UNIVERSIDADE FEDERAL RURAL DE PERNAMBUCO
PRÓ-REITORIA DE PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOCIÊNCIA ANIMAL
JOSÉ RICARDO DE OLIVEIRA SANTOS

PARÂMETROS HEMATOLÓGICOS, BIOQUÍMICA SÉRICA, ULTRAESTRUTURA DAS CÉLULAS SANGUÍNEAS E COMPORTAMENTO DEFENSIVO DE *Leptodactylus vastus* Lutz, 1930 EM FRAGMENTOS DE FLORESTA ATLÂNTICA DO NORDESTE BRASILEIRO

RECIFE – PE
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Tese apresentada à coordenação do Curso de Pós-Graduação em Biociência Animal da Universidade Federal Rural de Pernambuco, como requisito para a obtenção do título de Doutor em Biociência Animal.

Orientação: Prof. Dr. Geraldo Jorge Barbosa de Moura – UFRPE

Coorientação: Prof.ª Drª. Regina Célia Bressan Queiroz de Figueiredo – IAM/FIOCRUZ

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José Ricardo de Oliveira Santos
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Dedicatória

Dedico à minha mãe, Maria do Socorro de Oliveira Santos, que ao se movimentar, toda uma estrutura se movimentou junto com ela.

Epígrafe

“Com a firmeza justiceira e pacífica de Oxalá. A bravura e a coragem flamejante de Xangô, o temerário agadá transformador de Ogum e o amor próprio de Oxum, marchamos para frente” – *Abdias do Nascimento.*

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Apresentação

Os impactos ambientais provocados por atividades humanas, nos ecossistemas terrestres e aquáticos têm provocado declínios populacionais na comunidade de anuros no mundo todo. Com isso, a biologia da conservação, fisiologia comparada e medicina da conservação tem utilizado diversas abordagens para o monitoramento da biodiversidade de anuros. Dentre essas abordagens, a avaliação do estado fisiológico, através de fatores intrínsecos e extrínsecos tem sido utilizada para aumentar o conhecimento sobre o estado fisiológico das espécies de anuros.

Dentre os fatores intrínsecos, pode-se destacar a avaliação do hemograma e bioquímica sérica em anuros. Contudo, trabalhos sobre parâmetros hematológicos e bioquímica sérica dos anuros, publicados em periódicos têm sido amplamente realizados com espécies de importância comercial, principalmente os gêneros *Bufo, Lithobates e Xenopus*. Em contrapartida, resultados sobre intervalos de referência dos parâmetros sanguíneos para as espécies endêmicas do Brasil ainda são incipientes.

Além disso, a investigação da ocorrência de hemoparasitos também pode ajudar a aumentar as informações sobre o estado fisiológico dos anuros. Com isso, alguns trabalhos têm relatado a presença de hemogregarinas em espécies de anuros, dentre elas espécies pertencentes ao gênero *Hepatozoon*, parasitos caracterizados pela presença de gamontes em eritrócitos ou leucócitos no sangue periférico do hospedeiro.

Outro fator, e nesse caso, extrínseco que vem sendo utilizado para avaliação do estado fisiológico dos anuros é a descrição do repertório defensivo. São mais de 30 tipos de estratégias antipredatórias que podem ser utilizados por esses animais. Sendo assim, a associação da fisiologia e ecologia como métodos de análises da saúde dos ecossistemas e animais associados a eles são importantes para o desenvolvimento de projetos de conservação das espécies.

Sendo assim, utilizou-se *Leptodactylus vastus* Lutz, 1930 como modelo biológico do estudo. Essa espécie pertence ao grupo *L. pentadactylus* e

apresenta ampla distribuição no nordeste brasileiro, com alguns relatos na Amazônia boliviana. Assim como para as demais espécies do grupo, não há dados sobre o hemograma, bioquímica sérica e investigação da ocorrência de *Hepatozoon* spp. em *L. vastus,* o que dificulta a investigação do estado fisiológico dessa e outras espécies no mesmo ecossistema. Dessa forma, o acesso a essas informações sobre a espécie auxilia na construção da história natural e, posteriormente na criação de planos de conservação de *L. vastus* e das demais espécies de anuros no país.

Resumo

Os impactos ambientais provocados pelo somatório das ações antrópicas e da natureza têm alertado para a perda da biodiversidade de anuros. Com isso, a biologia e medicina da conservação, bem como a fisiologia comparada tem utilizado abordagens intrínsecas e extrínsecas para a avaliação do estado fisiológico desses animais. Dentre as abordagens intrínsecas, pode-se destacar a análise do hemograma, bioquímica sérica e a ocorrência de hemoparasitos, enquanto que a descrição do repertório defensivo tem sido considerada como uma abordagem extrínseca importante para a investigação dos displays comportamentais antipredatórios apresentados por sapos, rãs e pererecas. Nesse tocante, o objetivo desse estudo foi avaliar o estado fisiológico e ecológico de machos da espécie *Leptodactylus vastus* Lutz, 1930 em fragmentos de Floresta Atlântica no nordeste brasileiro. Para a análise dos fatores intrínsecos, foram coletados 30 machos de *L. vastus* no Jardim Botânico e no Parque Estadual Dois Irmãos. Para a descrição do repertório defensivo, 30 machos foram capturados na Unidade de Conservação Mata do Camocim, situado no munícipio de São Lourenço da Mata. As três áreas estão situadas na região metropolitana de Recife. Os machos de *L. vastus* apresentaram uma média de variáveis hematológicas e bioquímica sérica próximos aos relatados para outras espécies de anuros. Contudo, os valores elevados do hematócrito podem sugerir problemas de desidratação/hemoconcentração, bem como os valores aumentados de basófilos e anormalidades nos eritrócitos podem sugerir uma hipersensibilidade imunológica nos espécimes. Além disso, os valores das enzimas relacionadas com a função hepática sugerem possíveis lesões no órgão, mas é necessário a realização de outros exames para o diagnóstico. Sobre o comportamento defensivo, os machos exibiram seis tipos de comportamentos, divididos em (i) mecanismo visual: inflar o corpo (100%), empurrar (83,3%), esconder (23,3%), descarga cloacal (16,6%) e utilização de espinhos (6,6%); e (ii) mecanismo acústico: canto de agonia (33,3%). Desse modo, a análise dos parâmetros fisiológicos e ecológicos de anuros são ferramentas necessárias para auxiliar no monitoramento do estado fisiológico

das espécies de anuros e, com isso, auxiliar na criação de estratégias para a conservação desses animais e dos ecossistemas associados a eles.

Palavras-chave: ecofisiologia, hematologia, bioquímica sérica, anuro, comportamento antipredatório.

Abstract

The environmental impacts caused by the sum of anthropic actions and nature have alerted to the loss of anurans biodiversity. That way, conservation biology and medicine, and comparative physiology have used intrinsic and extrinsic approaches to assess the physiological state of these animals. Among the intrinsic approaches, the analysis of the blood count, serum biochemistry and the occurrence of hemoparasites can be highlighted, while the description of the defensive repertoire has been considered an important extrinsic approach to the investigation of antipredatory behavioral displays presented by frogs and treefrogs. In this regard, the objective of this study was to evaluate the physiological and ecological status of males of the species *Leptodactylus vastus* Lutz, 1930 in fragments of the Atlantic Forest in northeastern Brazil. For the analysis of intrinsic factors, 30 males of *L. vastus* were collected in Botanical Garden and Dois Irmãos State Park. For the description of the defensive repertoire, 30 males were captured in the Camocim Forest located in the municipality of São Lourenço da Mata. The three areas are located in the metropolitan region of Recife. The males of *L. vastus* presented an average of hematological variables and serum biochemistry close to those reported for other anuran species. However, elevated hematocrit values may suggest dehydration/hemoconcentration problems, as well as increased basophil values and erythrocyte abnormalities, which may indicate immunological hypersensitivity in the specimens. In addition, the values of enzymes related to liver function suggest possible damage to the organ, but other tests are necessary for the diagnosis. Regarding defensive behavior, males exhibited six types of behaviors divided into (i) visual mechanism: inflating the body (100%), pushing (83.3%), hiding (23.3%), cloacal discharge (16, 6%) and use of thorns (6.6%); and (ii) acoustic mechanism: distress call (33.3%). Thus, the analysis of anuran physiological and ecological parameters are necessary tools to assist in monitoring the physiological state of anuran species and, thus, assist in the creation of strategies for the conservation of these animals and the ecosystems associated with them.

Keywords: ecophysiology, hematology, serum biochemistry, anuran, antipredatory behavior.

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1. Introdução

Os estudos sobre os fatores intrínsecos e extrínsecos para a avaliação do estado fisiológico dos anuros têm contribuído para o debate sobre a conservação desses animais. Dentre os fatores intrínsecos, a avaliação dos parâmetros sanguíneos apresenta resultados sobre o hemograma (Franco-Belussi et al., 2021; Rodrigues et al., 2017), bioquímica sérica (Oliveira et al., 2019), e a análise da ocorrência de hemoparasitos (Ferreira et al., 2020; Úngari et al., 2021). No tocante aos fatores extrínsecos, pode-se destacar os estudos sobre os comportamentos antipredatórios destes animais (Guerra et al., 2018; Sena et al., 2021; Toledo; Sazima; Haddad 2011).

As células sanguíneas dos anuros exibem eritrócitos ovoides, nucleados, que geralmente são maiores do que aqueles encontrados em outras espécies de vertebrados (Thrall et al., 2012; Wright; Whitaker, 2001). Este tamanho, por sua vez, varia de acordo com a espécie, sexo, idade, estado fisiológico (Attademo et al., 2014), temperatura e habitat (López-Olivera et al., 2003). Os leucócitos dos anuros são divididos em granulócitos: que incluem os basófilos, eosinófilos e neutrófilos; e os agranulócitos: linfócitos e monócitos (Thrall et al., 2012). Além disso, ainda podem ser observadas a ocorrência de trombócitos, células ovoides com a presença de cromatina condensada no centro do núcleo, que apresentam função similar as plaquetas dos mamíferos (Forzán et al., 2017).

A contagem total dos tipos celulares sanguíneos, contagem diferencial de leucócitos, determinação da hemoglobina e índices hematimétricos, bem como a análise da ocorrência de hemoparasitos e dos parâmetros da bioquímica sérica dos anuros fornecem informações sobre a saúde tanto do animal quanto do meio ambiente, uma vez que esses animais são considerados bioindicadores – potencialmente confiáveis e eficientes – da saúde dos ecossistemas (Hopkins, 2007). Assim, a relação entre os parâmetros quantitativos e qualitativos das células sanguíneas com as flutuações ambientais têm demonstrado ser em uma ferramenta para avaliar o estado fisiológico dos animais e dos ecossistemas (Dickinson; Jarchow; Trueblood al., 2002; Zhelev; Angelov; Mollov, 2006).

Assim como para as espécies de mamíferos, é necessário um maior engajamento dos profissionais para o estudo dessas variáveis para as espécies de anuros tropicais, embora o pequeno tamanho de algumas espécies e os poucos locais de punção venosa tornem esse estudo uma tarefa desafiadora.

Já as estratégias antipredatórias desempenhas pelos anuros podem auxiliar no entendimento dos parâmetros extrínsecos, voltados para a ecologia. Para estes animais, são mais de 30 tipos de comportamentos defensivos (Haddad et al., 2013; Toledo; Sazima; Haddad 2011), muitos dos quais podem ser exibidos em diferentes sequências. A descrição dos comportamentos defensivos dos anuros são oportunistas e baseadas em observações naturalistas, não sistematizadas. (Wells, 2010).

Dessa forma, estudos que abordam interações predador-presa são considerados relevantes para a ecologia em decorrência das evidências que a pressão seletiva tem produzido na evolução dos mecanismos antipredatórios dos anuros. Muitos estudos mostraram interações entre anuros e seus predadores, como artrópodes e quase todos os grupos de vertebrados, desde peixes a mamíferos (Vitt; Caldwell, 2013).

Com isso, estudos que abordem mecanismos fisiológicos e ecológicos para a sobrevivência de anuros tropicais, como a espécie *Leptodactylus vastus* Lutz 1930, bem como o acompanhamento desses resultados ao longo dos anos, com possíveis mudanças em resposta as oscilações na temperatura, umidade, fragmentação de habitat e disponibilidade de corpos d'água são relevantes para a ampliação de planos de conservação, principalmente mediante às intempéries provocadas pela ação humana nos ecossistemas terrestres e aquáticos associados aos anuros.

Leptodactylus vastus é uma espécie de anuro endêmica do nordeste brasileiro que pertence ao grupo *L. pentadactylus* (Heyer, 1972). Segundo a Lista Vermelha de Espécies Ameaçadas de Extinção (IUCN – Heyer, 2008), Ministério do Meio Ambiente (MMA) e a RESOLUÇÃO Nº 01, DE 09 DE JANEIRO DE 2015, *L. vastus* se encontra na categoria de LC (pouco preocupante). Vale ressaltar que, *L. vastus* foi comumente confundido com a espécie *L. labyrinthicus*, por

apresentar características morfológicas (comprimento rostro cloacal e coloração) e acústicas semelhantes (Heyer, 2005).

2. Revisão Bibliográfica

2.1. Fisiologia Comparada, Biologia da Conservação e Medicina da Conservação

A fisiologia comparada tem uma longa história de contribuições para a biologia da conservação, principalmente no que diz respeito a compreensão das adaptações que os organismos sofrem, em resposta às mudanças ambientais, resultantes da influência humana e da própria natureza (Wikelski; Cook, 2006). A biologia da conservação foi desenvolvida como um indicador de monitoramento da sexta grande extinção (Leakey; Lewin, 1997). Diferente do que ocorreu com as extinções anteriores, as tendências atuais estão relacionadas com as atividades humanas que resultam na conversão de habitat, isolamento de populações da fauna e invasão humana nos ecossistemas. Como uma subespecialidade da biologia da conservação, a medicina da conservação surge como uma demanda para a aplicação da medicina para contribuir com a conservação da vida silvestre e dos ecossistemas (Deem et al., 2000).

Embora as mudanças provocadas pela ação humana apresentem velocidade e potencial para afetar todos os grupos taxonômicos de animais, alguns organismos são mais suscetíveis do que outros (Mendelson et al., 2006). Anuros figuram entre os organismos mais sensíveis, conforme a redução de suas populações e extinção de algumas espécies (Pounds et al., 2006). Esse declínio populacional tem forte relação com as mudanças ecológicas e um dos focos da biologia da conservação é a promoção de ações para evitar a continuidade desse processo (Parmesan, 2006).

Os resultados dessas alterações ambientais são extinções locais, migrações e/ou ajustes fisiológicos (Donnelly; Crump, 1998; Helmuth; Kingsolver; Carrington, 2005). Esse ajuste significa a modificação dos parâmetros comportamentais e/ou fisiológicos para a adaptação às novas condições ambientais. Ajustes fisiológicos são comumente os únicos mecanismos para manter a estabilidade do sistema. Temperatura, pH, massa corporal, níveis de glicose e oxigênio são essenciais para a manutenção da vida, sendo essa estabilidade denominada de homeostase (Carey, 2005).

Outra demanda da biologia da conservação para o monitoramento da biodiversidade são as mudanças climáticas que têm provocado diversas respostas biológicas nos organismos e ecossistemas. Nesse caso, dependendo da previsão de que até 2100 haja um aumento de 1,5° – 2°C, sugere-se uma continuação dessas respostas, caso não haja profunda redução nas emissões de gases de efeito estufa, de acordo com o relatório “Climate Change 2021: The Physical Science Basis" (IPCC, 2021).

No Brasil, o domínio morfoclimático da Floresta Atlântica abrange a costa leste, sudeste e sul do país e apresenta alto grau de biodiversidade e endemismo. Contudo, em decorrência das conversões antropogênicas a Floresta Atlântica passou a ser considerada como um dos hotspots de biodiversidade no país (Mittermeier et al., 1999). Dentre os grupos de animais mais diversos da Floresta Atlântica, figuram-se os anuros que se distribuem em 529 espécies e apresentam 80% das taxas de endemismo (Haddad et al., 2013).

2.2. Impactos ambientais, conservação e fisiologia dos anuros

A biodiversidade do planeta está diminuindo a uma taxa alarmante, mesmo em ambientes que apresentam planos para a conservação e preservação (Barnosky et al., 2011). O declínio de espécies tem sido provocado, principalmente pelo somatório da interferência humana nos ambientes silvestres e as ações da própria natureza (Hooper et al., 2012; Rödder; Kielgast; Lötters, 2010). Dessa forma, é necessário que estudos voltados para os táxons que vêm sofrendo alto declínio populacional nos habitats, bem como os ecossistemas que alocam essas espécies, sejam realizados (Giraudo; Arzamendia, 2018; Foden et al., 2013).

Para os anfíbios, estudos mostram que nas últimas quatro décadas pelo menos 46% das espécies descritas diminuíram, ou foram extintas em todo mundo. Esse declínio tem sido provocado, principalmente pela dispersão de poluentes químicos nos ecossistemas aquáticos (Collins, 2010), fragmentação de habitat (Andersen; Fog; Damgaard, 2004), introdução de espécies exóticas (Mack et al., 2000) e disseminação de agentes infecciosos (Moura-Campos et al., 2021; O’hanlon et al., 2018).

2.2.1. Fragmentação do habitat

Um dos fatores que agravam o quadro de ameaça das espécies de anuros é a fragmentação de habitat. Esse fenômeno é resultado das alterações nas atividades físicas e humanas locais (Becker; Harris, 2010), que pode afetar o comportamento e a ecologia dos anuros (Becker et al., 2015) Além disso, a fragmentação pode reduzir a biodiversidade de artrópodes, possibilitando uma dieta que torne o anuro mais suscetível a perda de tamanho e condição corporal (Scheele et al., 2014).

Outra consequência é a redução da oportunidade de reprodução (Zimmerman; Bierregaard, 1986). Anuros apresentam uma ampla diversidade de modos reprodutivos (Haddad et al., 2013) e algumas espécies podem apresentar modos reprodutivos específicos, nos quais tanques e ninhadas dependem de fontes de água (Donnelly, 1998). Dessa forma, ambientes fragmentados podem não conter ecossistemas aquáticos apropriados para a reprodução, e, portanto, a migração de um corpo d'água para outro é uma alternativa que gera um desafio fisiológico, cuja magnitude está relacionada às condições de temperatura, disponibilidade de água e a distância entre os corpos d'água nesses fragmentos (Donnelly, 1998).

Alguns estudos ainda evidenciam que a ameaça de fragmentação de habitats para os anuros é agravada, em decorrência da fidelidade do local e da capacidade limitada de dispersão que esses animais apresentam (Alex Smith; Green, 2005; Blaustein; Wake; Sousa, 1994; Marsh; Pearman, 1997;). Outros também relacionam a fisiologia da locomoção ao perfil bioquímico das fibras musculares das pernas (Pough; Taigen, 1990; Taigen; Emerson, 1982;).

2.2.2. Espécies exóticas

Frequentemente, as mudanças ambientais provocadas pela ação humana têm sido historicamente associadas ao transporte deliberado ou acidental de espécies animais e vegetais para fora de suas áreas naturais, o que provoca alterações nos padrões locais da biodiversidade (Lonsdale, 1999; Mack et al., 2000; Mckinney, 2002). Uma das espécies exóticas que mais toleram essas mudanças antrópicas é a *Lithobates catesbianus* – rá-touro-americana (Shaw, 1802). Essa espécie foi introduzida no Brasil com o advento da

ranicultura na década de 30 e ocorre principalmente nas regiões do sul e sudeste nas áreas de Pampa e Floresta Atlântica (Nori et al., 2011).

Apesar de se alimentarem principalmente de insetos, os adultos podem predar pequenos vertebrados, incluindo espécies de anuros, além de competirem com espécies nativas (Kraus, 2015). Esses efeitos diretos/indiretos podem provocar mudanças na anurofauna, principalmente em decorrência da alta taxa de reprodução (Kaefer; Boetler; Cechin, 2007) que provoca um aumento na dispersão das rãs-touro (Quiroga et al., 2015).

Além disso, *L. catesbeianus* está comumente relacionada com a dispersão de agentes infecciosos, como ranavírus e o fungo *Batrachochytrium dendrobatidis* (Mazzoni et al., 2003; O'hanlon et al., 2018), e nesse caso, podem elevar o nível de ameaça para a comunidade de anuros local. Outros estudos ainda reforçam uma alta prevalência do fungo *Bd* nessa espécie de rã, em decorrência da morbidade em criatórios, o que reforça a hipótese de que a rã-touro é um reservatório eficiente do *Bd* (Adams et al., 2017; Greenspan et al., 2012; Schloegel et al., 2010). Com isso, a alta densidade de indivíduos na atividade de ranicultura e o seu comportamento generalista podem aumentar a proliferação do fungo *Bd*, através da disseminação dos indivíduos em áreas próximas, elevando a faixa de distribuição do patógeno.

Dessa forma, no que se refere a ranicultura o nordeste brasileiro apresenta uma espécie de rã que vem sendo utilizada na alimentação. *Leptodactylus vastus* pode atingir até cerca de 20 centímetros de comprimento rostro cloacal na fase adulta, apresentando alto teor de proteína e baixo teor de gordura. Apesar de sua importância comercial, os Ministérios do Meio Ambiente e Aquicultura não apresentam dados sobre distribuição desses centros de criação da rã na região, o que dificulta a difusão das informações a respeito de espécies nativas do Brasil que podem ser utilizadas nesse processo econômico. Vale ressaltar que, o Brasil já foi o segundo maior produtor mundial de carne de rã, quando produzia em média 600 toneladas de carne por ano (de Oliveira, 2015).

2.2.3. Hemoparasito

Outro marcador do estado fisiológico dos anuros é a análise da ocorrência de hemoparasitos, dentre eles a ocorrência de hemogregarinas (Apicomplexa: Adeleiorina). Esses parasitos são hemoprotozoários heteroxenos, intraeritrócitários ou interleucocitários, que infectam uma ampla gama de hospedeiros ectotérmicos, incluindo os anuros. As hemogregarinas são transmitidas, principalmente por vetores invertebrados hematófagos, como insetos dípteros, carrapatos, ácaros e sanguessugas (Davies; Johnston, 2000; Martin, 1994; Smith Desser).

As hemogrerarinas são divididas em quatro famílias: Dactylosomatidae Jakowska e Nigrelli, 1955, Haemogregarinidae Léger, 1911, Hepatozoidae Miller, 1908 e Karyolysidae Labbé, 1894. Em Hepatozoide, *Hepatozoon* Miller, 1908 são hemogregarinas mais comumente relatadas em anuros (Davies; Johnston, 2000; Leal et al., 2015; Netherlands et al., 2018). Esse gênero é caracterizado pela presença de gamontes em eritrócitos ou leucócitos, sem divisão merogônica e ocorre no sangue periférico dos hospedeiros. Esses gamontes emparelhados penetram a parede intestinal dos invertebrados (hemocele), onde ocorre o desenvolvimento esporogônico para a formação de grandes oocistos. Os oocistos apresentam paredes espessas e contém esporocistos com esporozoítos – estágio infeccioso do parasito – que surgem após a ingestão do hospedeiro, dando origem aos estágios merogônicos no fígado (Smith; Desser; Martin, 1994) (Figura 1).

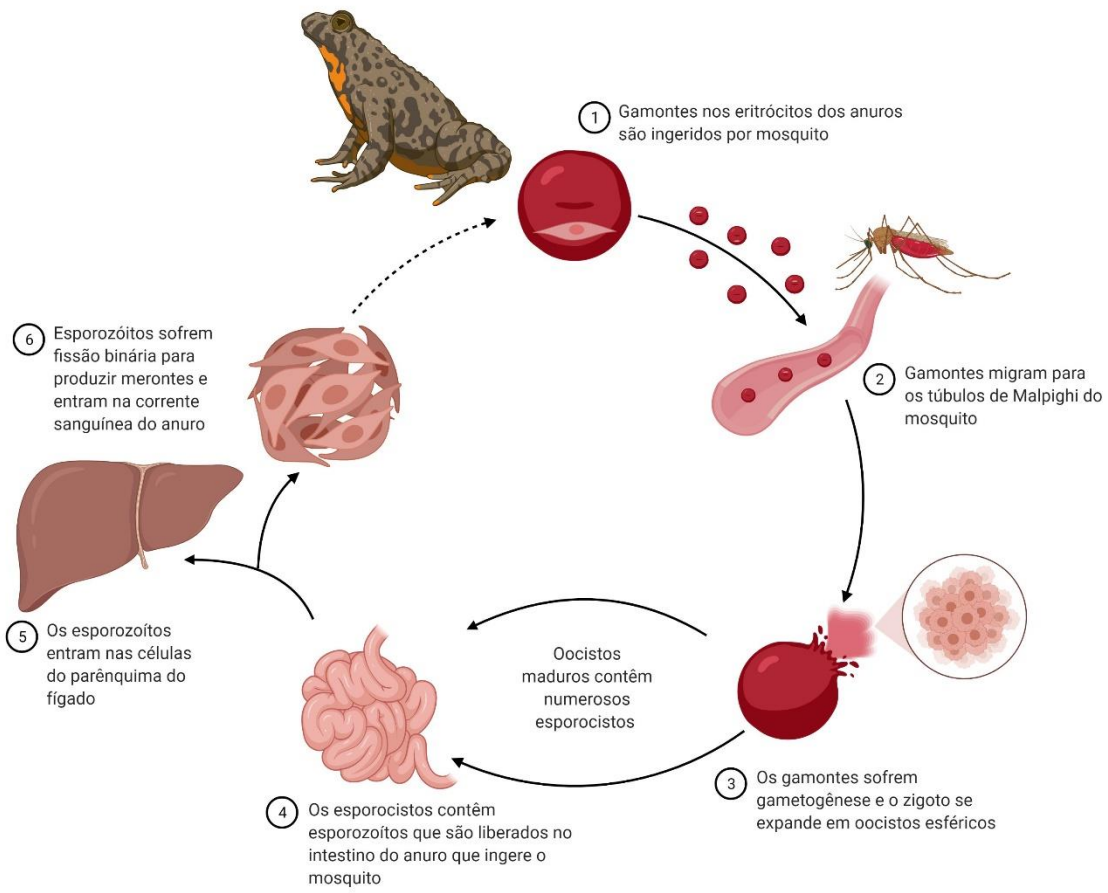


Figura 1. Representação diagramática do ciclo de vida de *Hepatozoon spp.* em anuro. Foto autoral criada no Biorender.

Cerca de 45 espécies de *Hepatozoon* foram descritas em anuros da África, Ásia e Europa (Netherlands et al., 2018). No Brasil, *Hepatozoon leptodactyli* (Lesage, 1908) Pessoa, 1970 e *Hemolivia stellata* Petit, Landau, Baccam e Lainson, 1990 têm sido relatadas em anuros brasileiros, incluindo espécies do gênero *Leptodactylus* Fitzinger, 1826 (Leal et al., 2015; Ferreira et al., 2020).

Costa et al. (1973) investigaram 90 espécimes de *L. latrans* (Steffen, 1815) e *L. pentadactylus* (Laurent, 1768), relatando 17 indivíduos infectados com *H. leptodactyli*. Contudo, não há registros de espécies de *Hepatozoon* em *L. vastus*. Além disso, alguns anuros podem servir como o primeiro hospedeiro intermediário de *Hepatozoon* spp., que podem ser transmitidos para um segundo hospedeiro intermediário após a ingestão do primeiro. Nesse tocante, Viana et al. (2012) afirmam que, *L. chaquensis* Cei, 1950 é

considerado como primeiro hospedeiro intermediário de espécies de *Caiman* spp.

2.3. Ferramentas para a conservação de anuros

2.3.1. Hematologia

Tendo em vista a crescente ameaça a biodiversidade de anuros no planeta, a necessidade da criação de estratégias para a conservação desses animais torna-se urgente. A avaliação hematológica pode ser utilizada para monitorar o estado fisiológico das espécies de anuros, desde que os intervalos de referência e as interpretações estejam alinhadas aos fatores bióticos (sexo, idade) e abióticos (temperatura, umidade). Com isso, a hematologia tem se tornado uma ferramenta útil na detecção de doenças que possam afetar as células sanguíneas, anemia, leucoponia, leucocitose e trombocitopenia (Campbell; Grant, 2015).

Enquanto a contagem total de células do sangue fornece informações básicas sobre a concentração de células sanguíneas e presença de patógenos (Allender; Fry, 2008), as análises bioquímicas, plasmáticas ou séricas, fornecem dados sobre o funcionamento dos órgãos, eletrólitos, proteínas, parâmetros nutricionais e metabólicos (Newman et al., 1997) (Figura 2).

As análises hematológicas, envolvendo a contagem total de eritrócitos e leucócitos, determinação da hemoglobina, contagem diferencial de leucócitos e determinação do hematócrito foram estabelecidos por volta de 1900 (Sato et al., 2018). Muito dos componentes utilizados para a contagem automatizada das células sanguíneas não podem ser utilizadas para os anuros, devido a presença de núcleo nos eritrócitos e trombócitos desses animais. Nesse caso, Sato et al. (2018), utilizaram o anticorpo monoclonal T12 e desenvolveram um sistema automatizado para a classificação e contagem das células sanguíneas de *Xenopus laevis* (Daudin, 1802) e *Xenopus tropicalis* (Gray, 1864), principalmente para diferenciar os trombócitos de outros tipos de células.

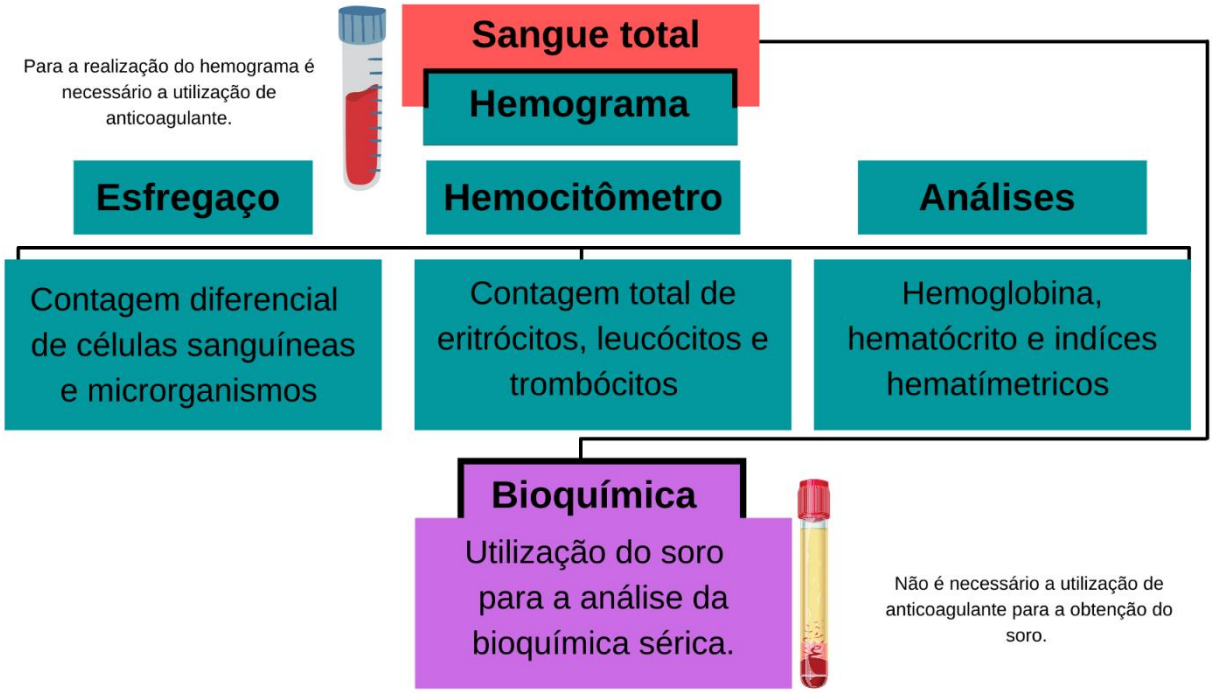


Figura 2. Diagrama do passo a passo para maximizar as análises, a partir do sangue periférico dos anuros. Foto autoral.

Levando em consideração a plasticidade de ambientes em que os anfíbios podem se distribuir (ecossistemas aquáticos e terrestres), os seus parâmetros hematológicos variam de acordo. Nesse caso, a interpretação do hemograma pode ser um desafio em alguns casos, no qual salamandras apresentam mais semelhanças com os peixes e sapos terrestres apresentam interpretações semelhantes às dos “répteis” (Campbell; Grant, 2015).

Nesse sentido, a determinação dos intervalos de referência para os parâmetros hematológicos e bioquímicos podem fornecer informações acerca do diagnóstico de doenças emergentes e na compreensão do quadro clínico dos animais (Campbell; Grant, 2015), bem como, verificar a influência dos diferentes níveis de antropização nas variáveis hematológicas dos anuros. E, apesar da sua importância, os trabalhos publicados sobre esses valores de referência têm sido realizados, principalmente com os gêneros de importância comercial, como *Bufo, Lithobates* e *Xenopus* (Bricker; Raskin; Densmore, 2012; Hadji-Azimi et al., 1987).

2.3.2. Formação das células sanguínea dos anuros

As células sanguíneas dos anuros possuem algumas semelhanças com as células sanguíneas dos peixes, “répteis” e aves. Assim como esses três grupos, os anuros apresentam eritrócitos nucleados (diferente dos eritrócitos dos mamíferos) e trombócitos (células equivalentes as plaquetas dos mamíferos) (Campbell; Grant, 2015). Essa semelhança pode ser útil para a compreensão dos padrões do estado fisiológico desses animais. Contudo, as principais diferenças entre anfíbios e mamíferos representam desafios para determinados diagnósticos, uma vez que os estudos hematológicos estão mais padronizados para este último grupo (Allender e Fry, 2008).

Durante o processo de metamorfose dos anuros, no qual larvas (girinos) se transformam em sapos, rãs ou pererecas, os tecidos do corpo aumentam e após a reestruturação desses tecidos o animal sofre uma redução no tamanho total (Davis, 2008). Como parte desse processo, as populações de eritrócitos se renovam e o número de leucócitos aumenta, durante a hematopoiese. Dessa forma, estudos sobre a reposição de populações de células sanguíneas de anuros, em ambientes aquáticos podem oferecer subsídios para os estudos sobre a contaminação nesses ecossistemas (Barni et al., 2007), principalmente quanto aos parâmetros dos leucócitos, os quais estão sujeitos a essa variação ambiental (Kiesecker, 2002; Davis et al., 2008).

A hematopoiese é o processo de crescimento, maturação, renovação e diferenciação de células-tronco hematopoiéticas, o que resulta na formação de células sanguíneas durante os estágios embrionários e adultos dos animais (Lüscher-Firzlaff et al., 2019; Till; Mcculloch; Siminovitch, 1964). Durante esse processo, as células-tronco hematopoiéticas darão origem as unidades formadoras de colônias mieloides e linfoides, na qual a unidade formadora mieloide será responsável pelos processos de eritropoiese, granulopoiese, trombopoiese e fator megacariócito, enquanto a unidade formadora linfoide resultará nos linfócitos (Adolfsson et al., 2005).

A diferenciação dessas células durante a hematopoiese depende da ativação do fator estimulador de colônias CSF-1 e Eritropoietina. CSF-1 é uma glicoproteína monomérica que funciona como uma citocina e estimula as células-

tronco hematopoiéticas (CTH) a produzirem granulócitos (basófilos, eosinófilos e neutrófilos) e monócitos que nos tecidos se desenvolvem em macrófagos e em células dendríticas (Root; Dale, 1999).

Por outro lado, a diferenciação dos precursores megacariócito/eritrócito depende da eritropoietina (Epo) (Nogawa-Kosaka et al., 2011). A eritropoietina é um hormônio produzido e secretado pelos rins, quando ocorre a diminuição de oxigênio na circulação sanguínea. Esse hormônio estimula a proliferação e diferenciação de precursores dos eritrócitos nos tecidos hematopoiéticos dando origem aos eritrócitos. Dessa forma, no processo de eritropoiese a Epo auxilia na manutenção do número de eritrócitos, tornando a concentração de hemácias suficiente para manter os níveis adequados de oxigênio nos tecidos (Sherwood; Klandorf; Yancey, 2005) (Figura 3).

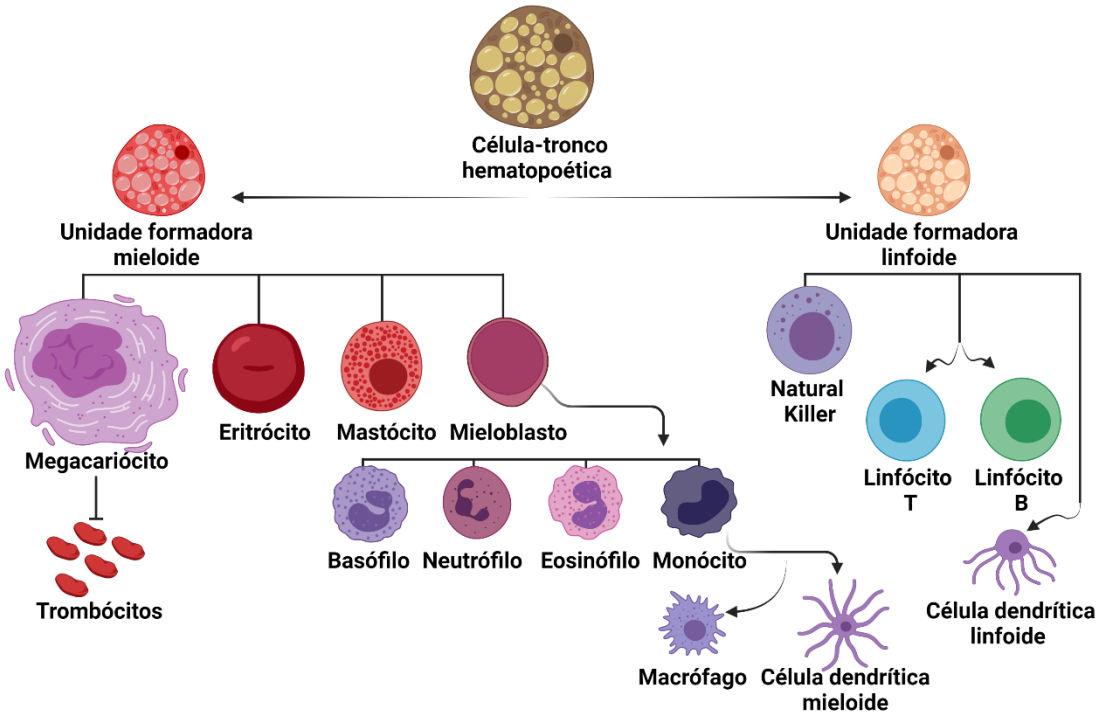


Figura 3. Ilustração do processo de formação dos tipos de células sanguíneas dos anuros (Hematopoiese). Foto autoral criada no Biorender.

Embora esse processo seja amplamente descrito para os mamíferos, cujas as células pluripotentes residem predominantemente na medula óssea, outros autores têm se debruçado nos estudos sobre esse processo nos anuros. Em *Lithobates catesbeianus* (Shaw, 1802), o processo de formação e diferenciação das células sanguíneas ocorre no baço, denominado como principal órgão para

esta espécie (De Abreu Manso; De Brito-Gitirana, 2009). Estes mesmos autores ainda conseguiram verificar que, durante a primavera o baço se torna o principal sítio para a eritropoiese, enquanto a hematopoiese migra para a medula óssea, o que sugere que esse processo em anuros é amplamente influenciado pela sazonalidade.

Em *Xenopus laevis* (Daudin, 1802), células hematopoiéticas foram encontradas em diversos tecidos, como o fígado, medula óssea femoral e baço. Nesse caso, o fígado, de acordo Hadji-Azimi et al. (1987) funciona como o principal sítio hematopoiético, enquanto a medula óssea é primária para esse processo. Ainda para essa espécie, Cumano e Godin (2007) relatam que o rim, o baço e o timo são locais hematopoiéticos definitivos. Mais recentemente, Yaparla, Reeves e Grayfer (2020) descreveram os processos de hematopoiese para *X. laevis* e verificaram que, apesar da presença de células pluripotentes (CTH) na medula óssea dos animais, o fígado é o principal sítio para a formação das células sanguíneas.

O uso da medula óssea como local hematopoiético para os anfíbios parece ter coevoluído com a vascularização do corpo dos animais, em decorrência da adaptação para o ecossistema terrestre (Tanaka, 1976). Apesar de algumas espécies de anuros aquáticos – como *Bombina* spp. e *Xenopus* spp. – apresentarem vascularização rudimentar na medula óssea, outras espécies de hábitos terrestres (*Bufo* spp.) possuem medula óssea com vascularização mais pronunciada, semelhante a observada em mamíferos (Tanaka, 1976).

No processo de eritropoiese também ocorre diferença entre larvas e adultos. Enquanto os indivíduos na fase larval desenvolvem seus eritrócitos no fígado e rins, os indivíduos adultos desenvolvem na medula óssea, bem como no baço e fígado. Ainda nos indivíduos adultos, o baço é o principal local para a formação dos eritrócitos, apesar do fígado ser considerado como um órgão secundário para esta função (Arikan; Cicek, 2014). Além disso, os eritrócitos dos anfíbios são grandes, quando comparado com os demais grupos dos vertebrados (Thrall et al., 2012; Wojtaszek; Adamowicz, 2003; Wright; Whitaker, 2001), dentre os anfíbios pode-se destacar o comprimento médio dos eritrócitos das espécies de *Amphiuma* Garden, 1821, cerca de 70 µm (Garrison, 1949).

Essas diferenças no tamanho dos eritrócitos dos anfíbios estão frequentemente associadas ao tamanho do genoma da espécie: quanto mais longo o seu genoma, maior o eritrócito. Portanto, anuros, cujo girinos habitam lagoas temporárias e apresentam tempo reduzido para completar a metamorfose, têm genomas mais curtos e eritrócitos menores (Gregory, 2001).

2.3.3. Tipos de células sanguíneas dos anuros

As células sanguíneas dos anuros consistem em eritrócitos, leucócitos e trombócitos. Os leucócitos por sua vez podem ser subdivididos em granulócitos e agranulócitos. Como granulócitos tem-se os neutrófilos, eosinófilos e basófilos. Já os agranulócitos são os monócitos e linfócitos.

Os eritrócitos apresentam morfologia oval-elíptica e núcleo redondo e basofílico. (Figura 4). Na fase larval dos anuros os eritrócitos são mais alongados, enquanto na forma adulta eles são menores e mais redondos (Benbassat, 1970), tendo como principal função o transporte de gases associados a respiração. O tempo de vida dos eritrócitos é longo, quando comparado ao tempo de vida dos eritrócitos das aves e mamíferos, entre 400-700 dias (*Rhinella marina*) (Altland; Brace, 1962).

Os trombócitos apresentam forma oval a redondo, com presença de núcleo com cromatina densa. Ao contrário dos linfócitos, a cromatina não é aglomerada aleatoriamente, mas condensada no centro do núcleo e suas bordas citoplasmáticas raramente são lisas. Os trombócitos dos anuros são análogas as plaquetas dos mamíferos, funcionando como componente para a coagulação do sangue (Forzán et., 2017) (Figura 4).

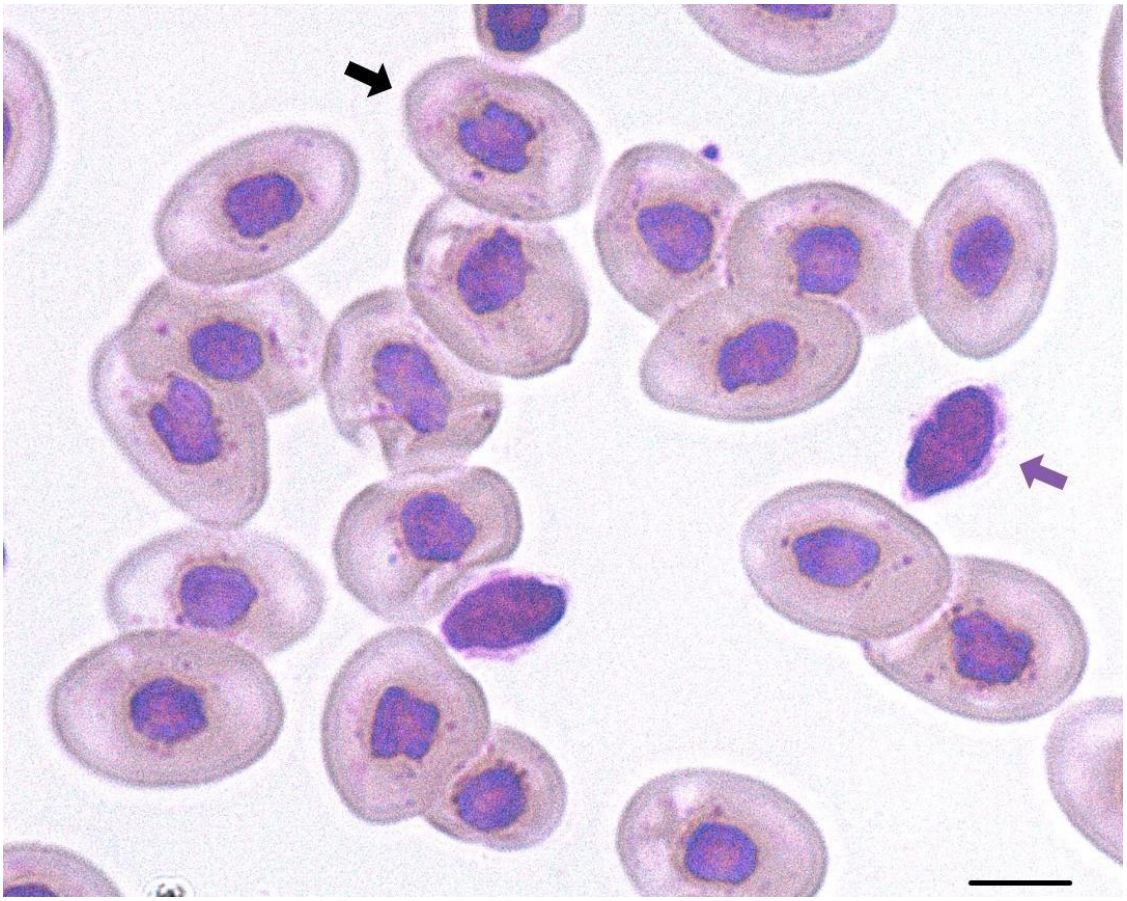


Figura 4. Células do tecido sanguíneo de *Leptodactylus vastus* Lutz, 1930 coradas com May Grunwald-Giemsa. Eritrócitos maduros (seta preta). Trombócito (seta violeta). A barra preta corresponde a 10 µm. Foto autoral.

Os neutrófilos, geralmente são células redondas, polimorfonucleares, nos quais os grânulos apresentam mieloperoxidases e citoplasma incolor quando corado com giemsa (Forzán et al., 2017). Em anuros, os neutrófilos são frequentemente positivos para a peroxidase, mas a atividade da fosfatase varia de acordo com a espécie (Turner, 1988). Em algumas espécies, um pequeno número de células pode se assemelhar aos neutrófilos, porém apresentam pequenos grânulos citoplasmáticos eosinofílicos distintos, sendo denominadas de heterófilos. Os neutrófilos dos anuros se assemelham as células encontradas em mamíferos com síndrome de Chediak-Higashi (Campbell; Grant, 2015) (Figura 5).

Os eosinófilos apresentam núcleo segmentado, grânulos citoplasmáticos eosinofílicos redondos a ovais e citoplasma ligeiramente basofílico. O tamanho dos eosinófilos é relativamente maior do que os neutrófilos. Os grânulos eosinofílicos encontrados nos eosinófilos exibem ultraestrutura cristaloide, típica

da morfologia ultraestrutural de eosinófilos de mamíferos (Turner, 1988; Pfeiffer; Pyle; Asashima et al., 1990) (Figura 5).

Os basófilos são células redondos a ovais que não apresentam núcleo com lóbulos segmentados, mas possuem grânulos citoplasmáticos basofílicos metacromáticos que, geralmente cobrem o núcleo. O seu tamanho pode variar de acordo com a espécie (Campbell; Grant, 2015). (Figura 5).

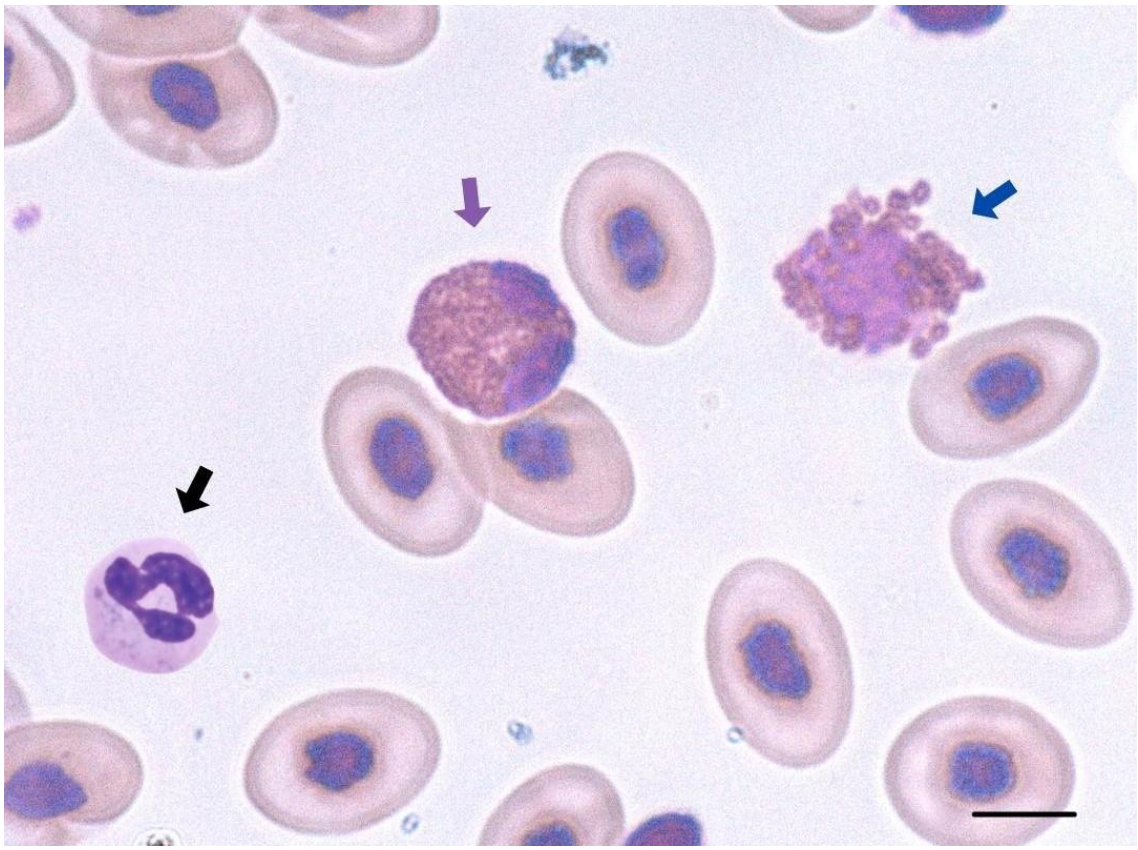


Figura 5. Células do tecido sanguíneo de *Leptodactylus vastus* Lutz, 1930 coradas com May Grunwald-Giemsa. Neutrófilo (seta preta). Eosinófilo (seta violeta). Basófilo (seta azul). A barra preta corresponde a 10 µm. Foto autoral.

Os monócitos são células redondas, nos quais pseudópodes podem estar presentes, com coloração azul-acinzentado claro, citoplasma abundante, pequenos grânulos azurófilos e de forma irregular (Campbell; Grant, 2015) (Figura 6). A morfologia dos linfócitos dos anuros se assemelham aos de outros vertebrados. São células pequenas e redondas com citoplasma azul claro e núcleo redondo e escuro. A cromatina do núcleo é densa e aglomerada, o que a diferencia dos trombócitos que não apresentam cromatina aglomerada. Os linfócitos dos anuros ainda se diferenciam dos linfócitos dos mamíferos, porque

são negativos para b-glucuronidase e arilsulfatase (Campbell; Grant, 2015; Turner, 1988) (Figura 6).

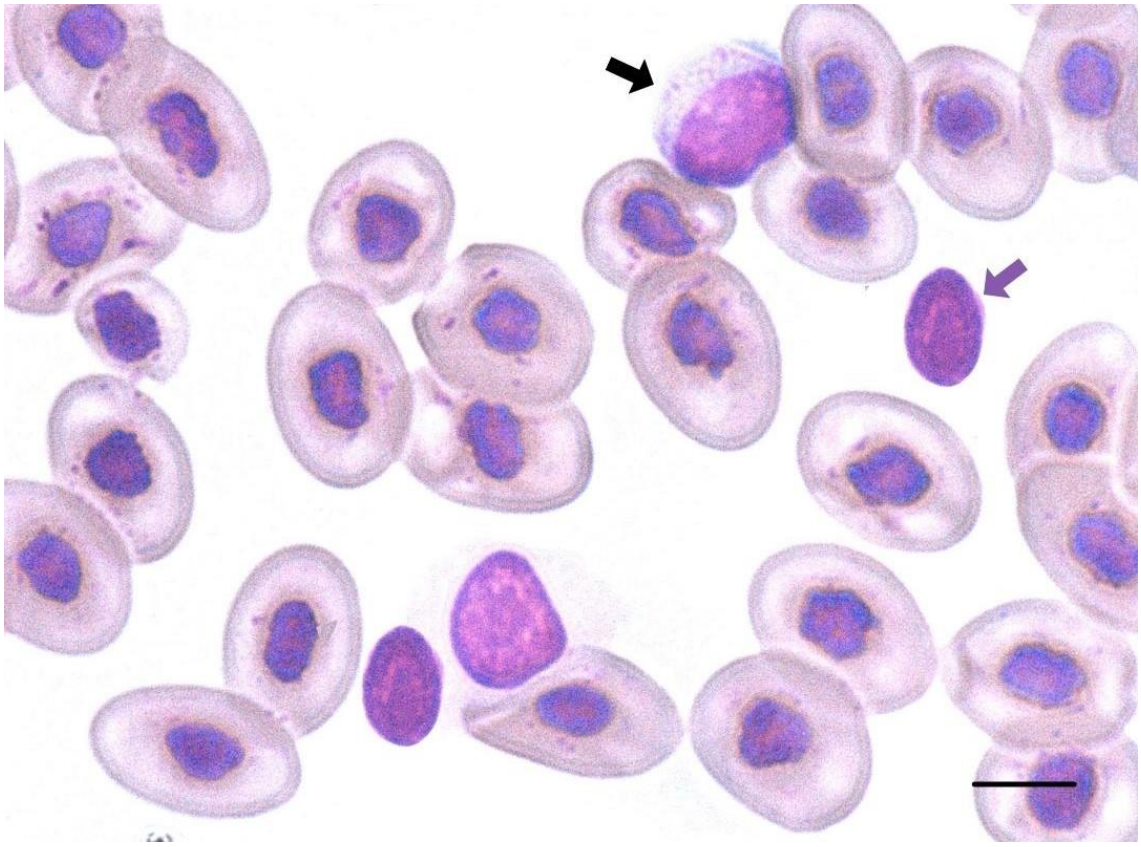


Figura 6. Células do tecido sanguíneo de *Leptodactylus vastus* Lutz, 1930 coradas com May Grunwald-Giemsa. Monócito (seta preta). Linfócito (seta violeta). A barra preta corresponde a 10 µm. Foto autoral.

A função imunológica dos leucócitos foi revisada extensivamente (Jain 1986) e podem apresentar diversas aplicações inflamatórias e imunológicas semelhante as de outros vertebrados (Duellman; Trueb, 1994). Além disso, a análise da razão Neutrófilo:Linfócito (N:L) pode estar associada com o aumento do cortisol plasmático, e, portanto, pode ser considerado como um indicador de estresse no animal (Garcia Neto et al., 2020).

Em anuros, linfócitos e neutrófilos constituem a maioria dos leucócitos, correspondendo a 80%, respectivamente, (Cathers et al., 1997; Davis et al., 2008; Thrall, 2012) (Tabela 1). Neutrófilos são leucócitos fagocíticos primários e se distribuem em maior quantidade na circulação sanguínea do animal, em resposta a infecções, inflamações e estresse (Jain, 1993; Campbell e Grant, 2015). Os linfócitos estão envolvidos em diversas funções imunológicas, sendo

classificados como células B produtoras de imunoglobulina, ou T que tem papel fundamental na modulação da defesa (Campbell; Grant, 2015).

Tabela 1 – Perfil leucocitário de espécies de anfíbios. Os resultados estão apresentados em porcentagem.

Espécie	Linfócito	Neutrófilo	Eosinófilo	Basófilo	Monócito	Autoria
Lithobates catesbianus (Shaw, 1802)	62,9%	22%	8,9%	2,5%	0,6	Cathers et al., 1997
Boana albopunctata (Spix, 1824)	63%	12%	7%	10%	8%	Titon et al., 2016
Boana faber (Wied-Neuwied, 1821)	63,6%	22,4%	1,4%	6,2%	6,4%	Barsotti et al., 2017
Rhinella icterica (Spix, 1824)	71,6%	8,9%	11,96%	1,04%	7,74%	Assis; Titon; Gomes, 2019

Os 20% restantes dos leucócitos são constituídos por eosinófilos, que desempenham importante função durante os processos de inflamação nos animais (Jain, 1993) e na defesa contra parasitos (Kiesecker, 2002). Os monócitos apresentam tempo de vida longo, enquanto células fagocíticas e estão associados a defesa contra infecções bacterianas (Campbell; Grant, 2015). Os basófilos contêm grânulos com mucopolissacarídeos ácidos (glicosaminoglicanos) que são menos sulfatados do que os grânulos dos basófilos de mamíferos, e nesse caso o conteúdo de histamina é inferior também (Turner, 1988). Os basófilos podem ser considerados como células que desempenham um papel significativo na vigilância imunológica (Campbell; Grant, 2015).

2.3.4. Bioquímica sérica dos anuros

A partir do soro ou plasma sanguíneo dos anuros pode-se obter informações sobre padrões bioquímicos. Alguns painéis foram determinados para algumas espécies de anuros (Tabela 2), exceto para espécies brasileiras. Apesar de tais valores, pouco se sabe sobre o diagnóstico das enzimas hepáticas, pancreáticas, musculares e cardíacas no sangue dos anuros.

Graças a esses resultados, geralmente fragmentados, sabe-se que anuros tolerantes ao congelamento, como a *Lithobates sylvaticus* (LeConte, 1825), *Pseudacris crucifer* (Wied-Neuwied, 1838) e *Pseudacris triseriata* Wied-Neuwied, 1838 acumulam níveis de carboidratos de baixo peso molecular nos tecidos e no sangue: glicose e glicerol (Storey; Storey, 1986). Os resultados da concentração de glicose em anuros que hibernaram recentemente podem ser maiores que 600 g/dL, enquanto que a glicose em espécimes que não hibernaram é inferior a 2,4 g/dL (Storey; Storey, 1986).

Para *Dryophytes versicolor* (LeConte, 1825), por exemplo, os resultados absolutos dependem da idade do animal, no qual em indivíduos adultos a glicose permanece inalterada durante a hibernação, enquanto as concentrações de glicerol aumentam de 6,8 para 423 mmol/L. Já as pererecas imaturas apresentam um ligeiro aumento de glicerol (0,1 – 16,3 mmol/L) e glicose (2,3 g/dL – 41,7 g/dL) (Storey; Storey, 1986).

Tabela 2. Painel bioquímico de espécies de anfíbios. Os números estão representados pela média, mínimo e máximo (quando fornecidos pelos respectivos autores). Abreviações: ALT (Alanina-aminotransferase), AST (Asparatoaminotransferase), GGT (Gama glutamiltransferase), LDL (*Low density lipoprotein*) e HDL (*High density lipoprotein*).

Bioquímica Sérica		**Espécies de anuros/Autores do estudo**				
Analito	**Unidade convenciona**	***Lithobates catesbeianus* Coppo; Mussart; Fioranelli et al. (2005)**	***Xenopus laevis* Wilson et al. (2011)**	***Ranoidea caerulea* Young et al. (2012)**	***Xenopus tropicalis* Maxham et al. (2016)**	***Lithobates catesbeianus* Peng et al. (2016)**
Albumina	g/dL	1,58 (1,49–1,67)	1 (0 – 3,58)	-	-	19,82 (14,71 –25,31)
ALT	U/L	12,4 (10–14,28)	21 (0 – 46,8)	-	-	14,5 (5,7–26,3)

AST	U/L	48,1 (42,8 – 53,4)	453 (0 – 1587)	91 (30 – 362)	-	111,5 (30,3–191,7)
Colesterol total	mg/dL	62 (56 –67)	232 (0 – 541,2)	-	-	475,83 (403,5–529,12)
Creatinina	mg/dL	0,48 (0,4–0,55)	0,4 (0 – 2,98)	-	-	0,21 (0,01–0,02)
Globulina	g/L	-	2,3 (0 – 4,88)	-		9,38 (9,10–9,69)
Glicose	mg/dL	50 (45–54)	53 (1,46 – 104,54)	64,9 (34,2 – 108,1)	-	35,85 (20,18–39,64)
Fosfatase alcalina	U/L	-	148 (59 – 282)	-		-
GGT	U/L	9,2 (7,8 –10,6)	4 (1 – 19)	-		5,09 (3,01–8,43)
LDL	mg/dL	39 (34–44)	114 (21 – 240)	-	-	-

HDL	mg/dL	4 (3–5)	36 (14 – 63)	-		-
Relação Albumina/ Globulina		0,54 (0,50 – 0,58)	0,7 (0 – 3,28)	-	-	-
Proteínas totais	g/dL	4,34 (4,19 – 4,49)	3,3 (0,72 – 5,88)	3,5 (1,8–5,6)	6,2 (3,9 – 8,59)	3,9 (2,42 – 5,4)
Triglicerídeos	mg/dL	43 (34–52)	117 (0 – 297,38)	-	-	-
Ureia	mg/dL	84,2 (76,1–92,4)	10,81 (0 – 66,68)	-	-	-

Ainda sobre dados a respeito da bioquímica sérica de anuros, de acordo com Shilton et al. (2001) *Osteopilus septentrionalis* (Duméril and Bibron, 1841), criados em cativeiro podem desenvolver obesidade e lipidose corneana, em decorrência do aumento significativo de colesterol e triglicerídeo séricos. Os indivíduos acometidos com lipidose corneana apresentam uma média de 27,5 mmol/L de colesterol, enquanto os espécimes silvestres não afetadas apresentam uma média de 3,86 mmol/dL.

Nesse contexto, percebe-se a necessidade de trabalhos que utilizem analitos bioquímicos para a investigação da fisiologia dos anuros endêmicos do Brasil. Funções hepáticas, renais e energéticas, bem como enzimas hepáticas são analitos que podem auxiliar no diagnóstico de hipersensibilidade imunológica associada a presença de parasitos que podem acometer o funcionamento dos órgãos, além de subsidiar a investigação da influência da pressão antrópica direcionada a saúde dos anuros e dos ecossistemas.

2.3.5. Repertório defensivo dos anuros

Os anuros apresentam mais de 30 estratégias de defesa que são utilizadas para evitar o risco de predação. Esses comportamentos variam desde permanecer imóvel até a fuga do ambiente (Toledo; Sazima; Haddad, 2011). Entre esses dois comportamentos, os anuros ainda podem exibir diversos mecanismos sinérgicos para aumentar suas chances de fuga, em ambientes nos quais quase todos os organismos carnívoros podem predar uma rã (Toledo, 2005; Toledo; Ribeiro; Haddad, 2007).

Toledo, Sazima e Haddad (2011) classificaram as diferentes fases da predação em: localização, identificação, abordagem, subjugação, ingestão e digestão. Já as estratégias defensivas dos anuros podem ser exibidas de forma primária: não dependem da presença do predador, ou secundária: desencadeadas pela presença do predador. Esses dois mecanismos podem ter evoluído para evitar a tentativa de predação (Edmunds, 1974) e, portanto, um anuro críptico pode evitar a sua visualização e um anuro venenoso pode evitar a sua ingestão.

Nesse caminho, o estudo sobre estratégias defensivas dos anuros funciona como uma ferramenta para o entendimento da ecologia desses animais nos

ecossistemas. Apesar disso, a maioria dos estudos sobre esses comportamentos são geralmente publicados em notas curtas (Figueiredo et al., 2021; Santos; Moura; Souza, 2020; Sena et al., 2021) o que dificulta o aprofundamento da discussão. Vale ressaltar que, para o comportamento antipredatório dos anuros há apenas as revisões feita pelo Toledo, Sazima e Haddad (2011) e Ferreira et al. (2019). As revisões reúnem mais de 30 comportamentos defensivos, conforme a tabela 3.

Tabela 3. Estratégias de defesa de anuros pós metamórficos e suas possíveis funções para evitar a predação (Toledo; Sazima; Haddad, 2011; Ferreira et al., 2019).

#	Comportamento	Variação	Possível função
1	Imobilidade		Evita a visualização
2	Permanecer agachado		Evita a imobilização
3	Tanatose ou fingir-se de morto		Evita imobilização
4	Contrair o corpo		Evita lesões durante a imobilização e ingestão
5	Flexionar a cabeça em direção ventral		Evita imobilização
6	Fragmose		Evita imobilização
7	Inflar o corpo		Evita imobilização
8	Levantar o corpo	Pernas esticadas verticalmente Pernas alongadas lateralmente	Evita imobilização

9	Inclinar o corpo		Evita imobilização
10	Manter as pernas rígidas		Evita a visualização
11	Curvar a cabeça para cima		Evita imobilização
12	Proteger os olhos com as patas		Evita lesões durante a imobilização e ingestão
13	Entrelaçar de pernas		Evita imobilização
14	Virar a parte posterior do corpo para o predador		Evita imobilização
15	Esconder		Evita a visualização
16	Escavar		Evita a visualização
17	Fugir		Evita imobilização
18	Descarga cloacal	Líquido	Evita imobilização
		Sólido (defecação)	
19	Atacar o predador		Evita imobilização
20	Bater com a cabeça		Evita imobilização
21	Morder		Evita imobilização
22	Manter a boca aberta		Evita imobilização
23	Protrair a língua		Evita imobilização
24	Agredir o predador	Morder Chute Punção	Contra ataque

25	Regurgitar		Evita ingestão
26	Vocalizar	Canto de socorro	Evita ingestão
		Canto de alerta	Evita imobilização
		Canto de alarme	Evita imobilização ou ingestão
27	Produzir secreções	Odorífero	Evita ingestão
		Adesivo	
		Nocivo	Evita ingestão / digestão
		Escorregadio	
28	Camuflar-se	Coloração parecida com o micro-habitat	Evita a detecção e previne ataques
		Camuflagem perturbadora	
29	Mostrar cores aposemáticas	Expor cores	Previne ataques
30	Brigar		Evita ingestão

2.3.6. Bioacústica para a análise do comportamento defensivo

A bioacústica é uma importante ferramenta utilizada para o estudo do comportamento dos anuros. Para esses animais, a comunicação acústica é um canal de transferência de informação intra e interespecífica (Wells; Schwartz, 2007). O canto pode estar associado a diversas funções sociais, desde a defesa do território até a atração de parceiras (Toledo et al., 2015). Os sinais acústicos são predominantemente emitidos pelos machos, e, estes por sua vez, apresentam um aparato vocal complexo, incluindo um saco vocal e uma

estrutura adaptada para a ressonância do som (o crânio) (Wells; Schwartz, 2007).

No contexto reprodutivo, as fêmeas emitem sinais acústicos para anunciar a receptividade para o macho, durante o acasalamento (Kokubum et al., 2009; Márquez; Verrell, 1991). Além disso, as fêmeas podem emitir sinais acústicos em interações defensivas (Toledo; Haddad, 2009) e agressivas (Preininger et al., 2016)

No que diz respeito a história evolutiva dos anuros, a comunicação acústica apresenta um papel crucial no reconhecimento e discriminação de indivíduos da mesma espécie, o que se denomina como variação intra e interindividual (Gerhardt, 1991; Morais et al., 2012; De Oliveira-Santos et al., 2020). Além disso, os sinais acústicos estão sujeitos a influência dos fatores bióticos (presença de outras espécies, barreiras naturais) e abióticos (temperatura, umidade e precipitação), resultando em diferenças interindividuais e interpopulacionais (Morais et al., 2012; Tessarolo et al., 2016).

Dentre os cantos emitidos pelos anuros, tem-se observado que o canto de socorro (*distress call*) é um display comportamental utilizado por esses animais no momento em que são interceptados pelos predadores (Toledo; Haddad, 2009). Esse canto (grito) também pode ser utilizado para alertar os vizinhos, o que resulta na interrupção das atividades de vocalização das espécies coespecíficas (Forti et al., 2017). Para esse tipo de canto, quanto mais alto e duradouro, ele se torna mais eficaz contra diferentes tipos de predadores (Toledo; Haddad, 2009). Apesar disso, as espécies do grupo *Leptodactylus pentadactylus* não seguem esse modelo, tendo em vista que são animais que emitem cantos de socorro de curta duração (Toledo; Haddad, 2009).

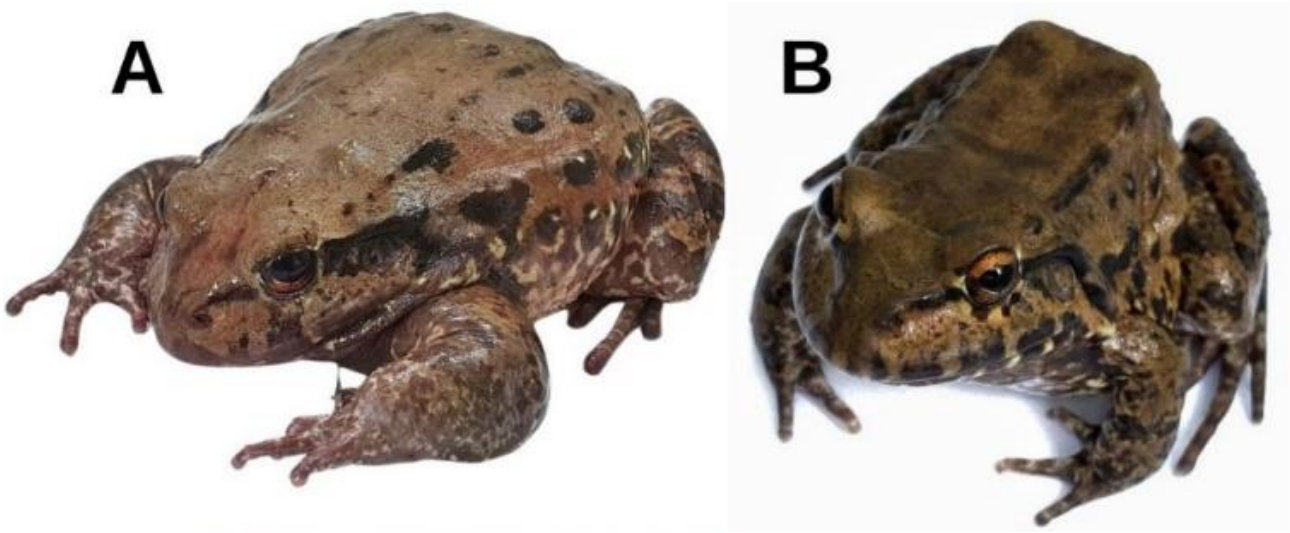
Desse modo, a bioacústica é uma importante abordagem para estudos sobre etologia (Gerhardt; Huber, 2002) ecologia (Oseen; Wassersug, 2002), evolução e conservação (Sugai; Llusia, 2019), além de detectar a influência das mudanças climáticas na biologia térmica desses animais (Llusia et al., 2013). O estudo sobre o repertório defensivo dos anuros, baseados em comportamentos observados em campos naturalistas tem produzido resultados que podem

auxiliar na construção da história natural (Ferrante; Najar; Kaefer, 2020) e em propostas para a conservação das espécies em seus respectivos ecossistemas.

2.4. *Leptodactylus vastus* Lutz, 1930

O gênero *Leptodactylus* Fitzinger, 1826 compreende 83 espécies distribuídas entre o sul da América do Norte e América do Sul, bem como ocidente da Índia (Frost, 2021). O gênero é dividido em quatro grupos: *L. fuscus, L. pentadactylus, L. latrans* e *L. melanonotus* (de Sá et al., 2014; Magalhães et., 2020). As espécies de anuros pertencentes a este último grupo se distribuem pela América Central e América do Sul, ocupando uma grande diversidade de nichos florestais (Heyer, 2005). Atualmente, o grupo apresenta 14 espécies, dentre elas *Leptodactylus vastus* Lutz, 1930.

No Brasil, *L. vastus* se distribui pelo nordeste brasileiro e, assim como as demais espécies do grupo pode apresentar até 20 cm de comprimento rostro cloacal (CRC) (Heyer, 2005) (Figura 7). De acordo com a Lista Vermelha de Espécies Ameaçadas de Extinção (IUCN) (Heyer, 2008), Ministério do Meio Ambiente (MMA) e a RESOLUÇÃO Nº 01, DE 09 DE JANEIRO DE 2015, *L. vastus* se encontra na categoria de LC (pouco preocupante). Vale ressaltar que, assim como no Brasil, *L. vastus* foi comumente confundido como *L. labyrinthicus* na Bolívia, por apresentar características morfológicas (CRC e coloração) e até acústicas muito parecidas (Heyer, 2005).



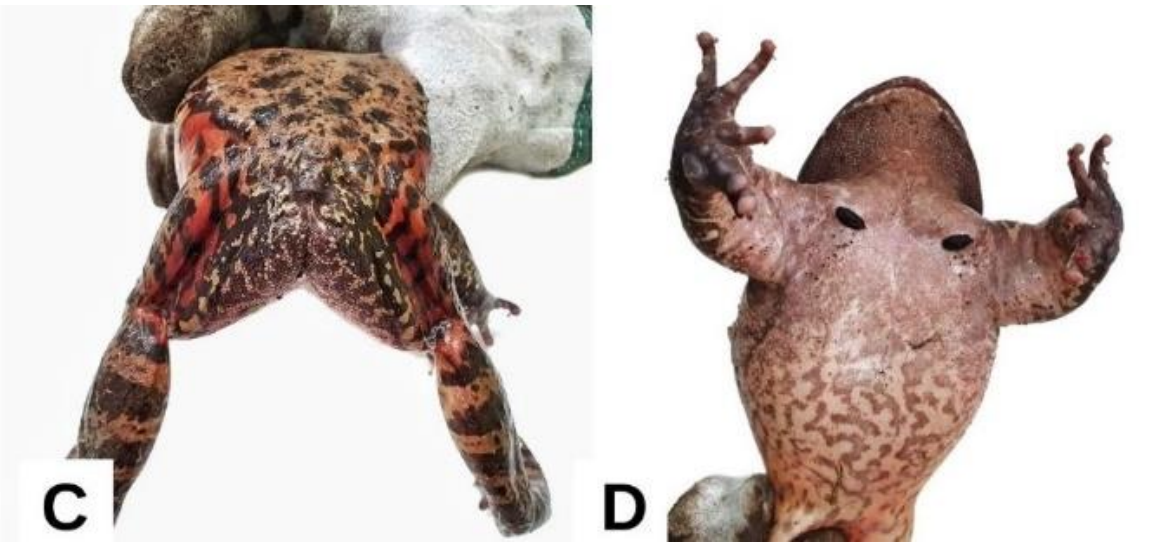


Figura 7. Espécimes de *Leptodactylus vastus* Lutz, 1930 registrados no Parque Estadual de Dois Irmãos e na Mata do Camocim, Pernambuco, Brasil. A) macho em repouso (CRC: 17,08 cm; peso: 300g). B) Vista anterior. C) Vista posterior, evidenciando as pernas com coloração aposemática. D) Macho em posição ventral, evidenciando os espinhos (calos nupciais) na região peitoral. (CRC: 15,01 cm; peso: 270g). Fotos: Jonathas Lins.

Assim como boa parte das espécies do gênero *Leptodactylus,* não há estudos sobre hemograma e bioquímica sérica de *L. vastus*. Contudo, análises sobre o canto de socorro de fêmeas foram investigados por Toledo e Haddad (2009) e o canto de anúncio foi definido por Jansen e Schulze (2012). De Castro et al. (2017) ainda reuniram três comportamentos defensivos de machos de *L. vastus* em uma nota: tanatose, levantamento do corpo e liberação de secreção tóxica pela epiderme. Nenhum desses trabalhos verificou a variação intra e interindivual do canto de socorro.

No contexto das ameaças para a diversidade de anuros no mundo, surge a necessidade do desenvolvimento de abordagens para o monitoramento do estado fisiológico das populações desses animais. Com isso, o biomonitoramento constitui um valioso instrumento de avaliação, uma vez que utiliza estudos de campo com animais bioindicadores para a compreensão da extensão temporal e espacial da contaminação ambiental e seus efeitos nos animais (Huggett et al., 2018).

E, apesar da hematologia dos anuros ser desafiadora, em decorrência da combinação de diversos fatores, como o comprimento rostro cloacal pequeno para algumas espécies, poucos locais para a punção venosa, falta de dados hematológicos que sirvam de base para a determinação de intervalos para a contagem e diferenciação dos diferentes tipos celulares sanguíneos nestes animais (Heatley; Johnson, 2009), é necessário que haja aprofundamento nos estudos sobre esses parâmetros fisiológicos para espécies de anuros brasileiros. Além disso, conhecer a história natural, através dos seus mecanismos de defesa podem auxiliar na construção de propostas ainda mais robustas, que possam mitigar as consequências que têm levado ao declínio das populações nos ecossistemas.

3. Objetivos

3.1. Objetivo Geral

Analisar o estado fisiológico de machos de *Leptodactylus vastus* Lutz, 1930, a partir de fatores intrínsecos e extrínsecos em remanescentes de Floresta Atlântica na região metropolitana de Recife, Pernambuco, Brasil.

3.2. Objetivos Específicos

- Determinar o perfil hematológico dos animais;
- Definir os parâmetros bioquímicos séricos dos animais;
- Descrever as características ultraestruturais das células sanguíneas;
- Verificar a ocorrência de hemoparasitos;
- Investigar o comportamento defensivo dos animais.

4. Material e Métodos

4.1. Área de Estudo

Os estudos foram realizados em três fragmentos de Floresta Atlântica semidecidual, situados na região metropolitana de Recife, Pernambuco, Brasil. Os machos de *Leptodactylus vastus* Lutz, 1930 foram capturados através do método de coleta ativa, no Jardim Botânico do Recife (8°04'00"S, 34°58'59"W, datum= SAD69, datum= SAD69 – 15 machos), no Parque Estadual Dois Irmãos (8°00'16"S, 34°57'10"W, datum= SAD69 – 15 machos) e na Unidade de Conservação Mata do Camocim (8°02'25"S, 35°11'48" W, datum= SAD69 – 30 machos) (Figura 8). As chuvas se concentraram entre os meses de março a agosto, e de acordo com a classificação climática Köppen-Geiger o clima da região é do tipo tropical (As) (Alvares et al., 2013). Os três fragmentos sofrem interferência antrópica, em decorrência de serviços ecossistêmicos voltados para o turismo e pesquisa. Com isso, os machos de *L. vastus* têm se concentrado em pequenos corpos d'água, situados em manchas florestais isoladas.

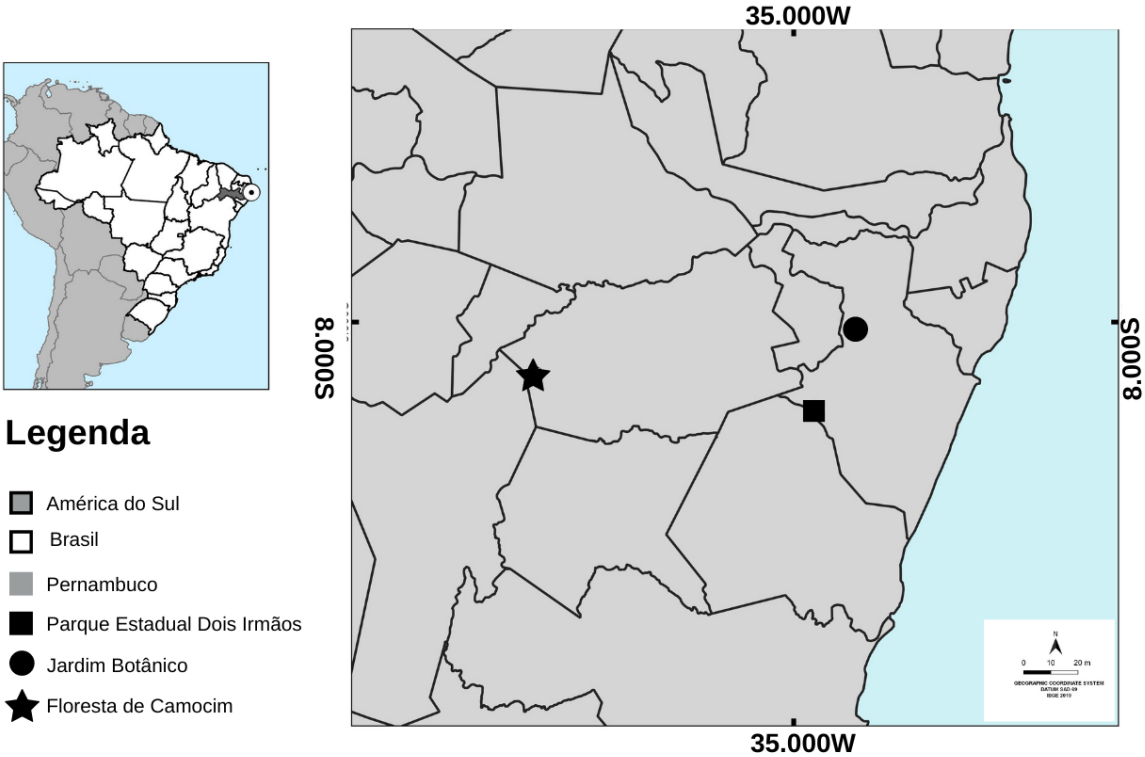


Figura 8. Mapa de localização das áreas de coleta de *Leptodactylus vastus* Lutz, 1930. As três áreas estão situadas na Região Metropolitana do Recife, Pernambuco, Brasil. Foto autoral.

4.2. Análise dos parâmetros intrínsecos

4.2.1. Coleta de sangue

O sangue dos animais foi coletado, através de punção cardíaca por incisão de seringa descartável (5 mL/22G) (Figura 9). Antes do procedimento foi utilizado lidocaína 5% no local da punção, de acordo com Forzán e Horney (2020). O sangue foi distribuído em tubos MiniCollect®CE contendo EDTA (ácido etilenodiaminotetracético) para o hemocitômetro e em tubos com gel separador para a obtenção do soro e realização da análise da bioquímica sérica. Tendo em vista a realização da análise da correlação entre os fatores bióticos e abióticos com as variáveis hematológicas e bioquímica, foram tomados dados como comprimento rostro cloacal (0,01mm precisão), massa corporal (0,01g precisão), além da temperatura (ºC) e umidade (%) ambiental. Após a coleta do sangue e análise da morfometria, os animais foram liberados no local da captura.

Figura 9. Coleta de amostra biológica e dados morfométricos. A e B) Coleta de sangue por punção cardíaca em um macho de Leptodactylus vastus Lutz, 1930. C) Análise do comprimento rostro clocal (CRC). D) Balança digital mostrando o peso do animal (g). Fotos autorais.

4.2.2. Contagem diferencial de leucócitos e parasitemia

Foram realizados três esfregaços sanguíneos de cada animal e, estes por sua vez, deixados secar em temperatura ambiente durante 10 min. As lâminas foram fixadas em metanol e coradas pelo método de May Grunwald-Giemsa para a contagem diferencial de 100 leucócitos (da Silva et al., 2020). Os esfregaços sanguíneos foram observados em objetiva de 100x (óleo de imersão) em microscópio óptico, em um padrão de zig-zag. Apenas campos de visão contendo células uniformemente distribuídas foram examinados e contadas por um único observador (Figura 10). A parasitemia foi determinada pela contagem do número de parasitos em 2.000 eritrócitos, divididos em 20 campos de 100 eritrócitos e expressa em porcentagem (Godfrey et al., 1987).

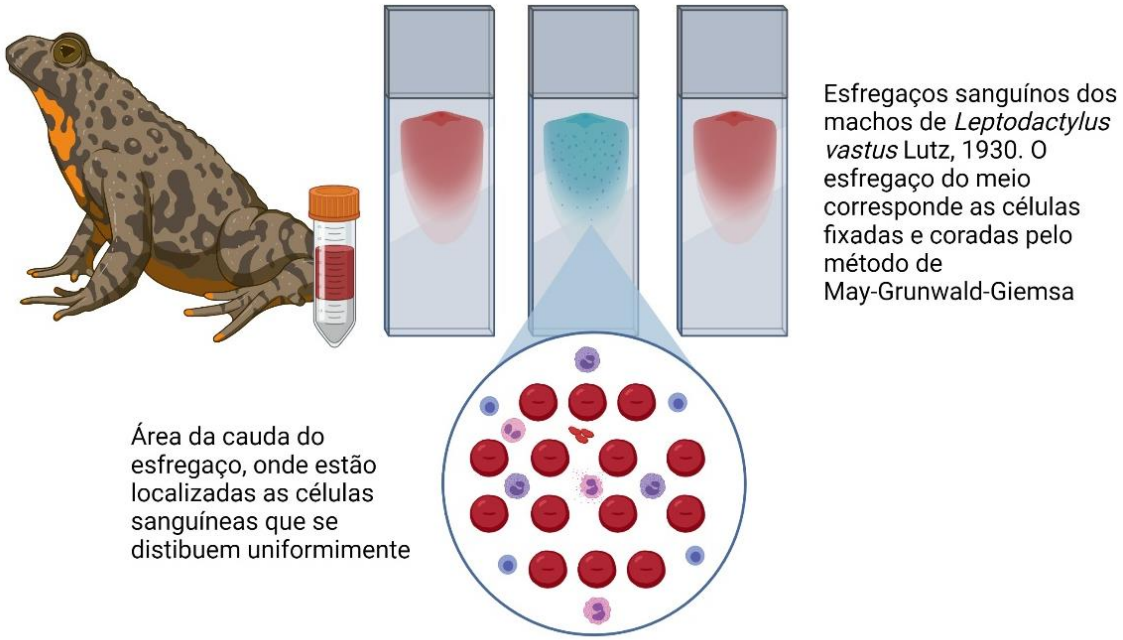


Figura 10. Ilustração do método de contagem diferencial das células sanguíneas de *Leptodactylus vastus* Lutz, 1930. Foto autoral criada no Biorender.

4.2.3. Contagem total de células sanguíneas

A contagem total de eritrócitos, leucócitos e trombócitos foi realizada através de hemocitômetro. O sangue foi diluído em solução de Natt Herrick (1:100) (Natt; Herrick, 1952) e, após, foram adicionados em uma câmera de Neubauer para a contagem. A contagem total de eritrócitos se deu a partir da

equação: N × 10.000 = total de eritrócitos em mm^3, a contagem total de leucócitos a partir da equação: (N + 10%) × 200 = total de leucócitos em mm^3 e a contagem total de trombócitos se deu a partir da equação: N × 2.000 = total de trombócitos em mm^3 (Thrall *et al.,* 2012; Wright; Whitaker, 2001).

4.2.4. Concentração de hemoglobina

A concentração de hemoglobina foi determinada pelo método de oxiemoglobina, a partir da diluição de 1:250 do sangue em solução do kit hemoglobina (Labtest®) (Drabkin; Austin 1935). As amostras foram incubadas por 5 minutos e, após este período, alíquotas de 100 µL foram adicionadas em placa de 96 poços para a leitura em absorbância de 540 nm na GloMax® Discover Microplate Reader (Figura 11). Ainda foi realizado uma curva de absorbância com o sangue padrão do kit hemoglobina (Labtest®), o que resultou uma equação da regressão: y = 0,1261x + 3E-05 e um coeficiente de correlação R2= 1. O cálculo da concentração de hemoglobina seguiu a seguinte equação:

$$\frac{(Absorbância\ do\ teste - 0{,}00003) \times 10}{0{,}1261} = \mathrm{xg/dL}$$

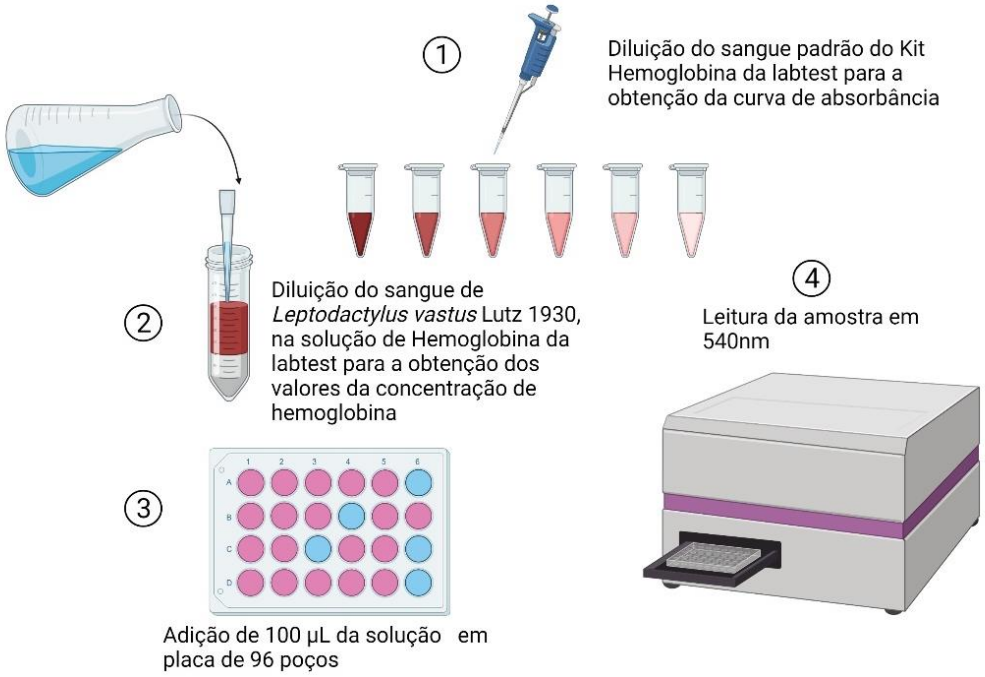


Figura 11. Ilustração do método de dosagem da concentração de hemoglobina de *Leptodactylus vastus* Lutz, 1930. Foto autoral criada no Biorender.

4.2.5. Hematócrito e índices hematimétricos

O hematócrito foi realizado a partir do preenchimento de ¾ de três tubos microcapilares não heparinizados, para cada animal. Os três tubos foram colocados dentro de um tubo Falcon de 50 ml para a centrifugação em centrífuga comum (Figura 12). Em seguida, as amostras foram lidas em uma régua manual de hematócrito (Figura 13). A partir dessas análises foi possível definir os valores hematimétricos de Volume Corpuscular Médio (VCM): (Ht/He mm^3) × 10 = VCM (fL); Hemoglobina Corpuscular Média (HCM): (Hb/He mm^3) × 10 = HCM (pg) e Concentração de Hemoglobina Corpuscular Média (CHCM): (Hb/Ht) × 100 (Campbell; Ellis, 2013).

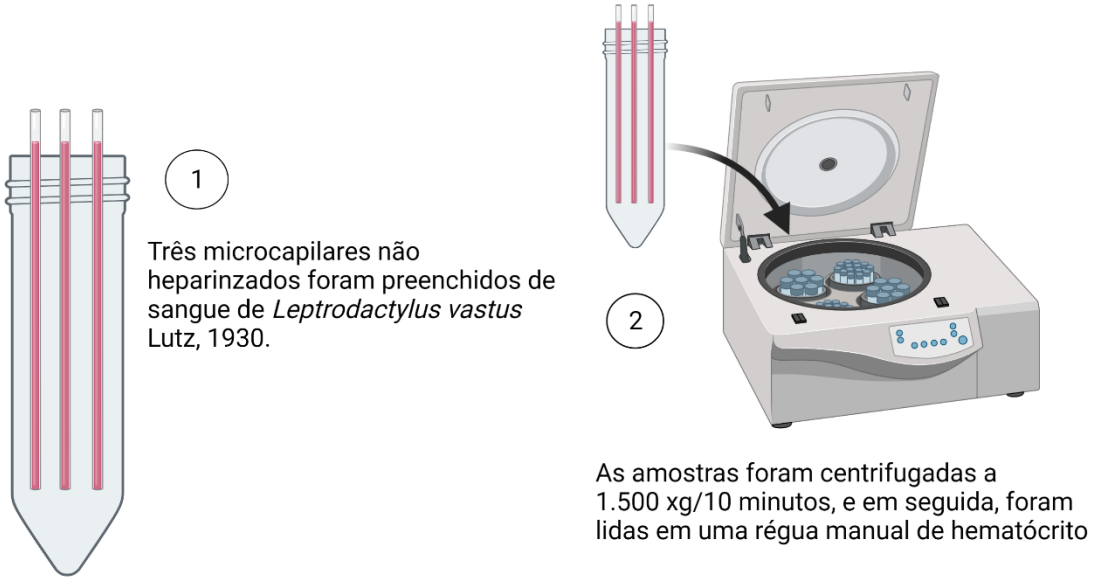


Figura 12. Ilustração do método de centrifugação de microcapilares para a obtenção dos valores do hematócrito de *Leptodactylus vastus* Lutz, 1930. Foto autoral criada no Biorender.

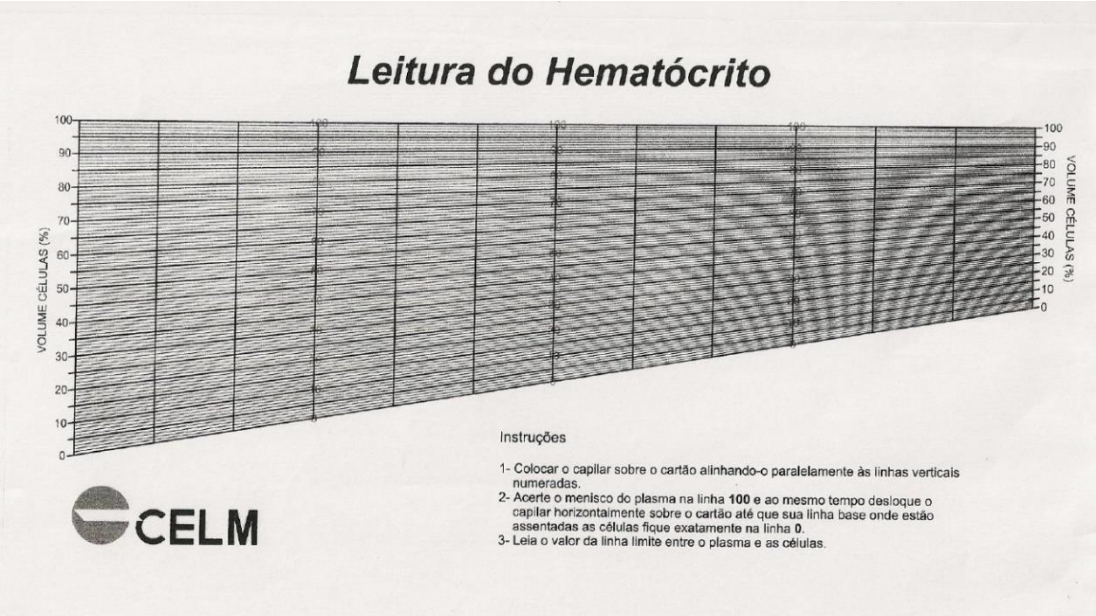


Figura 13. Régua para a leitura manual do hematócrito (Disponível em: google imagens).

4.2.6. Microscopia eletrônica de transmissão

O sangue total foi fixado em uma solução contendo 2.5% de glutaraldeido, 4% de paraformaldeido e 0,1 M de tampão cacodilato (pH= 7,4). Após cada etapa as células foram centrifugadas e lavadas com tampão cacodilato 0,1 M (pH 7,2). Em seguida, as células foram desidratadas em séries crescentes de acetona, infiltradas e incluídas em resina EPON (Sigma Aldrich, USA). Cortes ultrafinos foram obtidos e coletados em grades de níquel de 300 mesh. Após a obtenção dos cortes, as amostras foram contrastadas com acetato uranila 5% em água por 30 minutos e citrato de chumbo por 3 minutos. Os materiais foram então visualizados no microscópio de transmissão (Met – FEI Tecnai G2 Spirit Biotwin) (Figura 14). As análises hematológicas e ultraestruturais foram realizadas no Laboratório de Biologia Celular de Patógenos do Departamento de Microbiologia, Instituto Aggeu Magalhães – IAM/FIOCRUZ-PE.

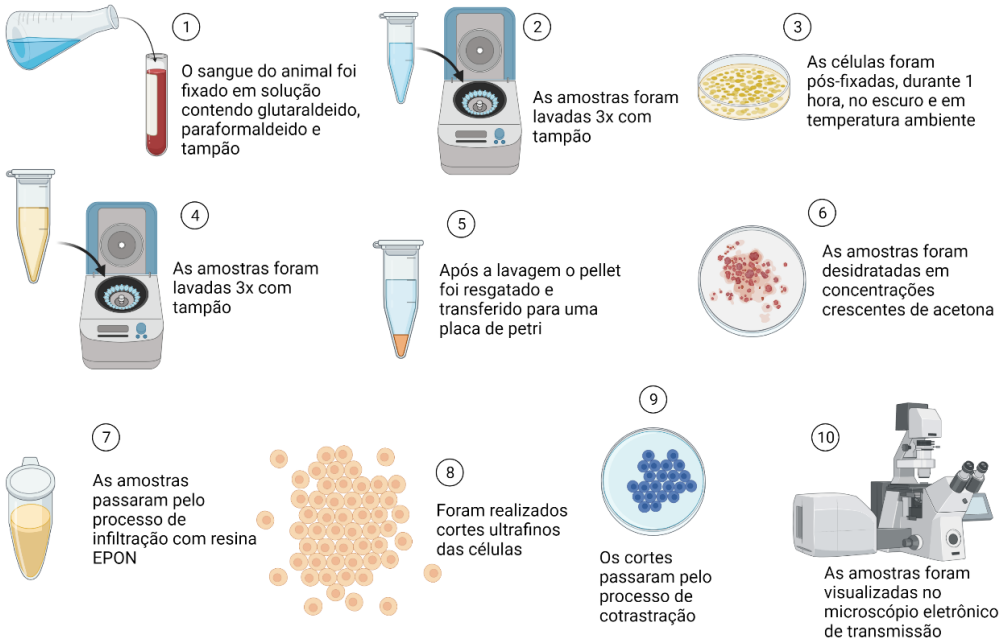


Figura 14. Ilustração do método de visualização dos tipos de células sanguíneas de Leptodactylus vastus Lutz, 1930 em microscópio eletrônico de transmissão. Foto autoral criada no Biorender.

4.2.7. Bioquímica sérica

Os tubos com gel separador permaneceram em repouso, durante 40 minutos até a coagulação. Em seguida, foram centrifugados por 10 minutos a uma velocidade de 1.500 xg. O soro foi armazenado em eppendorfs e congelado a -80ºC no Deep Freeze até a realização das análises. Foi utilizado o analisador automático Labmax 240® e reagentes Labtest® para determinação da função proteica através da Creatina, Ureia, Proteína Total; função energética: Albumina, Glicose, Triglicerídeos, Colesterol total, Colesterol HDL, Colesterol LDL; e função enzimática através do Asparato aminotransferase sérica (AST), Gama GT, Alanina aminotransferase (ALT) e Fosfatase alcalina (FA). As determinações da Bioquímica foram realizadas no Laboratório de Doenças Nutricionais e Metabólicas do Centro de Apoio à Pesquisa do Departamento de Medicina Veterinária da UFRPE.

4.3. Análise dos parâmetros extrínsecos

4.3.1. Repertório defensivo de *Leptodactylus vastus* Lutz, 1930

Os displays comportamentais dos machos de *L. vastus* foram investigados, através de três tipos de estímulos experimentais: (1) agarrar inesperadamente o animal pelos membros posteriores; (2) sujeitar a rã a movimentos erráticos, segurando-o pelos membros posteriores e (3) emitir o playback do canto de socorro (Figura 15). Cada um dos experimentos foi randomizado para cada animal e teve duração de 3 minutos. Quando o animal não apresentava algum tipo de display comportamental, este era colocado em uma caixa de contenção para repouso de 10 minutos. Em seguida, um dos experimentos era repetido (adaptado – Toledo; Haddad 2009). Após a realização dos experimentos, foram tomados dados do CRC e massa corporal dos animais.

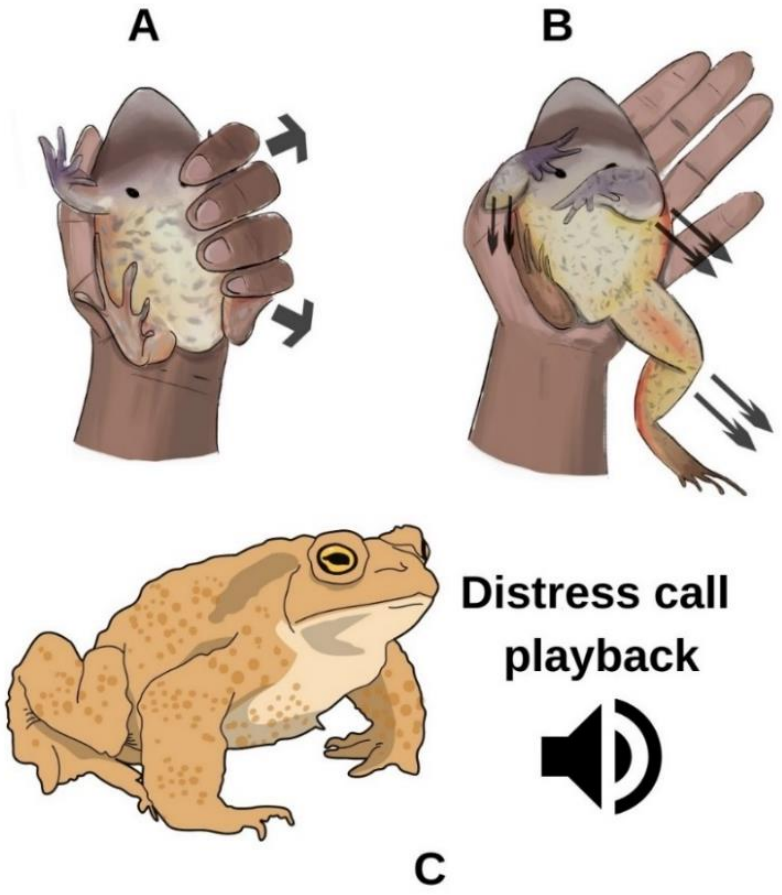


Figura 15. Ilustração dos experimentos para a investigação dos displays comportamentais de *Leptedocatylus vastus* Lutz, 1930. A e B) Estimulo através de movimentos erráticos. C) Emissão do playback do canto de socorro. Foto autoral.

4.3.2. Bioacústica

O canto de socorro de 10 machos foi registrado com auxílio de um microfone direcional HT-81 acoplado em um gravador digital Tascam DR-40, a 50 cm dos animais. Os parâmetros acústicos, como duração da chamada (s), o intervalo entre as notas (s), a frequência mínima (kHz), a frequência máxima (kHz), a amplitude da frequência (kHz), a frequência dominante (kHz), notas/min e harmônicos foram analisados no software Raven 1.6 e estão de acordo com Köhler et al. (2017). Para a construção do playback do canto de socorro foram selecionadas 10 notas de 3 animais, totalizando 30 notas previamente gravadas. O playback foi feito no software Audacity 1.3 e reproduzido em uma caixa de música portátil JBL Charge 3 (65Hz - 20kHz,> 80 dB) (Experimento 3).

4.4. Análise de dados

4.4.1. Hemograma e Bioquímica Sérica

Foi realizado um teste de Shapiro-Wilk para verificar a distribuição dos dados. Em seguida, foi realizada a Regressão Linear Simples para investigar a relação entre parâmetros morfológicos/variáveis hematológicas e parâmetros morfológicos/bioquímica sérica. Ainda foi realizado a análise da razão de neutrófilo/linfócito, de acordo com Davis et al. (2009). Todas as análises seguiram um nível de significância de $p<0,05$ e foram processadas, de acordo com Zar (2013), utilizando o Graphpad Prism 7.0.

4.4.2. Repertório defensivo

Foi realizado um teste de Shapiro-Wilk para verificar a distribuição dos dados. Em seguida, a Anova foi realizada para verificar se havia preferência dos machos por um dos estímulos experimentais. O coeficiente de variação intraindividual (CV_{intra}) e interindividual (CV_{inter}), de acordo com Gerhardt (1991), foi feito para investigar possíveis variações no canto de socorro. Ademais, a

análise de Regressão Linear Múltipla foi realizada para verificar a correlação entre os parâmetros acústicos e as variáveis morfológicas. Todas as análises seguiram um nível de significância de $p<0,05$ e foram processadas, de acordo com Zar (2013), utilizando o Graphpad Prism 7.0.

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ARTIGO I

PHYSIOLOGY AND ULTRASTRUCTURE OF THE BLOOD CELLS OF MALES OF *LEPTODACTYLUS VASTUS* LUTZ, 1930 (ANURA: LEPTODACTYLIDAE) FROM NORTHEAST OF BRAZIL

José Ricardo de Oliveira-Santos, Vanderlan Nogueira Holanda, Pierre Castro Soares, Daniel Nunes Araújo Gonçalves, Geraldo Jorge Barbosa de Moura & Regina Célia Bressan Queiroz de Figueiredo

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Physiology and ultrastructure of the blood cells of males of *Leptodactylus vastus* Lutz, 1930 (Anura: Leptodactylidae) from Northeast of Brazil

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Running title: Physiological state of *Leptodactylus vastus*

Highlights*:*

- The first paper that proposes to describe the blood cells of a species of anura endemic from Brazil, through electron microscopy;
- Our results suggesting that anurans submitted to anthropic pression could present leukocytosis;
- Our results demonstrated the occurrence of hemogregarine in a specie of group *Leptodactylus pentadactylus*;
- The results demonstrate an elevation of hepatic enzymes in wild male *L. vastus.*

Abstract

The hematological and biochemical characterization of wild anurans has been considered an important parameter to evaluate the health of these animals in the wild, driving conservation decisions. In this paper, we aimed to investigate the hematological profile and the biochemical parameters, as well as characterize the different blood cell types of the anuran *Leptodactylus vastus* Lutz, 1930. The blood samples from 30 males of *L. vastus*, captured in the Botanical Garden and Dois Irmãos State Park at the Metropolitan Region of Recife municipality, Pernambuco, Brazil were collected by cardiac puncture and processed for hematological parameters and biochemistry analysis. To morphologically characterized the blood cell types the ultrastructural study was performed. Our results showed that the hematological parameters of *L. vastus* are close to the values reported for other anuran species. However, the packed cell volume value range was higher than expected, suggesting that these specimens were undergone dehydration or haemoconcentration. An increased percentage of basophils as well as abnormalities in erythrocyte morphology may be due to immunological hypersensitivity in frogs in response to environmental stressors. We also observed the presence of hemogregarine in these frogs. Comparing to the other species of anurans, an increase of hepatic enzymes may be correlated with environmental conditions, as fragmentation of the habitat and the reduction in the number of niches.

Keywords: *Leptodactylus pentadactylus group*, hematological profile, eletron microscopy, wild animal health

1. Introduction

Amphibians have experienced a massive, widespread decline of the population worldwide over the last decades, with thousands of species on the verge of extinction. The introduction of exotic species (Forti et al., 2017), agricultural activities (Borges et al., 2019), pollution (da Costa Araújo and Malafaia, 2020), habitat fragmentation (Assis et al., 2020) and the emergence of emerging diseases (Assis et al., 2021) have become the main precursors of this decline. In this sense, monitoring and conservation of wild animals have been intensified to mitigate the impacts caused by human activity on ecosystems (Pollo et al., 2012), mainly with the study of hematological parameters and serum biochemistry of these animals.

Such parameters have been widely reported for commercially important species such as *Lithobates pipiens* (Rouf, 1964), *Xenopus leavis* (Hadji-Azimi et al., 1987), *Lithobates catesbianus* (Peng et al., 2016), *Lithobates sylvaticus* (Forzán et al., 2016) and *Xenopus tropicalis* (Maxham et al., 2016). Despite the importance of monitoring the health of anuran population, little information is available on the hematological and serum biochemical parameters of anurans from Brazilian ecosystems.

Furthermore, studies on the occurrence of hemoparasites can also help to investigate the health state of these animals. Among the most common hemoparasites in anurans, hemogregarines can be highlighted. (Netherlands et al., 2014; Leal et al., 2015; Ferreira et al., 2020). Hemogregarines (Apicomplexa: Adeleiorina) are heteroxenous hemoprotozoa that infect a wide range of ectothermic hosts, including anurans. In Brazil, *Hepatozoon leptodactyli* (Lesage, 1908) Pessoa, 1970 and *Hemolivia stellata* Petit, Landau, Baccam and Lainson, 1990 have been reported as hemogregarins that infect blood cells of Brazilian frogs, including species of the genus *Leptodactylus* Fitzinger, 1826 (Leal et al., 2015; Ferreira et al., 2020).

Leptodactylus vastus Lutz, 1930 is a species of anuran belonging to the group *L. pentadactylus* (Heyer, 1972) that occurs in northeastern Brazil and the Bolivian Amazon, being in the category of LC (Least concern), according to the IUCN Red List of Threatened Species. As with the other species of the group,

there is no information on the hematological and serum biochemical variables for *L. vastus*, which makes it difficult to assess its physiological state. Furthermore, as *Lithobates catesbeianus* (Dubois, 2006), which is an exotic invasive species in Brazilian ecosystems, *L. vastus* also has high potential for the frog trade, as it is a species with high protein and low fat content. The species has been consumed in the northeast region of the country, despite the absence of frog farming, previously legalized.

Therefore, this study aimed to analyze the physiological status of males of *L. vastus* in two fragments of Semideciduous Atlantic Forest in northeastern Brazil, through (i) determination of blood count and (ii) serum biochemistry, (iii) analysis of the occurrence of hemogregarine and (iv) evaluation of the ultrastructure of blood cells. In this way, such data can provide subsidies for the creation of conservation plans that try to mitigate the consequences, as a result of anthropization, on anurans and ecosystems associated with them.

2. Material and Methods

2*.1. Study area*

Thirty males of *Leptodactylus vastus* Lutz, 1930 were actively captured in the rainy season (July and August) of 2021 at a fragment of Atlantic Forest in the Botanical Garden of Recife (8S04' 00", 34W58'59", datum= SAD69, 15 males) and in the Dois Irmãos State Park (8S00'16", 34E57'10", datum= SAD69, 15 males). These areas are located at the metropolitan region of Recife, Pernambuco, Brazil, which present a tropical (As) climate, accordingly to the Köppen-Geiger climate classification (Alvares et al., 2013) (Fig. 1).

2.2. Morphometric measurements of *Leptodactylus vastus*

The snout-vent length of *L.vastus* were measured with pachymeter (0.01mm of precision) and the weight with a digital scale (precision of 0.01g). The environment temperature and wetness were measured using a digital thermohygrometer HT210 with a precision of 0.1°C.

2.3. Analysis of hematological profile of L. vastus

All procedures were approved by SISBIO (63567) and CEUA-UFRPE (131/2019). Blood samples (total volume 1.5 mL/animal) were collected by cardiac puncture, using 5 ml disposable syringes and 22G needles, after application of 5% lidocaine at the puncture site (Forzán and Horney, 2020). The blood was distributed in MiniCollect®CE tubes containing EDTA (ethylenediaminetetraacetic acid) for the hemacytometer, and tubes with separator gel to obtain the serum and perform the serum biochemistry analysis.

Three blood smears were made for each animal and allowed to dry at room temperature for 10 minutes. The slides were fixed in methanol and stained using the May Grunwald - Giemsa method for the differential count of 100 leukocytes (Silva et al., 2020). Blood smears were observed in a 100x objective (immersion oil) under an optical microscope in a zig-zag pattern. Only fields of view containing evenly distributed cells were examined and counted by a single observer. Parasitemia was determined by counting the number of parasites in 2000 erythrocytes, divided into 20 fields of 100 erythrocytes and expressed as a percentage (Godfrey et al., 1987). The Neutrophil:Lymphocyte ratio (N:L) was calculated, according to Davis and Maerz (2011) as an indicator of tress response

Total erythrocyte, leukocyte and thrombocyte counts were performed in duplicate using a Neubauer camera with a 1:100 dilution of whole blood in Natt Herrick's solution (Natt and Herrick, 1952). The total erythrocyte count was based on the equation: N × 10.000 = total erythrocytes in mm^3, the total leukocyte count: (N + 10%) × 200 = total leukocytes in mm^3, and the total thrombocyte count: N × 2000 = total thrombocytes in mm^3, where N = number of cells counted (Wright and Whitaker, 2001; Thrall et al., 2012).

Hemoglobin concentrations were determined by the oxyhemoglobin method from a 1:250 dilution of blood in a hemoglobin kit solution (Labtest®) (Drabkin and Austin, 1935). The samples were incubated for 5 minutes and after this period 100 µL aliquots of the solution were added to a 96-well plate for reading at an absorbance at 540 nm. An absorbance curve was also performed with the standard blood from the hemoglobin kit (Labtest®) resulting in the regression equation: $y = 0.1261x + 3E\text{-}05$ and a correlation coefficient $R^2 = 1$. The

calculation of the hemoglobin concentration followed the following equation, which X = hemoglobin value in g/dL:

$$\frac{(Test\ absorbance - 0.00003) \times 10}{0.1261} = \mathrm{Xg/dL}$$

The packed cell volume (PCV) was performed by filling ¾ of three non-heparinized microcapillary tubes for each animal. Capillaries were centrifuged at 1500 x g/5 minutes (Blaxhall and Daisley, 1973). From these analyzes it was possible to define the values of Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH) and Mean Corpuscular Hemoglobin Concentration (MCHC) according to Campbell and Ellis (2013).

2.4. Transmission electron microscopy

About 2ml of total blood was fixed in a solution containing 2.5% glutaraldehyde, 4% paraformaldehyde and 0.1 M cacodylate buffer (pH = 7.4). After each step, the cells were centrifuged and washed with 0.1 M cacodylate buffer (pH 7.2). Then, the cells were dehydrated in increasing series of acetone, infiltrated and embedded in EPON resin (Sigma Aldrich, USA). Ultrathin sections were obtained and collected on 300 mesh nickel grids. After obtaining the sections, the samples were contrasted with 5% uranyl acetate for 30 minutes and lead citrate for 3 minutes. The materials were then visualized under a transmission microscope (Met – FEI Tecnai G2 Spirit Biotwin).

2.5. Serum biochemistry

Serum was obtained from blood packed in tubes with separator gel for 40 min until coagulation. Then, they were centrifuged for 10 minutes at a speed of 1500 × g. The serum was stored in eppendorfs and frozen at -80ºC in the Deep Freeze until use. To verify the biochemical values, the automatic analyzer Labmax 240® and Labtest® reagents were used to determine the ptotein function through Creatinine, Urea, Total Protein; energetic function: Albumin, Glucose, Triglycerides, Total Cholesterol, HDL Cholesterol, LDL Cholesterol; and enzymatic function through Aspartate Aminotransferase (AST), Gamma GT, Alanine Aminotransferase (ALT) and Alkaline Phosphatase (PA).

2.6. Data analysis

Descriptive statistics included summaries for the blood count and another for serum biochemistry, presenting the mean, standard deviation and variation values. Then, the Shapiro-Wilk test was used to verify the distribution of dependent variables, and, after Simple Linear Regression analysis, to verify the relationship between SLV/blood count, mass/blood count, SLV/serum biochemistry, mass/serum biochemistry. In addition, this analysis was used to investigate the relationship between hemoglobin/PCV and hemoglobin/hematimetric indices. Results were considered significant when $p < 0.05$. The analyzes were performed, according to Zar (2013), using Graphpad Prism 7.0.

3. Results and discussion

The hematological and biochemical analyses of blood are important and less invasive tool to access the health and physiological status wild animals, especially in anurans (Das and Mahapatra, 2014). These parameters are useful for diagnosing blood diseases and pathogens, as well as organs related disorders and other diseases that can affect animals (Campbell and Grant, 2015). It is well known that blood composition would be influenced by intrinsic physiological characteristic of the amphibian, such as sex, developmental stage, and size, as well as by environment conditions (temperature, water availability, anthropocentric pressure, etc.) (Coppo et al., 2005). In this work we described morphologically the blood cells from males of *L. vastus,* by light and electron microscopy and characterized their hematological and biochemical profiles.

The mean snout-vent length value (SLV) of adult males of *L. vastus* was 14.13 ± 0.92 cm (12 – 15.72 cm), whereas the mean weight was 256.0 ± 49.7 g (145 – 312 g) (Fig. 2). Simple linear regression analysis showed that morphometric data did not correlate with hematological variables. However, for serum biochemistry, showed a negative correlation with the enzyme alkaline phosphatase ($F = 15.06$, $p = 0.0013$, $n = 18$ males) (Fig. 3). Although only one male has a value outside the margin of specie, the larger the animal, the lower the availability of these enzymes in the tissues. The other biochemical analytes showed no significant correlation with the body mass of these animals.

The analysis of hematological and biochemical parameters of *L. vastus* were summarized in the Tables 1 and 2. The total number of erythrocytes has been related to the habitat where the anurans live. In this regard, terrestrial anurans have higher number of erythrocytes, hemoglobin concentration, PCV, MCV, MCH and MCHC than those that live exclusively in aquatic habitat (Gül et al. 2011; Zhelev et al., 2017). Although this study showed no correlation of size and weight with the total of erythrocytes, possibly this correlation can be observed when comparing *L. vastus* with other species.Our results showed that the total number of erythrocytes of *L. vastus* (1.27×10^6 mm^3) was higher than those reported for males of *Polypedates maculatus* (0.4×10^6 mm^3) *Polypedates teraiensis* (5.9×10^4 mm^3) *Xenopus leavis* (0.67×10^6 mm^3) and *Bufotes viridis* (0.9×10^6 mm^3) (Mahapatra et al., 2012; Das and Mahapatra 2014; Wilson et al., 2011; Zhelev et al., 2017). On the other hand, males of *Lithobates catesbianus* presented a higher average of total erythrocytes (0.25×10^{12} mm^3) (Peng et al., 2016).

The mean hemoglobin concentration in *L. vastus* (12.09 g/dL) was higher than that reported for other species. On the other hand, our data were similar to the results obtained for *Bufo bufo* (11.8 g/dL) (Dönmez et al., 2009), whereas Carmena-Suero et al. (1980) demonstrated that males of *Polypedates maculatus* e *Osteopilus septentrionalis* presented a mean concentration of 6.5 and 6.2 g/dL, respectively. These values are closer to those reported for Sinha (1983) for males of *Pelophylax* kl. *esculentus* (8,8 g/dL) and *Bombina bombina* (7.4 g/dL) (Wojtaszek and Adamowicz, 2003). Das and Mahapatra (2014) reported a mean concentration of hemoglobin of 5.9g/dL for males of *Polypedates teraiensis*.

High PCV and high hemoglobin concentration are commonly physiological characteristics that can function independently or in combination to increase the transport and delivery of oxygen to actively metabolizing tissues. The mean percentage of *L. vastus* PCV was 40.43 ± 4.99% with a variation of 30–45%. Accordingly with some authors a mean PCV ranging 20-30% is indicative of healthy amphibians. However, values of PCV below 20% can be indicative of anemia (Campbell and Grant, 2015; Cital and Goodnight, 2016). On the other hand, an increased PCV, as observed for *L. vastus* in this study, can be indicative of dehydration and/or hemoconcentration, which can increase the blood viscosity impairing the capacity of blood to carry oxygen. Furthermore, high PCV can

increase the cardiovascular effort intensity to maintain the oxygen transport during gas exchange (Brown et al., 2021).

When compared with other anuran species the value of PCV for *L. vastus* was higher than those reported by Mahapatra et al. (2012) for *Polypedates maculatus* (28.65%) and Peng et al. (2016) for *Lithobates catesbeianus* (20.24%). However, this value was lower than those reported by Das e Mahapatra (2013) for *Polypedates teraiensis* (50.62%). Studies on other vertebrates have demonstrated a positive correlation between the value of haematocrit and other factors such as: reproductive effort (Hõrak and Murumägi, 1998), response to a stress condition (Johnstone et al. 2012) and metabolic rates (Yap et al., 2019).

It has been reported that the hemoglobin/PCV ratio is an important predictor of the oxygen transport efficiency in anurans as in other animals (Coles et al., 2009; Johnstone et al. 2017). Our results showed no correlation between these parameters (F = 0.324, p = 0.5738), which suggest that the gas transport and exchange in *L. vastus* may be impaired by environmental imbalance such as: habitat fragmentation, decrease of hydric resources, parasitism, etc. Accordingly, the Botanic Garden of Recife and The State Park of Dois irmãos, where the animals were collected, are constituted by two forest spots, with low hydric availability. Thus, the habitat of *L.vastus* is restricted to a few permanent bodies of water in both locations.

The values of MVC **=** 32.12 ± 5,52 ft/L; MCH **=** 9.57 ± 1.01 pg and MCHC**=** 30.46 ± 5.27% were lower than those reported for males of other species such as: *Polypedates maculatus*, *Lithobathes catesbianus* e *Leptodactylus podicipinus* (Mahapatra et al., 2012; Peng et al., 2016; Franco-Belussi et al., 2021). The regression analysis showed that the haemoglobin concentration correlated positively with MCH (F = 4.213, *p* = 0.0496) and MCHC (F = 11.24, *p* = 0.0023) (Fig. 4). However, the haemoglobin did not present a significative correlation with PCV (F = 0.324, *p* = 0.5739) e MCV (F = 3.924, *p* = 0.0575).

The analysis of blood smears from *L. vastus* by light microscopy showed that basophils are the most abundant cell type amongst the white blood cells with mean value of 51 ± 8.7% and a variation of 24–64%, followed by lymphocytes with mean value of 33 ± 7.4% and a variation of 20–50%. The mean ratio Neutrophils:Lymphocytes was 0.09 ± 0.1%. The total of leukocytes of *L. vastus*

was 12.230 ± 1.53 mm^3 (8.800–15.400 mm^3). This value is higher than those values reported for *Rana macrocnemis* (3.445 mm^3) *Polypedates teraiensis* (2.051 mm^3) *Scinax x-signatus* (3.550 mm^3) and *Physalaemus cuvieri* (1.700 mm^3) (Arserim and Mermer, 2008; Das and Mahapatra, 2014; Garcia Neto et al., 2020). However, values above to those observed in *L. vastus* were reported in *Polypedates maculatus* (14.628 mm^3) and *Leptodactylus podicipinus* (27.000 mm^3) (Mahapatra et al., 2012; Franco-Belussi et al., 2021). Garcia Neto et al. (2020) state that, in altered environments, frogs may experience a reduction or increase in the number of leukocytes in peripheral blood.

Given their role in the response to stressors through the expression of specific antigens, lymphocytes are reported as the most common white blood cell type in the peripheral blood of frogs (Robert and Otha, 2009). However, our results showed a higher percentage of basophils for the males of *L. vastus* (> 50%), followed by lymphocytes (> 30%). The expressive percentage of basophils may be associated to the inflammatory processes and/or antitoxic processes due to the infection by parasites or the presence of chemical compounds in the habitats (Romanova and Egorikhina, 2006; Barni et al. 2007; Vasaruchapong et al., 2013).

Wild animals, such as anurans (frogs, tree frogs and toads) are exposed to numerous pathogens, including hemoparasites belong to the *Trypanosoma* and haemogregarine group (Leal et al., 2009, Young et al., 2012). The identification of these pathogens is usually made through stage observation of their morphology in the peripheral blood of the host (Leal et al., 2009). In our study we found a high rate of parasitized erythrocytes corresponding to 30.43 ± 12% of the total o mature erythrocytes, and all animals were parasitized. The occurrence of *Hepatozoon* spp. in species of *Leptdodactylus* genus has been already reported. Leal et al. (2015) reported the prevalence of 26.47% *L. chaquensis* and 31.17% of *L. podicipinus* parasitized. Úngari et al. (2021) observed an infection prevalence in *L. labyrinthicus* and *L. latrans* of 6.45% and 25%, respectively. Some authors have demonstrated a direct correlation between the occurrence of parasites, the environment, and the presence of potential parasite vectors (such as dipteran, ticks, mites, leeches and even gnathiid isopods) associated to the anuran habitat (Netherlands et al., 2015). It has demonstrated that aquatic species are more susceptible to the parasite infection.

(Netherlands et al., 2015; Ferreira et al., 2020). The results found for *L. vastus*, a semi-aquatic specie, are consistent with those found for other species of the same genus. Although these infections often show no symptoms in wild vertebrates, there is evidence of pathologies, such as anaemia, erythrocytic hypertrophy and loss of haemoglobin (Stokes and Firkin, 1971; Ferguson and Robert, 1975; Leal et al, 2009; Young et al., 2012).

Few studies have investigated biochemical panel of blood of anuran groups. Although the analysis of this panel is important to monitor the physiological state of the organs, the lack of more robust reference values for each analyte raises an alert for the interpretation of the *L. vastus* results. Biochemical analytes such as creatinine and urea are used for the diagnosis of renal failure, metabolic alterations and nutritional disorders (Coppo, 2001). Our results showed that the value of creatinine for *L. vastus* (0.013 ± 0,05 mg/dL) was lower to those reported for *Lithobates catesbeianus* (0.48 mg/dL), *Xenopus laevis* (0.4 mg/dL) and *Lithobates catesbeianus* (0.21 mg/dL) (Coppo et al., 2005; Wilson et al., 2011; Peng et al., 2016). According to Bogan et al. (2017) in anurans, creatinine is produced in the skeletal and cardiac muscles and can serve as excellent markers of activity of these tissues in vertebrates. The functions of these enzymes in amphibian are similar to those in mammals (Keller et al. 1985), fish (Anderson et al. 2010) and reptiles (Petrosky et al. 2015). With this, we can suggest that these animals do not have a high level of motor activity, considering that many of them were found close to the burrows. L. vastus only made an escape attempt, and possible release of creatinine in the muscles when it was intercepted by the researcher.

Urea is a product of protein metabolism and consequently is considered a non-protein nitrogenous (NPN) waste product. Amino acids derived from the breakdown of protein are deaminated to produce ammonia. Ammonia is then converted to urea via liver enzymes. Therefore, the concentration of urea is dependent on protein intake, the body's capacity to catabolize protein, and adequate excretion of urea by the renal system (Salazar, 2014). The value or this parameter in the serum of *L. vastus* (14.93 ± 8.7 mg/dL) was above those reported for *Lithobates catesbeianus* (8.42 mg/dL) and *Xenopus laevis* (10.81 mg/dL) (Coppo et al., 2005; Wilson et al., 2011). This result may indicate possible

problems during the metabolism of urea in the liver, or possible disorders in the animal's kidneys.

The proteinogram is used to analyse possible alterations such as nutritional deficiency, malabsorption, liver diseases, inflammation, coagulative and immunological dysfunctions in the kidneys (Coppo, 2001). The total of protein measured for *L. vastus* (3.9 ± 0.6 g/dL) were similar to those reported for other amphibians such as *Lithobates catesbeianus* (4.3 g/dL), *Cryptobranchus alleganiensis* (3.3 g/dL), *Xenopus laevis* (3.3 g/dL), *Nyctimystes purpureolatus* (3.5 g/dL), *Xenopus tropicalis* (3.9 g/dL) and *Lithobates catesbeianus* (2.9 g /dL) (Coppo et al., 2005; Huang et al., 2010; Wilson et al., 2011; Young et al., 2012; Maxham et al., 2016; Peng et al., 2016).

The mean albumin value (1.1 ± 5 g/dL) was also similar to those reported for *Lithobates catesbeianus* (1.58 g/dL) and *Xenopus laevis* (1 g/dL) (Coppo et al., 2005; Wilson et al., 2011). In amphibians, albumins circulating in the blood are synthesized by the hepatopancreas (Goldstein, 1982). Albumin is considered as an indicator of protein biosynthesis, as well as acting as a nutritional reserve of amino acids in skeletal muscles (Coppo, 2001). Plasma proteins help the acid-base balance, immunity, clotting, vitamins, lipids, bilirubin, calcium, zinc, iron and copper (Kolb, 1987).

Although the values of glucose concentration in *L.vastus* is within the range for other amphibian species, some variation could be observed. The concentration of glucose found in our study (31.7 ± 24 mg/dL) was below those reported in *Lithobates catesbeianus* (50 mg/dL), *Xenopus laevis* (53 mg/dL), *Nyctimystes purpureolatus* (59.4 mg/dL), and *Ranoidea caerulea* (64.9 mg/dL) (Coppo et al., 2005; Wilson et al., 2011; Young et al., 2012). However, *L. vastus* presented values above to those reported for *Cryptobranchus alleganiensis* (22.2 mg/dL – Huang et al., 2010) and close to the reported value for *Lithobates catesbeianus* (35.85 mg/dL – Peng et al., 2016). Physiologically, blood glucose varies according to the effects of the animal's life stage and physical exercise. Pathologically, it changes in response to malnutrition, stress, endocrine and liver problems (Coppo, 2001).

Lipidogram values may vary according to life stage, heredity, diet and may be associated with renal hepatic failure, malabsorption, stress, and infections (Coppo, 2001). The triglyceride level in males of *L. vastus* (64.5 ± 35 mg/dL) was

above to those reported for *Lithobates catesbeianus* (43 mg/dL), *Cryptobranchus alleganiensis* (35 mg/dL) and *Lithobates catesbeianus* (12 mg/dL) (Coppo et al. al., 2005; Huang et al., 2010; Peng et al., 2016), but below to the value presented for *Xenopus laevis* (117 mg/dL – Wilson et al., 2011).

The total cholesterol concentration in *L.vastus* (89 ± 47) was above to those found for *Lithobates catesbeianus* (62 mg/dL) (Coppo et al., 2005), but below the values reported for *Cryptobranchus alleganiensis* (149 mg/dL), *Xenopus laevis* (232 mg/dL) and *Lithobates catesbeianus* (475 mg/dL) (Huang et al., 2010; Wilson et al., 2011; Peng et al., 2016). High density lipoprotein (HDL) concentration was higher in *L. vastus* (8 ± 3) than in *Lithobates catesbeianus* (4 mg/dL) and lower to those reported for *Xenopus laevis* (36 mg/dL) (Coppo et al., 2005; Wilson et al., 2011). Low density lipoprotein (LDL) concentration was higher (55 ± 47 mg/dL) than those found for *Lithobates catesbeianus* (39 mg/dL) and lower than the value reported for *Xenopus laevis* (114 mg/dL) (Coppo et al., 2005; Wilson et al., 2011).

In *L. vastus* the AST enzymatic activity (187 ± 77 U/L) was similar to those found for *Cryptobranchus alleganiensis* (157 U/L) (Huang et al., 2010), but higher than those found for *Lithobates catesbeianus* (48 U/L), *Nyctimystes purpureolatus* (67 U/L), *Ranoidea caerulea* (91 U/L) and *Lithobates catesbeianus* (111 U/L) (Coppo et al. 2005; Young et al. 2012; Peng et al. al., 2016) and lower than those reported for *Xenopus laevis* (453 U/L – Wilson et al., 2011). The Mean of ALT activity was higher (39 ± 18 U/L) than those reported for *Lithobates catesbeianus* (12 U/L), *Xenopus laevis* (21 U/L) and *Lithobates catesbeianus* (14 U/L) (Coppo et al., 2005; Wilson et al. 2011; Peng et al. 2016). Although high ALT values suggest possible renal failure, this generalization should be seen with caution because enzymes released by damaged kidney cells tend to be eliminated in the urine rather than the bloodstream (Boyd, 1988). In this regard compared the increased activity of this enzyme with AST can be useful to a correct diagnosis (Divers and Cooper, 2000). Furthermore, additional tests are needed to confirm liver or kidney disease in this animal. The measure of bile acids, for instance, although is validated in reptiles but not for amphibian species, can be used for assess liver function in these animals (McBride et al., 2006).

The AP activity (137 ± 86 U/L) was similar to those that reported for *Xenopus laevis* (148 U/L – Wilson et al., 2011) and GGT enzymatic activity (2 ±

0.9 U/L) was close to those reported for *Xenopus laevis* (4 U/L) and *Lithobates catesbeianus* (5 U/L) (Wilson et al., 2011; Peng et al., 2016), but was lower than those reported for *Lithobates catesbeianus* (9 U/L – Coppo et al., 2005). Both analytes are used to evaluate liver and biliary injuries. Gama Glutamil Transferase (GGT) is an enzyme produced in the pancreas, heart, and liver, and when it is present in high levels it can indicate a possible liver injury. Alkaline phosphatase (AP) is present in several tissues, especially in the cells biliary ducts where they help in the digestion of fat. This enzyme also is responsible for formation and maintenance in the bones. When the values are high, they may indicate an obstruction in the bile flow.

It is still important to emphasize the need of standardization of biochemical analytes for the diagnosis of possible problems in the physiological state of amphibians. In addition, it is important to note the importance of the infrastructure that supports these results, such as specific equipment and standardization of reagents.

The morphological characterization of blood cells of anurans is also an important tool to evaluate the health status of animals. In this regard we further characterized morphologically the blood cells of *L.vastus* by light and electron microscopy.

The peripheral blood cells of anurans consist of erythrocytes, leukocytes, and thrombocytes. Granulocytic leukocytes can be subdivided as neutrophils, eosinophils and basophils, whereas agranulocytes can be subdivided as lymphocytes and monocytes (Forzán et al. 2020). All the cell types presented in anurans could be observed by light microscopy in blood smears from *L. vastus* (Figure 5). As expected, the erythrocytes are the most predominant cells in the bloodstream of *L. vastus*. Mature erythrocytes presented an elliptic shape with mean length of 14.33 μm. After stained with May-Gruwalld solution, these erythrocytes presented elliptic shape, acidophil cytoplasm with pinkness colour, whereas a bluish round to oval nucleus, centrally located was observed. Comparing with the mature RBC, the immature ones are usually characterized by more basophilic cytoplasm with round nucleus (Fig. 5 B). The presence of immature RBC can be a bioindicator of the presence of pollutants in habitat where the anuran lives. Consistently, Pollo et al. (2017) reported the presence of higher

number of immature RBC in *Rhinella arenarum* in response of effluents pollutants. This phenomenon can be also attributed to the increased erythropoiesis to circumvent the low oxygen availability in the polluted environments (Barni et al., 2017). Some erythrocytes presented an evident micronucleus characterized as round blue spot in the cytoplasm adjacent to the nucleus (Fig. 5B). The presence of micronucleus indicates the occurrence of numeric or structural chromosomes aberrations during the mitosis (Fenech et al. 2011). Although the biological meaning of this morphological abnormality in RBC of anurans is not well comprehended, some authors suggested that the presence of xenobiotics can interfere with DNA synthesis besides inducing genetic mutation in the structural constituents of RBC of nuclear envelope, leading to the formation of micronucleus (Beutler 1985; Strunjak Perovic et al. 2009; Silva et al., 2020). On the other hand, other authors reported that a nuclear budding during the interphase can also induces micronucleus, aiming to eliminate amplified genes in the nucleus (Shimizu et al. 1998; Crott et al. 2001). In the case of *L. vastus* analyzed, possibly the presence of pollutants in the collection sites (Botanical Garden and State Park Dois Imrãos) is causing interference during the differentiation of blood cells. But to affirm, it is necessary to carry out a physical-chemical analysis of the water bodies of the two locations.

Thrombocytes are spindle cells with blue-gray cytoplasm and a blue nucleus (Fig. 5C). Sometimes these cells were found clumped together in blood smears. Thrombocytes from non-mammalian vertebrates have the same function as mammalian platelets, participating in the coagulation and wound healing processes (Astill and Sharif, 2022).

The basophils have non-segmented nucleus. Its cytoplasm contains several basophilic granules with various sizes (Fig. 5A). On the other hand, eosinophils have acidophilic cytoplasm. The cytoplasmic granules are also distributed throughout the cytoplasm but are smaller when compared with those found in basophils. The nucleus of eosinophils is usually bilobed (Fig. 5D). Neutrophils are characterized by round-shape body with a purple color, a multilobed violet nucleus and condensed chromatin (Fig. 5D). Lymphocytes have an oval-spherical morphology with a high Nucleus/Cytoplasm ratio. A centrally located nucleus of this cells type is observed (Fig. 5A). Monocytes have a

relatively large kidney- or horseshoe-shaped nucleus. The nucleus presented a condensed chromatin (Fig. 5E).

Both, mature and immature gamonts of protozoan were present in the cytoplasm of *L. vastus* (Fig. 5). According to the Netherlands et al (2017) immature gamonts (Fig. 5F) have elongated with small, recurved tail, cytoplasm staining whitish purple, with rounded nucleus having loosely arranged chromatin located at the posterior half of parasite. Immature gamonts are seen inside tight or vaguely visible parasitophorous vacuole (PV). On the other hand, mature gamonts are elongated and oval, encase in large PV. Although is not frequently, extracellular, or free moving gamont could be observed (Fig. 5B, arrowhead). Usually both mature and immature gamonts cause a noticeable displacement of host nucleus. (Figure 5F). Trophozoites and meronts were not observed in our samples.

Transmission electron microscopy analysis (Fig. 6) was performed to better characterize morphologically the cell types in pooled blood samples from *L. vastus.* We identified the presence of four cell types: erythrocytes (Figs 6A-6C), monocyte (Fig. 6D), lymphocyte (Fig.6E) and basophil (Fig. 6F). The ultrastructure of these cells was similar to those reported for other reptiles and amphibians species (Gutierre et al., 2008; Zago et al 2010; Kindlovits et al., 2017; Sato et al.; 2018). As observed by light microscopy, mature erythrocytes are the most common cell type found in samples processed for MET. These cells are elliptic with homogeneous cytoplasm, few organelles and scarce mitochondria spread throughout the cytoplasm (Fig. 6A). The nucleus is oval, centrally located, and rich in heterochromatin. Retracted nuclear envelope membrane and the presence of micronucleus can be easily found in *L. vastus* erythrocytes, but not in other cell types, suggesting that it is not an artefact induced by the processing for MET. Only haemogregarines protozoan can be also seen inside large parasitophorous vacuoles of *L. vastus* erythrocytes (Fig 6C).

Small lymphocytes, characterized by the high nucleus to cytoplasm ratio, could be observed in the peripheral blood of *L. vastus*. This cell is usually oval or round shaped with large, indented central nucleus and prominent heterochromatin. The cytoplasm is homogeneous, rich in

mitochondria and vacuoles (Fig. 6E). On the other hand, monocytes are characterized by the presence U-shaped nucleus with predominance of heterochromatin over euchromatin and is displaced to the cell periphery. The cytoplasm is rich in endoplasmic reticulum, vacuoles, and dispersed mitochondria (Fig. 6D)

Basophils of *L. vastus* presented a round or oval shape, non-lobulate nucleus with loosed euchromatin and single spotted clumping heterochromatin (Figure 6F, arrow). Several electrodense granules, varying from small to large size, could be seen in the periphery of cytoplasm nearby to the plasma membrane (Figure 6F). An interesting finding was the presence of crystalline structure inside the large granules (Fig. 6F, asterisks). Gutierre et al. (2008) reported the presence of lamellar inclusions, but not crystalline structures in the granules of basophils from Siphonops annulatus (Amphibia, Gymnophiona). Although this type of inclusions is usually associated to eosinophils (Muniz et al, 2012), this cell type in *L.vastus* (data not shown) are smaller than basophils and its nucleus are often lobulated (Gutierree et al., 2008)

4. Conclusions

Our results reported the hematological and blood biochemical profiles of males of *Leptodactylus vastus* an anuran belonging to the group of *L pentadactylus*. Our results shed light on the physiological status, presence of hemoparasites and ultrastructure of blood cell types. The hematological parameter pointed to the presence of stressors, although the N:L ratio (a blood indicator of stress response) did not show any correlation with stress in these animals. Thus, is also important that other factors as the natural history and/or other physiological variables should be also investigated to determine specie-specific responses in *L. vastus*.

It is still possible to conclude that the high percentage of basophils in the peripheral blood, as well as abnormalities in the erythrocytes of the species, may be indicative of an inflammatory process in the animals, possibly due to the presence of hemogregarines and/or chemical pollutants in the water bodies.

In this study we reported, for the first time, the serum biochemistry profile of endemic Leptodactylidae species in Brazil. Our analysis showed that the biochemical parameters are within the range reported for other amphibian species, although some variations could be detected. The analysis of enzymatic activity suggested that the population is suffering of some liver injuries. Further works are needed to address the hematological and biochemical references that clarify the physiological state of anurans, directing conservation actions for this group.

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Author contributions

Oliveira-Santos conceived the idea presented, designed and planned the experiments. In addition, he captured the animals and carried out the field experiments, wrote the manuscript, with the support of the other authors. Holanda designed and planned the laboratory experiments and assisted in the interpretation of the results. Soares and Gonçalves carried out the serious biochemistry experiments. Moura and Figueiredo supervised and contributed to the interpretation of results and the final version of the manuscript.

Declaration of competing interes

The authors declare that there are no conflicts of financial and personal interests that could have influenced the progress of the manuscript.

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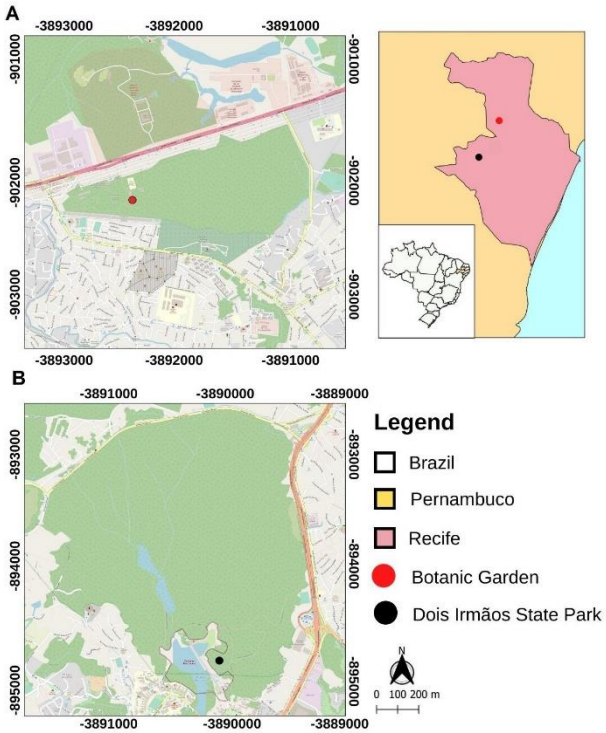

Fig. 1. Map of the location where the males of *Leptodactylus vastus* Lutz, 1930 were captured. The two areas are located at the metropolitan region of Recife, Pernambuco, Brazil.

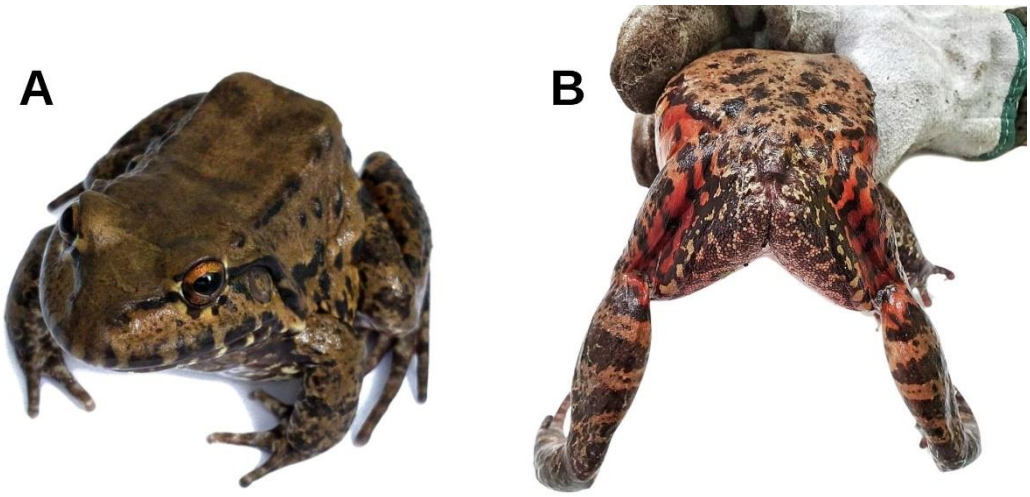

Fig. 2. General view of *Leptodactylus vastus* Lutz, 1930 male captured at the Dois Irmãos State Park, Pernambuco, Brasil. A) frontal view of adult male. B) back view showing the aposematic colours on the legs and ventral region (arrow) (CRC: 13.46 cm; body weight mean: 300 g). Photography by Jonathas Lins.

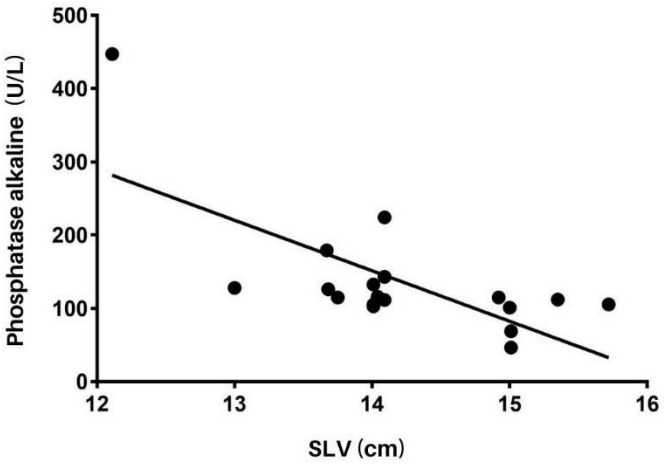

Fig. 3. Comparison of the snout-vent length with the phosphatase alkaline production in males of *Leptodactylus vastus* Lutz, 1930 by regression analysis.

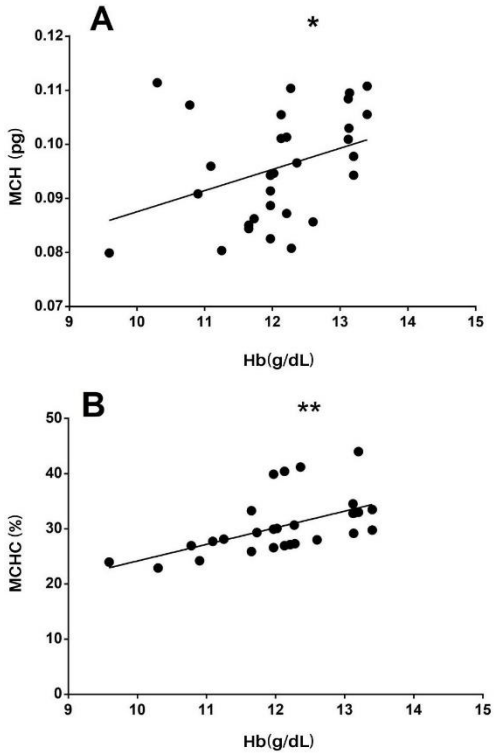

Fig 4. Simple Linear Regression between A) haemoglobin and MCH. B) haemoglobin and MCHC from males of *Leptodactylus vastus* Lutz, 1930.

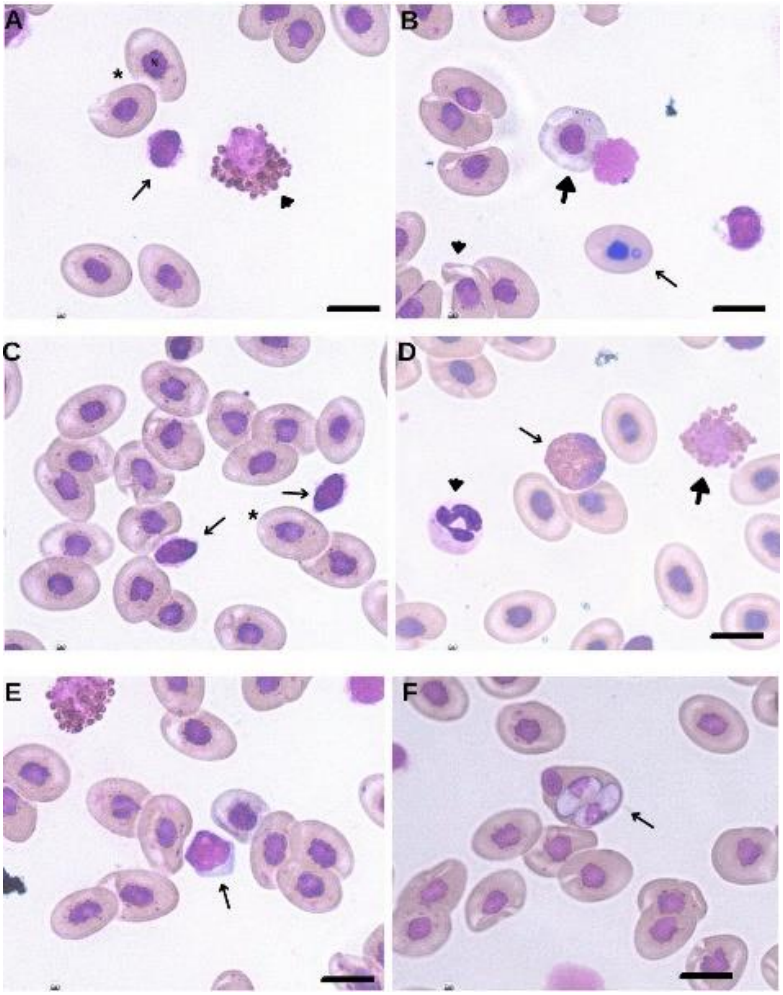

Fig. 5. Light microscopy of blood cells from *Leptodactylus vastus* Lutz, 1930. A) Detail of *L. vastus* blood smear showing several mature erythrocytes (*) presenting oval shape, pinkish cytoplasm and centrally located nucleus (N). Lymphocytes (arrow) with a basophil nearby (arrowhead). B) presence of immature erythrocyte with bluish cytoplasm and rounded nucleus (thick arrow). Note the presence of erythrocytes presenting a blue spot corresponding to a micronucleus (thin arrow). A mature gamont (arrowhead) can be also observed leaving the erythrocyte C) Thrombocytes (black arrow) near to mature erythrocytes (*). D) Eosinophil (arrow). Note the presence of a neutrophil presenting a multilobate nucleus (arrowhead) and a basophil (thick arrow) E) A monocytes (arrow) can be observed in the vicinity of mature erythrocytes. (F) Detail of blood smear showing the presence of two intracellular gamonts an erythrocyte. Bars = 10 µm.

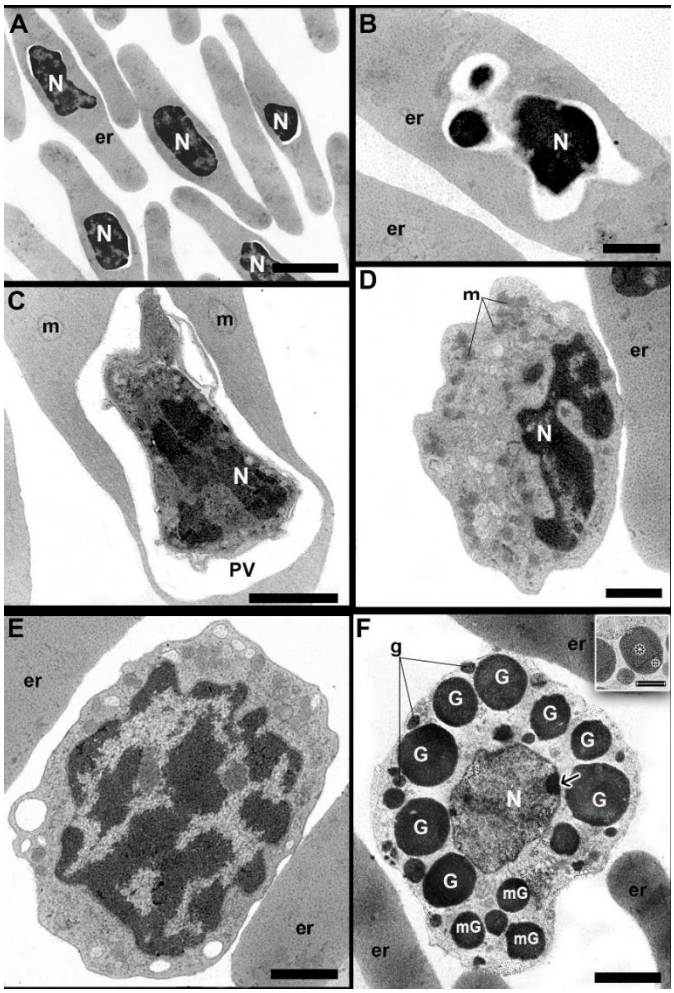

Fig. 6. Transmission Electron Microscopy of blood samples from *Leptodactylus vastus*. (A-C) Ultrastructure of *L. vastus* erythrocytes. (A) General view of erythrocytes (B) Detail of fragmented nucleus and formation of micronucleus (C) Detail of haemogregarine protozoa inside large parasitophorous vacuole (PV). (D) Details of monocyte of L.vastus presenting kidney-shaped nucleus displaced to the cell periphery and a number of mitochondria (m) spread throughout the cytoplasm. (E) detail of a lymphocyte with large nucleus and a thin cytoplasm (F) Large magnification of basophil with intracellular small (g) medium (mG) and large granules (G) widespread in the cytoplasm. Some of these large granules have crystalline inclusions (inset, asterisks). Only a single clumped heterochromatin spot could be observed associated to the nuclear envelope (arrow). Scarce mitochondria could be also observed (m). Nucleus (N); erythrocytes (er) Bars: A=5μm; B, C, D, E and F = 1μm; inset=1 μm.

Table 1. Hematological profile for males of *Leptodactylus vastus* Lutz, 1930 from Atlantic forest fragments. The value represents the mean ± SD of blood samples from 30 animals.

***Leptodactylus vastus* (n=30)**			
Hematological variables	**Mean**	**SD**	**Min-max value**
Red blood cells (mm^3)	1.27×10^6	131.86	9.24×10^4-1.52×10^6
haemoglobin (g/dL)	12.09	0.9	9.59-13.40
Haematocrit (%)	40.43	4.99	30-45
MVC (fL)	32.12	5.52	22.22-48.67
MCH (pg)	9.57	1.01	7.99-11.14
MCHC (%)	30.46	5.27	22.89-44
Thrombocytes mm^3	55.700	11.07	36.000-92.000
Leucocytes mm^3	12.230	1.53	8.800-15.400
Neutrophils (%)	3	2.5	0-8
Eosinophils (%)	3	2.9	0-8
Basophils (%)	51	8.7	24-64
Lymphocytes (%)	33	7.4	20-50
Monocytes (%)	9	4.5	2-20
Neutrophil:Lymphocytes	0.09	0.1	0-0.33
Haemogregarines (%)	30.43	12	14-56

Table 2. Biochemical analysis of males of *Leptodactylus vastus* Lutz, 1930 from Atlantic forest fragments. The value represents the mean ± SD of blood samples from 18 animals.

	***Leptodacytylus vastus* (n=18)**		
Biochemical variables	**Mean**	**SD**	**Min-max value**
Creatinine (mg/dL)	0.013	0.05	0.054-0.24
Urea (mg/dL)	14.493	8.709	3.340-28.108
Total protein (g/dL)	3.994	697	2.369-4.768
Albumin (g/dL)	1.126	5	1.122 -1.129
Glycose (mg/dL)	31.741	24.655	1.217-87.578
Triglycerides (mg/dL)	64.550	35.444	6.109-122.762
Total Cholesterol (mg/dL)	89.110	47.950	6.109-173.990
HDL (mg/dL)	8.576	3.389	1.965-14.859
LDL (mg/dL)	55.024	19.102	47.012-149.464
Asparate aminotransferase (U/L)	187.933	77.141	48.563-354.611
Alanine aminotransferase (U/L)	39.601	18.192	2.899-76.731
Alkaline phosphatase (U/L)	137.696	86.028	46.362-447.052
Gamma-glutamyl Transferase (GGT) (U/L)	2.295	913	1.036-3.938

ARTIGO II

THE DEFENSE REPERTOIRE OF MALES OF *Leptodactylus vastus* Lutz, 1930 IN A FRAGMENT OF THE ATLANTIC FOREST IN NORTHEASTERN BRAZIL

José Ricardo de Oliveira-Santos, Regina Célia Bressan Queiroz de Figueiredo and Geraldo Jorge Barbosa de Moura

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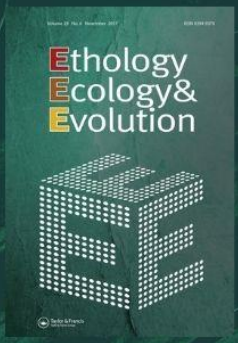

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Keywords:	Anti-predatory behavior, Defensive behavior, Distress call, Leptodactylus pentadactylus group

The defense repertoire of males of *Leptodactylus vastus* Lutz, 1930 in a fragment of the Atlantic Forest in northeastern Brazil

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Highlights

Studies on the defensive behavior of anurans have been presented information that can help in the construction of the natural history of the species in this group. The results show that males of *Leptodactylus vastus* exhibited five types of defensive behaviors of which 4 were of visual origin: puffing up the body, push, hiding, cloacal discharge, and spinal aggression; and one type of acoustic behavior: distress call. The behaviors were observed through three types of external stimuli in which during the data analysis it was evident that there is no male preference for a specific type of stimulus. Through the intra-individual and inter-individual coefficient of variation that there are no significant variations in the distress call being an unprecedented result for works that involve the distress call.

Abstract

Anurans present different defense mechanisms against predator attacks. There are more than thirty defense strategies that these animals can display in the different environments where they can be preyed upon. We analyzed the defense repertoire of the species *Leptodactylus vastus* Lutz, 1930, including the male distress call and its intra- and inter-individual coefficients of variation. We carried out this study in a fragment of Semideciduous Atlantic Forest at the Camocim Conservation Unit in the metropolitan region of Recife, Pernambuco, northeastern Brazil. The defensive displays of 30 males to different stimuli simulating a predator were analyzed. We observed five types of strategies which were used by these animals separately or concomitantly: puffing up the body (100%), push movement (83.3%), distress call (33.3%), hiding (23.3%), cloacal discharge (16.6%) and spine aggression (6.6%). The analysis of intra- and inter-individual coefficient of variation showed that there was no acoustic variation in the distress call of this species. Understanding the defensive mechanisms of a species is important to identify the anti-predatory strategies used by animals especially in face of emerging threats and to help to produce proposals for the conservation of neotropical species.

KEY WORDS: anti-predatory behavior, defensive behavior, distress call, *Leptodactylus pentadactylus* group.

Running head: Antipredatory behaviors of *Leptodactylus vastus* Lutz, 1930

INTRODUCTION

The pursuit for survival has selected several defense strategies for animals (Edmunds 1974; Toledo et al. 2011; Nunes et al. 2014). Some of these strategies have evolved in response to various interactions between prey and predators (Alcock 2016). For anurans, the high pressure exerted by predation is the main factor for their broad defensive repertoire (Haddad et al. 2013) which allows them to defend themselves against different predators (Ferrante et al. 2014).

Among the defense behaviors exhibited by anurans, the distress call has been widely studied. This call is a high-intensity noise emitted by prey when subdued. Apparently, this noise surprises and frightens the predators especially those with a sensitive acoustic system allowing the prey to escape (Bogert 1960; Toledo & Haddad 2009).

Many species of anurans are capable of emitting the distress call as a defensive signal when intercepted by predators (Wells 2010; Forti et al. 2018; Carrillo & Dena 2019). This type of vocal behavior can alert conspecific neighbors to the presence of a predator, ceasing the vocalization of nearby animals (Forti et al. 2017). Toledo and Haddad (2009) stated that to be successful the sound must be loud, sustained for as long as possible, and present a wide frequency range. After all, the louder the sound the lesser its degradation (Brémond & Aubin 1992). On the other hand, small frogs may not be able to produce this call, because the intensity and duration of the call are correlated with the size of the animal, as observed by Toledo and Haddad (2009).

Besides the distress call, other defense strategies are exhibited by anurans in environments where there is a high possibility of predation (Toledo et al. 2007). These antipredatory strategies include protruding the distal finger phalanges, puffing up the body, pushing with legs and releasing smelly secretions (Toledo et al. 2011).

Leptodactylidae Werner, 1896 (1838) is one of the most diverse anuran families in Brazil. Currently, this family comprises 232 species (Frost 2021) divided in four groups. *Leptodactylus vastus* Lutz, 1930 belongs to the *L. pentadactylus* group and is widely distributed in northeastern Brazil and in the Bolivian Amazon (De Sá et al. 2014). Thirteen species of this genus have had

their distress call described, and of these, four are from the *L. pentadactylus* group, including females of *L. vastus* (Toledo & Haddad, 2009). Thus, this study aimed to analyze (1) the defense repertoire of *L. vastus* males from an Semidecidual Atlantic Forest fragment, in the northeastern of Brazil; (2) their distress call and (3) intra- and inter-individual variation in the distress call of this species.

MATERIALS AND METHODS

Study area

Thirty males of *Leptodactylus vastus* Lutz, 1930 were captured between October and December 2019 in the Camocim Conservation Unit, located in the municipality of São Lourenço da Mata, metropolitan region of Recife, Pernambuco, Brazil (8S02'25", 35W11 '48", datum= SAD69) (Fig. 1). This site has an area of 200ha and is covered by Semideciduous Atlantic Forest. The climate of this area is characterized as tropical (As), according to the Köppen-Geiger climate classification (Alvares et al. 2013), with rainfall between February and September.

Behavioral defense display of Leptodactylus vastus in response to simulated predator threats

Thirty males of *L. vastus* were randomly selected and subjected for 3 min to one of the following experimental stimuli in the field: (1) unexpectedly grabbing the male by the hind limbs, (2) causing erratic movements by holding the individual's hind limbs, or (3) emitting distress call playbacks. When the animal displayed one or more defensive behavior in one of the experiments it was released. When there was no behavioral display the animal was kept in a containment box for 10 min for resting and then one of the experiments was randomly repeated (adapted - Toledo & Haddad 2009). After the experiments, we measured the SVL (snout–vent length, 0.01mm precision) and the body mass (0.01g precision) of all handled animals. We also measured the environment

temperature (ºC) and humidity (%) using a HT-210 digital thermo hygrometer (0.1°C accuracies). The defense behaviors analyzed in response to predator threatening were described in the ethogram.

Distress call

The distress call of ten males, submitted to the above-mentioned stimulus, was recorded with a TASCAM DR-40 recorder (uncompressed format, sample rate = 44.1kHz, 16 bits) coupled to a Yoga HT-81 directional microphone (100 Hz - 16 kHz), positioned at 0.5 m of the animals. Twelve calls were analyzed for each animal, totaling 120 calls. We analyzed the acoustic parameters using Raven Pro 1.6 software and produced the graphics using the Seewave package in Rstudio. For the construction of the playback, we chose 10 notes of distress call (Audacity 1.3 Software) which were reproduced using a portable box music JBL Charge 3 (65Hz – 20kHz, > 80 dB).

The following acoustic variables established by Köhler et al. (2017) were analyzed: duration of the call (s), interval between notes (s), minimum frequency (kHz), maximum frequency (kHz), frequency amplitude (kHz), dominant frequency (kHz), notes/min, and harmonics. The recorded calls were deposited at Coaxar, a library of anuran vocalizations of the Laboratório de Estudos Herpetológicos e Paleoherpetológicos in Universidade Federal Rural de Pernambuco, northeastern Brazil (SCLEHP 01018 – 01028).

Data analysis

We performed the Shapiro-Wilk test to verify data normality. Posteriorly, we performed an ANOVA analysis to test whether the frogs would have a preference for a specific type of stimulus. The intra- and inter-individual coefficients of variation were calculated according to Gerhardt (1991) using the following equation: $CV = SD/\overline{X} \times 100$. Where, CV = coefficient of variation; SD = standard deviation and $\overline{X}$ = mean.

We calculated the intra-individual coefficient (Cv_{intra}) using the mean and standard deviation of the acoustic parameters of each individual, whereas for the

inter-individual level (Cv_{inter}) we used the values of all individuals. Acoustic parameters were classified as static when the intra-individual variation was < 5% and dynamic when Cv_{intra} > 12%. For the inter-individual variation, values < 10% were considered as static and > 20%, dynamic. Values between these ranges (dynamic – static) were considered intermediate for both Cv_{intra} and Cv_{inter}. Then, a Multiple Linear Regression was performed for each acoustic parameter using the body mass and SVL of the animals as independent variables. We followed a significance level of $p < 0.05$ (Zar 2013) for the tests and used Graphpad Prism 7.0 software to perform the aforementioned analyses.

RESULTS

All the tested frogs displayed defensive behaviors regardless of the type of stimulus ($F = 3.1109$, $p = 0.0805$). Five behaviors were identified in males of *Leptodactylus vastus,* and all of them were considered as antipredator behavior: The most prevalent was puffing up the body (100%), followed by push movement (83.3%), distress call (33.3%), hiding (23.3%), cloacal discharge (16.6%) and spine aggression (6.6%) (Figs 2-3; Table 1).

The distress call was composed of a harmonic structure with, on average, 20 ± 0.83 harmonics. It also had a wide frequency range of 1.91 ± 0.06 kHz – minimum frequency 0.86 ± 0.06 kHz, maximum frequency 2.77 ± 0.07 kHz, and dominant frequency 2.66 ± 0.33 kHz. The call duration was 0.95 ± 0.03 s, the interval between notes was 2.78 ± 0.51 s and it also had, on average, 12 ± 0.32 notes/min (Fig. 4; Table 2).

There was low variation in the distress call of *L. vastus.* For Cv_{intra}, we found that call duration, interval between notes, maximum frequency, frequency amplitude, harmonics and notes/minute were static parameters, whereas dominant frequency was a dynamic parameter; minimum frequency had intermediate values. For Cv_{inter}, we found that call duration, minimum frequency, maximum frequency, frequency amplitude and harmonics were static parameters; the interval between notes and dominant frequency had intermediate values. As observed in our multiple linear regression models, there was no

relationship between either the animal's body mass or SVL and the acoustic parameters (Table 2).

DISCUSSION

Due to the need of understanding the ecological relationships between anurans and ecosystems, the defensive repertoire appears as one of the main parameters for evaluating the various antipredatory mechanisms of these animals in response to predator attacks. The behaviors exhibited by *Leptodactylus vastus* males, such as puffing up the body and the push movement, were also observed by other authors (Toledo et al. 2011; Ferrante et al. 2014; Kowalski et al. 2018; Ferrante et al., 2020). Toledo et al. (2011) stated that during lung deflation, some bufonids may emit a noise produced by expiration. In our study, males of *L. vastus* emitted a noise after the simulated stimuli of a possible predator (stimuli 2 and 3, which included handling the animals). We suggest that this sound could be a secondary defensive behavior, that is, it is only used when there is direct contact with the anuran.

The hiding behavior was observed when the researcher approached the animals for capture. Males were observed close to burrows, which were approximately 1 m deep, and when the animals noticed any approach, they entered the burrow. The burrows were close to the temporary pond, which suggests that these microhabitats may also be used for the reproduction of these animals. Shahrudin (2014) observed that males of *Microhyla berdmorei* hid under the leaves after being manipulated. In contrast, males of *L. vastus* usually perform this behavior when they perceive the presence of a threat, without necessarily having direct contact with the predator. Hiding under leaves or litter has also been observed in *Leptobrachium hendricksoni* (Shahrudin 2016).

The cloacal discharge, which can be liquid or solid, is released when the animal is intercepted or is escaping. This behavior was also observed by Kowalski et al. (2018) in frogs in response to predators and corresponded to 1.02% of the behavioral response to hedgehogs and 1.43% to rabbit attacks. In our study, this behavior was only performed at the moment of the animals' escape, which also suggests a secondary defense.

Spines on male hands and breasts can be used to deeply scratch a potential predator or a conspecific male during aggressive interactions (Toledo et al. 2007). They can also be used during reproductive mating interactions, as noted for specimens of *L. vastus.* However, not all species of anurans that have spines use them for defense, as seen by Toledo et al. (2011) for *Boana beckeri, Boana bischoffi, Boana caingua,* and *Boana prasine*. These authors divided spines use behavior into (i) using spines to pierce, (ii) using spines to scratch any reachable part of the predator's body, and (iii) causing trauma without piercing the predator's skin (Toledo et al. 2011).

We observed that some males presented more than one behavioral response concomitantly. For instance, five males (16.6%) exhibited the displays (1) distress call, (2) puffing up the body and (3) push movement, and 22 males (73.3%) displayed both displays 2 and 3. Although the concomitant use of different defense mechanisms results in a higher chance of escaping (Toledo et al. 2011), it may also represent a high spend of energy by the animal and the need for a resting period to recover.

Some acoustic parameters observed in our study diverged from the data of Toledo and Haddad (2009). Mean call duration (0.95 s vs. 0.75 s, respectively), minimum frequency (0.86 kHz vs. 0.17kHz), and dominant frequency (2.66 kHz vs. 1.64 kHz) were higher in the distress call of *L. vastus* males from our study than those from Toledo and Haddad (2009). Conversely, the maximum frequency (2.77kHz vs. 7.42kHz) was lower in our study. Such variation can be attributed to the use of males in our study and females in Toledo and Haddad's study (2009). In addition, the acoustic variables in the distress call of *L. vastus* also differed from those found in other species of the *L. pentadactylus* group, such as: *L. chaquensis* (Carrillo & Dena, 2019), *L. labyrinthicus* (Toledo et al. 2005), *L. pentadactylus*, and *L. savagei* (Toledo & Haddad, 2009). For instance, the duration of the distress call of *L. vastus* was the longest compared to those of other species of the *L. pentadactylus* group. However, the average dominant frequency was in the same range of the other species in the group, except for *L. pentadactylus* and *L. vastus* analyzed by Toledo and Haddad (2009).

Toledo and Haddad (2009) established a positive relationship between call duration and the size of some anuran species. However, this relationship may not apply to the distress call of some species in the *L. pentadactylus* group, as

observed by the same authors. For this group, larger species have shorter distress calls compared to smaller sized species (Toledo & Haddad 2009). We show in this study that the distress call of *L. vastus* corroborates this pattern, as both temporal and spectral parameters of their distress call did not correlate with their SVL and body mass. Toledo et al. (2007) argued that this pattern could be the result of different evolutionary pressures, such as sexual selection and predation.

We still know little about variation in the distress call of *L. vastus*. Our study shows that most of the acoustic parameters that we analyzed are static, which suggests that this call has little variation within and between individuals. In addition, the male distress call of this species has a short duration with no relationship with the animals' mass or size, as also demonstrated for other species of the *L. pentadactylus* group. In this regard, they cannot be included in the model proposed by Toledo and Haddad (2009).

De Castro et al. (2017) reported three additional defensive behaviors for *L. vastus*: thanatosis, in which the animal remained with an exposed abdomen and retracted the hind legs for about 7 minutes. According to Toledo et al. (2010) remaining immobile can lead the predator diverting its attention from the prey. The second behavior was the body-raising, in which the animal stretched its legs, raised its body, and kept its snout on the ground, exposing the reddish color of the leg for 5 minutes. Toledo et al. (2011) claimed that this behavior can be divided into (1) partial when the animal stretches its legs and touches its snout to the ground, and (2) total when the animal stretches its legs, raises its belly and snout. The last behavior was the release of toxic secretion by the epidermis. This type of secretion can cause burning in the eyes, mouth, and nostril (Toledo et al. 2011; Haddad et al. 2013), and that is why this species is popularly known as the pepper frog in the region.

Therefore, the study of defensive behavior of frogs, as *L. vastus*, provides fundamental information about agonistic interactions among species, which can be useful in promoting conservation actions to protect these species. *L. vastus* exhibited a variety of antipredator behaviors which are presented separately or simultaneously in response to predators.

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DISCLOSURE STATEMENT

No potential competing interest was reported by the authors.

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ETHICAL STANDARD

All the procedures were performed according to the SISBIO (63567) and the Ethics Committee on the Use of Animals from the Universidade Federal de Pernambuco (23082.006641/2019-29).

AUTHOR CONTRIBUTIONS

de Oliveira-Santos conceived the presented idea, designed, and planned the experiments. de Oliveira-Santos collected the animals, performed the field experiments, and wrote the manuscript, with the support of Figueiredo and Moura. Both Moura and Figueiredo contributed to the interpretation of the results and the final version of the manuscript. Moura supervised the project.

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Table 1.

The defense repertoire parameters evaluated in males of *Leptodactylus vastus* Lutz, 1930 of the Camocim Conservation Unit Forest, located in the municipality of São Lourenço da Mata in the Metropolitan Region of Recife, Pernambuco, Brazil. The description of the behaviors is in accordance with Toledo et al. (2011).

Behavior	**Description**	**Possible main function against predators**	**Other references**
Puffing up the body	The animal inhales air and inflates its body.	To appear larger than it really is, in order to scare the predator.	Mângia and Garda (2015); Meneses and Corrêa (2020)
Push movement	The animal uses its legs to push.	The animal uses the forelimbs and hindquarters to push and get rid of the predator's constriction.	de Toledo et al. (2017)
Distress call	The animal exhibits stereotyped calls with high intensity during the interception.	The call can frighten predators that have a sensitive acoustic system and warn conspecific animals of the presence of a threat.	Dorado-Rodrigues et al. (2012); Lopes et al. (2021)
Hiding	The animal uses burrows to hide.	When the animal perceives the approach of a threat it hides inside burrows that present an average of 1m in depth.	Ferreira et al. (2019); Sena et al. (2021)

Cloacal discharge	The animal releases solid material through the cloaca when it is intercepted.	The solid material released may present unpalatable taste causing the predator to lose interest in ingesting the prey	Toledo, Sazima and Haddad (2011)
Spine aggression	The animal uses the spines located on the thumbs and pectoral region to scratch or pierce.	The spines located in the phalanx and pectoral region can injure the predator, causing it to release the frog.	Toledo, Sazima and Haddad (2011)

Table 2.

Analyzes of the acoustic parameters and intra- and inter-individual variation coefficients of the distress call of males of *Leptodactylus vastus* Lutz, 1930 of the Camocim Conservation Unit Forest, located in the municipality of São Lourenço da Mata in the Metropolitan Region of Recife, Pernambuco, Brazil.

Acoustic parameters	**Mean ± SD**	**Variation**	**Cv_{intra} (%)**	**CV_{inter} (%)**	**Multiple Linear Regression**	
					SLV	Mass
Call duration (s)	0.95 ± 0.03	0.85 – 1.00	2.30	3.54	F = 1.8, *p* = 0.2165	F = 0.3, *p* = 0.5769
Interval between call (s)	2.82 ± 0.36	2.12 – 3.65	4.07	12.68	F = 4.9, *p* = 0.0575	F = 0.4, *p* = 0.5431
Minimum frequency (kHz)	0.86 ± 0.06	0.60 – 0.98	5.58	6.57	F = 1.4, *p* = 0.3158	F = 0.5, *p* = 0.4649
Maximum frequency (kHz)	2.77 ± 0.07	2.45 – 2.98	2.02	2.52	F = 0.14, *p* = 0.7121	F = 0.7, *p* = 0.4092
Frequency range (kHz)	1.91 ± 0.06	1.58 – 2.15	1.95	3.35	F = 0.5045, *p* = 0.4977	F = 0.02, *p* = 0.8739
Dominant frequency (kHz)	2.66 ± 0.33	1.63 – 2.88	12.7	12.2	F = 1.5, *p* = 0.2490	F = 0.7, *p* = 0.4003

Notes/min	12 ± 0.32	12 – 13	2.61	-	F = 4.3, *p* = 0.0703	F = 2.1, *p* = 0.1839
Harmonics	20 ± 0.83	17 – 22	1.69	4.15	F = 3.6, *p* = 0.0930	F = 1.2, *p* = 0.2895

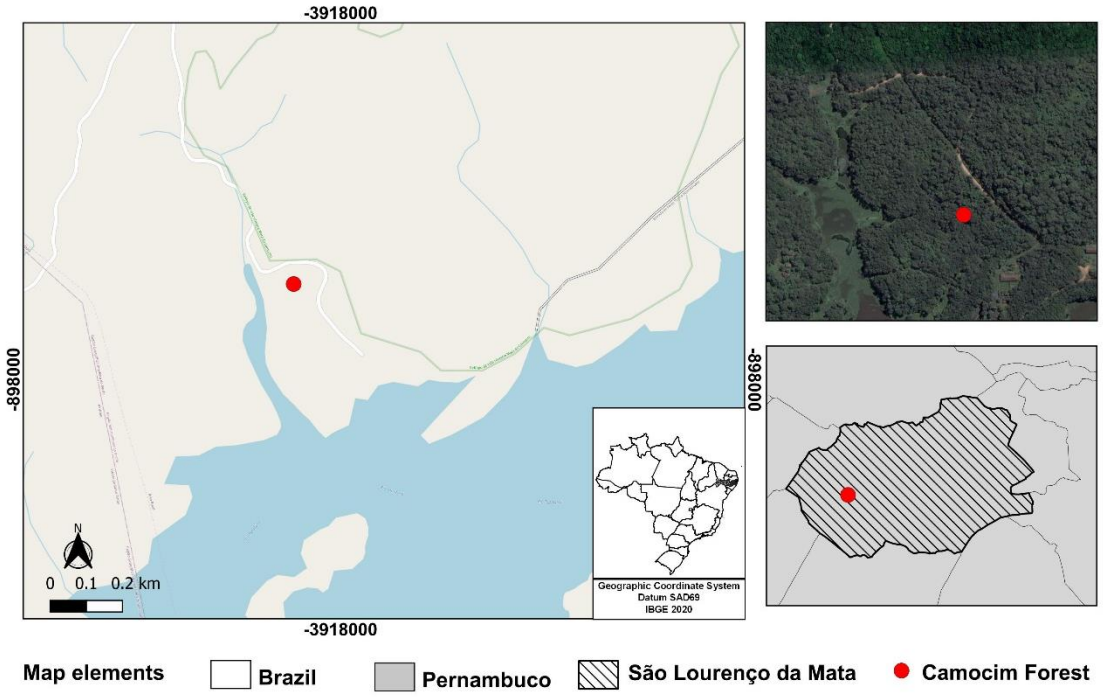

Fig. 1. – Location map of the Camocim Conservation Unit Forest, located in the municipality of São Lourenço da Mata in the Metropolitan Region of Recife, Pernambuco, Brazil

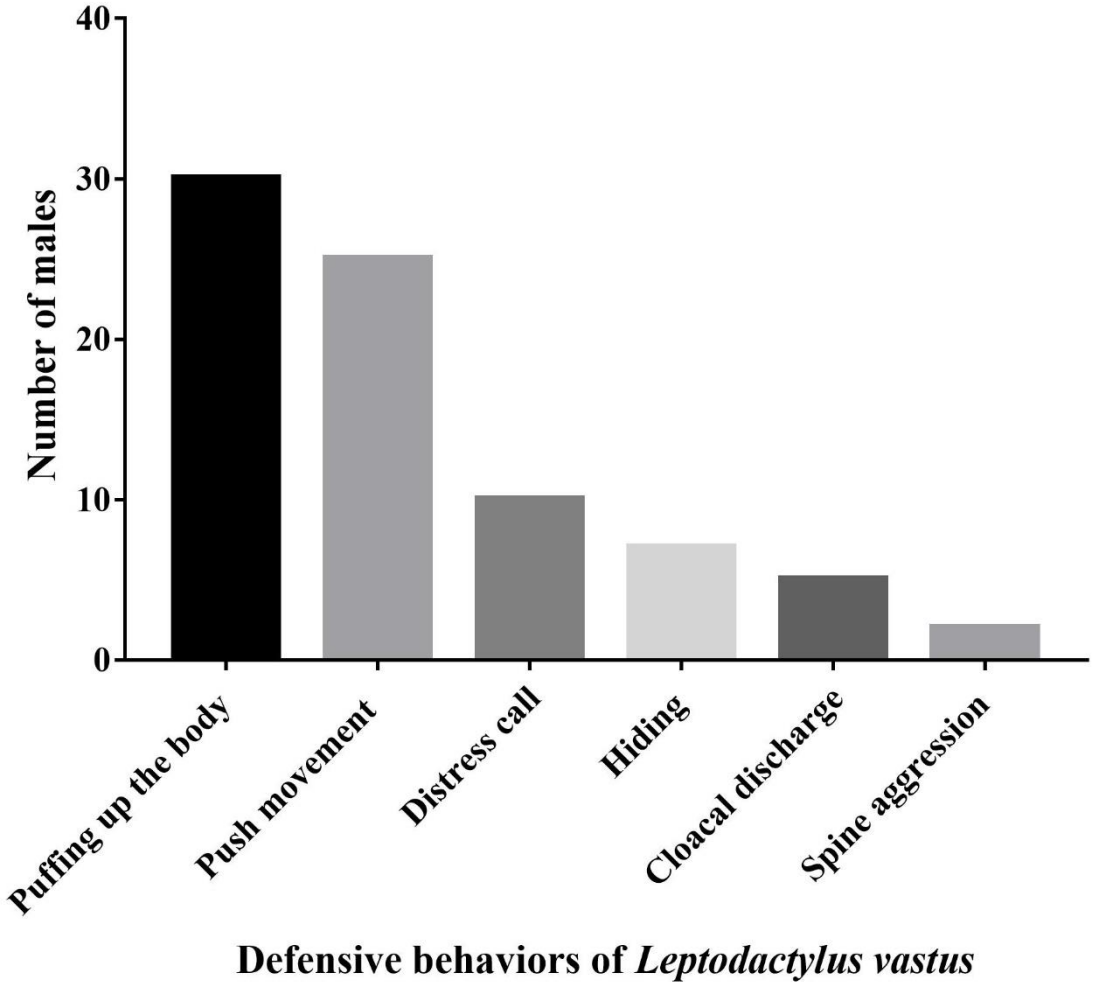

Fig. 2. – Defensive behavior of males of *Leptodactylus vastus* Lutz, 1930 of the Camocim Conservation Unit Forest, located in the municipality of São Lourenço da Mata in the Metropolitan Region of Recife, Pernambuco, Brazil.

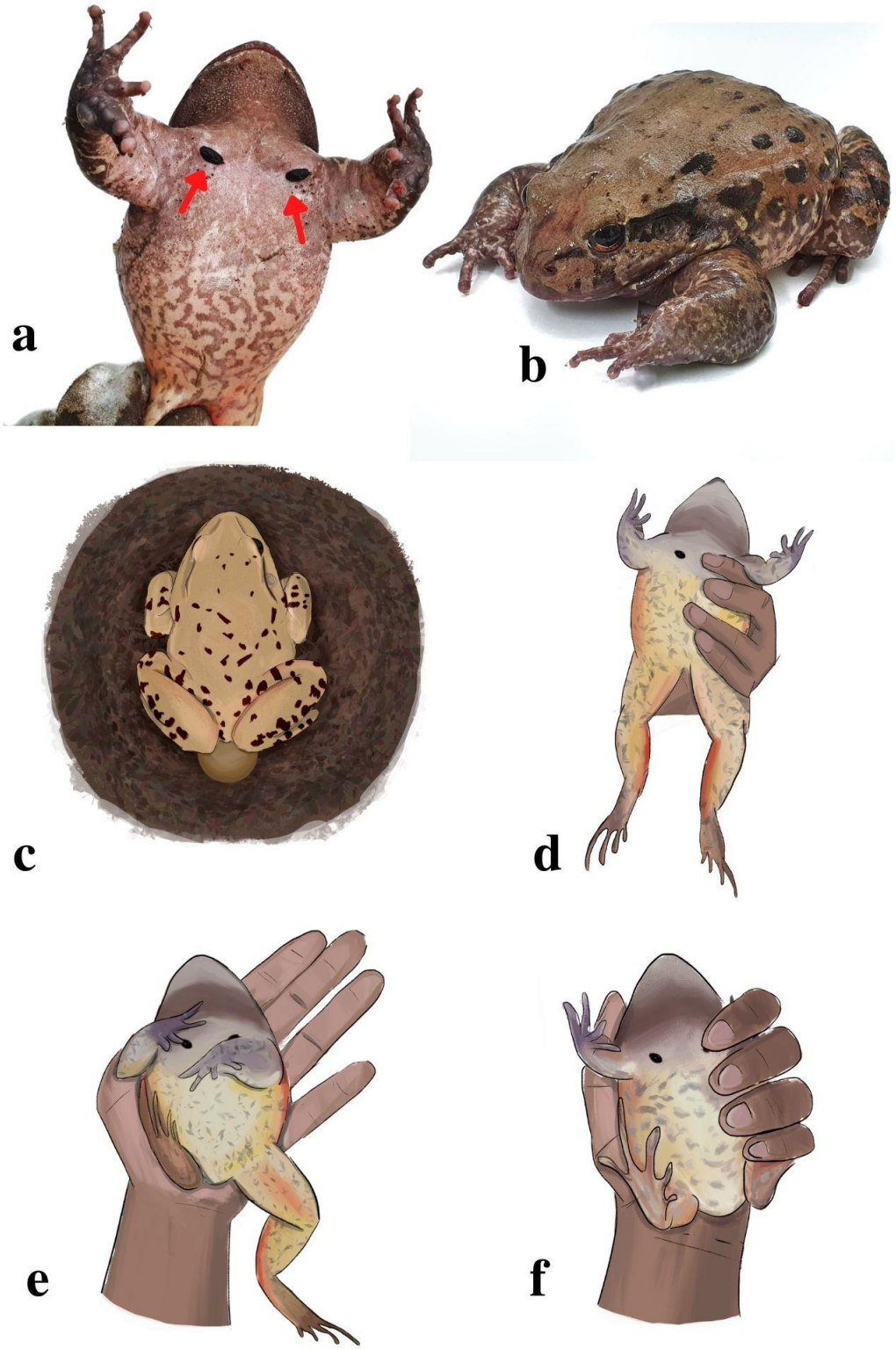

Fig. 3.– Illustrative representation of behavioral display presented by male of *Leptodactylus vastus* Lutz, 1930 submitted to a thereat stimulus. (A) Ventral view of animal body showing the presence of spines on the pectoral region (red arrows). (B) Male puffing up the body (SLV: 13,46 cm; body

mass: 300 g). (C) Illustration of male cloacal discharge of solid material as seen in the field. Illustration sequence demonstrating leg pushing behavior: (D) Male with outstretched legs. (E) Male curling his legs. (F) Male using legs to push. Photo by Jonathas Lins and illustration by Marcelo Brandt Feijó.

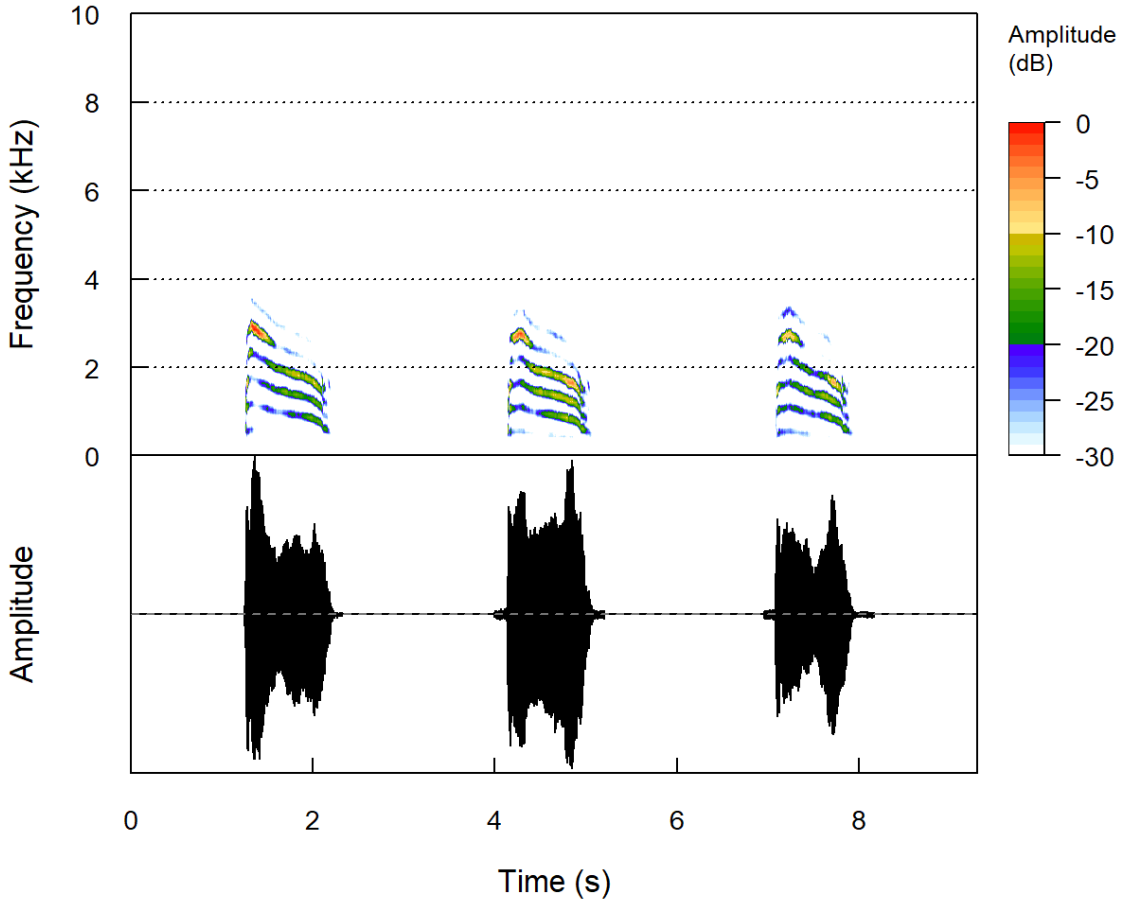

Fig. 4. – The Oscillogram and spectrogram representation of the distress call of *Leptodactylus vastus* Lutz, 1930 of the Camocim Conservation Unit Forest, located in the municipality of São Lourenço da Mata in the Metropolitan Region of Recife, Pernambuco, Brazil. Air temperature: 27 8°C; air humidity: 85%. SLV= 13,46 cm; body mass = 300g. (Individual voucher: SCLEHP01018)

Considerações finais

O estudo sobre o estado fisiológico de uma população de *Leptodactylus vastus*, através da análise dos parâmetros hematológicos e da bioquímica sérica evidenciou que esses animais têm apresentado anormalidades morfológicas nas células sanguíneas, além do número elevado de basófilos e das enzimas hepáticas. Tais resultados podem estar relacionados as atividades antrópicas que vêm sendo desempenhadas no Jardim Botânico e Parque Dois Irmãos, o que pode ter provocado a ocorrência de hemogregarinas nos eritrócitos desses animais. Esse hemoparasito, quando em excesso pode provocar anemia. Além disso, a presença de eritrócitos imaturos e micronúcleos nos eritrócitos maduros podem estar associados a poluentes nos ecossistemas aquáticos, nos quais esses animais estão distribuídos.

Com isso, esse estudo deixa, enquanto proposta futura a análise físico-química da água para verificar possíveis distúrbios que possam provocar declínios populacionais nas espécies de anuros do Brasil. Essa relação ambiental com o estado fisiológico, através da hematologia, pode trazer possíveis respostas acerca de doenças relacionadas ao sangue e todo o sistema, além dos analitos bioquímicos para a verificação do funcionamento dos órgãos. Tais resultados ajudarão a compor valores de referências para a padronização de estudos. Ainda em relação aos parâmetros intrínsecos, esse estudo torna-se o primeiro a descrever, através da microscopia eletrônica, as diferentes células sanguíneas de uma espécie de anuro endêmica do nordeste brasileiro.

Em relação aos parâmetros extrínsecos, a descrição do repertório defensivo mostrou que *L. vastus* apresentou seis tipos de comportamentos, que foram categorizados em comportamentos visuais e acústicos. Quando somado aos trabalhos já publicados para a espécie, observa-se que esses animais desempenham 9 tipos de comportamentos antipredatórios. Esse tipo de trabalho pode auxiliar na compreensão da ecologia desses animais e de como eles tem se adaptado, em resposta as diversas mudanças ambientais.

Anexos

BIOLOGICAL CONSERVATION

AUTHOR INFORMATION PACK

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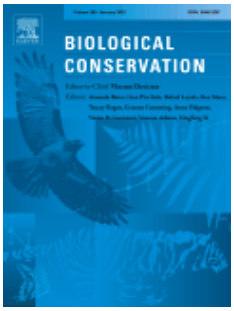

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