, 1947) with a x 100 oil immersion lens by light microscopy.

2.3.3 Isolation of leukocytes

Two previously described density gradient methods to isolate leukocytes were applied and compared: (1) A discontinuous ficoll-paque PLUS (GE Healthcare Life Sciences, Uppsala, Sweden) gradient at 1.070 g/mL and 1.055 g/mL was prepared as reported earlier (ROSSI et al., 2009). Briefly, 2 mL of 1.070 g/mL density ficoll were pipetted into a 15 mL conical tube (TPP, Trasadingen, Switzerland), followed by 2 mL of 1.055 g/mL ficoll, which was carefully overlaid. Next, 1 mL of blood diluted with 1 mL of RPMI medium (Gibco, Life Technologies, New York, NY, USA) was carefully overlaid. The gradient was then centrifuged (Eppendorf 5804R, Hamburg, Germany) for 45 min at 450g, at 18°C, with acceleration and brakes at 0. The tubes were carefully removed from the centrifuge, and the leukocyte layer between the 2 ficoll densities was carefully pipetted off; (2) A one-step percoll (GE Healthcare Life Sciences) gradient as described earlier (HARMS; KELLER; KENEDY-STOSKOPF, 2000) and adapted later (MUÑOZ et al., 2009) was prepared by the placing 2 mL of a 57% percoll stock solution in Hanks' balanced salt solution 1 x (Gibco, Life technologies) in a 15 mL conical tube, followed by slowly overlaying 2 mL of blood diluted in RPMI as described for the ficoll gradient. This gradient was then centrifuged at 1280g for 5 min, at 18°C, with acceleration and

brakes at 0. The leukocyte layer between plasma and percoll was carefully recovered by using an automatic pipette. The isolated leukocytes were washed once in 10 mL PBS, followed by centrifugation at 300g for 10 min, and the resulting cell pellet was resuspended in 1 mL of Phosphate Buffered Saline (PBS) for further analysis.

Viable leukocytes were counted after mixing 10 μL of the leukocyte suspension with 90 μL of a 0.4% stock solution of trypan blue and incubated at room temperature for 5 minutes. The cells were counted in a Neubauer chamber. Cells with blue cytoplasm were considered nonviable.

An aliquot of the leukocyte suspension was prepared and stained like a blood smear for a differential count at x 100 oil immersion lens by light microscopy.

2.3.4 Flow cytometric analysis

About 1-5 x 10^5 cells were used for flow cytometric analysis using a dual laser FACSCalibur (Becton Dickinson, Burlington, MA, USA) and CellQuest Pro acquisition software (Becton Dickinson, San Jose, CA, USA). A total of 10^4 total events were acquired. Forward (FSC) and side scatter (SSC) properties representing size and internal complexity, respectively, were evaluated at linear and logarithmic scales to determine the most efficient criteria to separate the leukocyte populations. Voltage and amplitude values determined for FSC and SSC detectors were: E-1 voltage, 1.70 amplitude (linear scale) for FSC, and 315 for SSC voltage. These values were defined such that a maximal number of cells were included. Dot plot cytograms were used for leukocyte relative frequency analysis, which was performed using FlowJo software (Tree Star Inc., San Carlos, CA, USA) version 7.6.4 for Windows.

From one snake per species, flow activated cell sorting of 10^7 cells resuspended in 3 mL of PBS plus 1% bovine serum albumin (Sigma-Aldrich, Saint Louis, MO, USA) was performed using a FACSAria IIu (Becton-Dickinson, Bedford, Massachusetts, USA). Sorted cells were spread out on a microscope slide, stained using the Rosenfeld method (ROSENFELD, 1947), and phenotyped based on morphologic appearance.

2.3.5 Statistical analysis

Prism version 5 (GraphPad Software Inc., San Diego, CA, USA) software was used for all statistical analyses and graphs. Continuous data were initially compared by Kolmogorov–Smirnov and Shapiro–Wilk tests for normality and ranked according to their adherence to the Gauss curve. Data values were expressed as median, interquartile ranges, and minimum and maximum values. When comparing more than 2 groups of samples, Kruskal–Wallis with Dunn's posttest was used. Data from each category were represented by relative frequency (%). Differences were considered statistically significant at $P < .05$. Population homogeneity was calculated by analysis of skewness and kurtosis, and their respective standard errors (SE). Data were considered homogenous when skewness and kurtosis values did not exceed 2 SE.

2.4 RESULTS

2.4.1 Microscopic whole blood differential leukocyte count

All 3 species (n = 10 for each species) had a differential cell count dominated by lymphocytes (mean 58.6–78.2%), followed by azurophils (15.0–24.8%) and heterophils (6.6–17.1%) (Figure 1). Basophils were very rare or absent when counting 100 leukocytes.

2.4.2 Viability and differential leukocyte count following density gradient isolation

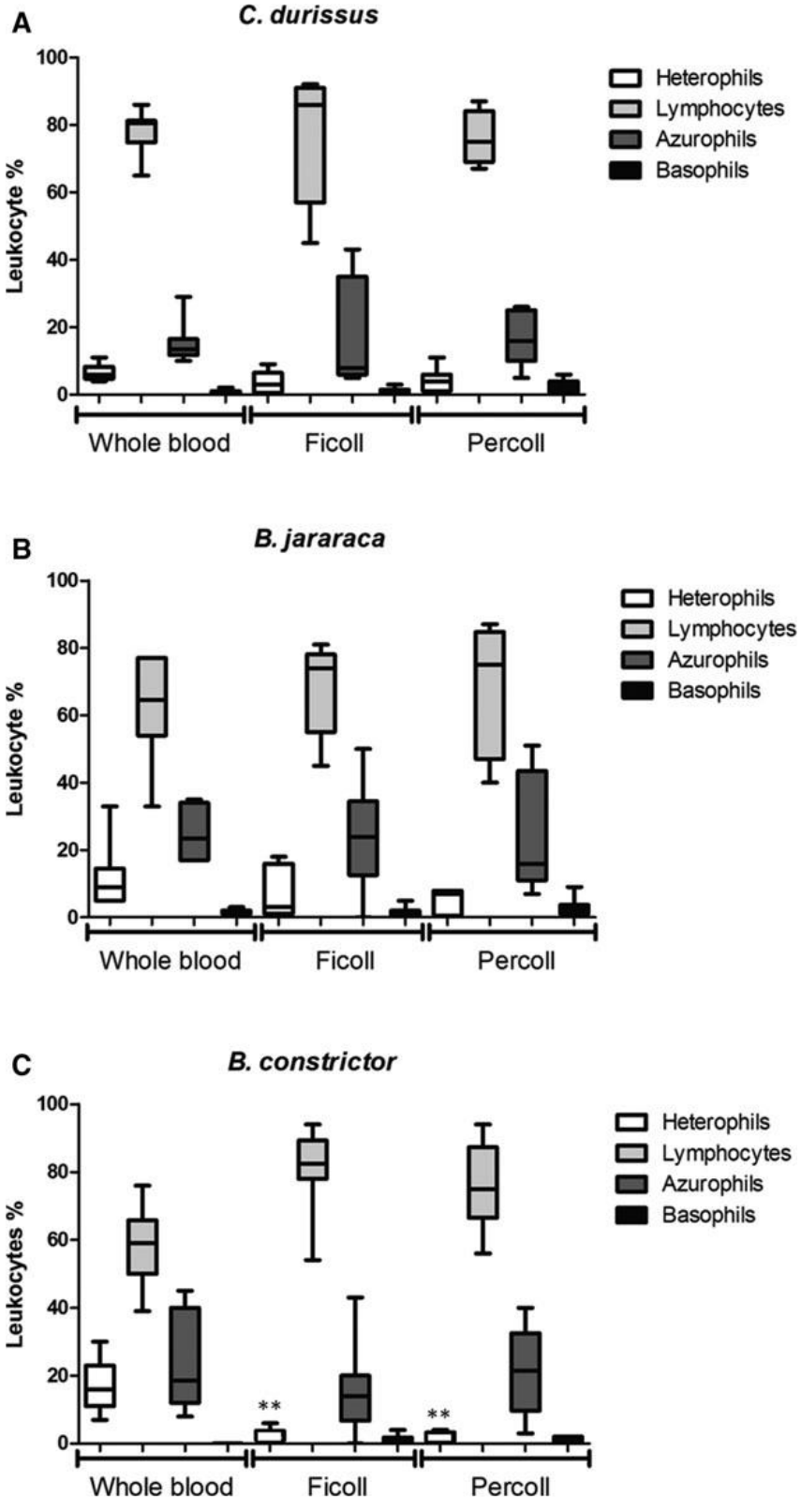
After ficoll or percoll density gradient isolation, the leukocyte viability was consistently > 95% (mean ± SD: 96.58 ± 1.2) based on the trypan blue exclusion test in all 30 specimens.

There was no significant statistical difference between relative differential leukocyte counts in whole blood and after density gradient isolation (Kruskal–Wallis–Dunn's, $P > 0.05$), with the exception of significantly lower relative heterophil counts from *Boa constrictor* isolated with ficoll or percoll (Kruskal–Wallis–Dunn's, $P < 0.01$) (Figure 1C). There was no

significant difference between ficoll or percoll gradient isolation (Kruskal–Wallis–Dunn's, $P > 0.05$) (Figure 1).

Thrombocytes were identified and differentiated from lymphocytes. Thrombocytes represented < 10% of total counted cells, therefore they were not considered of statistical relevance.

Figure 1 – Microscopic differential count of peripheral blood leukocytes on blood smear and after density gradient separation. Box plots show interquartile ranges, the line indicates the median, and whiskers show minimum and maximum leukocyte percentage. (A) *C. durissus*, (B) *B. jararaca*, and (C) *B. constrictor*. **statistically significant difference between whole blood heterophil counts vs ficoll- and percoll-isolated heterophil counts (Kruskal–Wallis–Dunn's, $P < .01$)



2.4.3 Flow cytometric definition of different leukocyte populations based on FSC and SSC

Four gated cell populations, G1 to G4, were set as defined cell clusters representing relatively homogeneous cell populations based on cell size/FSC and internal complexity/SSC (Figures 2 and 3). Graphs of leukocyte populations are illustrated on representative cytograms. Cytograms depicted in Figure 2A–F applied a logarithm size (FSC) scale, while cytograms in Figure 3A–F were created in linear FSC scale. The relative number of cells in the respective gates was comparable, regardless of density gradient isolation media and FSC scale (Figure 4, logarithmic scale data).

Figure 2 – Representative cytograms of leukocyte populations based on size (forward scatter [FSC], logarithmic scale) and internal complexity (side scatter [SSC]) for ficoll-isolated (left panel) and percoll-isolated (right panel) leukocytes of (A and B) *C. durissus*, (C and D) *B. jararaca*, and (E and F) *B. constrictor*

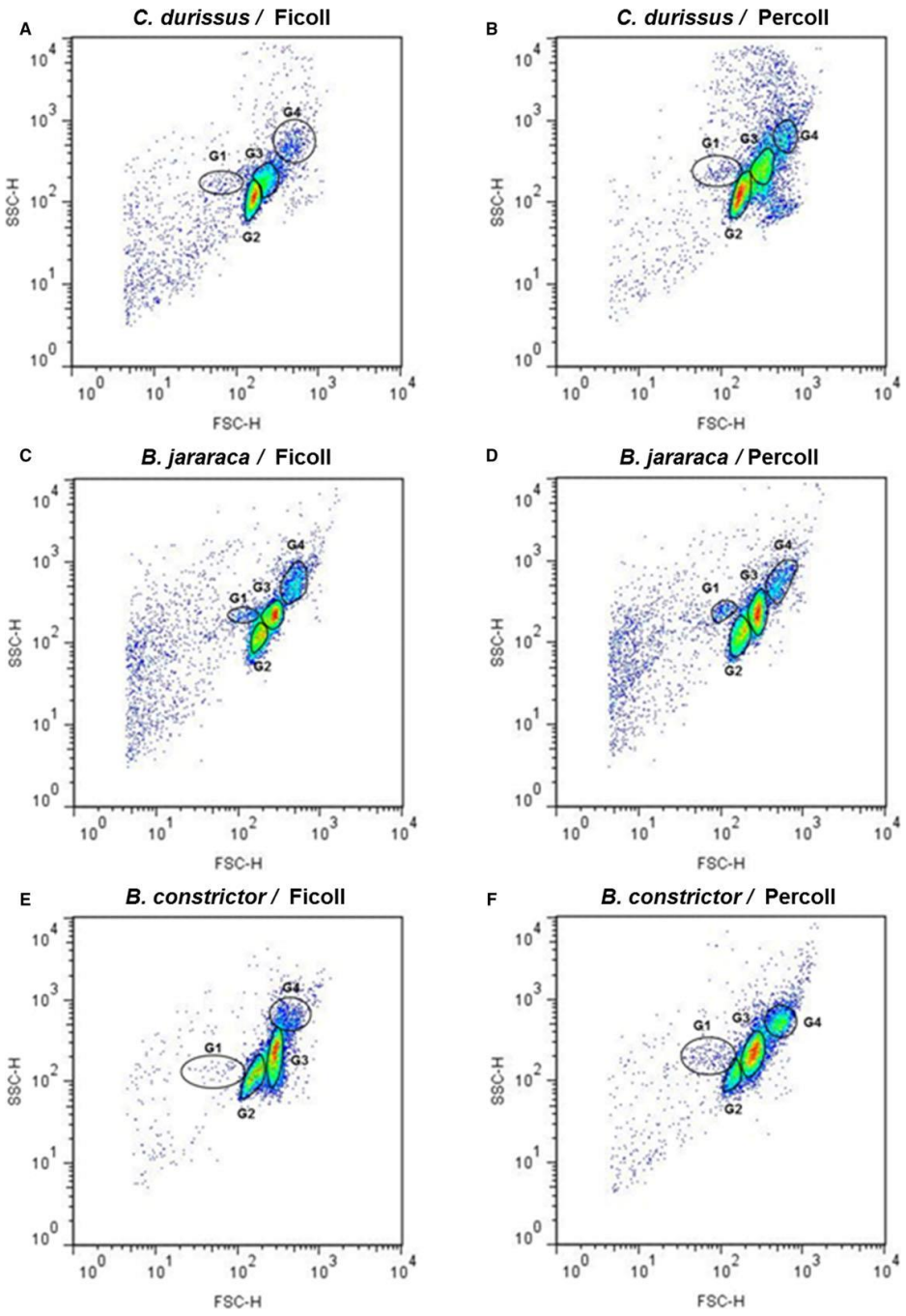


Figure 3 – Representative cytograms of leukocyte populations based on size (forward scatter [FSC], linear scale) and internal complexity (side scatter [SSC]) for ficoll-isolated (left panel) and percoll-isolated (right panel) leukocytes of (A and B) *C. durissus*, (C and D) *B. jararaca*, and (E and F) *B. constrictor*

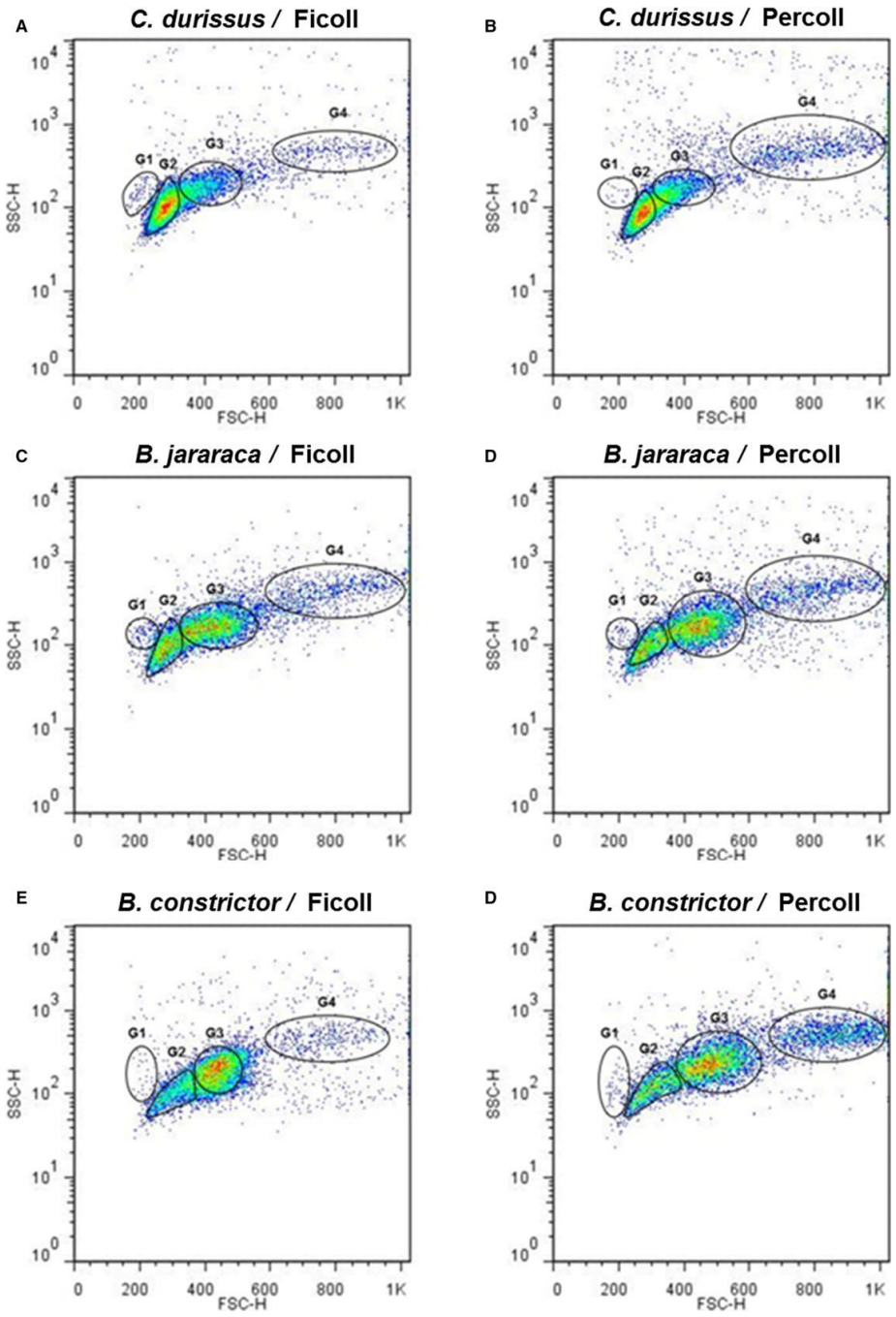
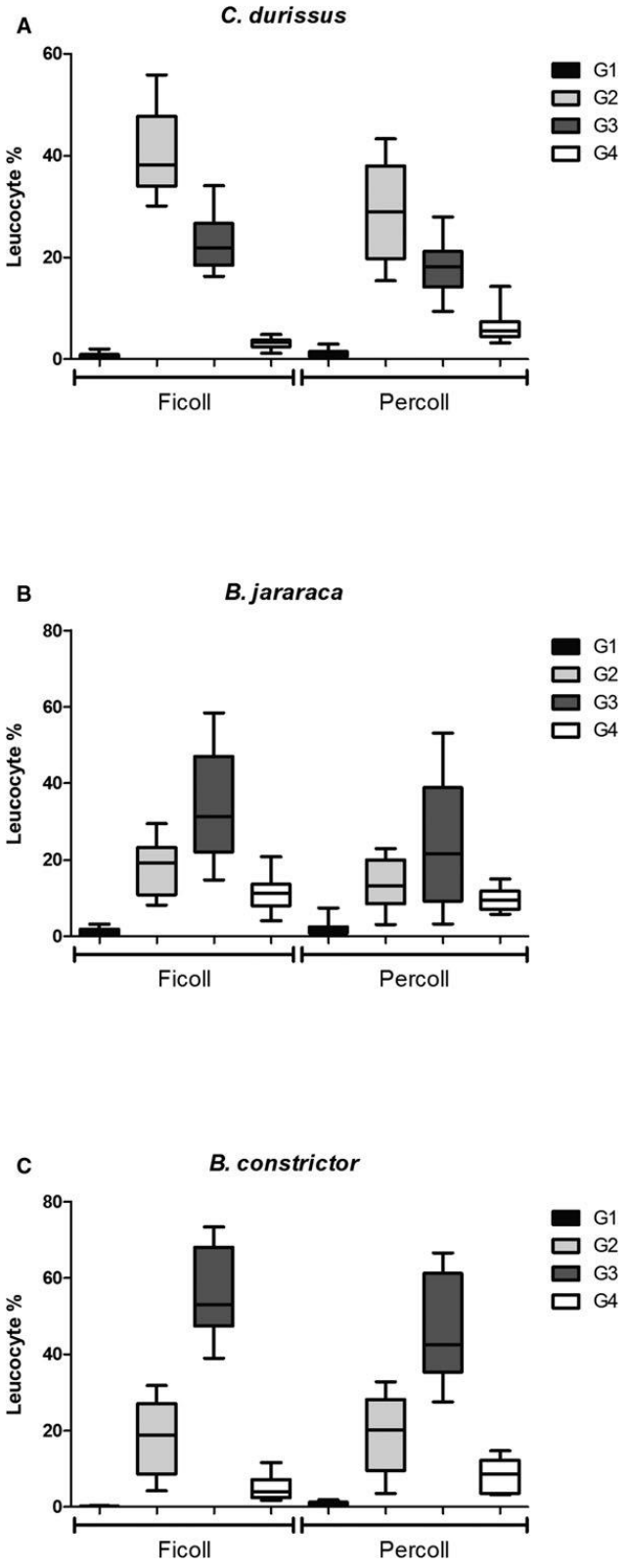


Figure 4 – Frequency of leukocyte types in population clusters G1–G4 in logarithmic FSC and SSC scales, after ficoll or percoll isolation. Box plots show interquartile ranges, the line indicates the median, and whiskers show minimum and maximum leukocyte percentage: (A) *C. durissus*, (B) *B. jararaca*, and (C) *B. constrictor*. Leukocyte differential counts were statistically similar between ficoll- and percoll-isolated cells from one species



2.4.4 Identification of populations obtained by cell sorting

One leukocyte suspension from each species was sorted via the 4 defined gates G1 through G4, and the leukocytes present in these 4 fractions were identified based on their light microscopic appearance on slide preparations, which were stained as blood smears (Figure 5). Although the morphology of the 4 sorted leukocyte types was poorly preserved, heterophils, lymphocytes, and azurophils were recognized based on their Rosenfeld staining characteristics, morphology, and refringence. Tables 1–3 summarize the relative frequencies of sorted leukocyte fractions in gates G1–G4 after ficoll or percoll gradient isolation for each snake species. Figure 5 shows representative images of postsorting leukocytes isolated by percoll, and insets showing their corresponding image in blood smear. No basophils were sorted.

Table 1 – Representative differential leukocyte count based on smears of cells sorted from FACSAria leukocyte gates (G1–G4) defined by size/forward (FSC) and complexity/side scatter (SSC). Input cells were obtained after density gradient leukocyte isolation with ficoll and percoll from peripheral blood of a rattlesnake, *Crotalus durissus* (n = 1). Microscopic differential count after gradient isolation of the same sample before cell sorting is also listed

	Heterophils (%)	Small Lymphocytes (%)	Large Lymphocytes (%)	Azurophils (%)	Basophils (%)
Ficoll					
Microscopic differential count*	9	71	15	5	0
G1	94	6	0	0	0
G2	0	100	0	0	0
G3	7	93	0	0	0
G4	0	0	64	36	0
Percoll					
Microscopic differential count*	11	68	7	14	0
G1	97	3	0	0	0
G2	0	100	0	0	0
G3	0	0	33	67	0
G4	0	0	88	12	0

*Isolated cells before sorting

Table 2 - Representative differential leukocyte count based on smears of cells sorted from FACSAria leukocyte gates (G1–G4) defined by size/forward (FSC) and complexity/side scatter (SSC). Input cells were obtained after density gradient leukocyte isolation with ficoll and percoll from peripheral blood of an American pit viper, *Bothrops jararaca* (n = 1). Microscopic differential count after gradient isolation of the same sample before cell sorting is also listed.

	Heterophils (%)	Small Lymphocytes (%)	Large Lymphocytes (%)	Azurophils (%)	Basophils (%)
Ficoll					
Microscopic differential count*	15	70	5	10	0
G1	86	14	0	0	0
G2	0	100	0	0	0
G3	0	0	100	0	0
G4	0	0	26	74	0
Percoll					
Microscopic differential count*	8	74	7	7	4
G1	97	3	0	0	0
G2	0	100	0	0	0
G3	0	0	100	0	0
G4	4	0	27	69	0

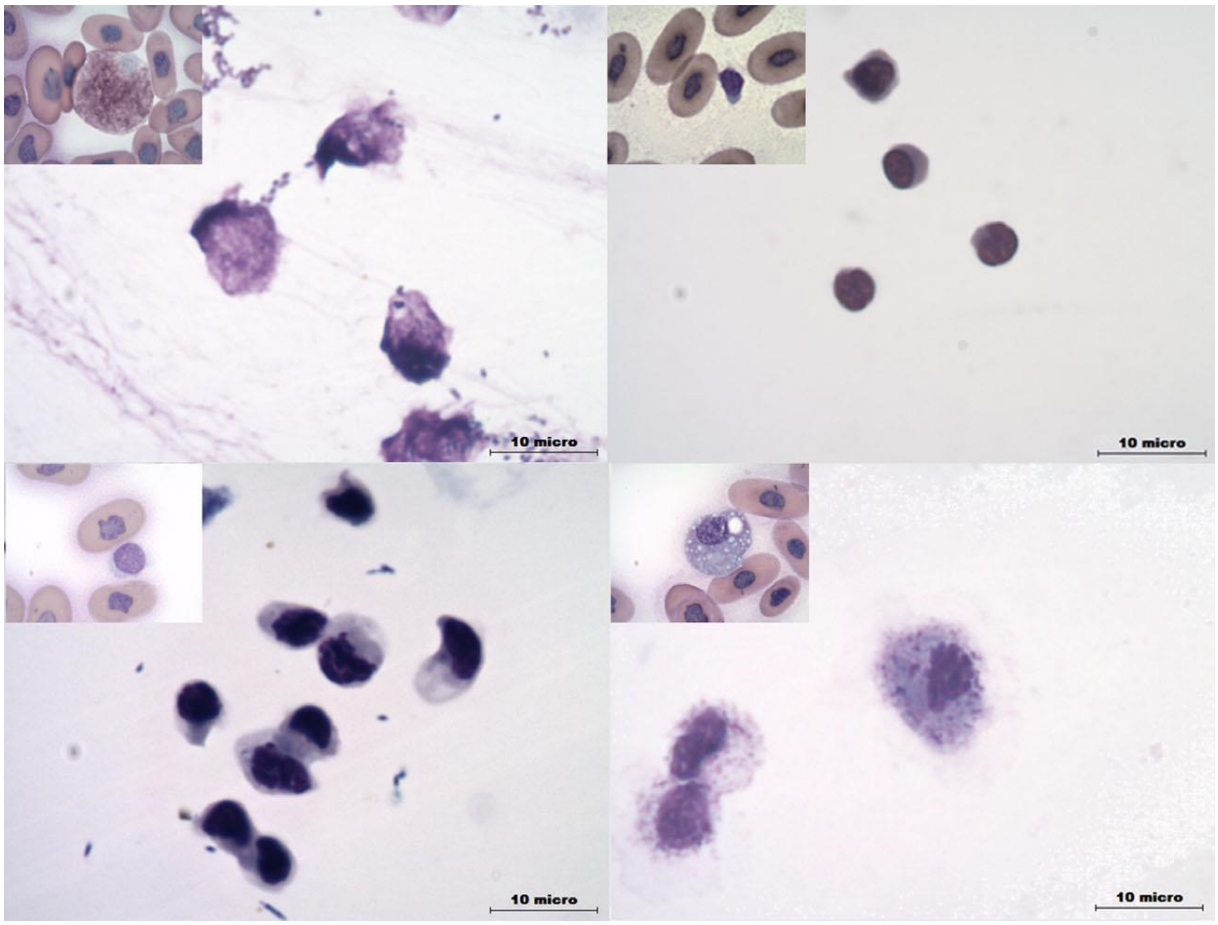
*Isolated cells before sorting

Table 3 - Representative differential leukocyte count based on smears of cells sorted from FACSAria leukocyte gates (G1-G4) defined by size/forward (FSC) and complexity/side scatter (SSC). Input cells were obtained after density gradient leukocyte isolation with ficoll or percoll from peripheral blood of a boa, *Boa constrictor* (n = 1). Microscopic differential count after gradient isolation of the same sample before cell sorting is also listed

	Heterophils (%)	Small Lymphocytes (%)	Large Lymphocytes (%)	Azurophils (%)	Basophils (%)
Ficoll					
Microscopic differential count*	1	72	7	20	0
G1	2	98	0	0	0
G2	15	85	0	0	0
G3	0	0	85	15	0
G4	0	0	10	90	0
Percoll					
Microscopic differential count*	3	71	14	12	0
G1	0	100	0	0	0
G2	2	98	0	0	0
G3	4	0	96	0	0
G4	0	0	28	72	0

*Isolated cells before sorting

Figure 5 – Representative images of sorted leukocytes from blood of 3 species of snakes. (A) Heterophils, with eccentric nucleus and granular eosinophilic cytoplasm (*B. jararaca*, G1). (B) Small lymphocytes, with high nucleus to cytoplasmic ratio, scant basophilic cytoplasm, and round nucleus (*C. durissus*, G2). (C) Large lymphocytes, with high nucleus to cytoplasmic ratio, and slightly more abundant basophilic cytoplasm (*B. jararaca*, G3). (D) Azurophils, round cells with vacuolated and granular, grayish blue cytoplasm (*B. constrictor*, G4). Insets: respective cell type, from the same species, in blood smear samples.



2.5 DISCUSSION

Besides red blood cell lysis, the purification of peripheral blood mononuclear cells is one standard procedure for isolating leukocytes in mammals, allowing their use for diagnostic approaches, functional studies, or therapeutic protocols (JANEWAY et al., 1999). The use of density gradients for the study of avian, reptilian, and amphibian leukocytes is also relevant as their nucleated erythrocytes are not easily lysed. In addition, protocols for erythrolysis can lead to leukocyte damage and dysfunction, while a large number of free nuclei can affect the results (TSUJI et al., 1999).

Therefore, in this study, we tested 2 density gradient protocols and compared relative leukocyte differential counts in one boa and 2 viper snake species with the relative frequency of heterophils, lymphocytes, azurophils, and basophils based on blood smears evaluation. Overall, the ranges in our study corroborate with data previously published (CAMPBELL; ELLIS, 2007). Specifically, in all 3 species, the dominating leukocyte was the lymphocyte, followed by azurophil and heterophil. Interestingly, density gradient isolation also yielded similar proportions of azurophils and heterophils as in native blood, which is in contrast mostly to the mononuclear leukocyte isolates generated with ficoll or percoll in mammals (HARMS; KELLER; KENMEDY-STOSKOPF, 2000; MUÑOZ et al., 2009). Nonetheless, it is worth noting that we found a significant loss of heterophils after leukocyte gradient preparation of *Boa constrictor*. As the results were comparable for ficoll or percoll isolation, percoll is preferable as it is simpler to prepare and requires a shorter centrifugation period.

The actual distribution of the flow cytometric leukocyte gates differed slightly between the 3 species of snakes, demonstrating similarity between the vipers *C. durissus* and *B. jararaca*, and an apparent difference in comparison to *B. constrictor*. This can potentially be explained by the phylogenetic distance between Boidae and Viperidae families (FRANCO, 2003); however, there is currently no detailed study comparing size and/or complexity in flow cytometric evaluation between the leukocytes of these species.

After cell sorting, the blood leukocytes isolated from one boa presented 2 lymphocyte subpopulations, and the G1 gate did not include a heterophil population as seen for the 2 viper samples. Possibly, this was due to the significantly lower gradient recovery of heterophils for this species, and the gate included some lymphocytes from adjacent clusters. The lack of a basophil gate in the cytograms is attributed to the very small proportion of these cells (0–5%) in the peripheral blood of healthy snakes (RASKIN, 2000).

The quality of leukocyte morphology after flow cytometric sorting was poor and not comparable with the appearance on peripheral blood smears, likely due to the drying process in a drop of PBS with high osmolality and salt crystallization (ALBERTI, STOVEL, HERZENBERG, 1984). Nonetheless, we were confident that the leukocyte populations could be properly identified microscopically.

Heterophils are large cells measuring around 10–23 μm (RASKIN, 2000), yet in the leukocyte suspension of *B. jararaca* and *C. durissus*, they were the dominant cell type isolated from G1, the gate with the smallest cells. We suspect that osmotic changes could account for this finding, possibly caused by high osmolality (above 290 mOsm/kg) of density gradients used for leukocyte isolation (TIMOMEN et al., 1982), causing dehydration and cell shrinking.

This should be taken into account when evaluating snake heterophils in flow cytometry. On the other hand, lymphocytes and azurophils were represented in cytograms in expected locations according to size and internal complexity characteristics described by light and electron microscopy (ALLEMAN; JACOBSON; RASKIN, 1999; CAMPBELL, 2006; ZANOTTI, 2007).

Flow cytometric analysis as described in the present study may offer an important method for the understanding of the leukocyte series in snakes, allowing further morphologic and functional studies, and leading to advances in research from ecoimmunology to clinical diagnosis.

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ATTACHMENT B Statements of co-authors

São Paulo, 02 de maio de 2018

DECLARAÇÃO

Eu, Nicolle Gilda Teixeira Queiroz Hazarbassanov, CPF: 340.119.978-11, co-autora do artigo "Flow cytometric characterization of peripheral blood leukocyte populations of 3 neotropical snake species: *Boa constrictor*, *Bothrops jararaca*, and *Crotalus durissus*", publicado em 2016 pela revista:"Veterinary Clinical Pathology", declaro estar **de acordo** com a inclusão e publicação do acima referido artigo na Tese de Doutorado intitulada "Functional analysis of peripheral blood leucocytes of snakes (Boidae and Viperidae) during the process of adaptation to captivity" de autoria de Marcelo Pires Nogueira de Carvalho, aluno de Doutorado do Programa de Patologia Experimental e Comparada do Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo.

Atenciosamente,

Nicolle Queiroz

Nicolle Gilda Teixeira Queiroz Hazarbassanov

São Paulo, 02 de maio de 2018

DECLARAÇÃO

Eu, Cristina de Oliveira Massoco Salles Gomes, CPF: 167643398-80, co-autora do artigo "Flow cytometric characterization of peripheral blood leukocyte populations of 3 neotropical snake species: *Boa constrictor*, *Bothrops jararaca*, and *Crotalus durissus*", publicado em 2016 pela revista:"Veterinary Clinical Pathology", declaro estar **de acordo** com a inclusão e publicação do acima referido artigo na Tese de Doutorado intitulada "Functional analysis of peripheral blood leucocytes of snakes (Boidae and Viperidae) during the process of adaptation to captivity" de autoria de Marcelo Pires Nogueira de Carvalho, aluno de Doutorado do Programa de Patologia Experimental e Comparada do Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo.

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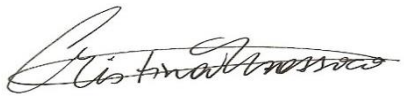

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Cristina de Oliveira Massoco Salles Gomes

02/Maio/2018

DECLARAÇÃO

Eu, Silmara Rossi, CPF:.257.342.368-96, co-autora do artigo "Flow cytometric characterization of peripheral blood leukocyte populations of 3 neotropical snake species: *Boa constrictor*, *Bothrops jararaca*, and *Crotalus durissus*", publicado em 2016 pela revista:"Veterinary Clinical Pathology", declaro estar **de acordo** com a inclusão e publicação do acima referido artigo na Tese de Doutorado intitulada "Functional analysis of peripheral blood leucocytes of snakes (Boidae and Viperidae) during the process of adaptation to captivity" de autoria de Marcelo Pires Nogueira de Carvalho, aluno de Doutorado do Programa de Patologia Experimental e Comparada do Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo.

Atenciosamente,

Silmara Rossi

Silmara Rossi

São Paulo, 02 de maio de 2018

DECLARACÃO

Eu, Sávio Stefanini Sant'Anna, CPF: 130.023.398-22, co-autor do artigo "Flow cytometric characterization of peripheral blood leukocyte populations of 3 neotropical snake species: *Boa constrictor*, *Bothrops jararaca*, and *Crotalus durissus*." publicado em 2016 pela revista: "Veterinary Pathology", declaro estar **de acordo** com a inclusão e publicação do acima referido artigo na Tese de Doutorado intitulada "Functional analysis of peripheral blood leucocytes of snakes (Boidae and Viperidae) during the process of adaptation to captivity" de autoria de Marcelo Pires Nogueira de Carvalho, aluno de Doutorado do Programa de Patologia Experimental e Comparada do Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo.

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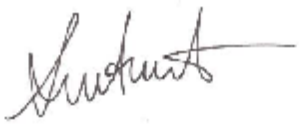

Sávio Stefanini Sant'Anna

São Paulo, 02 de maio de 2018

DECLARAÇÃO

Eu, José Luiz Catão Dias, CPF 02959788800, co-autor do artigo "Flow cytometric characterization of peripheral blood leukocyte populations of 3 neotropical snake species: *Boa constrictor*, *Bothrops jararaca*, and *Crotalus durissus*", publicado em 2016 pela revista:"Veterinary Clinical Pathology", declaro estar **de acordo** com a inclusão e publicação do acima referido artigo na Tese de Doutorado intitulada "Functional analysis of peripheral blood leucocytes of snakes (Boidae and Viperidae) during the process of adaptation to captivity" de autoria de Marcelo Pires Nogueira de Carvalho, aluno de Doutorado do Programa de Patologia Experimental e Comparada do Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo.

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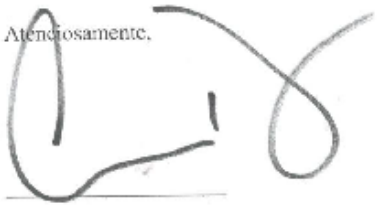

José Luiz Catão Dias

São Paulo, 02 de maio de 2018

DECLARACÃO

Eu, Kathleen Fernandes Grego, CPF: 851.044.347-53, co-autora do artigo "Flow cytometric characterization of peripheral blood leukocyte populations of 3 neotropical snake species: *Boa constrictor*, *Bothrops jararaca*, and *Crotalus durissus*" publicado em 2016 pela revista:"Veterinary Clinical Pathology", declaro estar **de acordo** com a inclusão e publicação do acima referido artigo na Tese de Doutorado intitulada "Functional analysis of peripheral blood leucocytes of snakes (Boidae and Viperidae) during the process of adaptation to captivity" de autoria de Marcelo Pires Nogueira de Carvalho, aluno de Doutorado do Programa de Patologia Experimental e Comparada do Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo.

Atenciosamente,

Kathleen Fernandes Grego

3 FUNCTIONAL CHARACTERIZATION OF NEOTROPICAL SNAKES PERIPHERAL BLOOD LEUKOCYTES SUBSETS: LINKING FLOW CYTOMETRY CELL FEATURES, MICROSCOPY IMAGES AND SERUM CORTICOSTERONE LEVELS

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3.1 ABSTRACT

Reptiles are the unique ectothermic amniotes, providing the key link between ectothermic anamniotes fish and amphibians, and endothermic birds and mammals; becoming an important group to study with the aim of providing significant knowledge into the evolutionary history of vertebrate immunity. Classification systems for reptiles' leukocytes have been described by their appearance rather than function, being still inconsistent. With the advent of modern techniques and the establishment of analytical protocols for snakes' blood by flow cytometry, we bring a qualitative and quantitative assessment of innate activities presented by snakes' peripheral blood leukocytes, thereby linking flow cytometric features with fluorescent and light microscopy images. Moreover, since corticosterone is an important immunomodulator in reptiles, hormone levels of all blood samples were measured. We provide novel and additional information which should contribute to better understanding of the development of the immune system of reptiles and vertebrates.

Keywords: Flow cytometric analysis (FACS). Immune system. Oxidative burst. Phagocytosis. Reptile.

3.2 INTRODUCTION

Knowledge of the mechanisms underlying the immune defense, and how they have adapted and changed to pathogens and environmental challenges is fundamental to understand the evolution of life on our planet. Despite its importance, development of the field of evolutionary immunobiology is far from mature, with several information gaps being readily identified, preventing this field from achieving its full plenitude (WARR; CHAPMAN; SMITH, 2003). In this context, the immune system of certain important classes of animals are still poorly understood due to limited investigation. An example is the reptile group, about which little is known, from an immunological perspective (ORIGGI, 2007; WARR; CHAPMAN; SMITH, 2003).

Reptiles are the unique ectothermic amniotes, providing the key link between ectothermic anamniote fish and amphibians, and endothermic birds and mammals, thus becoming an important group to study with the objective of providing important information into the evolutionary history of vertebrate immunity, as well as the growing field of eco-immunology (ZIMMERMAN; VOGEL; BOWDEN, 2010). Moreover, it is impossible to completely comprehend the immune systems of higher groups, such as mammals and birds, without information on the immunological features of the ancestries from which they derived (WARR; CHAPMAN; SMITH, 2003).

The immune system response comprises a complex process which includes specialized cells, effector molecules and hormones. Many of these cells interactions and effector functions are likely to exist in the immune system of reptiles, but only a few of these have been adequately studied and identified (ORIGGI, 2007).

The classification systems available for reptiles' leukocytes are still inconsistent because variable criteria have been used to categorize these cells; in addition, morphological studies on reptilian leukocytes have typically utilized only a restricted number of techniques to define such cells, leading to both unsubstantiated interpretations and inconsistent reports (CLAVER; QUAGLIA, 2009; SYPEK; BORYSENKO, 1988). Furthermore, leukocytes in reptiles have been described according to their morphology rather than their function. Because of the lack of functional knowledge, the nomenclature applied to certain reptilian leukocytes should be regarded as tentative (CLAVER; QUAGLIA, 2009; SYPEK; BORYSENKO, 1988).

To cover these gaps in knowledge on reptiles' immune system, very interesting studies have recently been published, successfully describing some phenotypic and functional

characteristics of these cells, mainly in species from the Testudines Order (PASMANS et al., 2001; MUÑOZ et al., 2009; ROSSI et al., 2009; ROUSSELET et al., 2013; ZIMMERMAN et al., 2009, 2013). However, considering the heterogenicity and phylogenetic distance between reptile orders (PYRON et al., 2013), further studies involving other species of reptiles are required to better elucidate their immune system characteristics.

With the advent of more modern techniques and the establishment of analytical protocols for snakes' blood leukocytes by flow cytometry (CARVALHO et al., 2016), here we describe, for the first time, a qualitative and quantitative assessment of innate (phagocytosis and oxidative burst) cellular activities presented by leukocytes isolated from the peripheral blood of three species of neotropical snakes, by coupling flow cytometric features with fluorescent and light microscopy images. Yet, as it is known that corticosterone is a powerful regulator of the immune function in reptiles (MEYLAN et al., 2010), we measured the serum corticosterone levels of sampled snakes. We describe novel and additional information which should contribute to better understanding of the immune system development in reptiles and vertebrates.

3.3 MATERIALS AND METHODS

3.3.1 Snakes

The present study was approved by the School of Veterinary Medicine and Animal Science, University of São Paulo, Ethics Committee on Animals Use, (Registry Number 8322070714), and by SISBIO (Authorization System and Information in Biodiversity) license 44990-5.

Three species of Brazilian snakes, two vipers (*Crotalus durissus* and *Bothrops jararaca*) and one boa (*Boa constrictor*), of the Laboratory of Herpetology, Butantan Institute, Brazil, were included in the study. The snakes were housed in acclimatized standard rooms with defined lighting cycles, at temperatures between 25º C and 28º C, and 60% relative humidity. The snakes were maintained in individual plastic containers (60 x 40 x 15 cm) with corrugated fiberboard substrate and water *ad libitum*, supplied by plastic water fountain drinker.

Blood samples were collected from 15 males and 15 females from each vipers' species and 6 males and 6 females from boas. The snakes were assumed to be adapted to captivity due to their over 12 months captivity period; and clinically healthy based on feeding routine, behavior, successful shedding, body score, hemogram and leukogram values.

3.3.2 Blood collection

Blood samples were collected in a maximum period of 3 minutes after the beginning of physical restraint, from the ventral coccigeal vein in vipers or from the paravertebral sinus in boas, using heparinized disposable hypodermic needles (Becton Dickinson) attached to plastic syringes (Becton Dickinson). Samples were immediately transferred to tubes containing sodium heparin (Becton Dickinson) and transported at room temperature, within a maximum of 30 minutes to the laboratory for processing and analysis.

3.3.3 *In vitro Staphylococcus aureus* and Zymosan A qualitative phagocytosis assay in fresh blood

For qualitative analysis of phagocytosis activity in snakes' leukocytes, two phagocytosis assays per species (n = 6) were performed by adding (1) 10 μL of Zymosan A (Sigma, St Louis. Mo, USA) suspension (4 x 10^6 particles) in 300 μL of fresh blood and (2) 200 μL of inactivated *Staphylococcus aureus* Pansorbin® (Calbiochem, Merck Millipore) stained with propidium iodide (Sigma, St Louis. Mo, USA) (SAPI) suspension (0.5 mg/mL) in 300 μL of fresh blood volume as describe for Zymosan A assay. Samples were incubated for 60 min, in agitation over a 450 rpm shaker, at room temperature (25º C-28º C), then washed with 2 mL of 3 mM ethylenediamine tetraacetic acid (EDTA) solution to stop phagocytosis activity and to wash off the excess of free Zymosan A and *Staphylococcus aureus* particles. Samples were then suspended in 300 μL of Phosphate Buffered Saline (PBS). Smears (n = 6) were then prepared and air dried followed by the May-Grünwald-Giemsa/Rosenfeld staining technique (CAMPBELL, 2006; ROSENFELD, 1947). Blood films were completely analyzed by light

microscopy using a 100 x oil immersion objective. Phagocytosis images were captured with Image-Pro 3DS 6.0 software (Media Cybernetics Inc., Silver Spring, MD, USA) using a photographic camera model Go-5 Datasheet-QImagingn, coupled to an Olympus BX-50 optical microscope (Olympus Optical, Tokyo, Japan). Leukocytes were classified as heterophils, lymphocytes, azurophils and basophils, as described by Alleman et al. (1999).

3.3.4 Isolation of leukocytes

Peripheral blood leukocytes were isolated using a one-step Percoll (GE Healthcare Life Sciences) gradient, as previously described (CARVALHO et al., 2016). This gradient was prepared by placing 2 mL of a 57% Percoll stock solution in 1x Hanks' Balanced Salt Solution (HBSS) (Gibco, Life Technologies) in a 15 mL conical tube, followed by slowly overlaying 2 mL of blood diluted (1:1) in 1x HBSS. The gradient was centrifuged at 1280g for 5 min, at 18º C, with acceleration and brakes at 0. The leukocyte layer between plasma and Percoll was carefully recovered using an automatic pipet. The isolated leukocytes were washed once in 10 mL of PBS, followed by centrifugation at 300g for 10min, and the resulting cell pellet was suspended in 1 mL of PBS for further analysis. Cell numbers and viability were evaluated using a Neubauer chamber and the trypan-blue exclusion method.

3.3.5 Phagocytosis assays

Phagocytosis activity was evaluated *in vitro* as previously described (ROSSI et al., 2009), with slight modifications (*B. jararaca*, n = 30; *C. durissus*, n = 30; *B. constrictor*, n = 12). Viable cells were counted in Neubauer counting chamber using trypan-blue (10 μL of cell suspension in 90 μL of trypan-blue). At least 95% of the cells had to be viable in order to be subjected to functional assays. After determining the number of viable cells, samples of cell suspensions were adjusted to 4 x 10^5 leukocytes/100 μL. As bacterial challenge for phagocytosis, SAPI was used in a proportion of approximately 100 bacterial particles per leukocyte. As fungi challenge, Zymosan A (Sigma, St Louis. Mo, USA) marked with 10 mM

carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) was used in a proportion of 10 particles per leukocyte. Cell suspensions were incubated for 60 min at room temperature (25º C-28º C), in agitation on a 450 rpm shaker, in the dark. After the incubation period, samples were washed in 2mL of 3mMEDTA solution, at 300g for 7 min. Cells were then suspended in 300 mL of PBS and analyzed by flow cytometry. For each sample, the phagocytosis assays were organized as follows:

- Condition A (control): cell suspension;
- Condition B (bacterial stimulus): cell suspension + SAPI suspension;
- Condition C (fungi stimulus): cell suspension + Zymosan A suspension.

3.3.6 Oxidative burst assays

Oxidative burst was evaluated in vitro as previously described (ROSSI et al., 2009), with slight modifications (*B. jararaca*, n = 30; *C. durissus*, n = 30; *B. constrictor*, n = 12). Viable cells were counted using a Neubauer counting chamber and trypan-blue (10 μL of cell suspension in 90 μL of trypan-blue). At least 95% of the cells had to be viable in order to be submitted to the functional assays. After determining the number of viable cells, samples of the cell suspensions were adjusted to 4 x 10^5 leukocytes/100 μL. As bacterial challenge for oxidative burst, inactivated SAPI was used in the same proportion described above. As a chemical challenge for oxidative burst, phorbol 12-myristate 13-acetate (PMA) was applied. An intermediate solution was prepared at a 2 μg/mL concentration, while final PMA assay concentration was 0.2 μg/mL or 324 nM/mL. Cell suspensions were incubated for 60 min at room temperature (25º C-28º C), under agitation over a 450 rpm shaker, in the dark, with 2,7-dichlorofluorescin diacetate (DCFH-DA) (Sigma, St Louis. Mo, USA) solution prepared at a 304 μM in PBS, resulting in a final concentration of 60.8 μM DCFH. After incubation, samples were washed in 2 mL of 3 mM EDTA solution, at 300g for 7min. Cells were then suspended in 300 μL of PBS and analyzed by flow cytometry. For each sample, the oxidative burst assays were organized as follows:

- Condition D (control): cell suspension + DCFH-DA solution;
- Condition E (chemical stimulus): cell suspension + PMA solution + DCFH-DA solution;

- Condition F (bacterial stimulus): cell suspension + SAPI suspension + DCFH-DA solution.

3.3.7 Fluorescence microscopy of SAPI and Zymosan A phagocytosis assays

To confirm the quality of the fluorescence of antigenic particles and phagocytosis activity, fluorescence microscopy was performed in one snake per species (n = 3) with SAPI and Zymosan A phagocytosis assays.

For samples from the SAPI assay, an aliquot of snake leukocytes was fixed in PBS/1-2% paraformaldehyde, followed by washing with 0.1 M tris-glycine for 5min to block aldehyde groups, and then washed with 2 mL of PBS. Cells were permeabilized with PBS/0.1% Triton X-100 for 20min and then incubated with PBS/0.01% Phalloidin-FITC (Molecular probes®, Thermo Fisher Scientific) for 30min. Samples were then washed 3 times with PBS for 5min each. To assemble the microscope slides Vectashield (Vector Laboratories, Burlingame, CA) mounting medium containing 4,6'-diaminido-2-phenylindole (DAPI) was used.

After Zymosan A phagocytosis assay, snake leukocytes were stained as described for the SAPI assay with the substitution of phalloidin by acridine orange, at a final concentration of 10 ng/mL.

3.3.8 Fluorescence microscopy and image analysis

Images were captured using a fluorescence microscope (Nikon Eclipse 80i and x 100 oil-immersion objective) and Nikon ACT-1C software (Nikon Instruments Inc., Melville, NY, USA).

3.3.9 Flow cytometric analysis and cell sorting

After the functional assays, cell suspensions (about 1-5 x 10^5 cells) were used for flow cytometric analysis in a dual laser FACSCalibur (Becton Dickinson, Burlington, MA, USA) and CellQuest Pro acquisition software (Becton Dickinson, San Jose, CA, USA). A minimum of 10^4 events were acquired from leukocytes gates for oxidative burst and phagocytosis analysis. Forward (FSC) and side scatter (SSC) parameters representing size and internal complexity, respectively, were evaluated using logarithmic scale. Voltage and amplitude values determined for FSC and SSC detectors were the same as reported in Carvalho et al. (2016). Dot plot cytograms were analyzed using FlowJo software (Tree Star Inc., San Carlos, CA, USA) version 7.6.4 for Windows. To assess phagocytic activity of snakes' leukocytes, we compared the immune responses from the same leukocyte population with different challenges, as well as the immune response of different leukocyte populations with the same challenge. In both situations, we evaluated the frequency of cells that underwent phagocytosis (cell percentages) and the mean fluorescence intensity (MFI) of each leukocyte subset. After SAPI and Zymosan A phagocytosis assays, from one snake per species, flow activated cell sorting of 10^7 cells suspended in 300 μL of PBS was carried out using a FACSAria IIu (Becton-Dickinson, Bedford, Massachussetts, USA). Sorted cells were allowed to spread on a microscope slide pretreated with 1% BSA (Bovine Serum Albumin) (ICN Biomedicals, Inc., Aurora, Ohio) for 24 h. Immediately after sorted cells were spread onto microscope slides, 5 μL of methanol were carefully placed over the drop of sorted cells. Samples were then stained with adapted May-Grünwald-Giemsa/Rosenfeld staining technique (CAMPBELL, 2006; ROSENFELD, 1947) and analyzed by light microscopy.

3.3.10 Measurement of serum corticosterone

For serum corticosterone measurement, heparinized blood samples (n = 72) were centrifuged at 2000g for 10min for plasma separation. Plasma fractions were recovered using an automatic pipet and stored at -80º C. DetectX® Corticosterone Enzyme Immunoassay Kit (Arbor Assays, Ann, MI, USA) was used to quantify corticosterone serum levels following the

protocol suggested by the manufacturer. Plasma dilution was tested, with the best results being achieved at 1:100.

3.3.11 Statistical analysis

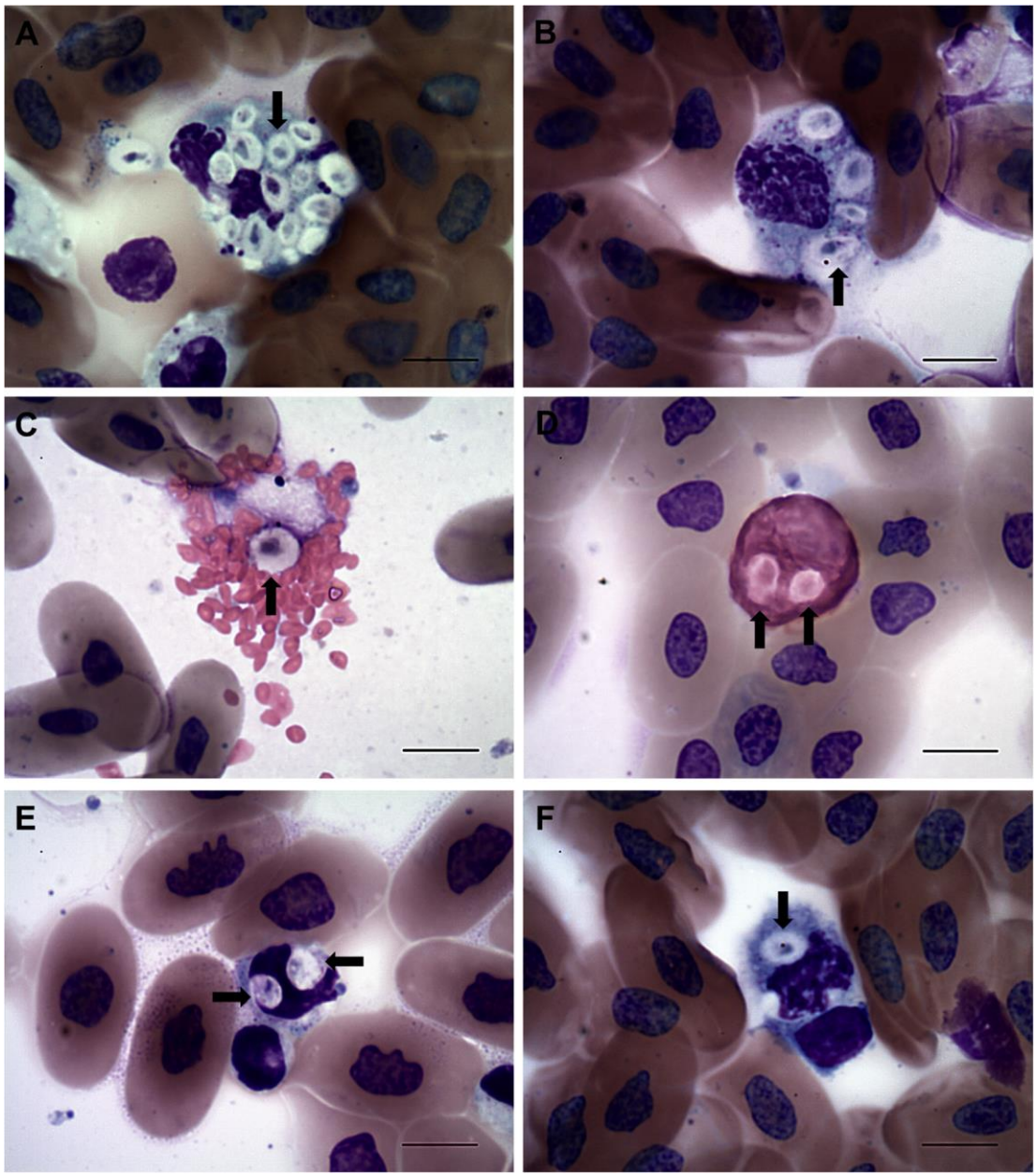
All statistical analyses and graphs were performed in Prism version 5 (Graph Pad Software Inc., San Diego, CA, USA) software. Continuous data were compared by Kolmogorov-Smirnov tests for normality and ranked according to their adherence to a Gaussian curve. Data were expressed as median, interquartile ranges, and minimum and maximum values. Same populations exposed to either SAPI or Zymosan stimuli where compared with Unpaired t tests, or Mann-Whitney tests if distribution was non-gaussian. When comparing more than two groups of samples, Kruskal-Wallis with Dunn's posttest was used. Differences were considered statistically significant at $p < 0.05$.

3.4 RESULTS

3.4.1 Qualitative analysis of snake leukocytes phagocytosis activity in blood smears

By examining the blood smears (n = 6), heterophils, lymphocytes and azurophils of all snake species were observed to present phagocytosis activity in the presence of Zymosan A particles (Figure1). Neither thrombocytes nor basophils were found with internalized or phagocytized particles. Leukocyte types could not be identified in SAPI phagocytosis assays due to the dark-purple staining of engulfed bacteria throughout the cytoplasm and nucleus.

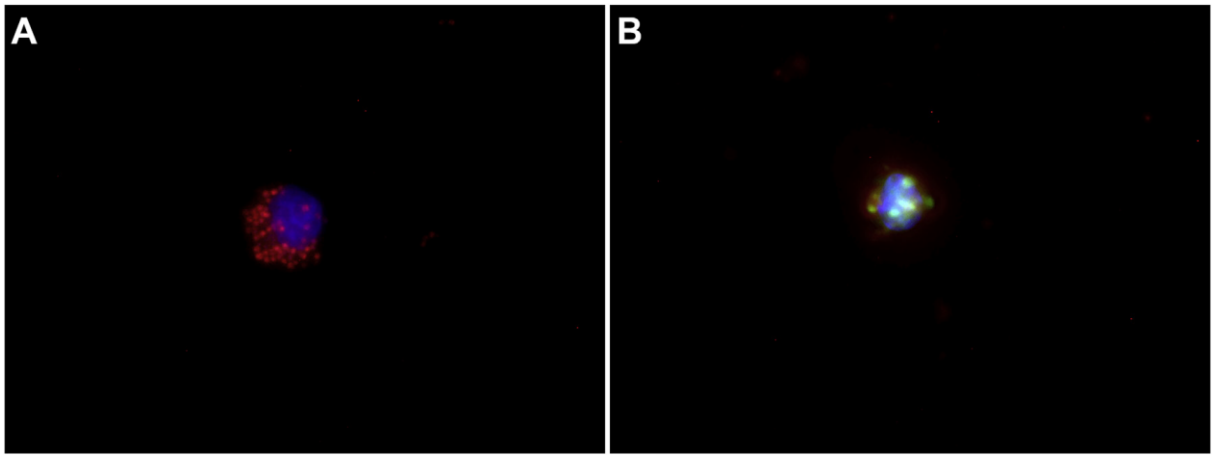
Figure 1 – *In vitro* phagocytosis activity presented by snakes' fresh blood leukocytes upon Zymosan A particles. (A and B) *Crotalus durissus* azurophils showing engulfed particles (arrows). (C) *Boa constrictor* heterophil with engulfed particle (arrow) and presenting degranulation. (D) *Boa constrictor* heterophil with two engulfed particles (arrows) with no degranulation. (E) *Bothrops jararaca* small lymphocyte with two engulfed particles pressing the cell nucleus. (F) *Bothrops jararaca* large lymphocyte with one engulfed particle. Scale bars: 8 mm



3.4.2 Fluorescence microscopy of phagocytosis assays

To confirm the quality of the fluorescence of antigenic particles and phagocytosis activity, fluorescence microscopy was performed in one SAPI and one Zymosan A phagocytosis assays per species. After images analysis, we verified that both stimuli were fluorescent and engulfed by snake leukocytes (Figure 2).

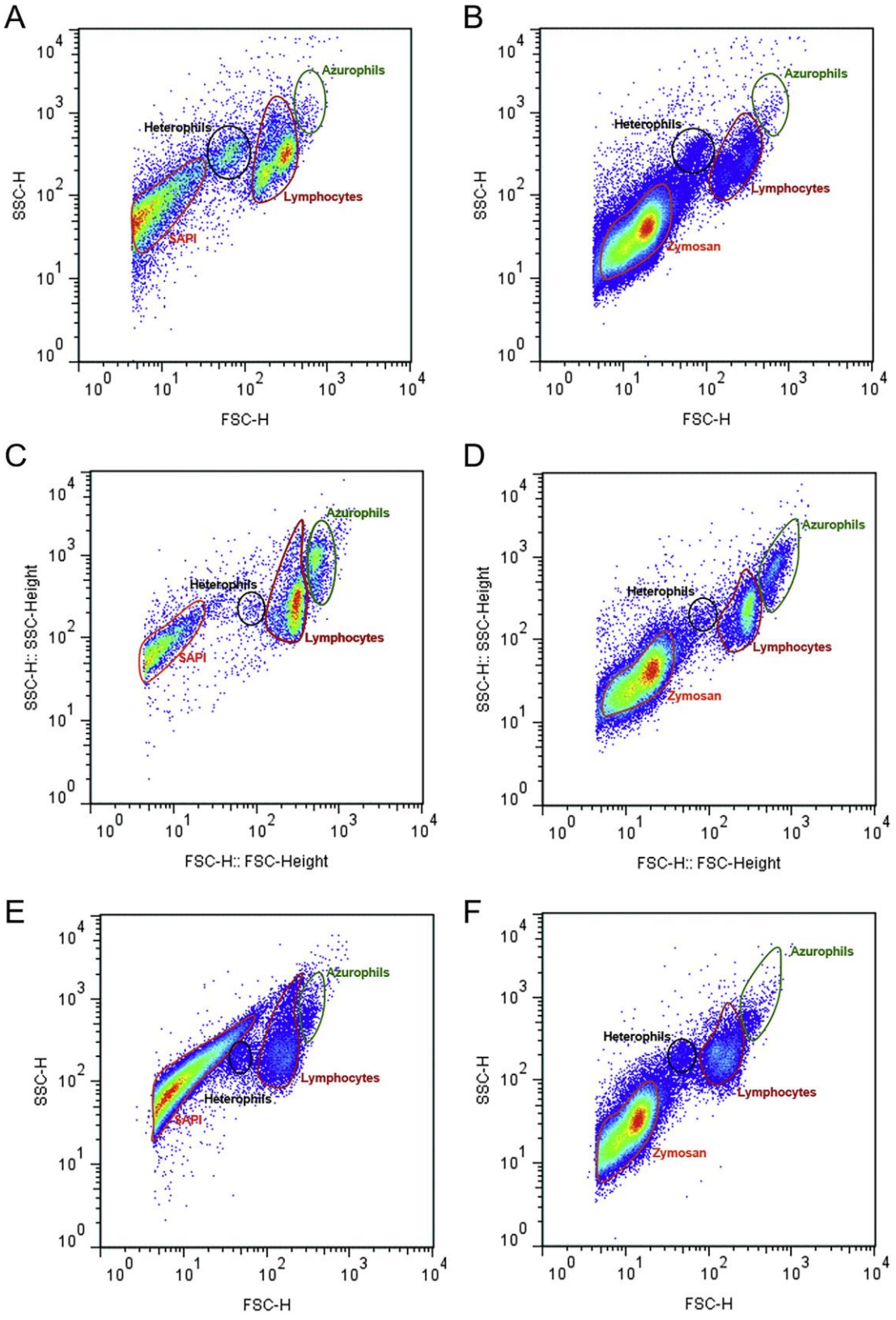
Figure 2 – Fluorescence microscopy of phagocytosis assays. (A) *Crotalus durissus* leukocyte with engulfed SAPI particles (red). (B) *Bothrops jararaca* leukocyte with engulfed Zymosan A particles (green). Nuclei are staining in blue (DAPI). Immersion oil 100x objective



3.4.3 Flow cytometric profiles and cell sorting

Three clusters of cell populations were defined representing relatively homogenous cell populations based on cell size/FSC and internal complexity/SSC. One leukocyte sample from each species was sorted via the three defined clusters and could be identified based on their light microscopic morphology. Although the morphologies were poorly preserved, heterophils, lymphocytes and azurophils were properly identified. Graphs of leukocyte populations with phagocytosis challenges are illustrated in representative cytograms per snake species (Figure 3). Representative dot plots of size and fluorescence channel (FSC x FL1 or FSC x FL2) with and without stimuli, for each cell population are provided for *Bothrops jararaca* (n = 1) (Supplementary material).

Figure 3 – Representative cytograms of phagocytosis assays with SAPI (left column) and Zymosan A (right column) stimuli based on size (FSC) and internal complexity (SSC). (A and B) *Crotalus durissus*, (C and D) *Bothrops jararaca*, and (E and F) *Boa constrictor*



3.4.4 Detection of phagocytosis activity of snakes' leukocytes subsets by flow cytometry: frequency and mean fluorescence intensity

Considering the percentage of cells that undergo phagocytosis activities upon SAPI treatment, heterophils presented the highest phagocytic frequency, when compared to azurophils and lymphocytes (Kruskal-Wallis-Dunn's, $p < 0.05$). With Zymosan A particles, heterophils and azurophils populations presented a major phagocytic frequency, when compared to lymphocytes (Kruskal-Wallis-Dunn's, $p < 0.05$). Results patterns were similar in all three snake species (Figure 4A, C and E).

When the same leukocyte subset was analyzed with different challenges, we verified that heterophils and lymphocytes presented higher frequency of phagocytosis with the SAPI than with the Zymosan A challenge (Mann-Whitney, $p < 0.05$) in all three species of snakes (Figure 4A, C and E). However, azurophils presented a different behavior according to snakes' species, showing no statistical difference of phagocytosis frequency between SAPI and Zymosan A challenges in *Boa constrictor* and *Bothrops jararaca* (Unpaired t-test, $p > 0.05$) (Figure 4B and C), but higher frequency of phagocytosis activity with SAPI than with Zymosan A in *Crotalus durissus* (Mann-Whitney, $p < 0.05$) (Fig. 4A). The phagocytosis frequency medians and standard errors for heterophils, lymphocytes and azurophils with SAPI and Zymosan A challenges are plotted in Table 1.

After analysis of the results obtained for MFI, we verified that there is no statistical difference in the MFI presented by azurophils with SAPI or Zymosan A challenges (Mann-Whitney, $p > 0.05$) and that lymphocytes engulfed statistically more particles of SAPI than Zymosan A in all snakes' species (Mann-Whitney, $p < 0.05$) (Figure 4B, D and F). In vipers, heterophils presented no statistical difference of MFI between SAPI and Zymosan A stimuli (Mann-Whitney, $p > 0.05$) (Fig. 4B and D). However, *Boa constrictor* heterophils presented a higher MFI with SAPI than with Zymosan A particles (Mann-Whitney, $p < 0.05$) (Figure 4F).

Comparing the same challenge with different leukocyte populations, we verified that lymphocytes presented the lowest MFI with SAPI, when compared to azurophils and heterophils (Kruskal-Wallis-Dunn's, $p < 0.05$) (Fig. 4B, D and F). With Zymosan A stimulus, lymphocytes presented the lowest MFI in vipers' species (Figure 4B and D), however, in *Boa constrictor* heterophils and lymphocytes presented lower MFI when compared to azurophils (Kruskal-Wallis-Dunn's, $p < 0.05$) (Fig. 4F). The phagocytosis mean fluorescence intensity

medians and standard errors for heterophils, lymphocytes and azurophils upon SAPI and Zymosan A challenges are plotted in Table 2.

Figure 4 - *In vitro* phagocytosis activity presented by snakes' leukocytes. Box plots show quartile ranges, middle line indicates the median, and whiskers show minimum and maximum obtained values. Percentages of leukocytes undergoing phagocytosis are found in A, C and D, while mean fluorescence intensities (MFI) are represented in B, E and F. Asterisks indicates statistically significant differences found between percentages of phagocytosis or mean fluorescence intensities in the same leukocyte population (Mann-Whitney, **p < 0.01, ***p < 0.001). Letters and symbols indicate the presence (when different; Kruskall-Wallis-Dunn's, p < 0.05) or absence (when identical; Kruskall-Wallis-Dunn's, p > 0.05) of statistical differences in leukocytes populations' response considering the same challenge

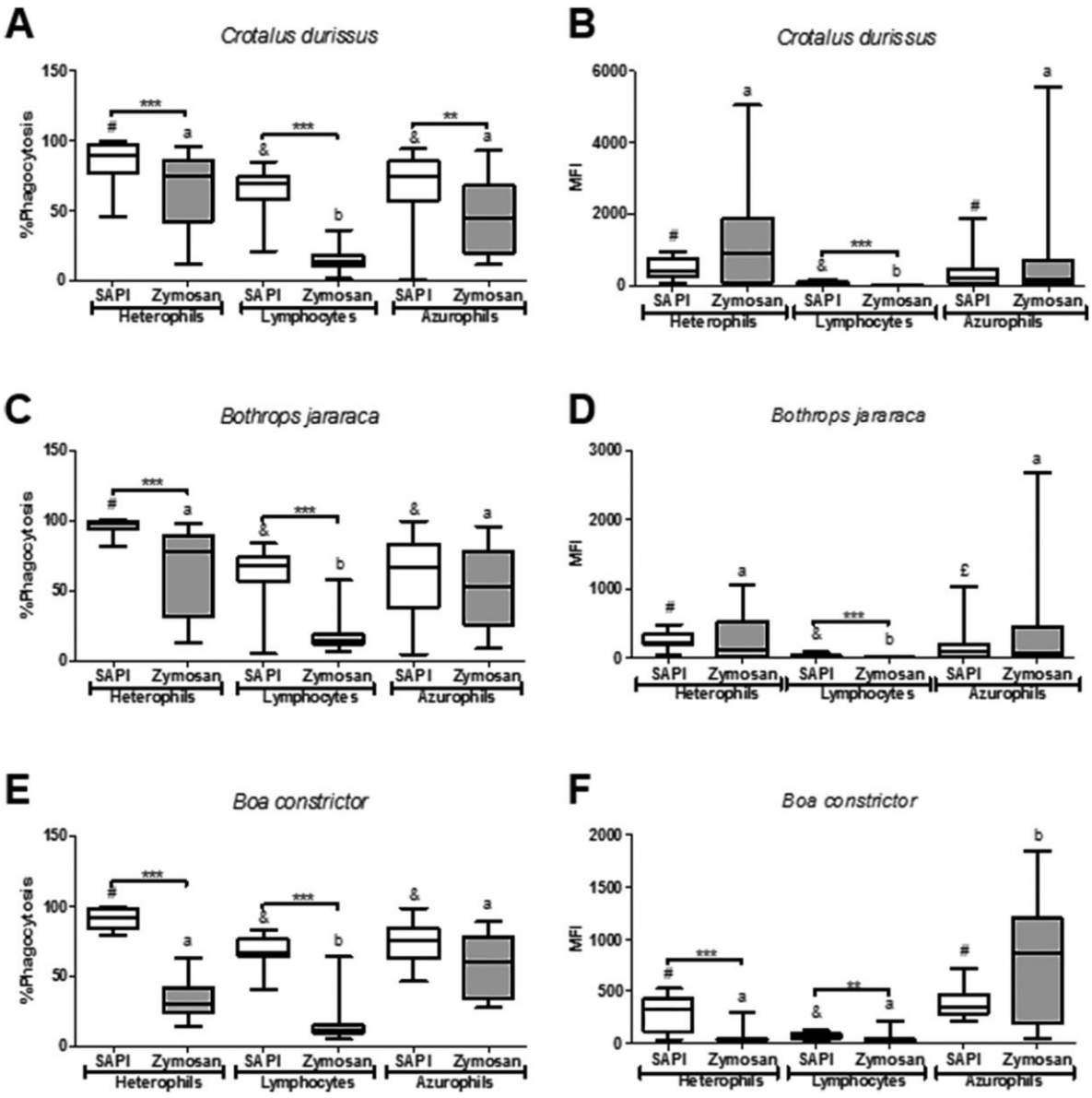


Table 1 – Phagocytosis frequency medians and standard errors for heterophils, lymphocytes and azurophils upon SAPI and Zymosan A challenges

Species	SAPI			Zymosan A		
	Heterophils	Lymphocytes	Azurophils	Heterophils	Lymphocytes	Azurophils
C. durissus	89.45 ± 2.86	69.10 ± 3.08	74.05 ± 4.47	73.90 ± 4.96	13.40 ± 1.24	44.30 ± 4.82
B. jararaca	97.70 ± 0.94	68.45 ± 3.24	66.45 ± 4.93	77.40 ± 5.38	14.00 ± 1.84	53.05 ± 5.00
B. constrictor	92.20 ± 2.08	66.75 ± 3.79	74.09 ± 4.31	29.95 ± 4.03	11.50 ± 4.47	59.85 ± 6.31

Table 2 – Phagocytosis mean fluorescence intensity medians and standard errors for heterophils, lymphocytes and azurophils upon SAPI and Zymosan A challenges

Species	SAPI			Zymosan A		
	Heterophils	Lymphocytes	Azurophils	Heterophils	Lymphocytes	Azurophils
C. durissus	424.0 ± 50.5	65.65 ± 6.5	201.0 ± 75.9	881.5 ± 251.7	14.8 ± 0.7	165.5 ± 200.7
B. jararaca	224.5 ± 18.6	32.65 ± 3.1	94.20 ± 38.6	111.5 ± 64.6	4.705 ± 0.5	67.75 ± 125.8
B. constrictor	334.0 ± 47.6	58.0 ± 9.2	346.5 ± 43.4	37.70 ± 22.4	12.40 ± 16.4	878.0 ± 172.5

3.4.5 Detection of oxidative burst activity of snakes' leukocytes subsets by flow cytometry

Flow cytometric analysis of snakes' leukocytes oxidative burst demonstrated that azurophils reflected strong oxidative burst activity against both bacteria and PMA challenges, and were the only cells to show a statistically significant difference between baseline and activated oxidative burst with PMA in all snake species (Mann-Whitney, $p < 0.05$). Lymphocytes and heterophils generated low oxidative burst activity with no statistical difference with their respective baseline burst (Mann-Whitney, $p > 0.05$) (Figure 5).

3.4.6 Measurement of serum corticosterone

Since corticosterone is a powerful regulator of leukocytes' function in reptiles, we measured the serum corticosterone of all snake samples. Yet, we were careful to include, in the data set of this study, only blood samples that were collected within a maximum of 3min (ROMERO; REED, 2005) from the start of physical restraint, preventing the influence of

corticosterone released by restraint stress. Serum corticosterone mean levels were, respectively: 34.16 ng/mL for *Crotalus durissus*, 40.50 ng/mL for *Bothrops jararaca* and 9.09 ng/mL for *Boa constrictor*.

The serum corticosterone levels (ng/mL) of the 72 sampled snakes are shown, according to the species, in figure 6.

Figure 5 – *In vitro* oxidative burst activity presented by snakes' leukocytes. Box plots show interquartile ranges, middle line indicates the median, and whiskers show minimum and maximum geometric mean fluorescence intensity. Asterisks indicate statistically significant differences between baseline respiratory burst and PMA stimulated respiratory burst activity. For vipers (Mann-Whitney, *p < 0.05); for *Boa constrictor* (Unpaired t-test, *p < 0.05). Legend: BB = basal burst

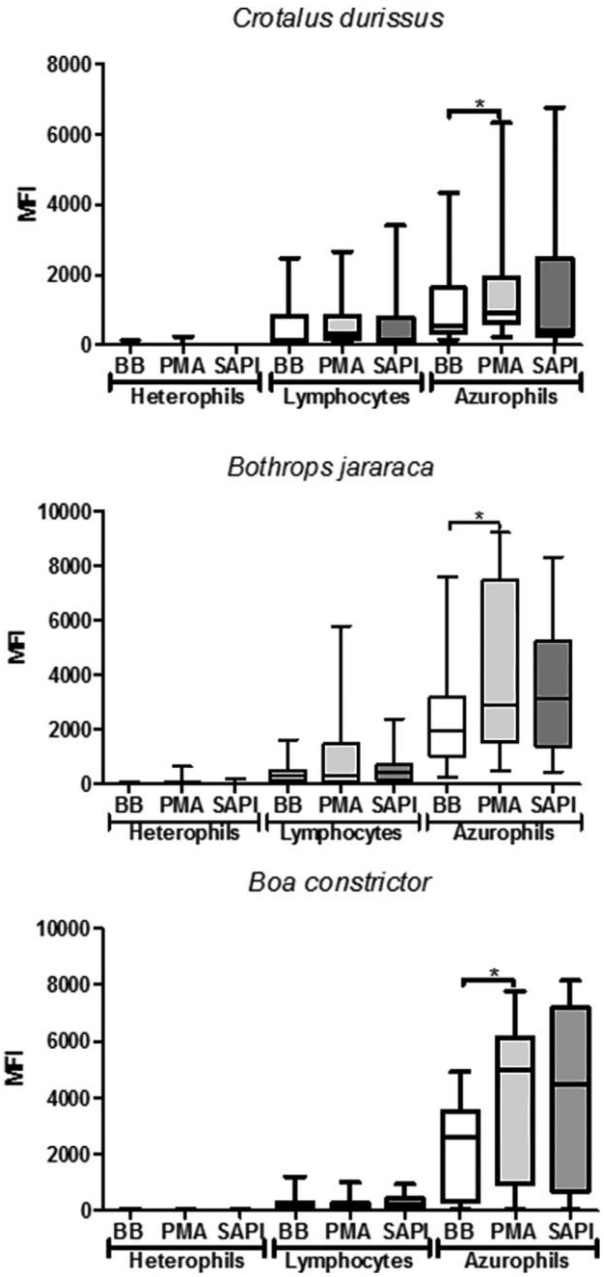
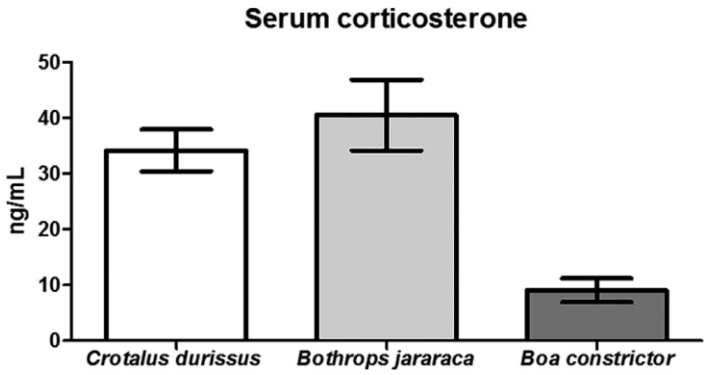


Figure 6 – Snakes' serum corticosterone levels according to snake species. Bars represent mean ± standard error



3.5 DISCUSSION

Phagocytosis and oxidative burst are considered to be fundamental mechanisms of the innate immune defense against microbes, being preserved across many species, rendering them as potentially useful tools to assess wildlife health and to establish phylogenetic studies and new experimental models (MUÑOZ et al., 2014; NAGASAWA et al., 2014). In spite of their importance, little is still known about phagocytic and oxidative burst responses of snakes' leukocytes.

To the best of our knowledge, this is the first study that successfully demonstrates and documents with photos, the phagocytic abilities of snakes' peripheral blood azurophils, heterophils and lymphocytes. Of these, azurophils and heterophils are traditionally considered to be the major phagocytic cell types of reptiles (ZIMMERMAN et al., 2009; JURD, 1994); nevertheless, the phagocytic activity of snakes' lymphocytes has not been previously demonstrated or measured. The data here reported constitute the first evidence that snakes' lymphocytes display phagocytic abilities even with particles of about 3 μm diameter.

Assessment of phagocytosis in blood smears assay is a very simple and cost-effective assay that resulted in excellent cell morphology preservation, with leukocyte types being easily identified. Therefore, it could potentially be useful to qualitatively characterize the existence of phagocytosis activities in cells of reptile species not yet investigated. Although some authors suggest the phagocytosis capacity of thrombocytes in fish, amphibians, reptiles, and birds (NAGASAWA et al., 2014; WIGLEY et al., 1999; FRYE, 1991), we did not find any

thrombocyte with engulfed particles in the blood smears analyzed. Further studies directed to snakes' thrombocytes should be undertaken to further investigate the complex functions of these cells in ophidians.

Subjecting the cells to fluorescence microscopy prior to flow cytometric analysis, was useful to confirm that bacterial and Zymosan A particles were, in fact, internalized by the snakes' leukocytes during our *in vitro* assays. The use of fluorescence microscopy in conjunction with flow cytometry has been described by several authors in phagocytosis studies in order to determine whether the particles have been internalized or were, in fact, only adhered to the cell membrane (DREVETS; CAMPBELL, 1991).

Besides the qualitative analysis of cell innate immunity, quantitative characterization of cellular phagocytic activity and oxidative burst is considered to be a useful indicator of the status of important components of the immune system (KANIA, 2008). Therefore, to further elucidate and better characterize phagocytosis and oxidative burst activities of snakes' leukocytes, flowcytometric evaluation of these cellular functions was undertaken.

Functional flow cytometric cell features, obtained by FSC and SSC parameters, are demonstrated here, for the first time, for snakes' peripheral blood leukocytes. Cell sorting with post leukocyte subsets identification by light microscopy was essential to confirm the dominant cell type in each cluster. Even with a poor and altered morphology, due to the cell sorting process and to the presence of phagocytosed particles, leukocytes were properly identified as heterophils, lymphocytes and azurophils (ALLEMAN et al., 1999), corroborating previous data (CARVALHO et al., 2016). Basophils did not form a cytogram population, probably due to the low number of this leukocyte type in the peripheral blood of healthy snakes (0-4%) (CAMPBELL, 2006; FRYE, 1991) and even for its lower gradient recovery (CARVALHO et al., 2016). Eosinophils also did not form clusters in the analyzed cytograms, but since they were described in only a few elapid species, such as: *Naja kaouthia*, *Naja naja*, *Ophiphagus hannah* and *Bungarus candidus* (PARIDA et al., 2014; VASARUCHAPONG et al., 2014; SALAKIJI et al., 2002; ALLEMAN et al., 1999), it is quite possible that they are not present in the peripheral blood of the vipers and boa species samples used here.

Comparing the functional cytograms with the previously nonfunctional ones published (CARVALHO et al., 2016), it is possible to verify that events which performed phagocytosis apparently shifted up and right in the dot plot graphs, demonstrating an increase in cell volume and internal complexity, typical of cells that have undergone phagocytosis (LEHMANN et al., 2000). In order to quantitatively evaluate phagocytosis and oxidative burst functions, the percentage of leukocytes subsets which are capable of phagocytosing and the mean number of

engulfed particles (MFI) per leukocyte subset, in addition to the strength of their oxidative burst response (MFI), were measured and analyzed.

In this work, heterophils, lymphocytes and azurophils were involved in the phagocytosis response to bacterial particles, with the heterophils population presenting the highest percentage of phagocytosing cells. This corroborates the literature, since heterophils in reptiles and birds are considered to be functionally equivalent to mammalian neutrophils, playing an important role in the bacterial phagocytic process and inflammation (MUÑOZ et al., 2014; ZHOU et al., 2013). Upon the Zymosan A stimulus, we verified that the azurophils and heterophils populations displayed a higher frequency of phagocytosis, when compared to lymphocytes, probably due to the size of the Zymosan A particles (about 3 μm), whereas lymphocytes are small cells (5-10 μm) and may have difficulty in phagocytosing large particles. This hypothesis is sustained when one takes into account that a higher percentage of lymphocytes phagocytosed bacterial particles (which are smaller-about 0.6 μm) than Zymosan A. In cases of bacteremia, lymphocytes containing engulfed microorganisms are often observed (FRYE, 1991). Considering the MFI results, we observed that heterophils and azurophils internalized, in general, more particles, when compared to lymphocytes subjected to both stimuli, suggesting that leukocytes phagocytosis capacity in vitro may be directly related to leukocytes size.

Oxidative burst, also named respiratory burst, is an effector mechanism of microbial killing performed by some phagocytic cells (HÕRAK; SILD, 2010). This mechanism destroys microorganisms by producing reactive oxygen species, mediated by the NADPH oxidase enzyme, which constitutes the primary source of oxidants required to kill microorganisms (FREITAS et al., 2009; HÕRAK; SILD, 2010). In humans, the inactivity of NADPH oxidase results in a chronic granulomatous disease (a genetic disease) due to the inability of phagocytes to produce microbicidal oxidants (FREITAS et al., 2009). In reptiles, the oxidative burst ability has already been attributed to monocytes and lymphocytes in sea turtles (ROSSI et al., 2009; ROUSSELET et al., 2013) and to macrophages in *Trachemys scripta* (PASMANS et al., 2001).

In the present work, only azurophils presented a significant and strong oxidative burst activity, when compared to their respective burst baseline. This agrees with the expected function of snakes' azurophils, since oxidative burst activity is a characteristic function of cells specialized in phagocytosis, such as monocytes, azurophils and neutrophils (HEARD et al., 2004; FREITAS et al., 2009). Furthermore, snakes' azurophils stain positive for benzidine peroxidase, Sudan Black B and PAS, and have been proven to mount an oxidative burst, like mammalian neutrophils (HEARD et al., 2004; ALLEMAN et al., 1999).

Based on cytochemical staining and ultrastructural studies, reptilian heterophils are assumed to be functionally equivalent to mammalian neutrophils with their primary functions being phagocytosis and microbicidal activity (STRIK et al., 2007). Although snakes' heterophils seem to be a specialized phagocytic cell, cytochemical studies describe that heterophils granules from most reptile species stain negative for peroxidase enzyme. Therefore, its function may be closely involved to non-oxygen dependent destruction mechanisms of phagocytosed microorganisms (BOUNOUS et al., 1996; STRIK et al., 2007), corroborating the absence of oxidative burst activity found for the heterophils populations described in this work.

Since it is indisputable that glucocorticoids are powerful regulators of the immune function and potential immunosuppressant in reptiles (BERGER et al., 2005; MEYLAN et al., 2010), we undertook the task of evaluating the leukocytes' immune response only from blood samples collected in less than 3min from the beginning of the physical restraint of the snakes (ROMERO; REED, 2005), so that the immune functions analyzed were not underestimated by the effect of restraint stress. Furthermore, we reported the serum values of this hormone for the species studied, so that future researchers may compare their results with serum corticosterone concentrations, paralleling functional results with serum corticosterone levels.

This work is the first to report on the functional characterization of innate responses of snakes' leukocytes subsets, linking flow cytometry cell features, microscopic images, and corticosterone levels. These methods exploit cell-specific characteristics inherent to snakes' leukocytes subpopulations: heterophils, lymphocytes and azurophils. The results obtained here may offer important and reproducible methods for better understanding of the leukocyte series in reptiles, allowing further studies and leading to advances in research in immunobiology, eco-immunology and clinical diagnosis.

Funding

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Acknowledgements

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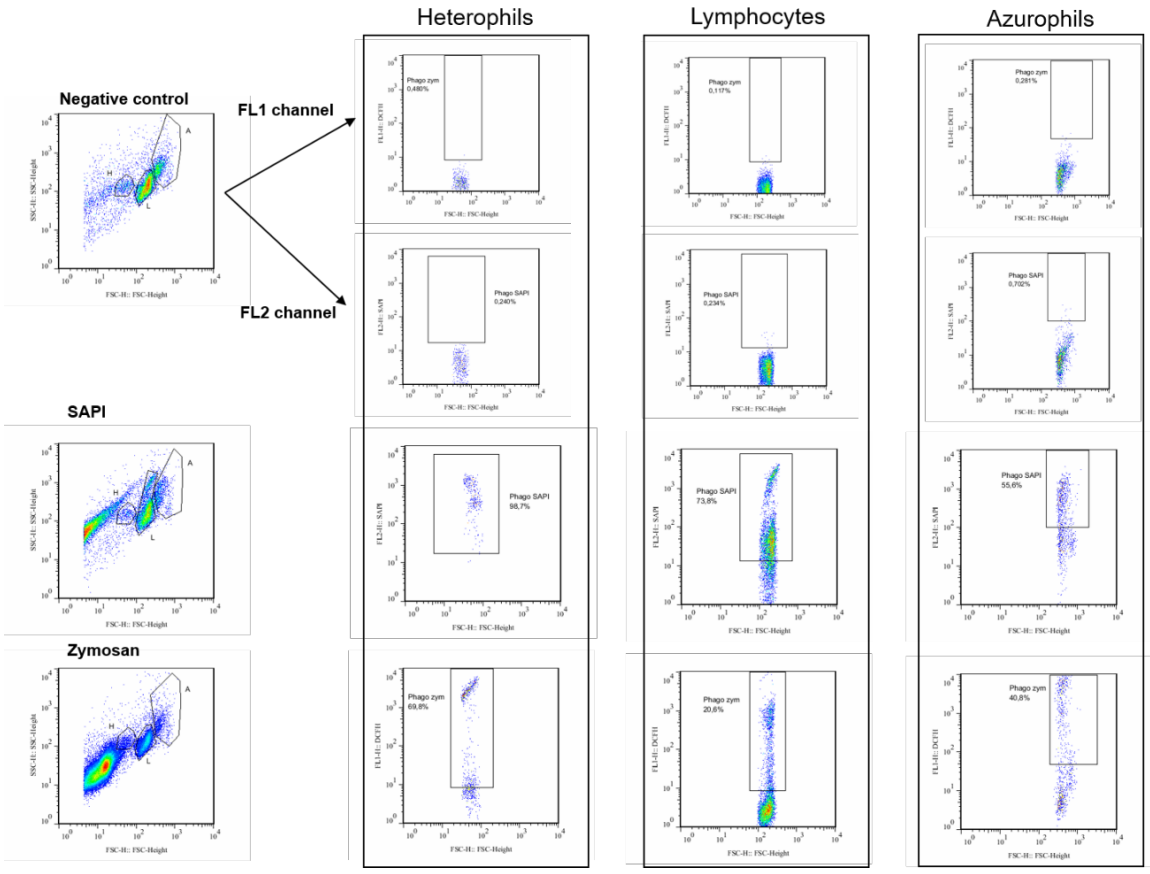
APPENDIX A Supplementary data.

Supplementary data related to this article can be found at: http://dx.doi.org/10.1016/j.dci.2017.04.007



Supplementary material: Far left panels – representative size and scatter dot plots of the same sample without stimuli or with either SAPI or Zymosan A. Right panels – Representative dot plots of size and fluorescence channel (FSC x FL1 or FSC x FL2) of the same sample with and without stimuli, for each cell population. N=1, *Bothrops jararaca*.

ATTACHMENT A Developmental and Comparative Immunology license.

ATTACHMENT B Statements of co-authors.

São Paulo, 02 de maio de 2018

DECLARAÇÃO

Eu, Nicolle Gilda Teixeira Queiroz Hazarbassanov, CPF: 340.119.978-11, co-autora do artigo "*Functional characterization of neotropical snakes peripheral blood leukocytes subsets: Linking flow cytometry cell features, microscopy images and serum corticosterone levels.*", publicado em 2017 pela revista:"Developmental and Comparative Immunology", declaro estar **de acordo** com a inclusão e publicação do acima referido artigo na Tese de Doutorado intitulada "Functional analysis of peripheral blood leucocytes of snakes (Boidae and Viperidae) during the process of adaptation to captivity" de autoria de Marcelo Pires Nogueira de Carvalho, aluno de Doutorado do Programa de Patologia Experimental e Comparada do Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo.

Atenciosamente,

Nicolle Gilda Teixeira Queiroz Hazarbassanov

São Paulo, 02 de maio de 2018

DECLARAÇÃO

Eu, Cristina de Oliveira Massoco Salles Gomes, CPF: 167643398-80, co-autora do artigo "*Functional characterization of neotropical snakes peripheral blood leukocytes subsets: Linking flow cytometry cell features, microscopy images and serum corticosterone levels*." publicado em 2017 pela revista:"Developmental and Comparative Immunology", declaro estar **de acordo** com a inclusão e publicação do acima referido artigo na Tese de Doutorado intitulada "Functional analysis of peripheral blood leucocytes of snakes (Boidae and Viperidae) during the process of adaptation to captivity" de autoria de Marcelo Pires Nogueira de Carvalho, aluno de Doutorado do Programa de Patologia Experimental e Comparada do Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo.

Atenciosamente,

_____

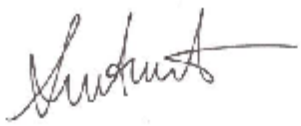
Cristina de Oliveira Massoco Salles Gomes

São Paulo, 02 de maio de 2018

DECLARACÃO

Eu, Sávio Stefanini Sant'Anna, CPF: 130.023.398-22, co-autor do artigo "*Functional characterization of neotropical snakes peripheral blood leukocytes subsets: Linking flow cytometry cell features, microscopy images and serum corticosterone levels.*" publicado em 2017 pela revista: "Developmental and Comparative Immunology", declaro estar **de acordo** com a inclusão e publicação do acima referido artigo na Tese de Doutorado intitulada "Functional analysis of peripheral blood leucocytes of snakes (Boidae and Viperidae) during the process of adaptation to captivity" de autoria de Marcelo Pires Nogueira de Carvalho, aluno de Doutorado do Programa de Patologia Experimental e Comparada do Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo.

Atenciosamente,



Sávio Stefanini Sant'Anna

São Paulo, 02 de maio de 2018

DECLARAÇÃO

Eu, Mariana Mathias Lourenço, CPF: 362310508-23, co-autora do artigo "*Functional characterization of neotropical snakes peripheral blood leukocytes subsets: Linking flow cytometry cell features, microscopy images and serum corticosterone levels.*" publicado em 2017 pela revista:"Developmental and Comparative Immunology", declaro estar **de acordo** com a inclusão e publicação do acima referido artigo na Tese de Doutorado intitulada "Functional analysis of peripheral blood leucocytes of snakes (Boidae and Viperidae) during the process of adaptation to captivity" de autoria de Marcelo Pires Nogueira de Carvalho, aluno de Doutorado do Programa de Patologia Experimental e Comparada do Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo.

Atenciosamente,

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Atenciosamente,

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Atenciosamente,

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São Paulo, 02 de maio de 2018

DECLARAÇÃO.

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Atenciosamente,

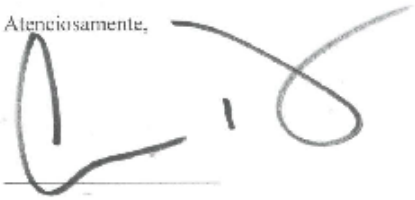
Kathleen Fernandes Grego

São Paulo, 02 de maio de 2018

DECLARAÇÃO

Eu. José Luiz Catão Dias, CPF 02959788800, co-autor do artigo "*Functional characterization of neotropical snakes peripheral blood leukocytes subsets: Linking flow cytometry cell features, microscopy images and serum corticosterone levels.*", publicado em 2017 pela revista:"Developmental and Comparative Immunology", declaro estar **de acordo** com a inclusão e publicação do acima referido artigo na Tese de Doutorado intitulada "Functional analysis of peripheral blood leucocytes of snakes (Boidae and Viperidae) during the process of adaptation to captivity" de autoria de Marcelo Pires Nogueira de Carvalho, aluno de Doutorado do Programa de Patologia Experimental e Comparada do Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo.

Atenciosamente,



José Luiz Catão Dias

4 CORTICOSTERONE-IMMUNE-HAEMATOLOGICAL INTERACTIONS DURING ACUTE AND CHRONIC CONFINEMENT IN TWO RECENTLY WILD-CAUGHT VIPERIDAE SPECIES: INSIGHTS INTO MEASURING AND UNDERSTANDING STRESS IN REPTILES

4.1 ABSTRACT

There is evidence from several vertebrate animals that the hypothalamic-pituitary-adrenal (HPA) axis interferes in various immune and haematological parameters. Consequently, researches that concomitantly measure components of both the HPA axis and the immune and haematological systems are required to better understand their physiologic and functional relationship in the face of environmental trials. Serum corticosterone levels are presumably raised by exposure to both acute and chronic stressors involved in the process of adapting to environmental changes. Stress-induced increases in serum corticosterone have several significant purposes, including modulation of innate leukocyte functions, such as phagocytosis and oxidative burst, and variations in the number and proportion of circulating leukocytes. In this context and with the improvement of flow cytometry methods comprising snake species, we report on innate cellular function (phagocytosis and oxidative burst) and haematological profile (differential leukocyte count and heterophil:lymphocyte ratios) interactions with serum corticosterone levels in recently caught snakes, at two time points in their adaption to captivity (5 and 60 days of captivity). Serum corticosterone levels showed no correlation with the oxidative burst activity of azurophils or with the heterophil: lymphocyte ratio in either viper species. For *Bothrops jararaca*, the percentage of phagocytosis displayed by heterophils with *Staphylococcus aureus* stained with propidium iodide (SAPI) at 5 days of captivity presented a positive correlation with serum corticosterone levels. However, the percentage of phagocytosis presented by azurophils with SAPI at 60 days of captivity presented a negative correlation with serum corticosterone concentrations. Additionally, a negative correlation was found between the quantity of Zymosan A ingested by lymphocytes and serum corticosterone levels at 5 days of captivity. For *Crotalus durissus*, the percentage of phagocytosis displayed by lymphocytes with SAPI at 60 days of captivity presented a negative correlation with serum corticosterone levels. This research, to the best of our knowledge, is the first to describe the functional characterization of non-specific cellular responses of peripheral blood leukocyte subsets in

wild-caught snakes, linking immune and haematological profiles with serum corticosterone levels, during the process of adaptation to captivity. Our results show that each subpopulation of leukocytes presents behaviours peculiar to the phases of adaptation to captivity, and the likely existence of concomitant factors other than corticosterone, which may modulate the functions of the immune cells in these viperid species.

Keywords: Corticosterone. Glucocorticoids. Heterophil: lymphocyte ratio. Immune system. Oxidative burst. Phagocytosis. Snakes

4.2 INTRODUCTION

Environmental stressors (e.g. habitat loss, pollution, climate change and captivity) represent important challenges to wild- or recently caught vertebrate populations and have the capacity to concurrently trigger two major organic responses that are usually required to cope with such stressors: the immune system and the hypothalamic-pituitary-adrenal (HPA) axis (GRAHAM et al., 2012). There is evidence from several vertebrate animals that the HPA axis interferes in various immune and haematological parameters (SAPOLSKY; ROMERO; MUNCK, 2000; MARTIN, 2009). Consequently, researches that concomitantly measure components of both the HPA axis and the immune and haematological system are required to better comprehend their physiologic and functional relationship in the face of environmental trials (GRAHAM et al., 2012).

Although the idea of stress is ambiguous and difficult to describe (ROMERO; DICKENS; CYR, 2009), the concentration of circulating glucocorticoids is frequently taken as a direct measurement of the level of stress an animal is exposed to in ecological sciences (HOFER; EAST, 1998). Glucocorticoids, such as corticosterone, play a vital role in composing biological reaction patterns determining adapted responses to stressful environments (VOITURON et al., 2017). Corticosterone is secreted from adrenocortical cells as part of the hormone cascade controlled mainly by the HPA axis (ROMERO, 2004; WEST; KLUKOWSKI 2018).

Serum corticosterone levels are presumably raised by exposure to both acute and chronic stressors involved in the process of adapting to environmental changes (MOORE;

JESSOP, 2003; TILBROOK et al., 2000). Stress-induced elevations of serum corticosterone have several significant purposes, including permitting the organism to deal with an acute stressor via enhanced energy mobilization and behavioural changes (WINGFIELD et al., 1998), enhancing other stress response systems, producing a negative feedback loop in the HPA axis, and preparing the animal for future stressors (ROMERO 2004; SAPOLSKY; ROMERO; MUNCK, 2000). Furthermore, glucocorticoids have been shown to alter innate leukocyte functions such as phagocytosis and oxidative burst (RODRÍGUEZ et al., 2001), and promote variations in the number and proportion of circulating leukocytes as well as in neutrophil or heterophil: lymphocyte ratios (MCKAY; CIDLOWSKI, 2003; GOESSLING et al., 2015).

This general description of stress-immune interaction was mostly established from studies in mammals (SAPOLSKY et al., 2000; GRAHAM et al., 2012), and supplementary researches in vertebrates other than mammals will likely contribute to our understanding of these interactions (SOMERO, 2000; GRAHAM et al., 2012). Additionally, how stressors interact with immunity in wild animals remains practically unstudied (MARTIN, 2009).

In this context and with the improvement of flow cytometry techniques comprising snake species (CARVALHO et al., 2016, 2017), we report on innate cellular function (phagocytosis and oxidative burst) and haematological profile (differential leukocyte count and heterophil: lymphocyte ratios) interactions with serum corticosterone levels in recently caught snakes, comprising two species of neotropical viperids, at two time points in their adaption to captivity (5 and 60 days of captivity). We describe novel and additional information that should contribute to a better understanding of how stress caused by environmental change can affect innate leukocyte functions and haematological parameters, as well as the presence or absence of a correlation between these profiles and serum corticosterone levels. The new data and methodologies reported may also be useful in facilitating the comprehension and measurement of stress in non-mammal vertebrates.

4.3 MATERIAL AND METHODS

4.3.1 Snakes

The present study was approved by the School of Veterinary Medicine and Animal Science, University of São Paulo, Ethics Committee on Animal Use (Registry Number 8322070714), and by SISBIO (Authorization System and Information in Biodiversity, licence number 44990). Two Brazilian viper species (*Bothrops jararaca* and *Crotalus durissus*) from the Laboratory of Herpetology, Butantan Institute, Brazil were included in the study. The snakes were captured in rural areas by citizens and voluntarily forwarded to the Butantan Institute, where they were housed in acclimatized standard rooms with natural lighting cycles, at temperatures of between 25 ºC and 28 ºC, and in 60 % relative humidity. The serpents were maintained in individual plastic containers (60 x 40 x 15 cm) with a corrugated fibreboard substrate and water *ad libitum*, supplied by a plastic water fountain drinker, and fed with 10–20 % of their live weight in rodents per month. Blood samples were collected from 15 males and 15 females from each viper species at two time points, after 5 and 60 days of captivity. Day zero of captivity was considered the day that each snake was caught in nature.

4.3.2 Blood Samples

All blood sampling was performed in the morning (8:00 to 10:00 am), in the summer, within a maximum period of three minutes after the beginning of physical restraint (FLOWER et al., 2015) from the ventral coccygeal vein, using heparinized disposable hypodermic needles (Becton Dickinson) attached to plastic syringes (Becton Dickinson). The samples were immediately transferred to tubes containing sodium heparin (Becton Dickinson) and transported at room temperature, within a maximum of 30 minutes, to the laboratory for processing and analysis.

4.3.3 Differential count of leukocytes, and heterophil: lymphocyte ratios

The differential leukocyte count of a total of 100 cells, including heterophils, basophils, lymphocytes, and azurophils, following the classification previously described (ALLEMAN; JACOBSON; RASKIN, 1999) was performed by an expert in reptile haematology on individual blood smears, carried out shortly after blood collection and stained according to an adapted May–Grunwald–Giemsa/Rosenfeld technique (ROSENFELD, 1947) with a x100 oil immersion lens by light microscopy. The heterophil: lymphocyte ratio was calculated by dividing the percentage of heterophils by the percentage of lymphocytes in all blood samples (n=120).

4.3.4 Isolation of leukocytes

Peripheral blood leukocytes were isolated using a one-step Percoll (GE Healthcare Life Sciences) gradient, as previously described (CARVALHO et al., 2016). This gradient was prepared by placing 2 mL of a 57 % Percoll stock solution in 1 x Hanks' Balanced Salt Solution (HBSS) (Gibco, Life Technologies), followed by slowly overlaying 2 mL of blood diluted (1:1) in 1 x HBSS, in a 15 mL Falcon tube. The gradient was centrifuged at 1280 g for 5 min, at 18ºC, with no acceleration or braking. The leukocyte layer obtained between plasma and Percoll was recovered using an automatic pipette. Isolated leukocytes were washed once in 10 mL of PBS, followed by centrifugation at 300 g for 10 minutes, and the resulting cell pellet was suspended in 1 mL of PBS. Cell numbers and viability were evaluated using a Neubauer chamber and the Trypan Blue exclusion method.

4.3.5 Phagocytosis assays

Phagocytosis assays were performed as previously described (CARVALHO et al., 2017). Viable cells were counted in a Neubauer counting chamber using Trypan Blue (10 μL

of cell suspension in 90 μL of Trypan Blue). At least 95% of the cells had to be viable in order to be subjected to functional assays. As a bacterial challenge for phagocytosis, inactivated *Staphylococcus aureus* Pansorbin® (Calbiochem, Merck Millipore) stained with propidium iodide (Sigma, St Louis, Mo, USA) (SAPI) was used in a proportion of approximately 100 bacterial particles per leukocyte. As a fungi challenge, Zymosan A (Sigma, St Louis, Mo, USA) marked with 10 mM carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) was used in a proportion of 10 particles per leukocyte. Cell suspensions were incubated for 60 minutes at room temperature (25 ºC – 28 ºC), under agitation on a 450 rpm shaker, in the dark. After the incubation period, the samples were washed in 2 mL of 3 mM EDTA solution, at 300 g for 7 minutes. The cells were then suspended in 300 mL of PBS and analysed by flow cytometry. For each sample, the phagocytosis assays were organized according to the conditions below:

- Condition A (control): cell suspension;
- Condition B (bacterial stimulus): cell suspension + SAPI suspension;
- Condition C (fungi stimulus): cell suspension + Zymosan suspension.

4.3.6 Oxidative burst assays

Oxidative burst was evaluated *in vitro* as previously described (ROSSI et al., 2009). Viable cells were counted using a Neubauer counting chamber and Trypan Blue (10 μL of cell suspension in 90 μL of Trypan Blue). At least 95% of the cells had to be viable in order to be submitted to the functional assays. As a bacterial challenge for oxidative burst, inactivated SAPI was used in the same proportion described above. As a chemical challenge for oxidative burst, phorbol 12-myristate 13-acetate (PMA) was applied. Cell suspensions were incubated for 60 minutes at room temperature (25 ºC – 28 ºC), under agitation on a 450 rpm shaker, in the dark, with 2,7-dichlorofluorescin diacetate (DCFH-DA) (Sigma, St Louis, Mo, USA) solution prepared at 304 mM in PBS, resulting in a final concentration of 60.8 mM DCFH. After incubation, samples were washed in 2 mL of 3 mM EDTA solution, at 300 g for 7 minutes. Cells were then suspended in 300 mL of PBS and analysed by flow cytometry. For each sample, the oxidative burst assays were organized as follows:

- Condition D (control): cell suspension + DCFH-DA solution

- Condition E (chemical stimulus): cell suspension + PMA solution + DCFH-DA solution;
- Condition F (bacterial stimulus): cell suspension + SAPI suspension + DCFH-DA solution.

4.3.7 Flow cytometric analysis

After functional assays, cell suspensions (about 1–5 x 10^5 cells) were used for flow cytometric analysis in dual-laser FACSCalibur (Becton Dickinson, Burlington, MA, USA) and CellQuest Pro acquisition software (Becton Dickinson, San Jose, CA, USA). A minimum of 10^4 events were acquired from leukocyte gates for oxidative burst and phagocytosis analysis. Forward (FSC) and side scatter (SSC) parameters representing size and internal complexity, respectively, were evaluated using a logarithmic scale. Voltage and amplitude values determined for FSC and SSC detectors were the same as reported in Carvalho et al. (2016). Dot plot cytograms were analysed using FlowJo software (Tree Star Inc., San Carlos, CA, USA) version 10 for Windows. To assess the phagocytic activity of snakes' leukocytes during the captivity time, we compared the immune responses from the same leukocyte subpopulation in the same individuals at two different time points (5 and 60 days of captivity). In both situations, we evaluated the frequency of cells that underwent phagocytosis (cell percentages) and the mean fluorescence intensity (MFI) of each leukocyte subset. Since, in these snake species, only azurophils perform oxidative burst (CARVALHO et al., 2017), these results were compared using the MFI index (stimulated/basal burst) of azurophils.

4.3.8 Measurement of total serum corticosterone

For total serum corticosterone measurement after 5 and 60 days of captivity, heparinized blood samples (n=120) were centrifuged at 2000 g for 10 min for plasma separation. Plasma fractions were recovered using an automatic pipette and stored at −80 °C. A DetectX®

Corticosterone Enzyme Immunoassay Kit (Arbor Assays, Ann, MI, USA) was used to quantify total serum corticosterone levels following the protocol suggested by the manufacturer.

4.3.9 Correlation of total serum corticosterone with immune and haematological profiles

Since corticosterone is recognized as a powerful regulator of the function of leukocytes in reptiles, we measured total serum corticosterone levels of all snake samples (n=120) and performed correlation statistical tests of corticosterone levels with immune (phagocytosis percentage and MFI of all leukocyte populations and oxidative burst of azurophils) and haematological (differential leukocyte count and heterophil: lymphocyte ratio) results.

4.3.10 Statistics

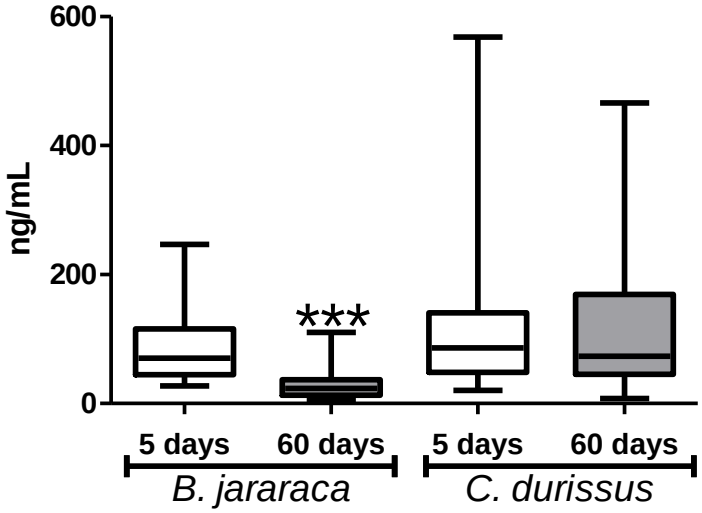
Prism version 5 (GraphPad Software Inc., San Diego, CA, USA) software was used for all statistical analyses and graphs. Continuous data were initially compared using Kolmogorov–Smirnov tests for normality and ranked according to their adherence to the Gauss curve. Data values were expressed as median, interquartile ranges, and minimum and maximum values. When comparing more than two groups of samples, Kruskal–Wallis with Dunn's post-test was used. Data from each category were represented by relative frequency (%). Differences were considered statistically significant at $p < .05$. The presence or absence of statistical correlation between serum corticosterone levels and haematologic and immunological results was evaluated using Spearman test with Prism version 5 (GraphPad Software Inc., San Diego, CA, USA).

4.4 RESULTS

4.4.1 Measurement of total plasma corticosterone at 5 and 60 days of captivity

Serum corticosterone levels (ng/mL) were statistically lower for *B. jararaca* at 60 than at 5 days of captivity (Wilcoxon signed rank test; $p < 0.0001$); for *C. durissus* no statistical difference was found (Wilcoxon signed rank test; $p > 0.05$). Mean serum corticosterone levels at 5 and 60 days of captivity, respectively, were 91.25 ng/mL and 28.89 ng/mL for *Bothrops jararaca*, and 113.2 ng/mL and 115.9 ng/mL for *Crotalus durissus*. The serum corticosterone levels (ng/mL) of the 120 samples are shown, according to the species, in figure 1.

Figure 1 – ELISA measurement of total plasma corticosterone in snakes at 5 and 60 days of captivity. Values in ng/mL are represented by medians, interquartile ranges, minimum and maximum obtained values. ***Statistically lower after 60 days, Wilcoxon signed rank test ($p < 0.0001$). N=120.



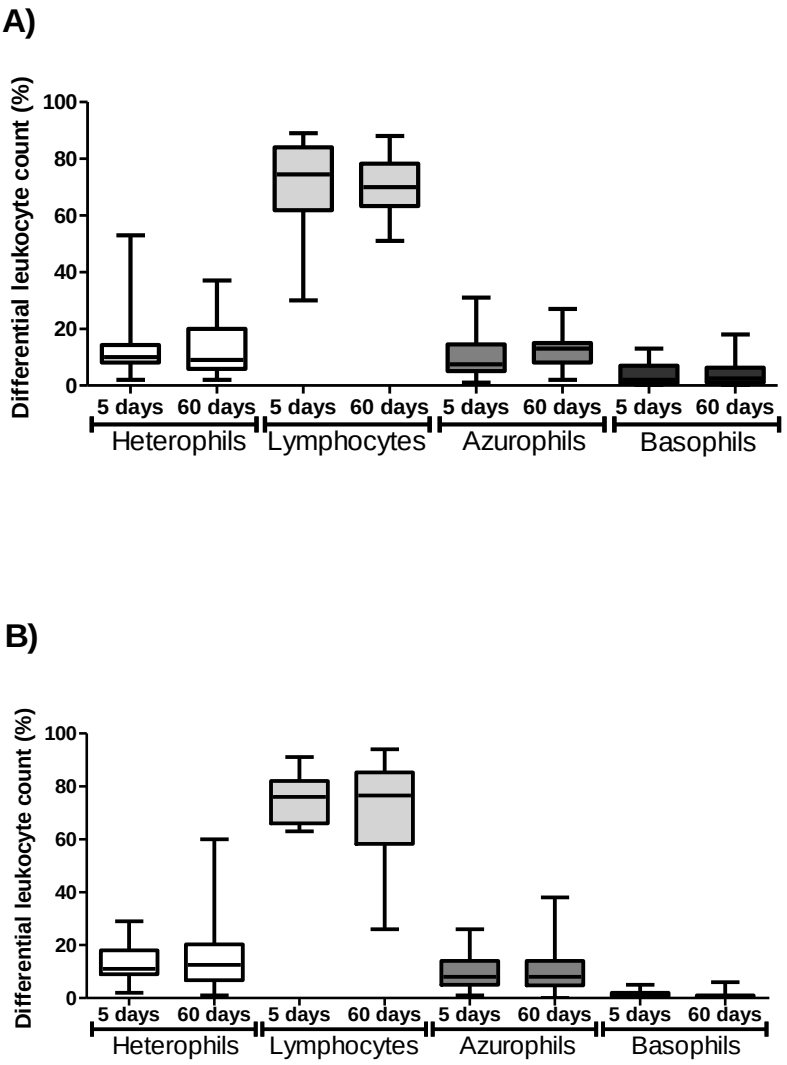
Source: (CARVALHO, M. P. N., 2018)

4.4.2 Haematological profiles of snakes at 5 and 60 days of captivity

4.4.2.1 Differential leukocyte count

Differential leukocyte count was performed for all blood samples (n=120). The results showed no statistical differences between the differential leukocyte count at 5 and 60 days of captivity (Wilcoxon signed rank test, $p > 0.05$) either viper species (Figure 2).

Figure 2 – Differential leukocyte counts in snakes at 5 and 60 days of captivity. Box plots show medians, interquartile ranges, minimum and maximum obtained values. A) *B. jararaca*. B) *C. durissus.* No statistically significant difference was found (Wilcoxon signed rank test, $p > 0.05$). N=120.

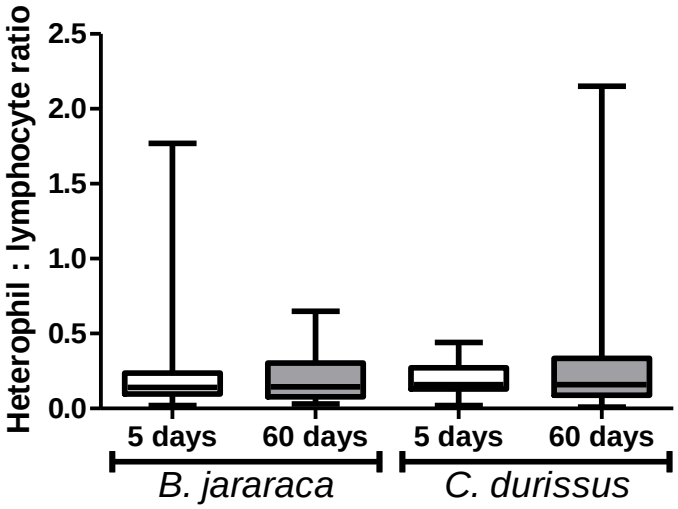


Source: (CARVALHO, M. P. N., 2018)

4.4.2.2 Heterophil: lymphocyte ratio

The heterophil: lymphocyte ratio was measured in all blood samples (n=120) at 5 and 60 days of captivity in both viper species. No significant statistical differences were found for *B. jararaca* and *C. durissus* (Wilcoxon signed rank test, $p > 0.05$) (Figure 3).

Figure 3 – Heterophil: lymphocyte ratio in snakes at 5 and 60 days of captivity. Box plots show medians, interquartile ranges, minimum and maximum obtained values. No statistically significant difference was found (Wilcoxon signed rank test, $p > 0.05$). N=120.



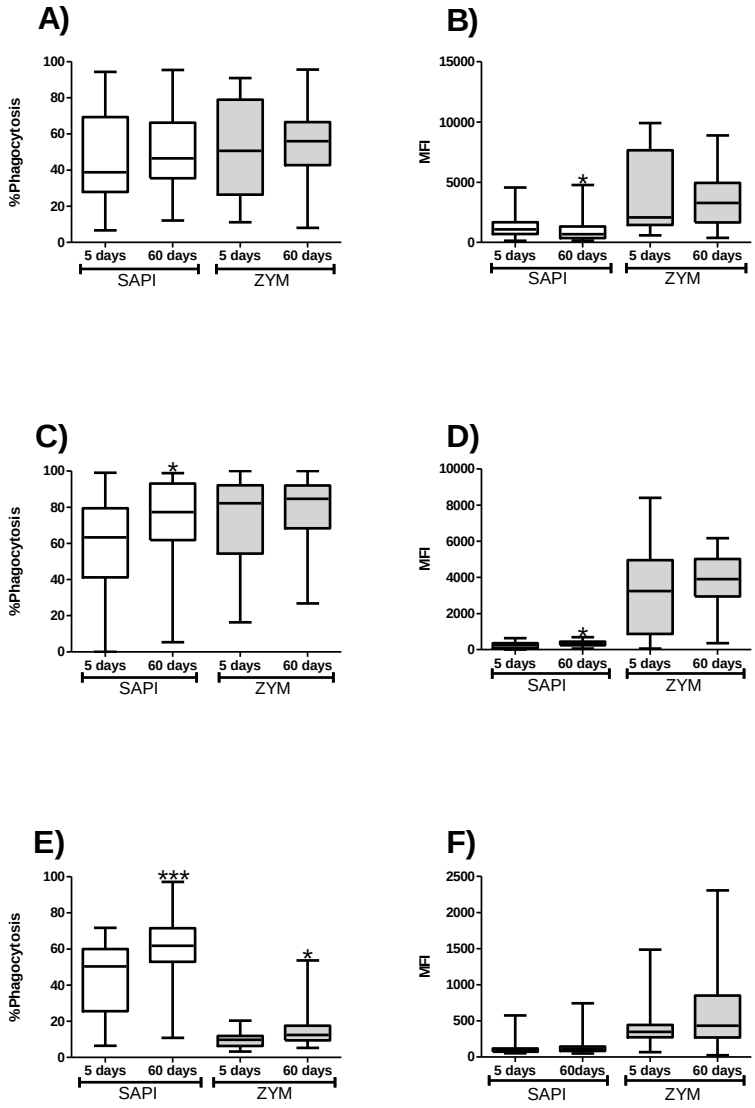
Source: (CARVALHO, M. P. N., 2018)

4.4.3 Detection of phagocytosis activity of snakes' peripheral blood leukocyte subsets by flow cytometry: frequency and mean fluorescence intensity (MFI) at 5 and 60 days of captivity

With regard to the percentage of phagocytosis for *B. jararaca* leukocytes, heterophils with SAPI, and lymphocytes with SAPI and Zymosan A, presented a higher phagocytic frequency at 60 than at 5 days of captivity (for heterophils, paired t-test, $p < 0.05$; for lymphocytes, Wilcoxon signed rank test, $p < 0.05$). However, azurophils presented a different behaviour, showing no statistical difference in phagocytosis frequency between 60 and 5 days of captivity (paired t-test, $p > 0.05$) (Figure 4A, C and E). According to the results obtained for

the MFI, we verified that *B. jararaca* azurophils engulfed a lower number of particles in snakes at 60 than at 5 days of captivity (Wilcoxon signed rank test, $p < 0.05$). However, heterophils presented a different performance with a higher MFI at 60 than at 5 days of captivity (paired t-test, $p < 0.05$). Furthermore, lymphocytes show no statistical difference in terms of MFI at 60 and 5 days of captivity (Wilcoxon signed rank test, $p > 0.05$) (Figure 4B, D and F).

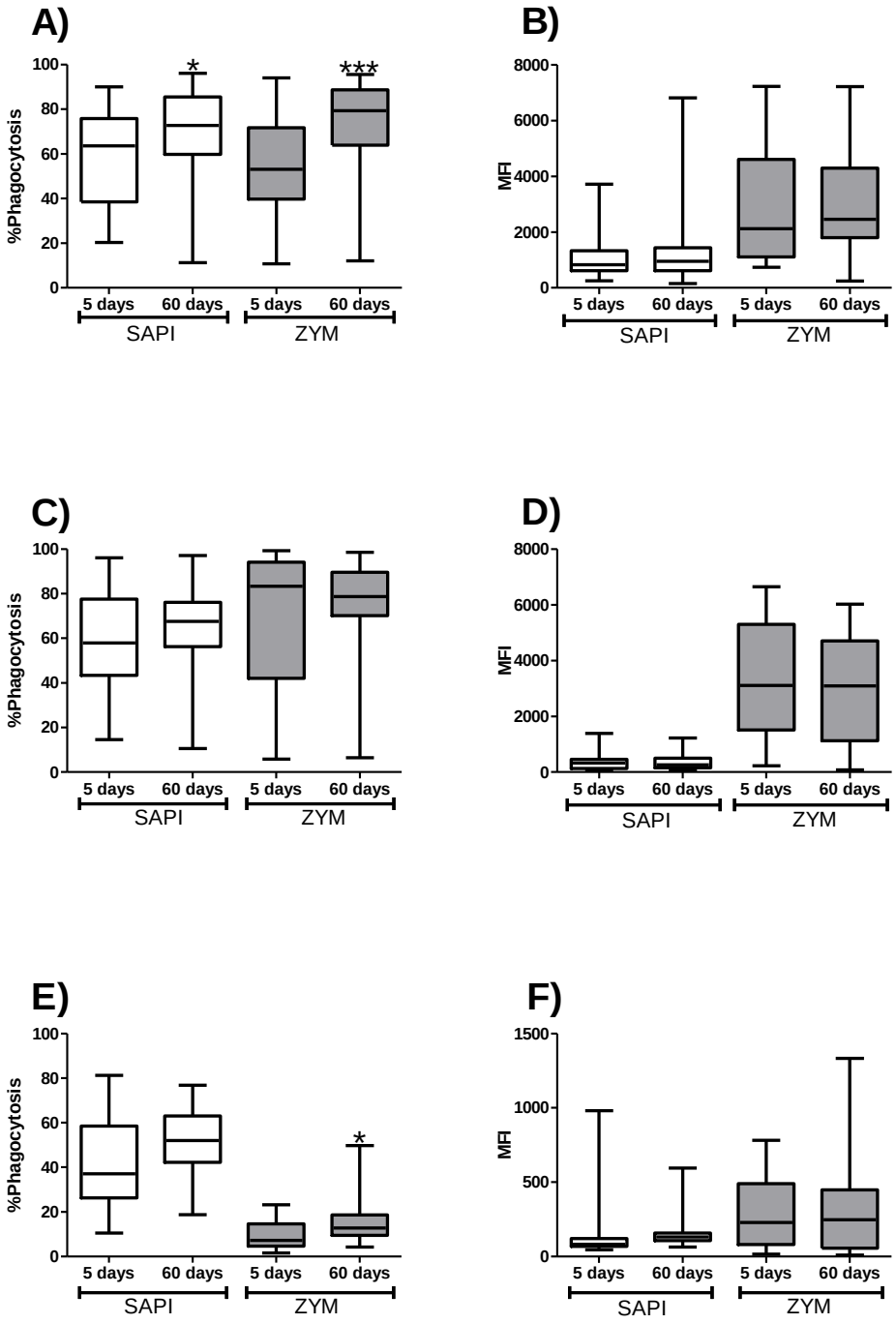
Figure 4 – *In vitro* phagocytosis activity presented by *B. jararaca* leukocytes. Percentage and mean fluorescence intensity (MFI) of phagocytosis in snakes at 5 and 60 days of captivity. Box plots show medians, interquartile ranges, minimum and maximum obtained values. A) Percentage and B) Mean fluorescence intensity of azurophil phagocytosis. C) Percentage and D) Mean fluorescence intensity of heterophil phagocytosis. E) Percentage and F) Mean fluorescence intensity of lymphocyte phagocytosis. For azurophils: *Statistically lower at 5 days, Wilcoxon signed rank test ($p < 0.05$). For heterophils: *Statistically higher at 5 days, paired t-test ($p < 0.05$). For lymphocytes: ***Statistically higher at 5 days, Wilcoxon signed rank test ($p < 0.0001$), *Statistically higher at 5 days, Wilcoxon signed rank test ($p < 0.05$). SAPI: *Staphylococcus aureus* stained with propidium iodide. ZYM: Zymosan A stained with CFSE. N=60.



Source: (CARVALHO, M. P. N., 2018)

After analysis of the percentages of phagocytosis results found for *C. durissus* leukocytes, we verified that azurophils presented a higher performance with SAPI and Zymosan A at 60 than at 5 days of captivity (paired t-test, $p < 0.05$). Similarly, lymphocytes presented a higher percentage of phagocytosis at 60 than at 5 days of captivity (paired t-test, $p < 0.05$) (Figure 5A, C and E). According to the results obtained for *C. durissus* leukocytes, no statistical difference in terms of MFI at 5 and 60 days of captivity was found (for azurophils, lymphocytes and heterophils with SAPI, Wilcoxon signed rank test, $p > 0.05$; for heterophils with Zymosan A, paired t-test, $p > 0.05$) (Figure 5B, D and F).

Figure 5 – *In vitro* phagocytosis activity presented by *C. durissus* leukocytes. Percentage and mean fluorescence intensity (MFI) of phagocytosis at 5 and 60 days of captivity. Box plots show medians, interquartile ranges, minimum and maximum obtained values. A) Percentage and B) Mean fluorescence intensity of azurophil phagocytosis. C) Percentage and D) Mean fluorescence intensity of heterophil phagocytosis. E) Percentage and F) Mean fluorescence intensity of lymphocyte phagocytosis. For azurophils: *Statistically higher at 5 days, paired t-test ($p < 0.05$); ***Statistically higher at 5 days, paired t-test ($p < 0.05$). For lymphocytes: *Statistically higher at 5 days, Wilcoxon signed rank test ($p < 0.05$). SAPI: *Staphylococcus aureus* stained with propidium iodide. ZYM: Zymosan A stained with CFSE. N=60.

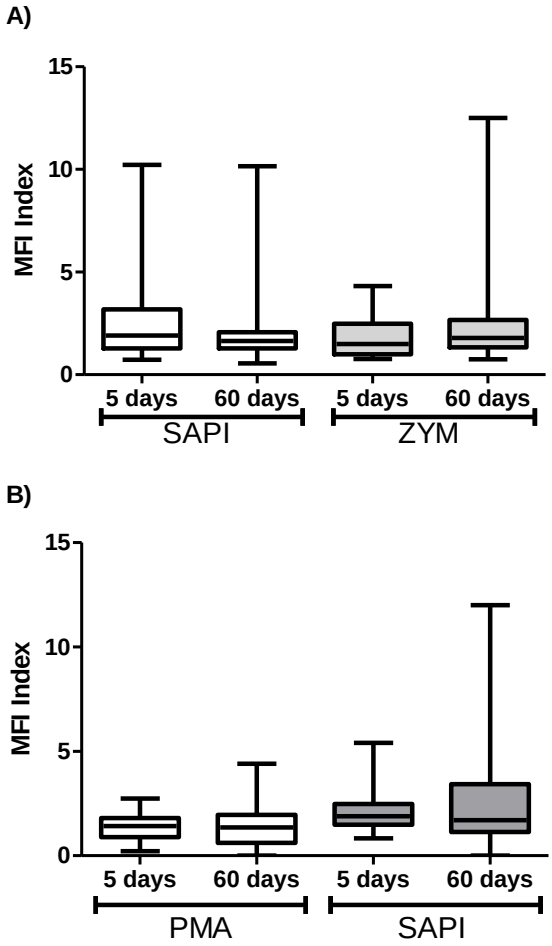


Source: (CARVALHO, M. P. N., 2018)

4.4.4 Detection of oxidative burst activity of azurophils by flow cytometry, at 5 and 60 days of captivity

Flow cytometric analysis of snakes' azurophil oxidative burst demonstrated no statistical difference at 5 and 60 days of captivity for either viper species (Wilcoxon signed rank test, $p > 0.05$). These results are shown in figure 6.

Figure 6 – Mean fluorescence intensity index for oxidative burst of azurophils in snakes at 5 and 60 days of captivity. Box plots show medians, interquartile ranges, minimum and maximum obtained values. A) *B. jararaca*. B) *C. durissus.* No statistically significant difference found (Wilcoxon signed rank test, $p > 0.05$). N=120.

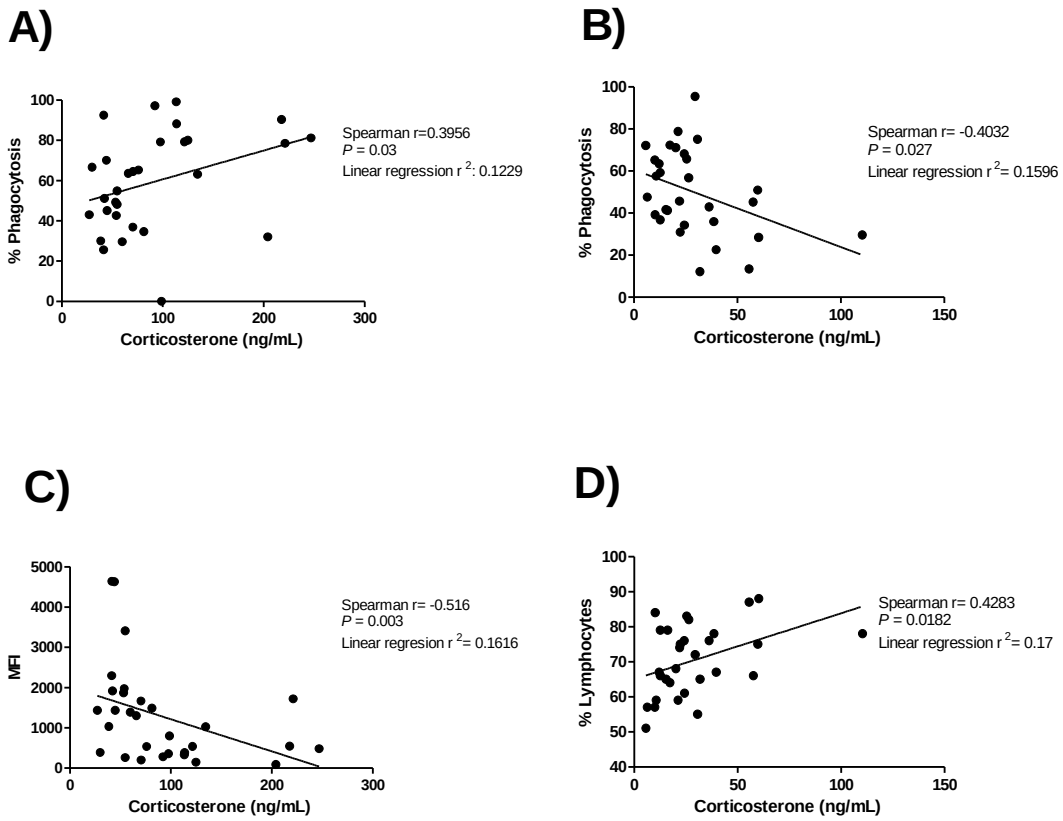


Source: (CARVALHO, M. P. N., 2018)

4.4.5 Correlation between serum corticosterone levels and immune and haematological profiles

After analysis of serum corticosterone level correlation with immune and haematological findings for *B. jararaca*, we verified the presence of a positive correlation between the percentage of phagocytosis performed by heterophils with SAPI and serum corticosterone levels, at 5 days of captivity (Spearman test, r = 0.3956, $p = 0.03$). However, at 60 days of captivity, a negative correlation between serum corticosterone levels and the percentage of phagocytosis achieved by azurophils with SAPI was found (Spearman test, r = -0.4032, $p = 0.027$). As regards the MFI, we verified a negative correlation between the MFI as performed by lymphocytes with Zymosan A and serum corticosterone concentrations at 5 days of captivity (Spearman test, r = -0.516, $p = 0.003$). Among haematological profiles, only the differential count of lymphocytes was positively correlated with the serum corticosterone levels at 5 days of captivity (Spearman test, r = 0.4283, $p = 0.0182$). These results are shown in figure 7.

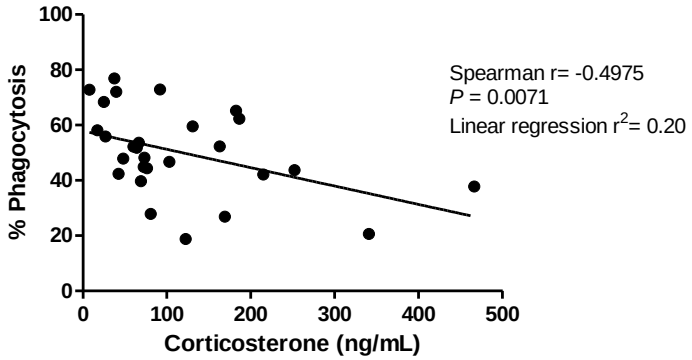
Figure 7 – Correlation between serum corticosterone levels and immune and haematological profiles found for *B. jararaca*. A) Positive correlation between serum corticosterone levels and percentage of phagocytosis presented by heterophils with SAPI in snakes at 5 days of captivity (Spearman test, r = 0.3956, p = 0.03). B) Negative correlation between serum corticosterone levels and the percentage of phagocytosis presented by azurophils with SAPI in snakes at 60 days of captivity (Spearman test, r = -0.4032, p = 0.027). C) Negative correlation between serum corticosterone and MFI levels performed by lymphocytes with Zymosan A in snakes at 5 days of captivity (Spearman test, r = -0.516, p = 0.003). D) Positive correlation between serum corticosterone concentrations and differential lymphocyte counts in snakes at 5 days of captivity (Spearman test, r = 0.4283, p = 0.0182). N=60.



Source: (CARVALHO, M. P. N., 2018)

For *C. durissus*, a negative correlation between serum corticosterone levels and the percentage of phagocytosis performed by lymphocytes with SAPI at 60 days of captivity was found (Spearman test, r = -0.4975, p = 0.0071) (Figure 8).

Figure 8 – Correlation between serum corticosterone levels and immune profiles found for *C. durissus*. Negative correlation between serum corticosterone levels and the percentage of phagocytosis presented by lymphocytes with SAPI in snakes at 60 days of captivity (Spearman test, r = -0.4975, *p* = 0.0071). N=60.



Source: (CARVALHO, M. P. N., 2018)

Serum corticosterone levels showed no correlation with the oxidative burst activity of azurophils and the heterophil: lymphocyte ratio, in either viper species.

4.5 DISCUSSION

The most commonly measured indicators of physiological stress in confined vertebrates are glucocorticoids, which generally increase after minutes of capture in wild-caught animals and may remain elevated with continued captivity (SPARKMAN et al., 2014). In reptiles, as in other vertebrates, exposure to confinement stress generally results in an increase in serum concentrations of glucocorticoids (JONES; BELL, 2004).

The means of serum corticosterone obtained by Carvalho et al., (2017) from *B. jararaca* and *C. durissus* individuals adapted to captivity (snakes with more than 1 year of captivity) were, respectively, 40.50 ng/mL and 34.16 ng/mL. Therefore, our experimental model for induction of captivity stress seems to be effective, since the serum corticosterone means obtained here (91.25 ng/mL for *B. jararaca* and 113.2 ng/mL for *C. durissus*, at 5 days of captivity) were more than twice those found in the same species individuals already adapted to captive conditions.

B. jararaca specimens sampled at 60 days of captivity exhibited a statistically lower serum corticosterone level than at 5 days of confinement, demonstrating a possible process of adaptation to captive conditions in this species within a period of 60 days. However, *C. durissus*

presented no statistical difference in serum corticosterone levels between 5 and 60 days of captivity, indicating that for *C. durissus* the period of adaptation to captivity appears to be longer than for *B. jararaca* species.

Some of the variation in reported responses to short- or long-term captivity stress may reflect different sensitivities to differences between species in their ability to adapt to captive conditions (JONES; BELL, 2004). For example, bearded dragons (*Pogona barbata*) show a surprising absence of alteration in serum corticosterone concentrations 3.5 hours after capture (CREE; AMEY; WHITTIER, 2000). Whereas some species do adapt, other reptiles do not adapt or take longer periods to do so. As evidenced by our results, *B. jararaca* seems to be a more plastic species than *C. durissus* in its ability to adapt to captivity. Such observations demonstrate the risk of inferring adrenal function information from one reptilian species to another (LANCE, 1994; JONES; BELL, 2004).

Much of the early literature points to a close link between leukocyte profiles and glucocorticoid levels. In particular, these hormones act to increase the number and percentage of heterophils, while decreasing the number and percentage of lymphocytes in reptiles and birds (DAVIS; MANEY; MAERZ, 2008). In contrast to Davis, Maneu and Maerz (2008), our results showed no statistical differences between differential leukocyte counts at 5 and 60 days of captivity for either viper species. Furthermore, our study demonstrates a positive correlation between serum corticosterone levels and the percentage of lymphocytes found in the blood of *B. jararaca* at 60 days of captivity.

Since the number of heterophils and lymphocytes is affected by stress in opposite directions, researchers have often considered the ratio of one to the other, which is the relative proportion of heterophils to lymphocytes, as a composite measure of the stress response in avians and reptiles (DAVIS et al., 2008). Although laboratory studies with exogenous corticosterone treatment or strong stressors found a positive correlation between circulating corticosterone and the heterophil: lymphocyte ratio, studies in free-living birds did not (MÜLLER; JENNI-EIERMANN; JENNI, 2011).

Corroborating the data published by Müller, Jenni-Eirmann and Jenni (2011), our results show that an endogenous increase in serum corticosterone levels, caused by captive stress, was not sufficient to alter the heterophil: lymphocyte ratios at 5 and 60 days of captivity in these species, which is reinforced by the absence of correlation found between corticosterone levels and heterophil: lymphocyte ratios. As described by Müller, Jenni-Eirmann and Jenni (2011), our results show that corticosterone level and heterophil: lymphocyte ratio cannot be used

interchangeably as indicators of stress, but together can provide a comprehensive panel regarding the stress status of a reptile in environmental change studies.

Several *in vivo* and *in vitro* researches have suggested that corticosterone modulates certain immune functions in vertebrates (RODRÍGUEZ et al., 2001; GRAHAM et al., 2012). Of particular interest is the finding that hormones, such as corticosterone, are able to modulate effects on non-specific cellular immunity, such as phagocytosis and oxidative burst (RODRÍGUEZ et al., 2001). With the advent of more advanced techniques and the establishment of analytical protocols for snake blood by flow cytometry (CARVALHO et al., 2017), we exploit cell-specific characteristics inherent to snakes' leukocyte subpopulations – heterophils, lymphocytes and azurophils – in recent wild-caught snakes, during the process of adaptation to captivity.

It is clear from leukocyte subpopulation phagocytosis results that each type of leukocyte presents peculiar behaviours during the phases of adaptation to captivity. The presence of a positive correlation between the percentage of heterophil phagocytosis and serum corticosterone levels and a negative correlation between the percentage of phagocytosis presented by azurophils and lymphocytes demonstrates that leukocyte subpopulations may be influenced in different ways by serum corticosterone concentrations. These results suggest a different reaction to the action of steroids on the diverse leukocyte subpopulations, as has been shown in other reptiles (SAAD et al., 1983, 1984), and the likely existence of other concomitant factors that modulate the phagocytosis response of the immune cells (MUÑOZ et al., 2000).

The negative correlations found between serum corticosterone levels with the percentage of phagocytosis activity presented by azurophils as well as with the phagocytosis efficiency of lymphocytes, may be related to the immunosuppression described in reptiles under the period of adaptation to captivity (GUILLETTE; CREE; ROONEY, 1995). Immunodeficiency related to mature or immature azurophils are associated with inflammatory or infectious disease (i.e., bacterial infections) in snakes, particularly in acute stages of disease (JACOBSON et al., 1997). Similarly, lymphocyte suppression may predispose viral and parasitic diseases (CAMPBELL, 1996; JACOBSON, 1999; GREGO, 2004).

As described by Carvalho et al. (2017), only azurophils present a significant and strong burst activity in this viper species. In our study, the absence of statistical differences between oxidative burst activities at 5 and 60 days of captivity, added to the lack of correlation between this activity and serum corticosterone levels, suggests an absence of effects of serum corticosterone on this leukocyte activity in both viper species, at 5 and 60 days of captivity. This result is different from what was described by Rodríguez et al. (2001), proving the

influence of serum corticosterone levels on oxidative burst activity in the heterophils of doves (*Streptopelia risoria*). To the best of our knowledge, there is no report indicating a correlation between snakes' azurophil oxidative burst activity and serum corticosterone levels.

Our results make it clear that depending on the nature of the question, and the timeline of the predicted response to a stressor, the best measures of the stress response may include several physiological parameters. Also, we verified that physiological behaviour in the face of environmental changes is different in each reptile species, even when the stressful environmental conditions are similar.

This research is the first to describe the functional characterization of innate responses of snakes' leukocyte subsets, linking leukocyte immunological functions and haematological profiles with serum corticosterone levels, during the process of adaptation to captivity. These methods exploit cell-specific characteristics inherent in snakes' leukocyte subpopulations – azurophils, heterophils and lymphocytes – in the face of a stressful condition, demonstrating how adrenal responses to environmental changes can influence haematological and immune profiles. The data presented here, therefore, may complement and support eco-physiological researches, enabling further studies and leading to advances in the comprehension and measurement of stress in lower vertebrates.

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5 FINAL CONSIDERATIONS

Due to the innumerable implications that the study of the immune system of reptiles has, either in relation to the intrinsic aspects of each species or to the important role in understanding the evolutionary characteristics of the vertebrate immune system, the objective of this study was to verticalize the analysis of data already available in the literature, with the intention of complementing the available information and opening new methodological possibilities directed toward the investigation of the immune system of three species of Brazilian snake.

In this regard, the specific objectives proposed were to validate methods for recovering leukocytes from the peripheral blood of snakes by means of density gradients commonly used in researches with mammalian models in order to enable the use of more advanced techniques, such as flow cytometry, in the evaluation of the morphological and functional characteristics of the leucocyte subpopulations described in the peripheral blood of snakes; as well as their interactions with physiological parameters closely correlated with the immunological response of vertebrates.

The density gradients, ficoll and percoll, were efficient in recovering the leukocytes from the peripheral blood of the snake species investigated and did not differ statistically from each other in cell recovery efficiency. Furthermore, it is worth mentioning that cellular viability after leukocyte recovery, in both recovery methods, remained above 95 percent, allowing further functional analysis. This leukocyte recovery technique is interesting for nucleated erythrocyte species, since leukocyte recovery methodologies involving the lysis of erythrocytes make it difficult to perform further analyses by flow cytometry due to the presence of large numbers of erythrocyte nuclei loose in the sample, which overlap the leukocyte populations obtained by cytograms, changing the analysis results.

The investigation of innate cellular functions presented by azurophils, heterophils, and lymphocytes, by flow cytometry, demonstrated that phagocytosis ability was present in all leukocyte subpopulations in all species investigated. However, the oxidative burst activity was evidenced only in azurophils. We believe that these data will be useful for a more assertive classification of the cells constituting the immune system of snakes. Additionally, the evidence that lymphocytes in these species presented phagocytosis activity corroborates the data previously described for reptiles of the Testudine order (ZIMMERMAN et al., 2010), determining strong evidence on the presence of phagocytic B cells in the constitution of reptiles'

immune system. To confirm this hypothesis for snake lymphocytes, future studies involving immunophenotyping techniques will be necessary.

The association between serum glucocorticoids levels and immunological responses has long been used as a sign of stress in reptiles, but the form or situations in which this happens at a cellular level are aspects that remain unclear. Thus, the study presents, in detail, the different innate functional performances presented by the circulating leukocytes subpopulations of *B. jararaca* and *C. durissus* in correlation with serum corticosterone levels during the period of adaptation to captivity.

Correlation analyses between the innate immunological functions of azurophils, heterophils, and leukocytes with plasma corticosterone levels during the process of adaptation in captivity of *B. jararaca* and *C. durissus* demonstrate the presence of specific correlations between cell types and serum corticosterone levels. However, the negative correlations found between plasma corticosterone concentrations, with the percentage of phagocytosis presented by azurophils, and the efficiency of phagocytosis presented by lymphocytes may be related to immunosuppression and to higher frequency in the appearance of infectious diseases in reptiles under adaptation in the captivity period.

The validation of the technique of flow cytometry for the blood of snakes, as well as the results from the application of this technique, will be useful for future studies involving questions about immunological aspects of reptiles in relation to the environmental disturbances of the Anthropocene, along with the verticalization of knowledge about aspects of reptile immunology, which will aid in understanding the evolution of the vertebrate immune system.

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