UNIVERSIDADE FEDERAL DE SÃO CARLOS

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA

E MONITORAMENTO AMBIENTAL

Elisabete Teixeira Tsukada

COMPARATIVE HISTOLOGICAL AND ANATOMICAL BIOMARKERS OF THE SPLEEN AND KIDNEYS IN AMPHIBIANS: A SPECIAL FOCUS ON *RHINELLA ICTERICA*

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Dissertação apresentada ao Programa de Pós-Graduação em Biotecnologia e Monitoramento Ambiental para obtenção do título de Mestre em Biotecnologia e Monitoramento Ambiental.

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Não tenho dúvidas que esqueci de colocar o nome de alguém, mas, como eu descobri durante esses anos de mestrado, é muito difícil lembrar de tudo sozinha. E mais, é impossível fazer ciência sozinha. A ciência é coletiva e se constrói a partir do trabalho de muitas mãos. Obrigada a todos que esqueci de mencionar nominalmente, e um agradecimento especial a todos os autores citados neste trabalho de 1923 a 2023, todos esses cientistas possibilitaram este texto com contribuições diversas, distintas, todas importantes, imprescindíveis para este estudo e para a construção do conhecimento.

“Biology is the study of complicated things that have
the appearance of having been designed with a purpose.”
Richard Dawkins

RESUMO

TSUKADA, Elisabete. Comparative Histological and Anatomical BIOMARKERS of the Spleen and Kidneys in Amphibians: A Special Focus On *Rhinella Icterica*. 2023. Dissertação de Mestrado em Nome do Programa de Pós-Graduação em Biotecnologia e Monitoramento Ambiental) – Universidade Federal de São Carlos, Sorocaba, 2023.

Os anfíbios pertencem a um grupo diversificado de vertebrados ectotérmicos e apresentam uma vasta gama de adaptações anatômicas e fisiológicas que lhes permite prosperar em vários habitats aquáticos e terrestres. O baço e os rins, dois órgãos essenciais no corpo dos anfíbios, têm recebido uma atenção considerável devido ao seu significado funcional. O baço é um componente do sistema linfático e está envolvido nas respostas imunitárias, na hematopoiese e na renovação dos eritrócitos. Os rins, por outro lado, são cruciais para a manutenção do equilíbrio hídrico e eletrolítico, bem como para a excreção de resíduos metabólicos.

A compreensão das características histológicas e anatômicas destes órgãos nos anfíbios é fundamental para o conhecimento das suas capacidades funcionais e adaptações evolutivas. O objetivo desta dissertação de mestrado foi realizar duas revisões bibliográficas detalhadas, uma centrada no baço e outra nos rins, em anfíbios. Estas revisões fornecerão uma síntese abrangente da literatura existente, analisando as variações histológicas e anatômicas observadas em diferentes espécies e identificando a potencial utilização destes órgãos como relevantes fontes de biomarcadores para estudos de monitoramento ambiental. Além disso, foi realizada a primeira descrição detalhada da histomorfologia do baço e dos rins de uma espécie neotropical de anfíbio anuro, *Rhinella icterica*. Ao examinar esta espécie, pretende-se contribuir para a compreensão das suas adaptações fisiológicas e fornecer informações valiosas para os campos da anatomia, e da histo-morfologia, bem como para o monitoramento da saúde ambiental. Em última análise, esta investigação contribuirá para a base de conhecimentos existente sobre anatomia e histologia dos anfíbios, particularmente no que diz respeito ao baço e aos rins. Os resultados deste estudo podem ter implicações para a compreensão da saúde dos anfíbios, compreender aspectos acerca da suscetibilidade a doenças, e fomentar esforços para a conservação dos anfíbios. Além disso, a caracterização detalhada da histomorfologia de *Rhinella icterica* servirá como uma referência para futuros estudos comparativos e pode lançar luz sobre potenciais correlações entre morfologia e função.

Palavras-chave: esplênico; renal; biomarcadores; descrição; espécie neotropical.

ABSTRACT

Amphibians belong to a diverse group of ectothermic vertebrates. These animals exhibit a wide range of anatomical and physiological adaptations that allow them to thrive in various aquatic and terrestrial habitats. The spleen and kidneys are two essential organs within the amphibian body. These organs received considerable attention due to their functional significance. The spleen is a component of the lymphatic system, and it is involved in immune responses, hematopoiesis, and erythrocyte turnover. The kidneys, on the other hand, are crucial for maintaining water and electrolyte balance, as well as excretion of metabolic waste products.

Understanding the histological and anatomical characteristics of these organs in amphibians is fundamental for gaining insights into their functional capabilities and evolutionary adaptations. The aim of this masters dissertation was to perform two detailed bibliographic reviews, one of them focusing on the spleen, and the other focusing on the kidneys, in amphibians. These reviews will provide a comprehensive synthesis of the existing literature, analyzing the histological and anatomical variations observed across different species, as well as identifying the potential use of these organs as relevant sources of biomarkers for environmental monitoring studies. Here we also present the first detailed description of the histomorphology of the spleen and kidneys in a neotropical anuran species, known as *Rhinella icterica*. By examining this species in detail, we aim to contribute to the understanding of its physiological and histo-morphological adaptations, and their potential uses in environmental health. Ultimately, this research will contribute to the existing knowledge base on amphibian anatomy and histology, particularly regarding the spleen and kidneys. Our study may have implications for the understanding of amphibian health, disease susceptibility, and conservation efforts. Additionally, the detailed characterization of the histomorphology of *Rhinella icterica* will serve as a valuable reference for future comparative studies and may shed light on potential functional correlations between organ structure and the selective pressures that act on the evolution of these organisms.

Keywords:splenic; renal; biomarkers; description; neotropical species.

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SUMÁRIO

1. INTRODUÇÃO GERAL

Os anfíbios são animais tetrápodes com ciclo de vida bifásico, ou seja, apresentam uma fase larval aquática, e ao longo da metamorfose desenvolvem modificações lhes permitem viver no ambiente terrestre (AMPHIBIAWEB, 2023; LIEDTKE *et al*., 2022; SCHMIDT-NIELSEN, 2002; VENCES; KOHLER, 2008). São encontrados em todos os continentes, com exceção dos pólos. Sua distribuição é mais abundante nas regiões tropicais, sendo o Brasil o país com a maior riqueza de espécies de anfíbios do planeta (AMPHIBIAWEB, 2023; FROST, 2023).

A classe dos anfíbios abarca três ordens: Anura, Caudata e Gymnophiona, com aproximadamente 8.500 espécies catalogadas (AMPHIBIAWEB, 2023; FROST, 2023). Esses grupos são distinguidos por sua morfologia corporal característica. As cobras-cegas ou cecílias, pertencentes a ordem Gymnophiona, não possuem membros e representam o grupo menos estudado, e consequentemente com menor número de espécies conhecidas (AMPHIBIAWEB, 2023; FROST, 2023). As salamandras, com suas caudas, nomeiam a ordem Caudata, enquanto os anuros, que compreendem os sapos, rãs, e pererecas, são a classe mais diversa que abarca a maior parte do táxon, com cerca de 7.500 espécies descritas (AMPHIBIAWEB, 2023; FROST, 2023).

Em relação à sua anato-fisiologia, os anfíbios possuem pele fina, bastante vascularizada e úmida para possibilitar as trocas gasosas e evitar a desidratação (HASLAM *et al*., 2014; LILLYWHITE, 2006). Além disso, apresentam capacidade de regular sua temperatura corporal através do comportamento, se afastando ou se aproximando de fontes de calor (BRATTSTROM, 1979). Essa relação intrínseca com os ambientes aquáticos, somada às peculiaridades anátomo-fisiológicas dos anfíbios, os tornam organismos bastante vulneráveis a alterações ambientais (AMPHIBIAWEB, 2023; CALDERON *et al*., 2022). Portanto, são organismos com alto valor ecológico e desempenham um papel fundamental em estudos de monitoramento ambiental de ecossistemas naturais e modificados como bioindicadores de qualidade ambiental, devido às características inerentes ao grupo que os tornam altamente sensíveis às variações ambientais (AMPHIBIAWEB, 2023; CALDERON *et al*., 2022; STUART *et al*., 2004).

Devido ao crescimento populacional e às ações antropogênicas inconsequentes, as quais têm resultado em grande impacto ao meio ambiente (GANIVET, 2020), registros mostram que desde a década dos anos 70 os cientistas vêm relatando uma redução expressiva na diversidade dos anfíbios em todo o planeta (KACOLIRIS *et al*., 2022). Diversas são as possíveis causas desses declínios, incluindo as mudanças climáticas, destruição e fragmentação de habitats, doenças, contaminação das águas e outros fatores (STUART *et al*., 2004; GREEN *et. al.,* 2020). Ademais, estudos recentes apontam que efeitos sub-letais não causam extinções em massa desses animais, porém podem reduzir o *fitness* adaptativo e mascarar uma perda gradual no tamanho das populações afetadas (FISHER; PASMANS, MARTEL, 2021). Além disso, alterações comportamentais (HOFFMAN; KLOAS, 2012); no tamanho dos indivíduos ou de seus órgãos (HAYES, 1995; BORGIOLI; FRANGIONE, 1997; FORZÁN *et al*., 2015) podem ser indicativos de que existe algum fator interferindo na estabilidade de uma população.

Nos anfíbios, o baço é o principal órgão linfóide e desempenha papel preponderante no sistema de defesa dos organismos (UDROIU; SGURA, 2017). Apesar de seu papel indispensável na resposta imunológica e em processos fisiológicos nos anfíbios, o baço é um órgão pouco estudado para o monitoramento ambiental. Doenças como a ranavirose e a quitridiomicose podem desencadear respostas imunes e gerar alterações no baço que podem ser quantificadas e comparadas para análises de ecotoxicidade (MORALES; ROBERT, 2007; RIBAS *et al*., 2009; GRAYFER; EDHOLM; ROBERT, 2014). Outro órgão muito importante para a ecotoxicologia é o rim, ele é crucial para a etapa de eliminação de toxicantes, além de participar diretamente do balanço eletrolítico e da homeostase (FORZÁN *et al.*, 2017). Os rins também podem ser um local de acúmulo de substâncias como os metais potencialmente tóxicos (DOBROVOLJC *et al.*, 2012; MAHMOOD *et al.*, 2016), que podem gerar respostas histomorfológicas no tecido renal (MAHMOOD *et al.*, 2016).

Nessa perspectiva, esta dissertação visa apresentar nos dois primeiros capítulos duas revisões bibliográficas sistemáticas com foco na histologia e morfologia desses órgãos (baço e rins) bem como em potenciais biomarcadores que podem ser fornecidos para a área da ecotoxicologia. Essas análises estão alicerçadas em mais de cem trabalhos ao longo de quase um século de pesquisas e descobertas acerca da histomorfologia esplênica e renal dos anfíbios. Além disso, o terceiro capítulo é composto da detalhada e inédita descrição histológica do rim e do baço do anuro *Rhinella icterica*, uma espécie neotropical, acompanhada da comparação com outras espécies de anfíbios descritas.

2. ARTIGO 1

The amphibian's spleen as a source of biomarkers:
systematic review, scientometric data and histological characteristics

Obs: Foi aceito para publicação em 20/07/2023 pela revista Science of the Total Environment (Qualis = A1, Fator de Impacto = 9.8)

Abstract

Amphibians are very sensitive to many environmental changes, so these animals are considered as good bioindicator models to ecotoxicology. Given the importance of the amphibian spleen for hematopoietic and immune responses, this can be a key organ for the evaluation of biomarkers to monitor the health of individuals in nature or in captivity. In this systematic review, we searched databases and summarized the main findings concerning the amphibian spleen as a source of possible biomarkers applied in different scientific fields. The searches resulted in 83 articles published from 1923 to 2022, which applied the use of splenic samples to evaluate the effects of distinc stressors on amphibians. Articles were distributed along more than twenty countries, with the USA, Europe and Brazil standing out among them. Publications focused mainly on anatomical and histomorphological characterization of the spleen, its physiology and development. Recently, the use of splenic biomarkers in pathology and ecotoxicology began to grow but many gaps still need to be addressed in herpetological fields. About 85% of the splenic biomarkers showed responses to various stressors, which indicates that the spleen can provide numerous biomarkers to be used in many study fields. The limited amount of information on morphological description and splenic anatomy in amphibians may be a contributing factor to the underestimated use of splenic biomarkers in herpetological research around the world. We hope that this unprecedented review can instigate researchers to refine herpetological experimentation, using the spleen as a versatile and alternative source for biomarkers in ecotoxicology.

Keywords: Splenic; Conservation; Pathology; Ecotoxicology; Toxic effects; Disease

2.1 Introduction

Amphibians are currently the most endangered vertebrate group on the planet (IUCN, 2022). About 40% of amphibian species have some degree of vulnerability and 16% of them still lack assessment data (IUCN, 2022). Considering only the groups with available information, about 48% of the amphibian species are currently at risk (IUCN, 2022). Information-deficient groups are possibly even most threatened, as it has been estimated that 85% of these species can be endangered (BORGELT *et al*., 2022). In other words, nearly half of the known amphibian species may be at risk.

This scenario made amphibians a group of great importance for environmental monitoring research, so many amphibian species have already been used as bioindicators of environmental changes (LONDERO *et al*., 2019; VELASCO *et al*., 2021), to evaluate the ecotoxicity of many contaminants (CALDERON *et al*., 2023; DING *et al*., 2022; GONÇALVES *et al*., 2017), and to understand the outcomes of emergings diseases (BLAUSTEIN *et al*., 2018; SCHEELE *et al*., 2019; FISHER *et al*., 2021).

Understanding the biology of these organisms is a crucial tool for environmental monitoring through the use of biomarkers (MCCARTHY; SHUGART, 2018). Several morpho-physiological functions have been used as sources of biomarkers in amphibians, such as the cardiac function (PELTZER *et al*., 2019; JONES-COSTA *et al*., 2018; SALLA *et al*., 2018), the hepatic function (ARAÚJO *et al*., 2020; SALLA *et al*., 2020; VAN METER *et al*., 2022), genotoxicity and mutagenicity measures (FERRIER *et al*., 2018; GONÇALVES *et al*., 2019; HEREK *et al*., 2021), hematological markers, among others (BRODEUR *et al*., 2020). Although the immune system has also been widely explored as a source of biomarkers to evaluate amphibian's health (SILVA *et al*., 2020; GAVEL *et al*., 2021), the study of the splenic function remains relatively unexplored, despite its recognized importance as a lymphoid organ (BRANNELLY *et al*., 2016).

The spleen is known as an important lymphoid organ in amphibians (DEMIRCAN *et al*., 2016). During larval stages, the spleen can also participate in hematopoietic functions (DEMIRCAN *et al*., 2016). In adult vertebrates, this organ can act in various tasks including blood storage and filtration, phagocytosis, destruction of old erythrocytes, iron metabolism, erythropoiesis and antibody production (DEMIRCAN *et al*., 2016). Once acting as part of the lymphatic system, the splenic function could be used as a sensitive biomarker to evaluate environmental changes and pathologies, possibly assisting various studies of risk assessment and amphibian conservation. In addition, increasing knowledge about the amphibian`s spleen morphology can help future comparative morphological and evolutionary studies of other vertebrates.

Thus, this paper aimed to review and discuss the published scientific literature about the anatomy and histomorphology of amphibian´s spleen and how these characteristics could be used as biomarkers. To that, the searches included morphological and histological characteristics of the amphibian spleen, studies of splenic toxicity in response to contaminants and environmental changes, splenic responses to amphibian pathologies and other molecular applications of evolutionary studies. In addition, bibliometric parameters (publication year, geographical distribution, species composition, and experimental types) were addressed and critically discussed. Overall, this systematic review is the first to compile ancient and current knowledge about the use of the amphibian splenic morphology as a source of biomarkers of multiple applications. Such information will contribute to the understanding of the amphibian splenic function, providing a basis for further comparisons of immune and/or hematopoietic processes among amphibians and other vertebrates, as well as it points out to future directions and perspectives for the various herpetological study areas.

2.2 Methodology

The search of the peer-reviewed studies was conducted in multiple databases, including "Scopus", "Web of Science", "PubMed", and "ScienceDirect" databases, and combining the keywords "spleen", AND "amphibian", OR anura, OR "frogs", OR "toads", OR "amphibia" (considering their singular and plural forms) from the first record found until 2022. Initially, the searches returned a total of 398 scientific manuscripts which were later sorted for eligibility purposes to include only the studies associated with the amphibian spleen. If other eligible papers were cited inside the selected papers, they were also included. The exclusion criteria consisted in non-English written papers, duplicated documents, review papers, technical reports, unpublished protocols, gray literature and studies that did not attend to the research aims. The final screening resulted in 83 manuscripts, which integrated the current review (Fig. 1). Graphic illustrations and the phylogenetic tree were produced in Adobe Illustrator software and in the Interactive Tree of Life (iTOL), and the map of global distribution of the studies was plotted in Excel software.

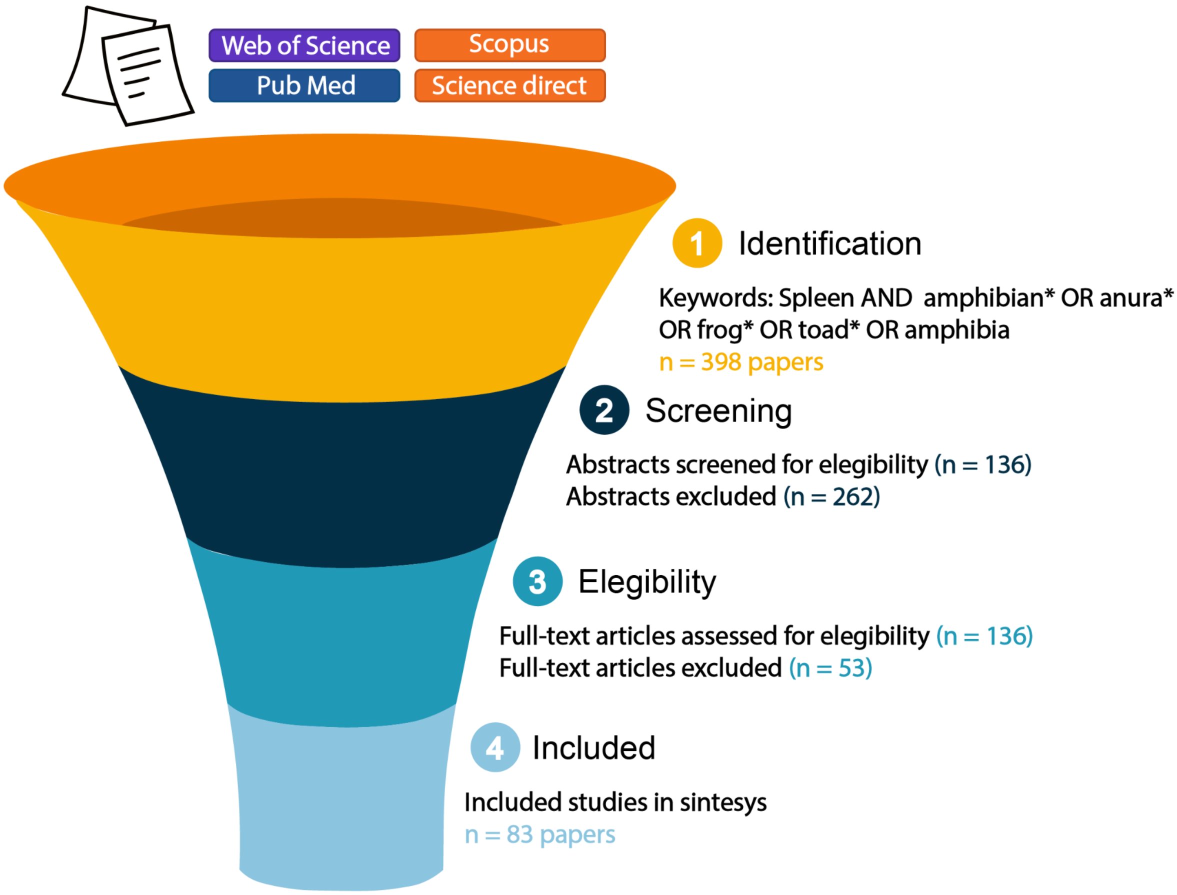

Figure 1. Methodological screening and selection of studies concerning the amphibian spleen as a source of biomarkers.

2.3 Results and Discussion

2.3.1. Scientometric data: chronology and global distribution

The first publication found through the filters dates back to 1923 (JORDAN; SEIDEL, 1923), followed by other punctual and temporally spaced publications until 1987, whose objectives varied from classical physiology studies to elucidating immunological and erythropoietic mechanisms, as well as other studies describing general morphological and nervous characteristics of the splenic amphibian tissue. From 1989 to 1993 publications increased to 2 articles per year, with the highlight of some European research groups that focused on studies of structural and functional biology and physiology of the amphibian spleen. In this same period, there was the publication of the first study that applied the use of the amphibian splenic tissue as a source of biomarkers in a pathological investigation (BRODKIN *et al.*, 1992). Precisely at that time (1989-1992), herpetologists had increased the concerns among the global declines of amphibian populations and numerous factors were suggested as possible causers of these declines (PHILLIPS, 1990). Thus, with that in mind, Brodkin *et al.* (1992) decided to investigate the pathogenesis of the bacterium *Pseudomonas aeruginosa* as a potential agent that could contribute to the mortality of amphibians worldwide, using splenic biomarkers to evaluate lymphoid responses. This particular paper represented an important milestone for the use of splenic biomarkers in amphibian studies. The following years were marked by two important growth peaks of publications: the first standing out in 1995, with emphasis on characterization studies of the amphibian spleen, which were done by Kinney and colleagues (KINNEY *et al*., 1994; 1996); and the second peak in 1997, with emphasis on the studies of two Italian scientists (FRANGIONI; BORGIOLI, 1996; 1997; BORGIOLI; FRANGIONI, 1997), who focused on understanding physiological adjustments of the amphibian spleen. From 1998 to 2000 studies have mainly focused on understanding the changes in blood cells that occur during the metamorphic development of the amphibians (GRANT *et al*., 1998; DUCOROY *et al*., 1999; HASEBE *et al*., 1999; TAMORI; WAKAHARA, 2000). In 2002 and 2003 splenic biomarkers were used to assess the effects of acid stress (SIMON *et al.*, 2002; BRODKIN *et al.*, 2003) and parasitic infestations (LAINSON *et al*., 2003). Between 2003 and 2004, the first studies in ecotoxicology appeared, using splenic biomarkers to assess the effects of pollutants (LINZEY *et al*., 2003; ARRIETA *et al.*, 2004). In line with the need for new models for ecotoxicology studies, Rollins-Smith *et al.* (2004) proposed an embryotoxicity test for *Xenopus laevis* tadpoles to assess the effects of xenobiotics on hematopoiesis. From that period until 2022, studies with the spleen in amphibians focused on ecotoxicological and disease-caused effects. Overall, the year of 2016 had the highest number of publications among all the years, reaching a total of 7 articles (Fig. 2).

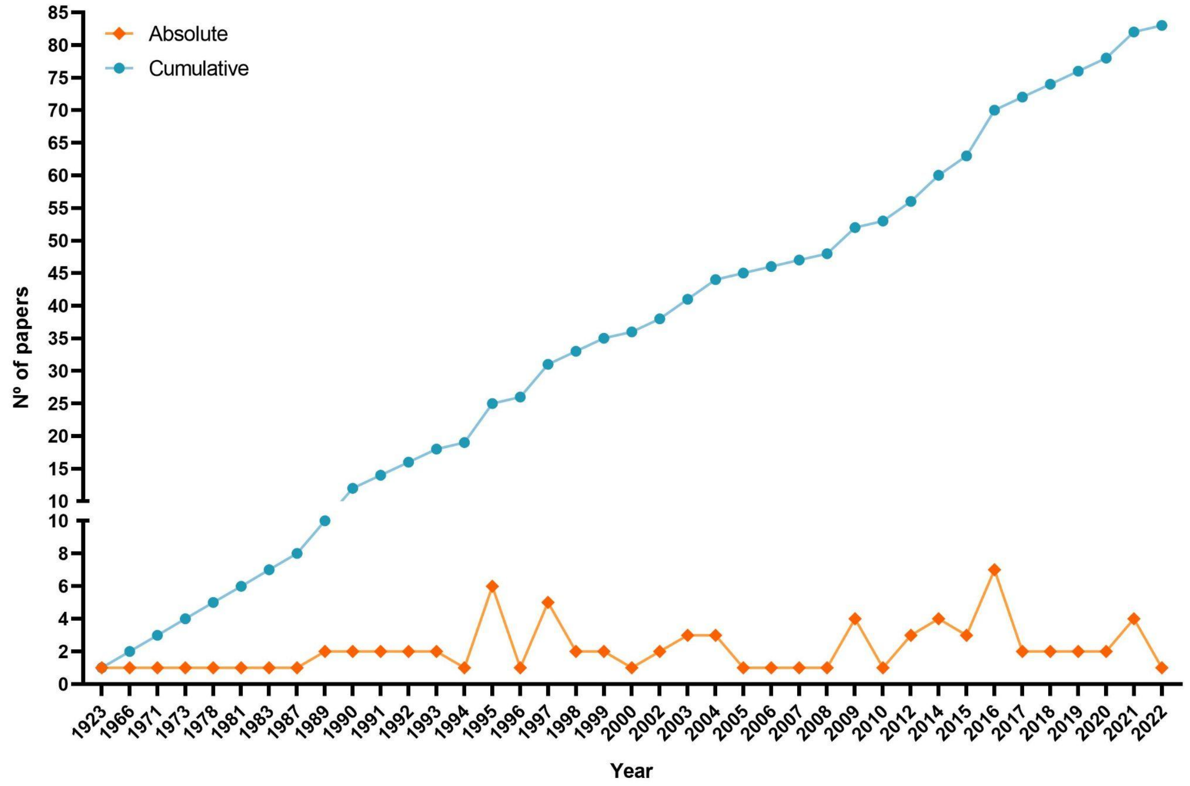

Figure 2. Absolute and cumulative number of articles investigating splenic biomarkers in amphibians.

Chronological analysis showed that publication trends in different areas of amphibian studies seem to vary over time. In 2006, Mccallum and Mccallum (2006) had already noticed a gradual reduction of studies dedicated to the natural history of species within herpetology (MCCALLUM; MCCALLUM, 2006). This trend can be explained by the fact that a greater number of herpetological studies are concentrated in the area of conservation (BRITO, 2008). Conservation studies have been highlighted within herpetologists due to the serious situation of decline in amphibian populations, and the consequent need to prioritize studies with this focus. In addition, research involving genetics and molecular biology usually receives greater attention from financial resources (MCCALLUM; MCCALLUM, 2006), which ends up generating greater competition with other areas of "basic biology" such as anatomy and morphology. This explains why morphological and descriptive articles were mostly published in the mid-1990s or earlier, while they are currently scarce.

The environmental area has been growing among researchers (LEIPOLD et al., 2019), and the application of morphological studies as sources of new biomarkers for environmental monitoring can be a good opportunity to expand the use of different samples, such as the splenic tissue in scientific research. Overall, although the use of amphibians as alternative model systems in animal experimentation has grown over the years, there is still a low employability of the spleen as a potential source of biomarkers. Thus, our results reinforce the need to encourage the application of these biomarkers that are considered as "less conventional", which end up being underestimated in terms of their relevance in scientific studies.

The global distribution analysis of the papers showed that the country with the absolute majority of publications was the United States (32.53%), followed by Italy (12.04%), Brazil (10.84%), and Australia (8.43%). The final 7.22% of the papers were distributed amongst Europe, Asia and North America (Figure 3).

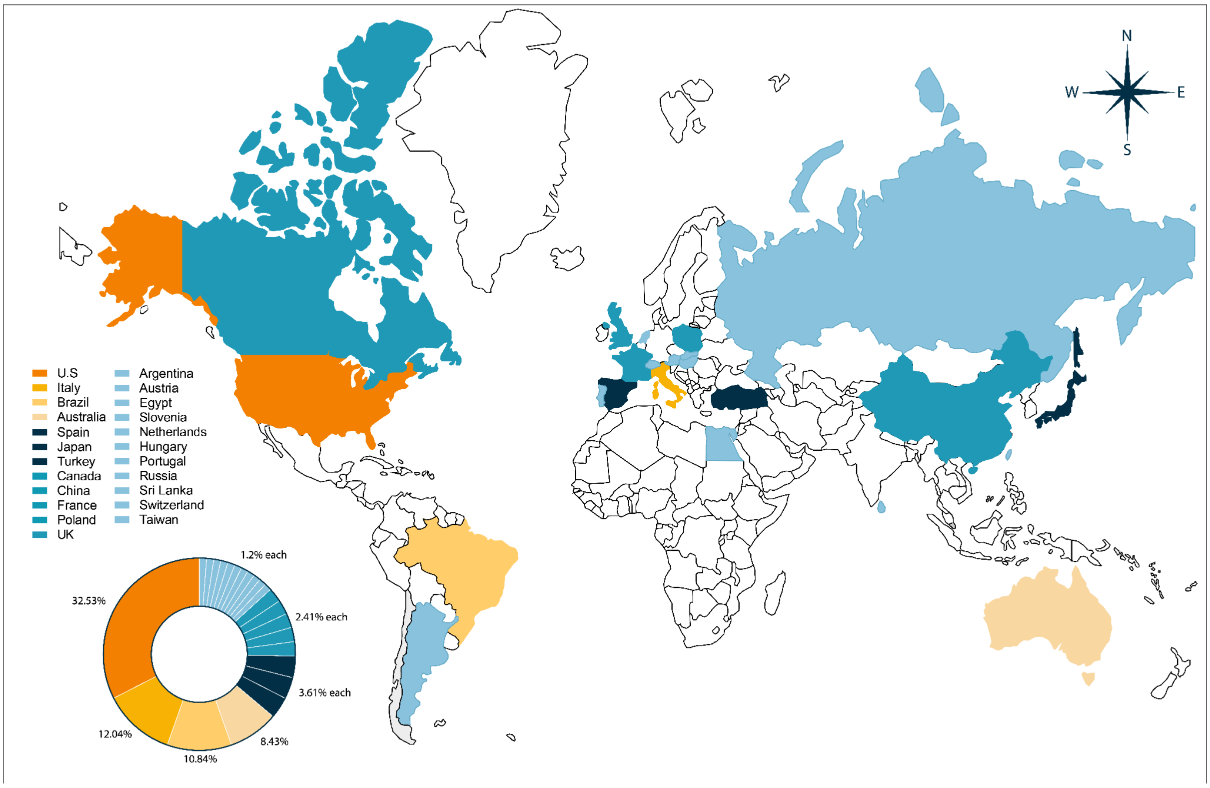

Figure 3. Global map of countries and the frequency of published papers concerning the amphibian splenic function.

Such a dominant trend of the publications from the United States, Europe and Australia has already been observed in other areas of herpetology, like conservation fields (HOULAHAN *et al.*, 2000). Considering that the Neotropical region holds almost half of all amphibian species richness in the world (DUELLMAN, 1999), in addition to representing the area where most of these populations are in decline (STUART *et al.*, 2004), neotropical countries still have a relatively small contribution of papers concerning amphibians (BRITO, 2008). In fact, even research with amphibians that are carried out in tropical regions, for the most part, are not conducted by native researchers, but mainly by scientists from developed countries (FAZEY *et al.*, 2005). According to Brito (2008), nowadays, language still represents a strong barrier for several developing countries to have their publications accepted in international journals, which ends up causing this great disparity in the number of articles published among countries. However, Brazilian studies are now starting to stand out among publications, and the use of amphibians as useful and alternative bioindicators might potentially grow, especially among environmental studies. Furthermore, not only Brazil, but many other countries also suffer from low investment in scientific research (GALVÃO-CASTRO *et al.*, 2022), which further limits the income of local researchers. Research areas that focus on the conservation of amphibians, as well as the monitoring of environmental health are crucial for the ecosystem services provided to humanity in an efficient and sustainable way. To this end, it is crucial that governments and institutions (national and international) increase research investment in these areas to improve the scientific capacities of developing countries and maximize scientific knowledge about the Neotropics. Such actions, in addition to promoting the scientific and social development of these regions, also expand knowledge about native species.

2.3.2. Taxonomy, Sex and Developmental Stage

Studies with anurans were the most abundant, followed by salamanders, and at last by caecilians. These results were already expected given that anurans are the most diverse group of amphibians (AMPHIBIAWEB, 2023; FROST, 2023). Concerning the developmental stage of the animals, 76% of the studies were carried out with adults, while 22% used tadpoles, and only 2% evaluated the embryonic stages. This fact can be explained by characteristics that are inherent to the splenic development during metamorphosis. The functional regions of the spleen are not yet well defined in tadpoles and begin to differentiate only during metamorphosis (KINNEY *et al.*, 1996; GRANT *et al.*, 1998; BLEYZAC *et al.*, 2005; LAMETSCHWANDTNER *et al.*, 2016), which makes it difficult to analyze the functional regions of the spleen during this early stage of development, so probably that is why adults were mostly chosen for the studies.

Considering sex, there was no information that pointed to morphophysiological differences between the spleens of males and females, which may be why about 90% of the studies did not inform the sex of the individuals or chose to use both genders. Females are usually less used in research with anurans, as males are easier to find and capture in the field due to vocalization (REBOUÇAS *et al*., 2019). The use of only females was limited to a single article and was not an arbitrary choice, as it only concerned the necropsy of a random individual with clinical signs of pathology in the study (TARIGO *et al.*, 2006). An overview of the taxonomy, sex and developmental stage data can be observed in Figure 4.

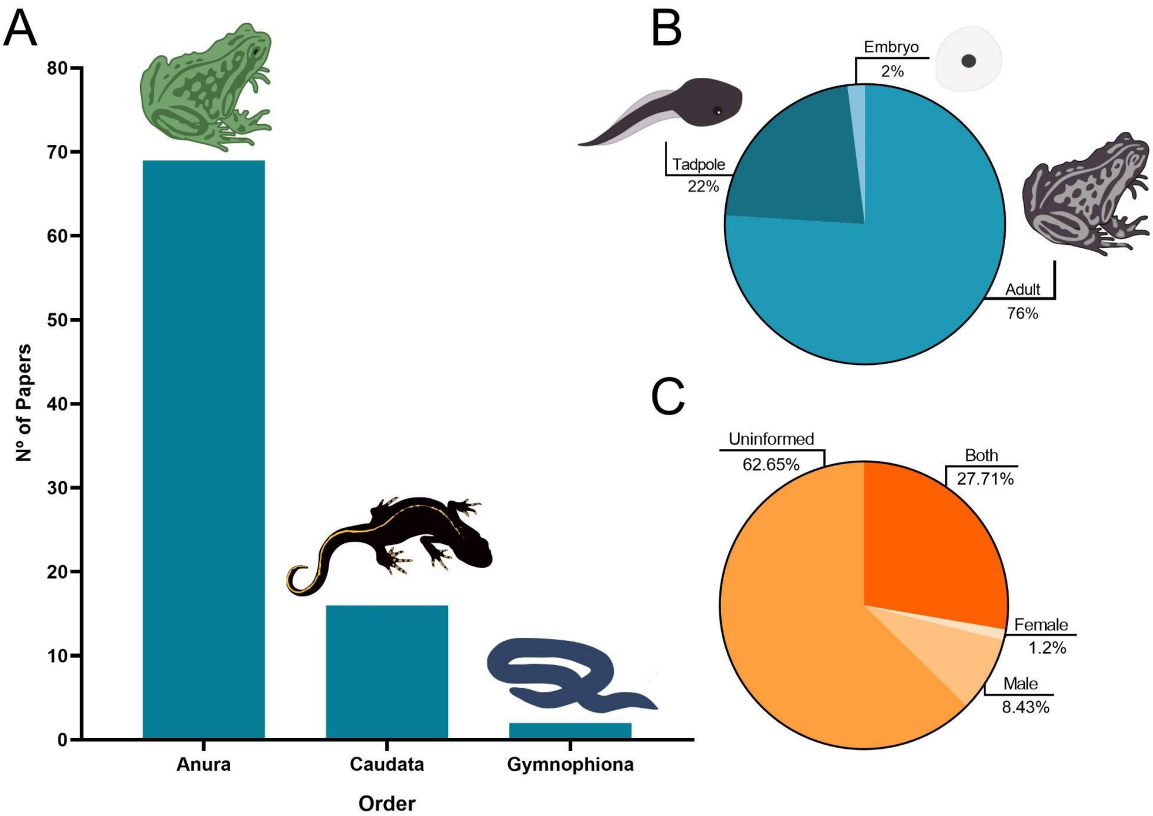

Figure 4. Number of studies on the amphibian spleen conducted with distinct taxonomic orders **(A)**, developmental stages **(B)**, and sex.

From the total of 49 amphibian species evaluated in the articles, most of them were Anurans (77.6%), distributed among 12 families: Bombinatoridae, Bufonidae, Dicroglossidae, Eleutherodactylidae, Leptodactylidae, Limnodynastidae, Myobatrachidae, Pelodryadidae, Pipidae, Ranidae, Rhacophoridae, Scaphiopodidae. The class of Caudata (20.4%) was less representative, including the families Ambystomatidae, Cryptobranchidae, Plethodontidae, Proteidae, Salamandridae, together with the final unique record of Gymnophiona (2%) with the family Typhlonectidae (Fig. 5).

The studies with Anurans were mainly conducted with *Xenopus* (e.g., *Xenopus tropicalis*, *Xenopus laevis*; 22.9%), *Lithobates* (e.g., *Lithobates sylvaticus*, *Lithobates pipiens*; 15.7%), *Rhinella* (e.g., *Rhinella arenarum*, *Rhinella marina*; 14.5%), and *Pelophylax* (e.g., *Pelophylax bedriagae*, *Pelophylax lessonae*; 9.6%) genders, while studies with Caudata and Gymnophiona were mainly conducted with *Ambystoma* (e.g., *Ambystoma tigrinum*, *Ambystoma mexicanum*; 7.2%), *Triturus* (e.g., *Triturus carnifex*, *Triturus cristatus*; 7.2%), and *Typhlonectes compressicauda* (2.4%), respectively (Fig. 5).

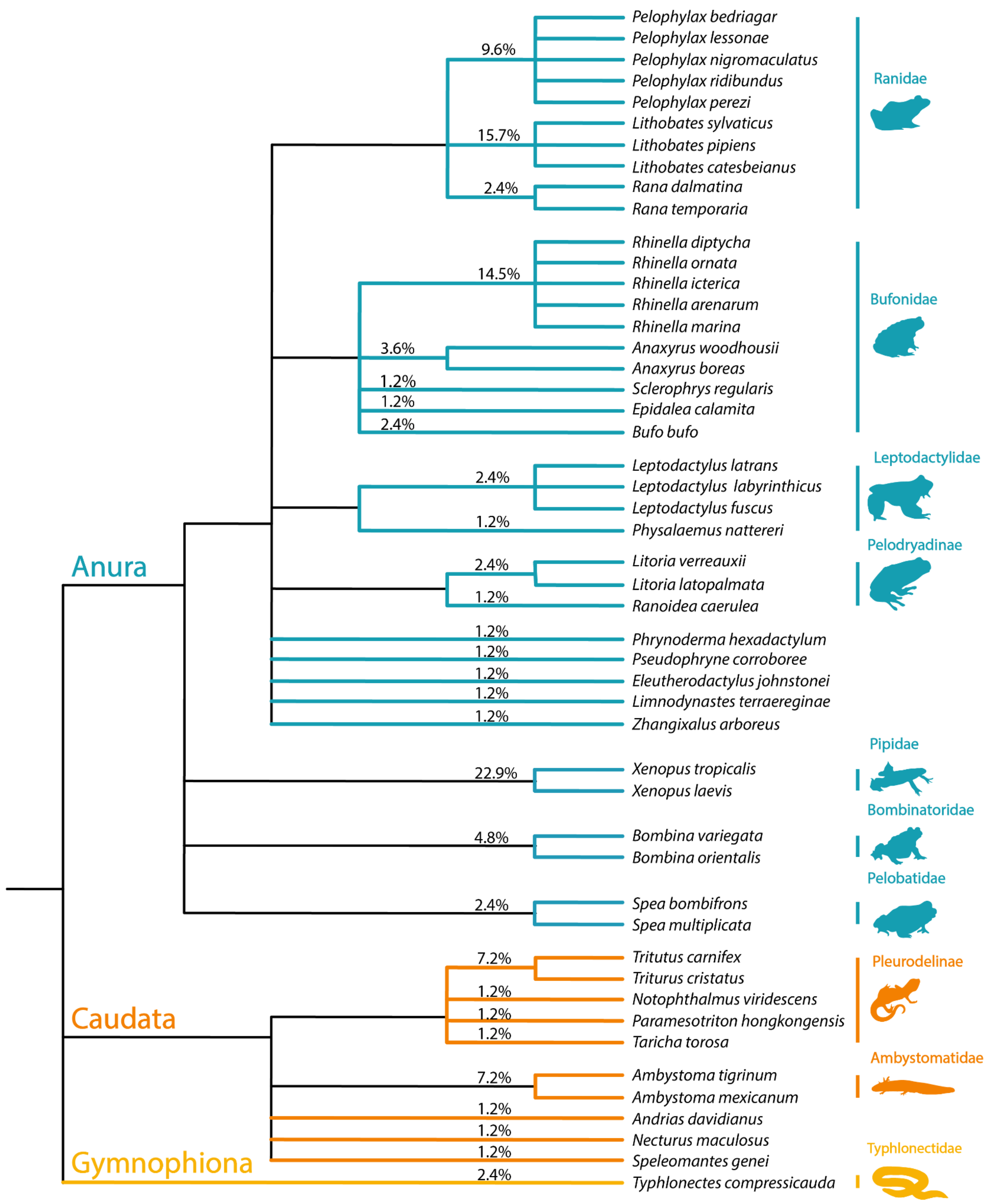

Figure 5. Number of studies conducted with amphibian genera (%) and species to access toxicity biomarkers in the spleen.

There was a predominance of studies that used classic model anuran species such as *X. laevis* and *X. tropicalis*, *L. pipiens*, *L. castebeianus*, *R. temporaria* and *Rhinella marina*. Such a tendency can be explained by the fact that amphibian model-species already have a well described biology, as well as other characteristics that facilitate its acquisition and maintenance in laboratory conditions (WAKE; KOO, 2018). The genus *Xenopus* is widely used in experimental biology due to the fact that these organisms are already known worldwide as experimental models, being used in the research areas of developmental biology (BECK; SLACK, 2001), cellular regeneration, molecular biology (BECK; SLACK, 2001; GRAINGER, 2012), immunology and toxicology (BURGGREN; WARBURTON, 2007; LIU *et al.*, 2016). The use of their embryonic stages has drawn the attention of scientists due to the ethical refinement that the use of initial and less complex stages of development provides when using organisms that are still underdeveloped (REDMOND, 2019). In addition, characteristics such as the ease of obtaining an abundant amount of eggs, the versatility of handling, and the resistance of the embryos to being kept in laboratory conditions make these animals excellent alternative models for animal experimentation (BURGGREN AND WARBURTON, 2007; LIU *et al.*, 2016).

Among the caudata, the axolotl *Ambystoma mexicanum* is also very frequently employed as a versatile model for regeneration, development and evolution studies (VOSS et al., 2009), which also explains its presence among the splenic studies. Overall, it was evident that neotropical species still need to have increased efforts to improve knowledge, especially considering the unique characteristics that reflect the diversity of their habitats, as well as the selective pressures that have contributed to their evolutionary process.

2.3.3. Morphophysiological and function description of the amphibian spleen

The spleen is the main lymphoid organ for amphibians, as they have no lymph nodes. In addition to lymphoid and immune functions, the spleen is related to erythrocyte cycling (UDROIU; SGURA, 2017) and also to hematopoiesis (HADJI-AZIMI *et al*., 1987; CUMANO; GODIN, 2007; DEMIRCAN *et al*., 2016). The spleen can be considered the main clearance site in the early phase of a bacterial invasion, before an adequate production of specific antibodies is initiated (JUNQUEIRA AND CARNEIRO, 2017; UDROIU AND SGURA, 2017). As a lymphoid organ, the spleen can act in phagocytic processes, antibody production (DEMIRCAN *et al*., 2016), as well as in lymphocyte recycling mechanisms (GRANT *et al*., 1998). Lymphocyte recycling varies along the developmental stages, with a low rate of splenic lymphocytic apoptosis during premetamorphosis, which rises in prometamorphic stages, reaching a peak at climax, and finally declining when metamorphosis is completed (GRANT *et al*., 1998). Such peak of lymphocytes' loss at the climax of the development (DU PASQUIER; HAIMOVICH, 1976) is associated with a significant increase in glucocorticoid hormones (ROLLINS-SMITH *et al*., 1997). The regulation of the development of macrophage-lineage cells also occurs through the expression of splenic genes (CSF1R), as observed for X. laevis (GRAYFER *et al*., 2014), which reinforces the role of the amphibian spleen as an indispensable organ to the immunological system. Similarly to other vertebrates (FELTEN; FELTEN, 1988), the amphibian splenic innervations also interact with the noradrenergic sympathetic nervous system (SNS) (KINNEY *et al*., 1996; KINNEY; COHEN, 2005), which reveals a phylogenetically ancient origin for the SNS-immune system communications (KINNEY; COHEN, 2005). These innervations were described as confined to the splenic white pulp of adult X. laevis (CLOTHIER *et al*., 1991), and could also be observed in tadpoles, although in a lower level, since prometamorphic stages (KINNEY *et al*., 1996).

The hematopoietic role of the spleen in amphibians is still a controversial fact since distinct studies have found variations on the hematopoietic activity. Although De Abreu Manso *et al.* (2009) did not find evidence of hematopoietic activity in the splenic tissue of adult Lithobates catesbeianus, older studies had previously reported that for adult bullfrogs the spleen was the main erythropoietic organ, while for tadpoles the hematopoiesis activity was reduced when compared to the bone marrow (JORDAN; SPEIDEL, 1923; HASEBE *et al*., 1999). Later, Maniatis and Ingram, (1971) corroborated the presence of hematopoietic tissue in the spleen of bullfrog tadpoles. For adult specimens of Xenopus laevis there was also evidence of splenic hematopoiesis (HADJI-AZIMI *et al*., 1987; CUMANO; GODIN, 2007). Such hematopoietic activity does not seem to be specific for anurans since neotenic axolotls also presented active hematopoiesis in the spleen (DEMIRCAN *et al*., 2016).

Apart from the lymphoid and hematopoietic functions, Borgioli and Frangioni (1997) had reported that the spleen of the Italian crested newt, Triturus carnifex, also performs a physiological compensation mechanism that helps to regulate their oxygen supply. When the newts are in repose and well-oxygenated, their spleen accumulates up to 50% of the red blood cells, releasing them into the circulation in case of hypoxic environment, or if metabolic needs get increased (FRANGIONI; BORGIOLI, 1989, 1993).

Although these studies have clarified primary mechanisms, which have great importance for the splenic function of amphibians, the comparative physiology of this organ still remains poorly studied, especially for non-model species and neotropical species of amphibians that may present interesting physiological adjustments to the environment. Deepening the knowledge about the splenic function of different species of amphibians could bring great insights for research on pathology and health monitoring of these organisms, so much research still needs to be done ahead.

2.3.4. The amphibian spleen: anatomy, histomorphology and its use as biomarker

2.3.4.1. Anatomy and histomorphology of the amphibian spleen

The amphibian spleen is a single lymphoid organ with oval or spherical shape in anurans, and elongate in apoda and caudata (MANNING, 1991); with colors that vary between red and dark brown (AKAT, 2018; FRANCO-BELUSSI; OLIVEIRA, 2016). This organ is located in the abdominal cavity, associated with the mesentery of the small intestine, and externally encapsulated by a connective tissue (FRANCO-BELUSSI; OLIVEIRA, 2016; LAMETSCHWANDTNER *et al.*, 2016; UDROIU; SGURA, 2017; AKAT, 2018). In most anurans, this connective tissue does not enter into the organ, so they don't present a trabecular structure throughout the parenchyma (FRANCO-BELUSSI; OLIVEIRA, 2016; LAMETSCHWANDTNER *et al.*, 2016; AKAT, 2018). However, a trabecular structure was recognized in the yellow-bellied toad (*Bombina variegata*) (DULAK, 1990), in the fire-bellied toad (*Bombina orientalis*) (DULAK *et al.*, 1993) and in the axolotl (*Ambystoma mexicanum*) (DEMIRCAN *et al.*, 2016), which indicates that multiple evolutionary pressures may have generated distinct morphological patterns among amphibian species.

For several anurans (FRANCO-BELUSSI; OLIVEIRA, 2016; LAMETSCHWANDTNER *et al.*, 2016; UDROIU AND SGURA, 2017; AKAT, 2018) and for the axolotl *Ambystoma Mexicanum* (DEMIRCAN *et al.*, 2016), the splenic parenchyma can be distinguished into two functional compartments: the red pulp and the white pulp (ALVAREZ, 1990; KINNEY *et al.*, 1996; BRICKER *et al.*, 2012; DEMIRCAN *et al.*, 2016; FRANCO-BELUSSI; OLIVEIRA, 2016; LAMETSCHWANDTNER et al., 2016; AKAT, 2018) (Fig. 6).

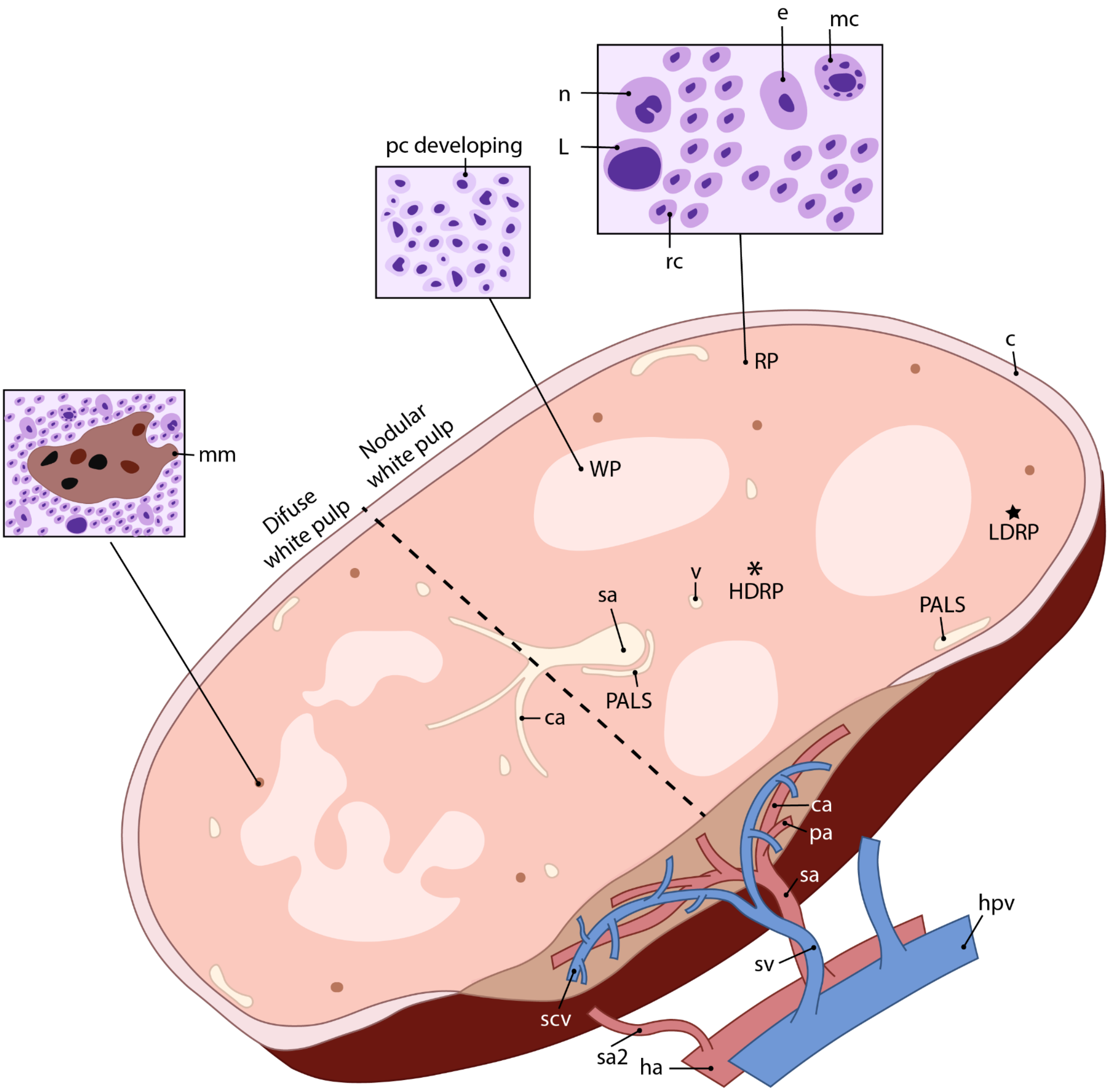

Figure 6. Schematic illustration of the amphibian spleen anatomy and histomorphology. Abbreviations: ca (central arteries); c (capsule); e (erythrocyte); ha (hemorrhoidal artery); hdrp (high density red pulp); hpv (hepatic portal vein); L (Lymphocyte); ldpr (lower density red pulp); mc (mast cell); mm (melanomacrophage); n (neutrophil); pa (penicillar arteries); PALS (periarterial lymphoid sheaths); pc (plasma cells); rc (reticular cells); rp (red pulp); sa (splenic artery); sa2 (second smaller splenic artery); scv (subcapsular vein); sv (splenic vein); v (vein); wp (white pulp).

The white pulp consists of clusters of lymphopoietic tissue (KINNEY *et al.*, 1996; FRANCO-BELUSSI; OLIVEIRA, 2016; AKAT, 2018), and it is also where the nerve network of the spleen can be found (KINNEY *et al.*, 1996). The red pulp, also named as the venous zone, is formed by chords and splenic sinuses filled mostly with erythrocytes, in addition to reticular cells, macrophages, plasma cells, lymphocytes, erythroid precursors and platelets (ALVAREZ, 1990; BRICKER *et al.*, 2012; DEMIRCAN *et al.*, 2016). This portion can be didactically divided into a region with higher cell density (medullary region) and another with more sparse cells (cortical region) (FRANCO-BELUSSI AND OLIVEIRA, 2016).

The white and red pulp can be gradually distinguishable throughout the metamorphosis process (KINNEY *et al.*, 1996; GRANT *et al.*, 1998; BLEYZAC *et al.*, 2005; LAMETSCHWANDTNER *et al.*, 2016) and it usually presents two different spatial distributions: nodular or diffuse. For *Lithobates catesbeianus* and *Pelophylax bedriagae*, which have diffuse architecture, the distinction of the pulps is not much pronounced, even in adults (BRICKER *et al.*, 2012; AKAT, 2018). For *Physalaemus nattereri*, a species that also shows diffuse cytoarchitecture, , in addition to the pulps being distinguishable, it is possible to observe subdivisions of the red pulp into medullary and cortical regions, even if the distribution pattern is diffuse (FRANCO-BELUSSI; OLIVEIRA, 2016). In species that present a nodular architecture, such as *Bufo sp*. and *Xenopus sp.*, the pulps have very clear boundaries, lymphoid tissue is distributed around a central artery in a very distinct way through the parenchyma (BARRUTIA *et al.*, 1983; DIENER; NOSSAL, 1966; BRICKER *et al.*, 2012; LAMETSCHWANDTNER *et al.*, 2016).

Species with more primitive characteristics may have less developed white pulp than more derived species (UDROIU; SGURA, 2017). Another taxonomic feature that can be observed by splenic histomorphology is the cytoarchitecture itself: the diffusely organized parenchyma is considered a basal feature (COOPER; WRIGHT 1976; FRANCO-BELUSSI; OLIVEIRA, 2016). In the table below, the species and their main histomorphological characteristics are summarized (Table 1).

Table 1 : Spleen histomorphology

Reference	Species	Family	Trabecula	Cytoarchitecture
Demircan *et al.*, 2016	*Ambystoma Mexicanum*	Ambystomatidae	Yes	Diffuse
Dulak *et al.*, 1993	*Bombina orientalis*	Bombinatoridae	Yes	Nodular
Dulak, 1990	*Bombina variegata*	Bombinatoridae	Yes	Nodular
Barrutia *et al.*, 1983	*Bufo calamita*	Bufonidae	No	Nodular
Diener & Nossal, 1966	*Bufo marinus*	Bufonidae	No	Nodular
Bricker *et al.*, 2012	*Lithobates catesbeianus*	Ranidae	No	Diffuse
Jordan & Seidel 1923	*Lithobates catesbeianus*	Ranidae	No	Diffuse
Akat, 2018	*Pelophylax bedriagae*	Ranidae	No	Diffuse
Franco Belussi & Oliveira, 2016	*Physalaemus nattereri*	Leptodactylidae	No	Diffuse
Alvarez, 1990	*Rana perezi*	Ranidae	No	Diffuse
Baldwin & Cohen, 1981	*Xenopus laevis*	Pipidae	No	Nodular
Bricker *et al.*, 2012	*Xenopus laevis*	Pipidae	No	Nodular
Hadji-Azimi *et al* 1987	*Xenopus laevis*	Pipidae	No	Nodular
Kinney *et al.*, 1996	*Xenopus laevis*	Pipidae	No	Nodular
Lametschwandtner *et al.*, 2016	*Xenopus laevis*	Pipidae	No	Nodular
Turner & Manning, 1973	*Xenopus laevis*	Pipidae	No	Nodular

Main histomorphological characteristics of the described species evaluated

Scattered throughout the hepatic parenchyma or organized in clusters, it is possible to observe melanomacrophage cells in the amphibian spleen. These are pigmented cells present in many visceral organs of amphibians, such as the liver, the kidneys, but also the spleen (SICHEL *et al.*, 1997; FRANCO-BELUSSI; OLIVEIRA, 2016). These cells are resident macrophages derived from hematopoietic stem cells and that are similar to Kupffer cells (SICHEL *et al.*, 1997). Their morphology is elliptical with dark brown colors due to the catabolic pigments found in these structures, mainly hemoglobin, lipofuscin, and hemosiderin, in addition to melanin inside the melanosomes (AGIUS; AGBEDE, 1984; HERRAEZ; ZAPATA, 1991; BARNI *et al.*, 2002). Melanomacrophage cells had been found scattered across the red pulp and/or near the splenic capsule in many amphibian species such as *Bufo calamita* (BARRUTIA *et al.*, 1983), *Pelophylax perezi* (ALVAREZ, 1990), *Bufo marinus* (DIENER; NOSSAL, 1996), *Pelophylax esculentus* (Rana esculenta) (GALLONE *et al.*, 2002), *L. catesbeianus* (BRICKER *et al.*, 2012), *X. laevis* (ZUASTI *et al.*, 1998; BRICKER *et al.*, 2012), *Physalaemus nattereri* (FRANCO-BELUSSI; OLIVEIRA, 2016), and *Pelophylax bedriagae* (AKAT, 2018). The frequency of these cells in the tissue might indicate detoxifying and immunological responses of the spleen to various factors such as stress, pollutants or pathogens. These morphological characteristics can provide insights of great relevance for studies of environmental health monitoring.

2.3.4.2. The amphibian spleen as a source of biomarkers in pathology

One of the major factors that has been aggravating the decline of natural amphibian populations is the increasing outbreaks of infectious diseases (CAREY *et al.*, 1999). In that sense, elucidating the pathophysiological roles of the amphibian spleen becomes especially relevant, since this organ can be intrinsically linked to immune responses to bacterial, viral and fungal pathologies (SPEARE *et al.*, 1997; FORZAN *et al.*, 2015; FORT *et al.*, 2016).

In this review, a major number of pathological studies have focused on the effects caused by bacterial diseases, mainly those related to *Mycobacterium* gender. Infected individuals, in addition to having relatively enlarged spleens, presented tissue lesions, necrosis, granulomas, white pulp reduction and an increased occurrence of melanomacrophages associated with bacteria infesting the splenic tissue (TARIGO *et al.*, 2006; FREMONT-RAHL *et al.*, 2011; HARIDY *et al.*, 2014; FORT *et al.*, 2016; LI *et al.*, 2017). Other bacteria such as *Elizabethkingia miricola* and *Pseudomonas aeruginosa* caused changes in cell density and splenic size in infected individuals (BRODKIN *et al.*, 1992; HUANG *et al.*, 2019).

Diseases caused by viruses lead to responses at a macroscopic level, such as hypertrophies (FORZÁN *et al.*, 2015), necrotic lesions, abscesses, hemorrhages and cellular changes (CULLEN *et al.*, 1995; CHENG *et al.*, 2014; MENG *et al.*, 2014; FORZÁN *et al.*, 2015). Changes in the spleen´s cytoarchitecture have also been reported in the face of viral pathologies, with emphasis on increased hematopoietic tissue and reduced occurrence of melanomacrophage cells (FORZÁN *et al.*, 2015). In addition to anatomical and histomorphological changes, it is also possible to assess immune responses and gene expression rates related to defense cells (GRAYFER *et al*., 2014; MORALES; ROBERT, 2007).

With regard to pathologies caused by fungi, many variables can influence the outcomes of the disease such as the load of infection, time and temperature (RIBAS *et al.*, 2009). In amphibians infected by the chytrid fungus, *Batrachochytrium dendrobatidis*, cell density and splenosomatic index were not significantly altered by the disease (PAETOW *et al*., 2012; BRANNELLY *et al.*, 2016), however there was an increase in proliferation of defense cells in the innate immune system (RIBAS *et al.*, 2009). The infection by the fungus *Mucor amphibiorum* reduced the pigmentation, enlarged the spleen, and increased the presence of granulomas in the splenic parenchyma (BERGER *et al.*, 1997; SPEARE *et al.*, 1997). Studies with Microsporidia, an intracellular parasitic fungi, caused a reduction in the spleen relative size and the occurrence of white spots on the splenic surface (PAPERNA; LAINSON, 1995).

These studies indicate that the spleen can play a crucial role in the immune responses of amphibians against different pathologies, acting from hematopoietic cell proliferation to local inflammatory responses. Despite the rare use of the amphibian spleen as a source for sensitive lymphoid and hematopoietic biomarkers in pathological studies, these findings reiterate the relevance of this organ as an alternative source of samples for these areas of study. A table with the main studies that used splenic samples to evaluate amphibian pathologies can be found in the supplementary material (Table 2 - APÊNCIDE A).

2.3.4.3. The amphibian spleen as a source of biomarkers in ecotoxicology

Amphibians have a biphasic life cycle and depend on the aquatic environment for their reproduction (WAKE; KOO, 2018), therefore these animals can act as sensitive sentinels to environmental changes, especially to contaminants in aquatic ecosystems (PYASTOLOVA *et al*., 1996). The toxicity that each pollutant exerts on these organisms will depend on the kinetic pathways (absorption, transport and elimination), as well as the toxicodynamic interactions between the toxicant and the target cells or organs (CASARETT, 2008). Therefore, for toxicants whose action results in effects on the hematopoietic and/or immunological functions of amphibians, the spleen may represent an important target for studies to recognize the action pathways of these toxicants. Hereby we have summarized the main findings of how distinct splenic biomarkers were used to elucidate the effects of different environmental stressors and/or contaminants in amphibians.

In one of the oldest articles found in this review (TURNER; MANNING, 1973), researchers have described the effect of hemolytic toxins on amphibian spleens and highlighted several interesting biomarkers such as the monitoring of the spleen size, and histological observations like the frequency of pyroninophilic cells, and the presence of vacuolization of red pulp cells. These alterations indicated relevant routes through which the amphibian spleen could act in hematological recycling.

Later, Hayes *et al*. (1995) described a splenic involution in larvae of the toads Bufo boreas and Bombina orientalis when exposed to corticosterone. These results showed that spleen weight indices may be useful biomarkers to evaluate physiological stress responses, such as those caused by corticosterone. Another study conducted with hormones (FRANCO-BELUSSI; OLIVEIRA, 2016) demonstrated that the α-Melanocyte-stimulating hormone (α-MSH) did not change the pigmented areas of the splenic parenchyma, but increased the volume of hemosiderin and lipofuscin contained in melanomacrophage centers. These results shed light on how these hormones may be linked to the control of splenic pigmentation mechanisms, which could also be used as an interesting biomarker in ecotoxicology studies with endocrine disruptors.

The relative mass of the spleen and the evaluation of pigmented cells such as melanomacrophages also proved to be sensitive biomarkers to some drugs. The pharmaceutical drug carbamazepine decreased the spleen size and melanin pigmentation in *Rana dalmatina* (BÓKONY *et al*., 2020). In another anuran species, *Bufo bufo*, carbamazepine increased melanin pigmentation in the spleen while *Rana dalmatina* had a reduction in melanomacrophagic centers, which, associated with a reduction in relative mass, may point to a immune system suppression (BÓKONY *et al*., 2020).

The environmental monitoring of areas affected by metal contamination (LINZEY *et al*., 2003; MARQUES *et al*., 2009; PRIYADARSHANI *et al*., 2015; FORT *et al*., 2016; ROMANOVA *et al*., 2021, VASCONCELOS-TEIXEIRA *et al*., 2022), industrial waste

(SANTANA *et al*., 2021), pesticides (LINZEY *et al*., 2003) and land use (MCMURRY *et al*., 2009) have also reported the use of splenic biomarkers of amphibians who inhabit these areas to assess the effects of these potential stressors. Decreases of the white pulp were observed in *Rhinella marin*a, *Bufo marinus* and *Eleutherodactylus johnstonei* collected in contaminated environments (LINZEY *et al*., 2003; FORT *et al*., 2016). In vitro studies also showed a decrease of spleen cell proliferative response in Bufo marinus of polluted areas (LINZEY *et al*., 2003), which reinforces that immunological measurements can act as a wide range biomarker to evaluate the effects of distinct pollutants.

Studies with metals have also described the use of various splenic biomarkers. Although tests conducted with *Rana perezi* collected from an uranium mine did not report alterations in splenic biomarkers (MARQUES *et al*., 2009), other studies with *Pelophylax ridibundus* and *Pelophylax lessonae* detected bioaccumulation of Cr and Fe in the spleen (ROMANOVA *et al*., 2021). This demonstrates that although the spleen does not represent an organ of frequent bioaccumulation of metals in amphibians, these studies raise a possible supporting role in the processes of bioaccumulation for some species.

The splenosomatic index also proved to be an excellent biomarker for studies with metals. Tests with *Euphlyctis hexadactylus* (Ranidae) showed a decrease of the splenossomatic index (SI; %) in urban wetlands contaminated with metals, suggesting immunotoxicity of the local residues (PRIYADARSHANI *et al.* 2015). *Rhinella diptycha* specimens collected from areas with high levels of contamination showed reduced spleens, which indicates an immunosuppressive effect (VASCONCELOS-TEIXEIRA *et al*., 2022). Similar results have also been observed in *Spea bombifrons* and *Spea multiplicata* collected in areas with different levels of pollution (MCMURRY *et al*., 2009). In contrast to these results, studies conducted in areas with historical industrial contamination, including fertilizer, cement, cellulose, chemical and petrochemical factories, reported significantly larger spleens in *Rhinella ornata* individuals than those living in places with less anthropic disturbance, suggesting an increase in the immunological responses of theses animals (SANTANA *et al*., 2021). Such variations indicate that the metals can act on the adjustment mechanisms of the amphibian spleen, leading to hypertrophic or atrophic responses, depending on the chemical nature of the toxicant.

Metals have also been tested in controlled laboratory exposures (ARRIETA *et al*., 2004; DOBROVOLJC *et al*., 2012). Under these conditions, although no changes were observed in splenic biomarkers of the species *Bufo arenarum*, *Bufo bufo* and *Necturus* *maculosus* (ARRIETA *et al.*, 2004; DOBROVOLJC *et al.*, 2012), there was splenic accumulation of lead in *Bufo arenarum* (ARRIETA *et al.*, 2004) and cadmium accumulation in *Necturus maculosus* (DOBROVOLJC *et al.*, 2012). Also, in *Bufo bufo* specimens exposed to Cd, Cu, Zn, Se and metallothioneins there was no splenic bioaccumulation (DOBROVOLJC *et al*., 2012). For those studies, the spleen was not their main target organ for bioaccumulation of the isolated metals, while other organs such as the liver and kidneys had more pronounced responses (ARRIETA *et al.*, 2004). Nevertheless, in most cases, amphibians collected in environments contaminated by metals revealed splenic responses (LINZEY *et al.*, 2003; MCMURRY *et al*., 2009; PRIYADARSHANI *et al*., 2015; FORT *et al.*, 2016; SANTANA *et al*., 2021; VASCONCELOS-TEIXEIRA *et al*., 2022), suggesting that the splenic responses can be related to synergic interactions with other compounds or environmental variables.

Agrochemicals are among the main drivers of amphibian declines worldwide (KENKO, 2022). For this class of contaminants, the amphibian spleen may represent an important source for elucidating the toxicity routes of different compounds. Studies like Rollins-Smith (2004) revealed that the pesticide Diazinon interfered with hematopoiesis by reducing the contribution of stem cells, which suggests that this pollutant can damage the developing immune system of the animals. To evaluate the toxicity of the insecticide deltamethrin in tiger salamanders (*Ambystoma tigrinum*), Froese *et al.,* (2009) applied the functional phagocytosis assay to measure the ability of neutrophils to protect from pathogens, and the mitogen assay to evaluate the proliferation rate of B lymphocytes, so these tests could predict the potential action of the toxicant on the immunological performance of organisms. Cytotoxicity routes were also studied through histopathological biomarkers in the spleen of *Pelophylax bedriagae* exposed to the insecticide carbaryl (ÇAKICI, 2018). In this study, the author described a dose-dependent degenerative alteration in the splenic tissue. The main alterations were vascular, as well as hemorrhage, enlargement of sinusoids, besides fibrosis and an increase of melanomacrophage cells (ÇAKICI, 2018).

Meanwhile, although deltamethrin did not trigger splenic alterations in *L. catesbeianus*, the 2,4-D (DMA® 806) significantly reduced the relative size of their spleen (VIRIATO *et al*., 2021). In *Lithobates pipiens*, the herbicides atrazine and glyphosate also did not cause alterations in the spleen, even if combined with the infection of the chytrid fungus (PAETOW *et al*., 2012). This indicates that the relative sensitivity of each species can also be a determining factor for the splenic responses of amphibians to different toxicants.

Another important toxicokinetic feature to consider is the fact that one single substance can cause opposite effects in the same species, depending on the route of exposure. Awaad (2020) tested the effects of iron oxide nanoparticles on the splenic tissue of *Bufo regularis reuss* through different routes. While oral injections of the nanoparticles led to an increase in the occurrence of melanomacrophage cells, peritoneal injections caused a reduction of these cells in the splenic parenchyma. These results highlight the importance of considering different exposure routes before choosing the biomarkers and endpoints to evaluate splenic alterations in amphibians.

Amphibians have increasingly been used as bioindicator organisms for assessing environmental quality (HOPKINS, 2007; WOMACK et al., 2022). Thus, splenic biomarkers such as the splenosomatic index, tissue injuries, fluctuations in the pigmentation of the organ and pigmented regions of the splenic parenchyma can act as useful tools for a comprehensive assessment of the health of amphibian individuals and populations. A summary of these studies and the main responses of the spleen to distinct pollutants were listed in supplementary material (Table 3 - APÊNDICE A).

2.3.4.4. Splenic biomarkers in response to environmental changes and physiological stress

Amphibians are ectothermic animals that present high sensitivity to environmental changes, such as temperature shifts (BODENSTEINER et al., 2021), oxygenation (PHILLIPS et al., 2022), and pH levels of the water (SCARAMELLA et al., 2022). Changes in these conditions can affect different physiological and homeostatic mechanisms in amphibians, including the splenic function itself. Thus, this session was devoted to describing the main findings concerning the splenic responses of amphibians to environmental changes.

In salamanders, the spleen has a compensatory mechanism that responds to situations in which the temperature increases or oxygen is scarce. In ideal situations, the spleen acts as an auxiliary blood reserve. However, when external conditions require more efficient tissue oxygenation, the spleen releases red blood cells into the bloodstream, therefore reducing the size of the organ (FRANGIONI; BORGIOLI, 1989; 1991; 1991b; 1993; 1996; BORGIOLI; FRANGIONE, 1997; FRANGIONI et al., 1997). However, this compensatory strategy was not observed in *Bufo woodhousii* (MALVIN et al., 1995), which indicates that these mechanisms were not evolutionarily conserved in anurans.

Stressful conditions related to pH changes usually require many physiological adjustments to ensure the maintenance of internal homeostasis (MALTBY, 1999). Studies with *Spea multiplicata* showed that reductions in water supply provided to froglets did not generate responses in the spleen (BAGWILL et al., 2016), but lowering pH levels altered cell density and efficiency of the immune responses, making them more susceptible to bacterial infections (BRODKIN et al., 2003; SIMON et al., 2002).

In addition to environmental physical factors, the stress generated by restraint during handling and transport can also affect amphibian responses. Indeed, Vasconcelos-Teixeira et al. (2022) found evidence that handling and transport of the amphibians can raise corticosterone levels, reducing their immune splenic responses. Eventually, wound healing processes also generate responses in the spleen. Franchini et al. (2016) found that central splenic artery dilation was associated with melanomacrophagic centers, increased mitosis, apoptosis, in addition to organ enlargement 14 days after injury events in *Xenopus laevis*. Experimental scenarios such as maintaining amphibians under laboratory conditions have also shown to reduce the spleen size of the animals, increased the occurrence of melanomacrophage cells, increased the connective tissue and reduced white pulp lymphocytes (DULAK; PLYTYCZ, 1989). These data show that the spleen is very sensitive to fluctuations in physicochemical parameters. The search for new ways to keep these organisms in captivity, with better conditions, as well as the development of new less invasive and stressful methodologies for studying amphibians still need to be encouraged among scientists. Thus, it is extremely relevant to consider the possible influence of all these variables on the results of an experiment, to guarantee greater reliability to the results of any research in herpetology. A summary of the main articles concerning amphibian splenic responses to physical stress and environmental changes can be observed in supplementary material (Table 4 - APÊNDICE A).

Overall, the various biomarkers provided by the spleen can provide interesting answers about the effects of diseases, environmental and physical changes. The analysis of splenic samples enables the evaluation of a wide range of biological levels, from macroscopic effects, with the use of parameters like the splenosomatic index and external pigmentation, to microscopic changes such as vascular alterations, cellular and cytoarchitecture disturbances of the splenic tissue, including apoptosis, fibrosis, necrosis, vacuolar degeneration, presence of granulomas, frequency of leucocytes, hemorrhagic areas, among others. A summary of the splenic biomarkers described in the articles can be observed in Figure 7. The study of splenic biomarkers can be used and interpreted in isolation to provide specific information on toxicity pathways of distinct toxicants. However, we recommend a reduction in the number of individuals allied to an increase in the number of organs and biomarkers for the composition of a more complete diagnosis, with more comprehensive responses requiring fewer euthanized animals. As mentioned above, improving transport and maintenance conditions of the animals still need to be addressed and improved in herpetological research, so that these parameters do not interfere with the results. Furthermore, amphibians are sentient beings (LAMBERT et al., 2022), so scientific society still needs to prioritize and encourage alternative methods of animal experimentation in order to refine environmental monitoring and provide more ethical research.

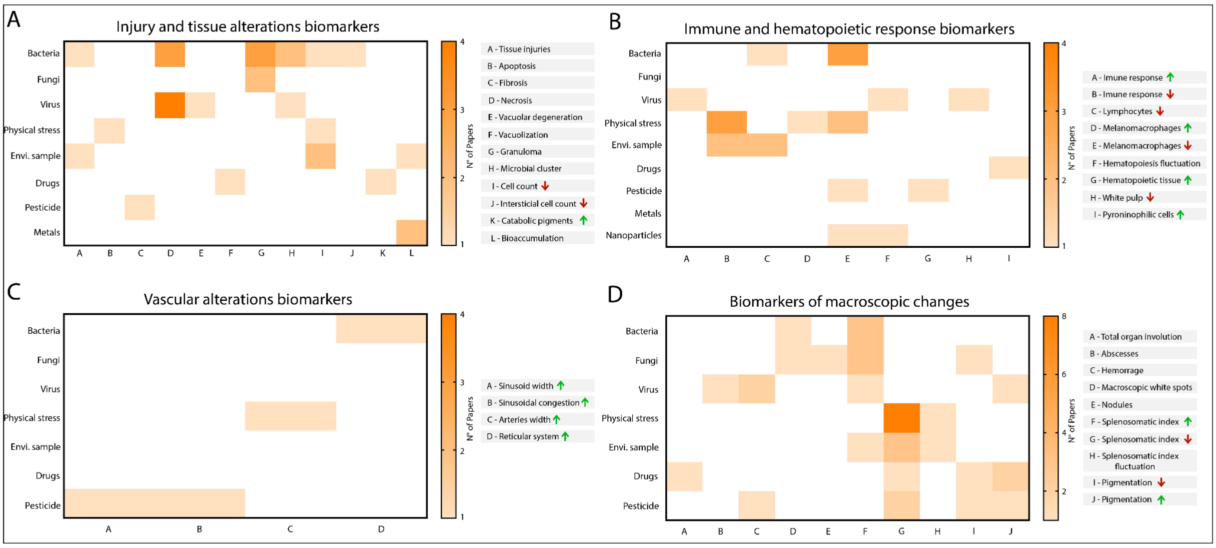

Figure 7. Number of studies focused on investigating injury and tissue alterations biomarkers **(A)**, immune and hematopoietic response biomarkers **(B)**, vascular alteration biomarkers **(C)**, and biomarkers of macroscopic changes **(D)** on amphibian spleen after exposure to microorganisms, pollutants, and after physical stress. Abbreviations: Envi. (environmental).

2.4 Conclusions and Knowledge Gaps

In Summary, more than 85% of the studies obtained significant responses using different biomarkers provided by the amphibian spleen. This demonstrates that this organ is very sensitive and also has a wide phenotypic plasticity, providing information for research in pathology, ecotoxicology, and environmental monitoring. Despite this efficiency, our systematic review showed that the spleen is still an underestimated sample in herpetological research, especially as a source of biomarkers (Fig 8).

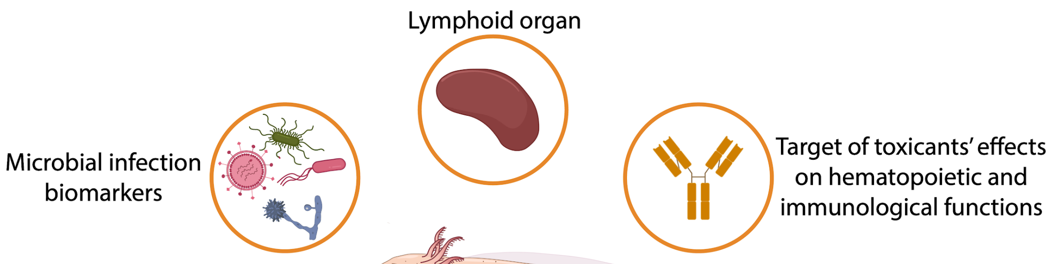

Figure 8. Graphical overview summarizing the main findings and information about the amphibian spleen as a source of biomarkers.

One of the main gaps in this field of knowledge is the lack of morphological descriptive work on the splenic tissue of amphibians with emphasis on their compositions and specific characterization of intraspecific particularities. Studies that describe structural densities of the components, as well as of the immune cells of the spleen for different amphibian species along the developmental stages should be encouraged.

REFERENCES

AGIUS, C.; AGBEDE, S. A. An electron microscopical study on the genesis of lipofuscin, melanin and haemosiderin in the haemopoietic tissues of fish. **Journal of Fish Biology**, v. 24, p. 471–488, 1984. Disponível em: https://doi.org/10.1111/j.1095-8649.1984.tb04818.x. Acesso em: 27 jun. 2023.

AKAT, E. Histological characteristics of the pancreas and spleen of Pelophylax bedriagae (Anura: Ranidae). **Biharean Biologist**, v. 12, p. 102–105, 2018.

ALVAREZ, R. An ultrastructural study of the spleen of the ranid frog Rana perezi. **Journal of Morphology**, v. 204, p. 25–32, 1990. Disponível em: https://doi.org/10.1002/jmor.1052040104. Acesso em: 27 jun. 2023.

AMPHIBIAWEB. Database accessible at https://amphibiaweb.org. University of California, Berkeley, CA, USA. Accessed 17 May 2023.

ARAÚJO, A. P. da C.; GOMES, A. R.; MALAFAIA, G. Hepatotoxicity of pristine polyethylene microplastics in neotropical physalaemus cuvieri tadpoles (Fitzinger, 1826). **Journal of Hazardous Materials**, v. 386, p. 121992, 2020. Disponível em: https://doi.org/10.1016/J.JHAZMAT.2019.121992. Acesso em: 27 jun. 2023.

ARRIETA, M. A. et al. Biosensors of Inorganic Lead Exposure and Effect in an Adult Amphibian. **Archives of Environmental Contamination and Toxicology**, v. 46, p. 224–230, 2004. Disponível em: https://doi.org/10.1007/s00244-003-2221-8. Acesso em: 27 jun. 2023.

AWAAD, A. The biodistribution of melanomacrophages and reactivity of PEG or amine-functionalized iron oxide nanoclusters in the liver and spleen of Egyptian toad after intraperitoneal or oral injections: Histochemical study. **Acta Histochemica**, v. 122, p. 151629, 2020. Disponível em: https://doi.org/10.1016/J.ACTHIS.2020.151629. Acesso em: 27 jun. 2023.

BAGWILL, A.L. et al. Effects of Water Loss on New Mexico Spadefoot Toad (Spea multiplicata) Development, Spleen Cellularity, and Corticosterone Levels. **Journal of**

Experimental Zoology Part A: Ecological Genetics and Physiology, v. 325, p. 548-561, 2016. DOI: https://doi.org/10.1002/JEZ.2049.

BARNI, S. et al. Mechanisms of changes to the liver pigmentary component during the annual cycle (activity and hibernation) of Rana esculenta L. **Journal of Anatomy**, v. 200, p. 185-194, 2002. DOI: https://doi.org/10.1046/j.0021-8782.2001.00011.x.

BARRUTIA, M.S.G. et al. Non-lymphoid cells of the anuran spleen: An ultrastructural study in the natterjack, Bufo calamita. **American Journal of Anatomy**, v. 167, p. 83-94, 1983. DOI: https://doi.org/10.1002/aja.1001670108.

BECK, C.W. and SLACK, J.M. An amphibian with ambition: a new role for Xenopus in the 21st century. **Genome Biology**, v. 2, p. 1-5, 2001. DOI: https://doi.org/10.1186/GB-2001-2-10-REVIEWS1029.

BERGER, L. et al. Mucormycosis in a Free-ranging Green Tree Frog from Australia. **Journal of Wildlife Diseases**, v. 33, p. 903-907, 1997. DOI: https://doi.org/10.7589/0090-3558-33.4.903.

BLAUSTEIN, A.R. et al. Effects of emerging infectious diseases on amphibians: A review of experimental studies. **Diversity**, v. 10, p. 1-21, 2018. DOI: https://doi.org/10.3390/d10030081.

BLEYZAC, P. et al. Morphological description of embryonic development of immune organs in Typhlonectes compressicauda (Amphibia, Gymnophiona). **Journal of Herpetology**, v. 39, p. 57-65, 2005. DOI: https://doi.org/10.1670/0022-1511(2005)039[0057:MDOEDO]2.0.CO;2.

BODENSTEINER, B.L. et al. Thermal adaptation revisited: How conserved are thermal traits of reptiles and amphibians? **Journal of Experimental Zoology Part A: Ecological and Integrative Physiology**, v. 335, p. 173-194, 2021. DOI: https://doi.org/10.1002/JEZ.2414.

BÓKONY, V. et al. Effects of two little-studied environmental pollutants on early development in anurans. **Environmental Pollution**, v. 260, 114078, 2020. DOI: https://doi.org/10.1016/j.envpol.2020.114078.

BORGELT, J., DORBER, M., HØIBERG, M.A., et al. More than half of data deficient species predicted to be threatened by extinction. **Commun. Biol**. 5, 1–9. https://doi.org/10.1038/s42003-022-03638-9

BORGIOLI, G., FRANGIONI, G. Blood and splenic respiratory compensation in larval newts. **Ital. J. Zool**. 64, 221–226. https://doi.org/10.1080/11250009709356200

BRANNELLY, L.A., WEBB, R.J., SKERRATT, L.F., et al. Effects of chytridiomycosis on hematopoietic tissue in the spleen, kidney and bone marrow in three diverse amphibian species. **Pathog. Dis**. 74, 1–10. https://doi.org/10.1093/femspd/ftw069

BRICKER, N.K., RASKIN, R.E., DENSMORE, C.L., et al. Cytochemical and immunocytochemical characterization of blood cells and immunohistochemical analysis of spleen cells from 2 species of frog, Rana (Aquarana) catesbeiana and Xenopus laevis. **Vet. Clin. Pathol**. 41, 353–361. https://doi.org/10.1111/j.1939-165X.2012.00452.x

BRITO, D. Amphibian conservation: Are we on the right track? **Biol. Conserv**. 141, 2912–2917. https://doi.org/10.1016/J.BIOCON.2008.08.016

BRODEUR, J.C., BAHL, M.F., NATALE, G.S., et al. Biomarker and hematological fieldwork with amphibians: is it necessary to sample all night? **Environ. Sci. Pollut. Res**. 27, 17152–17161. https://doi.org/10.1007/s11356-020-08313-2

BRODKIN, M.A., SIMON, M.P., DESANTIS, A.M., et al. Response of Rana pipiens to Graded Doses of the Bacterium Pseudomonas aeruginosa. **Society for the Study of Amphibians and Reptiles Stable**, 26, 490–495. https://doi.org/10.2307/1565128

BRODKIN, M., VATNICK, I., SIMON, M., et al. Effects of Acid Stress in Adult Rana pipiens. **J. Exp. Zool. Part A Comp. Exp. Biol**. 298, 16–22. https://doi.org/10.1002/jez.a.10270

BURGGREN, W.W., WARBURTON, S. Amphibians as animal models for laboratory research in physiology. ILAR J. 48, 260–269. https://doi.org/10.1093/ILAR.48.3.260

ÇAKICI, Ö. Histopathological analysis of carbaryl-induced toxicity in the spleen of Levantine frog, Pelophylax bedriagae (Anura: Ranidae). **Environ. Sci. Pollut. Res**. 25, 24917–24922. https://doi.org/10.1007/S11356-018-2538-5/TABLES/1

CALDERON, M.R., GONZÁLEZ, S.P., PÉREZ-IGLESIAS, J.M., JOFRÉ, M.B. Anthropogenic impacts on rivers: use of multiple indicators to assess environmental quality status. **Hydrobiologia** 850, 469–487. https://doi.org/10.1007/s10750-022-05090-6

CAREY, C., COHEN, N., ROLLINS-SMITH, L. Amphibian declines: An immunological perspective. **Dev. Comp. Immunol**. 23, 459–472. https://doi.org/10.1016/S0145-305X(99)00028-2

CASARETT, L.J. Casarett and Doull's toxicology: the basic science of poisons (Vol. 71470514). New York: McGraw-Hill, 2008.

CHENG, K., JANCOVICH, J.K., BURCHELL, J., SCHRENZEL, M.D., REAVILL, D.R., IMAI, D.M., KIRKENDALL, M., WOODS, L.W., PESSIER, A.P. Isolation of a Bohle-like iridovirus from boreal toads housed within a cosmopolitan aquarium collection. **Dis. Aquat. Organ**. 111, 139–152. https://doi.org/10.3354/DAO02770

CLOTHIER, R.H., RUBEN, L.N., JOHNSON, R.O., PARKER, K., SOVAK, M., GREENHALGH, L., OOI, E.E., BALLS, M. Neuroimmunomodulation: Neuroendocrine regulation of immunity: The effects of noradrenaline in Xenopus laevis, the South African clawed toad. **Int. J. Neurosci.** 62, 123–140. https://doi.org/10.3109/00207459108999766

COOPER, E.L., WRIGHT, R.K. The anuran amphibian spleen. An evolutionary model for terrestrial vertebrates. In: Battisto JR, Streilein JW, editors. Immuno-Aspects of the Spleen. Amsterdam: **Elsevier North-Holland Biomedical Press**, 1976, pp. 47–58.

CULLEN, B.R., OWENS, L., WHITTINGTON, R.J. Experimental infection of Australian anurans (Limnodynastes terraereginae and Litoria latopalmata) with Bohle iridovirus. **Dis.**

Aquat. Organ. 23, 83–92. https://doi.org/10.3354/DAO023083

CUMANO, A., GODIN, I. Ontogeny of the hematopoietic system. **Annu. Rev. Immunol**. 25, 745–785. https://doi.org/10.1146/annurev.immunol.25.022106.141538

DE ABREU MANSO, P.P., DE BRITO-GITIRANA, L., PELAJO-MACHADO, M. Localization of hematopoietic cells in the bullfrog (Lithobates catesbeianus). **Cell Tissue Res**. 337, 301–312. https://doi.org/10.1007/S00441-009-0803-0/FIGURES/15

DEMIRCAN, T., İLHAN, A.E., AYTÜRK, N., YILDIRIM, B., ÖZTÜRK, G., KESKIN, İ. A histological atlas of the tissues and organs of neotenic and metamorphosed axolotl. **Acta Histochem**. 118, 746–759. https://doi.org/10.1016/j.acthis.2016.07.006

DIENER, E., NOSSAL, G.J. V. Phylogenetic Studies on the Immune Response I. Localization Of Antigens And Immune Response In The Toad, Bufo Marinus. **Immunology** 10, 535, 1966.

DING, G.H., WANG, Z.Y., CHEN, J.Y., WU, Q.Q., ZHOU, Y.N. Comprehensive assessment of the ecological risk of exposure to triphenyl phosphate in a bioindicator tadpole. **Chemosphere** 308. https://doi.org/10.1016/j.chemosphere.2022.136242

DOBROVOLJC, K., FALNOGA, I., ŽNIDARIČ, M.T., MAZEJ, D., ŠČANČAR, J., BULOG, B. Cd, Cu, Zn, Se, and metallothioneins in two amphibians, Necturus maculosus (Amphibia, Caudata) and Bufo bufo (Amphibia, Anura). **Biol. Trace Elem. Res**. 150, 178–194. https://doi.org/10.1007/s12011-012-9461-2

DUELLMAN, W.E. Global distributions of amphibians: patterns, conservation and future challenges. In: Duellman, W.E. (Ed.), Patterns of Distribution of Amphibians: A Global Perspective. **Johns Hopkins University Press**, Baltimore, pp. 1–30, 1999.

DU PASQUIER, L., HAIMOVICH, J. The antibody response during amphibian ontogeny. **Immunogenetics** 3, 381–391, 1976. https://doi.org/10.1007/BF01576969

DUCOROY, P., LESOURD, M., PADROS, M.R., TOURNEFIER, A. Natural and induced apoptosis during lymphocyte development in the axolotl. **Dev. Comp. Immunol**. 23, 241–252, 1999. https://doi.org/10.1016/S0145-305X(99)00008-7

DULAK, J. The architecture of the spleen of the yellow-bellied toad, Bombina variegata. **Zool. Soc**. London 37, 1990–1991, 1990.

DULAK, J., BAUER, J., DÖPP, E.A., VAN ROOIJEN, N. Immune-complex-trapping cells in the spleen of the oriental fire-bellied toad, Bombina orientalis. **Cell Tissue Res**. 271, 155–158, 1993. https://doi.org/10.1007/BF00297553

DULAK, J., PŁYTYCZ, B. The effect of laboratory environment on the morphology of the spleen and the thymus in the yellow-bellied toad, Bombina variegata (L.). **Dev. Comp. Immunol**. 13, 49–55, 1989. https://doi.org/10.1016/0145-305X(89)90016-5

FAZEY, I., FISCHER, J., LINDENMAYER, D.B. Who does all the research in conservation biology? **Biodivers. Conserv.** 14, 917–934, 2005. https://doi.org/10.1007/S10531-004-7849-9/METRICS

FELTEN, D.L., FELTEN, S.Y. Sympathetic noradrenergic innervation of immune organs. **Brain Behav. Immun**. 2, 293–300, 1988. https://doi.org/10.1016/0889-1591(88)90031-1

FERRIER, V., GAUTHIER, L., ZOLL-MOREUX, C., L'HARIDON, J. Genotoxicity Tests in Amphibians — A Review. Microscale Test. **Aquat. Toxicol**. 507–519, 2018. https://doi.org/10.1201/9780203747193-35

FISHER, M.C., PASMANS, F., MARTEL, A. Virulence and Pathogenicity of Chytrid Fungi Causing Amphibian Extinctions. **Annu. Rev. Microbiol**. 75, 673–693, 2021. https://doi.org/10.1146/annurev-micro-052621-124212

FORT, D.J., MATHIS, M., FORT, C.E., FORT, H.M., FORT, T.D., LINZEY, D.W., BACON, J.P. Splenic immunotoxicity in developing cane toads (Rhinella marina) from Bermuda. **Environ. Toxicol. Chem**. 35, 2604–2612, 2016. https://doi.org/10.1002/ETC.3431

FORZÁN, M.J., JONES, K.M., VANDERSTICHEL, R.V., WOOD, J., KIBENGE, F.S.B., KUIKEN, T., WIRTH, W., ARIEL, E., DAOUST, P.Y. Clinical signs, pathology and dose-dependent survival of adult wood frogs, Rana sylvatica, inoculated orally with frog virus 3 (Ranavirus sp., iridoviridae). **J. Gen. Virol**. 96, 1138–1149, 2015. https://doi.org/10.1099/vir.0.000043

FRANCHINI, A., DELLA ROCCA, A., BERTOLOTTI, E. The spleen and skin wound healing in Xenopus adults. **J. Morphol**. 277, 888–895, 2016. https://doi.org/10.1002/jmor.20542

FRANCO-BELUSSI, L., DE OLIVEIRA, C. The spleen of Physalaemus nattereri (Amphibia: Anura): morphology, melanomacrophage pigment compounds and responses to α-melanocyte stimulating hormone. **Ital. J. Zool**. 83, 298–305, 2016. https://doi.org/10.1080/11250003.2016.1194488

FRANGIONI, G., BORGIOLI, G. Relationships between spleen and respiration in the newt. **J. Exp. Zool**. 252, 118–125, 1989. https://doi.org/10.1002/jez.1402520203

FRANGIONI, G., BORGIOLI, G. Effect of temperature on the splenic mechanism of respiratory compensation in the newt. **J. Exp. Zool**. 259, 316–323, 1991. https://doi.org/10.1002/jez.1402590306

FRANGIONI, G., BORGIOLI, G. Effect of spleen congestion and decongestion on newt blood. **J. Zool.** 223, 15–25, 1991. https://doi.org/10.1111/J.1469-7998.1991.TB04746.X

FRANGIONI, G., BORGIOLI, G. Splenic respiratory compensation and blood volume in the newt. **J. Zool**. 229, 505–514, 1993. https://doi.org/10.1111/J.1469-7998.1993.TB02651.X

FRANGIONI, G., BORGIOLI, G. Variations in the blood pressure of newts according to their respiratory conditions. **Ital. J. Zool**. 63, 201–206, 1996. https://doi.org/10.1080/11250009609356134

FRANGIONI, G., BRIZZI, R., BORGIOLI, G. Haematological changes by splenic respiratory compensation in the cave salamander, Hydromantes genei. **J. Zool**. 241, 175–183,

1997. https://doi.org/10.1111/j.1469-7998.1997.tb05507.x

FREMONT-RAHL, J.J., EK, C., WILLIAMSON, H.R., SMALL, P.L.C., FOX, J.G., MUTHUPALANI, S. Mycobacterium liflandii outbreak in a research colony of Xenopus (Silurana) tropicalis frogs. **Vet. Pathol.** 48, 856–867, 2011. https://doi.org/10.1177/0300985810388520

FROESE, J.M.W., SMITS, J.E.G., FORSYTH, D.J., WICKSTROM, M.L. Toxicity and immune system effects of dietary deltamethrin exposure in tiger salamanders (Ambystoma tigrinum). **J. Toxicol. Environ. Heal. - Part A** Curr. Issues 72, 518–526, 2009. https://doi.org/10.1080/15287390802706363

FROST, D.R. Amphibian Species of the World: an Online Reference. Version 6.1. Electronic Database accessible at https://amphibiansoftheworld.amnh.org/index.php. **American Museum of Natural History**, New York, USA, 2023. doi.org/10.5531/db.vz.0001

GALLONE, A., GUIDA, G., MAIDA, I., CICERO, R. Spleen and liver pigmented macrophages of Rana esculenta L. a new melanogenic system? **Pigment Cell Res.** 15, 32–40, 2002. https://doi.org/10.1034/j.1600-0749.2002.00052.x

GALVÃO-CASTRO, B., CORDEIRO, R.S.B., GOLDENBERG, S. Brazilian science under continuous attack. **Lancet** (London, England) 399, 23–24, 2022. https://doi.org/10.1016/S0140-6736(21)02727-6

GAVEL, M.J., YOUNG, S.D., BLAIS, N., FORBES, M.R., ROBINSON, S.A. Trematodes coupled with neonicotinoids: effects on blood cell profiles of a model amphibian. **Parasitol. Res**. 120, 2135–2148, 2021. https://doi.org/10.1007/s00436-021-07176-x

GLINSKI, D.A., HENDERSON, W.M., VAN METER, R.J., PURUCKER, S.T. Effect of hydration status on pesticide uptake in anurans following exposure to contaminated soils. **Environ. Sci. Pollut. Res.** 25, 16192–16201, 2018. https://doi.org/10.1007/s11356-018-1830-8

GONÇALVES, M.W., GAMBALLE, P.G., GODOY, F.R., ALVES, A.A., DE ALMEIDA REZENDE, P.H., DA CRUZ, A.D., MACIEL, N.M., NOMURA, F., BASTOS, R.P., DE MARCO, P., SILVA, D. DE M. The agricultural impact of pesticides on Physalaemus cuvieri tadpoles (Amphibia: Anura) ascertained by comet assay. **Zoologia** 34, 1–8, 2017. https://doi.org/10.3897/zoologia.34.e19865

GONÇALVES, M.W., DE CAMPOS, C.B.M., GODOY, F.R., GAMBALLE, P.G., NUNES, H.F., NOMURA, F., BASTOS, R.P., DA CRUZ, A.D., DE MELO E SILVA, D. Assessing Genotoxicity and Mutagenicity of Three Common Amphibian Species Inhabiting Agroecosystem Environment. **Arch. Environ. Contam. Toxicol.** 77, 409–420, 2019. https://doi.org/10.1007/S00244-019-00647-4/FIGURES/5

GRAINGER, R.M. Xenopus tropicalis as a Model Organism for Genetics and Genomics: Past, Present and Future. **Methods Mol. Biol.** 917, 3, 2012. https://doi.org/10.1007/978-1-61779-992-1_1

GRANT, P., CLOTHIER, R.H., JOHNSON, R.O., RUBEN, L.N. In situ lymphocyte apoptosis in larval Xenopus Laevis, the South African clawed toad. **Dev. Comp. Immunol.** 22, 449–455, 1998. https://doi.org/10.1016/S0145-305X(97)00057-8

GRAYFER, L., EDHOLM, E.S., ROBERT, J. Mechanisms of amphibian macrophage development: Characterization of the Xenopus laevis colony-stimulating factor-1 receptor. **Int. J. Dev. Biol**. 58, 757–766, 2014. https://doi.org/10.1387/ijdb.140271jr

HADIDI, S., GLENNEY, G.W., WELCH, T.J., SILVERSTEIN, J.T., WIENS, G.D. Spleen Size Predicts Resistance of Rainbow Trout to Flavobacterium psychrophilum Challenge. **J. Immunol**. 180, 4156–4165, 2008. https://doi.org/10.4049/jimmunol.180.6.4156

HADJI-AZIMI, I., COOSEMANS, V., CANICATTI, C. Atlas of adult Xenopus laevis laevis hematology. **Dev. Comp. Immunol.** 11, 807–874, 1987. https://doi.org/10.1016/0145-305X(87)90068-1

HARIDY, M., TACHIKAWA, Y., YOSHIDA, S., TSUYUGUCHI, K., TOMITA, M., MAEDA, S., WADA, T., IBI, K., SAKAI, H., YANAI, T. Mycobacterium marinum infection in Japanese forest green tree frogs (Rhacophorus arboreus). **J. Comp. Pathol.** 151, 277–289, 2014. https://doi.org/10.1016/J.JCPA.2014.04.014

HASEBE, T., OSHIMA, H., KAWAMURA, K., KIKUYAMA, S. Rapid and selective removal of larval erythrocytes from systemic circulation during metamorphosis of the bullfrog, Rana catesbeiana. **Dev. Growth Differ**. 41, 639–643, 1999. https://doi.org/10.1046/j.1440-169x.1999.00461.x

HAYES, T.B. Histological examination of the effects of corticosterone in larvae of the western toad, Bufo boreas (Anura: Bufonidae), and the oriental fire-bellied toad, Bombina orientalis (Anura: Discoglossidae). **J. Morphol.** 226, 297–307, 1995. https://doi.org/10.1002/JMOR.1052260306

HEREK, J.S., VARGAS, L., RINAS TRINDADE, S.A., RUTKOSKI, C.F., MACAGNAN, N., HARTMANN, P.A., HARTMANN, M.T. Genotoxic effects of glyphosate on Physalaemus tadpoles. **Environ. Toxicol. Pharmacol.** 81, 2021. https://doi.org/10.1016/J.ETAP.2020.103516

HERRAEZ, M.P., ZAPATA, A.G. Structure and function of the melano-macrophage centres of the goldfish Carassius auratus. **Vet. Immunol. Immunopathol.** 12, 117–126, 1986. https://doi.org/10.1016/0165-2427(86)90116-9

HOPKINS, W.A. Amphibians as models for studying environmental change. **ILAR J**. 48, 270–277, 2007. https://doi.org/10.1093/ILAR.48.3.270

HOULAAN, J.E., FIDLAY, C.S., SCHMIDT, B.R., MEYER, A.H., KUZMIN, S.L. Quantitative evidence for global amphibian population declines. **Nature** 404, 752–755, 2000. https://doi.org/10.1038/35008052

HUANG, X. et al. Candidate Animal Disease Model of Elizabethkingia Spp. Infection in Humans, Based on the Systematic Pathology and Oxidative Damage Caused by E. miricola in Pelophylax nigromaculatus. **Oxid. Med. Cell. Longev.**, v. 2019, 2019. Disponível em: https://doi.org/10.1155/2019/6407524. Acesso em: 2019.

IUCN SSC Amphibian Specialist Group | **IUCN**. Disponível em: https://www.iucn.org/our-union/commissions/group/iucn-ssc-amphibian-specialist-group. Acesso em: 30 abr. 2023.

JONES-COSTA, M. et al. Cardiac biomarkers as sensitive tools to evaluate the impact of xenobiotics on amphibians: the effects of anionic surfactant linear alkylbenzene sulfonate (LAS). **Ecotoxicol. Environ. Saf.**, v. 151, p. 184–190, 2018. Disponível em: https://doi.org/10.1016/J.ECOENV.2018.01.022. Acesso em: 2018.

JORDAN, H. E.; SPEIDEL, C. C. Studies on lymphocytes. I. Effect of splenectomy, experimental hemorrhage and a hemolytic toxin in the frog. **Am. J. Anat.**, v. 32, p. 155–187, 1923. Disponível em: https://doi.org/10.1002/AJA.1000320203. Acesso em: 1923.

JUNQUEIRA, L. C.; CARNEIRO, J. **Histologia básica**. 13ª edição. Rio de Janeiro: Guanabara Koogan, 2017.

KENKO, D. B. N. Effects of Pesticides on Amphibians and Tentative Solutions: Review. **J. Asian Sci. Res.**, v. 12, p. 218–236, 2022. Disponível em: https://doi.org/10.55493/5003.v12i4.4647. Acesso em: 2022.

KINNEY, K. S.; COHEN, N.; FELTEN, S. Y. Noradrenergic and peptidergic innervation of the amphibian spleen: Comparative studies. **Dev. Comp. Immunol.**, v. 18, p. 511–521, 1994. Disponível em: https://doi.org/10.1016/S0145-305X(06)80005-4. Acesso em: 27 jun. 2023.

KINNEY, K. S.; FELTEN, S. Y.; COHEN, N. Sympathetic innervation of the amphibian spleen: developmental studies in Xenopus laevis. **Dev. Comp. Immunol.**, v. 20, n. 1, p. 51-59, jan.-fev. 1996. DOI: 10.1016/0145-305X(95)00036-S. PMID: 8738936.

KINNEY, K. S.; COHEN, N. Increased splenocyte mitogenesis following sympathetic denervation in Xenopus laevis. **Dev. Comp. Immunol.**, v. 29, p. 287–293, 2005. Disponível em: https://doi.org/10.1016/j.dci.2004.08.001. Acesso em: 27 jun. 2023.

LAINSON, R.; PAPERNA, I.; NAIFF, R. D. Development of Hepatozoon caimani (Carini, 1909) Pessôa, De Biasi & De Souza, 1972 in the Caiman Caiman c. crocodilus, the frog Rana catesbeiana and the mosquito Culex fatigans. **Mem. Inst. Oswaldo Cruz**, v. 98, p. 103–113, 2003. Disponível em: https://doi.org/10.1590/S0074-02762003000100014. Acesso em: 27 jun. 2023.

LAMBERT, H.; ELWIN, A.; D'CRUZE, N. Frog in the well: A review of the scientific literature for evidence of amphibian sentience. **Appl. Anim. Behav. Sci.**, v. 247, p. 105559, 2022. Disponível em: https://doi.org/10.1016/J.APPLANIM.2022.105559. Acesso em: 27 jun. 2023.

LAMETSCHWANDTNER, A.; RADNER, C.; MINNICH, B. Microvascularization of the spleen in larval and adult Xenopus laevis: Histomorphology and scanning electron microscopy of vascular corrosion casts. **J. Morphol.**, v. 277, p. 1559–1569, 2016. Disponível em: https://doi.org/10.1002/jmor.20595. Acesso em: 27 jun. 2023.

LEIPOLD, S. et al. Discourse analysis of environmental policy revisited: traditions, trends, perspectives. **J. Environ. Policy Plan.**, v. 21, p. 445–463, 2019. Disponível em: https://doi.org/10.1080/1523908X.2019.1660462. Acesso em: 27 jun. 2023.

LI, W. T. et al. Mycolactone-producing Mycobacterium marinum infection in captive Hong Kong warty newts and pathological evidence of impaired host immune function. **Dis. Aquat. Organ.**, v. 123, p. 239–249, 2017. Disponível em: https://doi.org/10.3354/DAO03092. Acesso em: 27 jun. 2023.

LINZEY, D. W. et al. Role of environmental pollutants on immune functions, parasitic infections and limb malformations in marine toads and whistling frogs from Bermuda. **Int. J. Environ. Health Res.**, v. 13, p. 125–148, 2003. Disponível em: https://doi.org/10.1080/0960312031000098053. Acesso em: 27 jun. 2023.

LIU, L. S. et al. Research proceedings on amphibian model organisms. **Zool. Res.**, v. 37, p. 237, 2016. Disponível em: https://doi.org/10.13918/J.ISSN.2095-8137.2016.4.237. Acesso em: 27 jun. 2023.

LONDERO, J. E. L.; SANTOS, M. B. dos; SCHUCH, A. P. Impact of solar UV radiation on amphibians: focus on genotoxic stress. **Mutat. Res. Toxicol. Environ. Mutagen.**, v. 842, p. 14–21, 2019. Disponível em: https://doi.org/10.1016/J.MRGENTOX.2019.03.003. Acesso em: 27 jun. 2023.

MALTBY, L. Studying Stress: The Importance of Organism-Level Responses. **Ecol. Appl.**, v. 9, p. 431, 1999. Disponível em: https://doi.org/10.2307/2641131. Acesso em: 27 jun. 2023.

MALVIN, G. M. et al. Lymphatic regulation of hematocrit during hypoxia in the toad Bufo woodhousei. **Am. J. Physiol. - Regul. Integr. Comp. Physiol.**, v. 269. Disponível em: https://doi.org/10.1152/ajpregu.1995.269.4.r814. Acesso em: 27 jun. 2023.

MANIATIS, G. M.; INGRAM, V. M. ERYTHROPOIESIS DURING AMPHIBIAN METAMORPHOSIS I. Site of Maturation of Erythrocytes in Rana catesbeiana. **J. Cell Biol**., v. 49, p. 372–379, 1971. Disponível em: https://doi.org/10.1083/JCB.49.2.372. Acesso em: 27 jun. 2023.

MANNING, M. J. Histological organization of the spleen: implications for immune functions in amphibians. **Res. Immunol.**, v. 142, p. 355–359, 1991. Disponível em: https://doi.org/10.1016/0923-2494(91)90091-V. Acesso em: 27 jun. 2023.

MARQUES, S. M. et al. Histopathological changes and erythrocytic nuclear abnormalities in Iberian green frogs (Rana perezi Seoane) from a uranium mine pond. **Aquat. Toxicol.**, v. 91, p. 187–195, 2009. Disponível em: https://doi.org/10.1016/j.aquatox.2008.04.010. Acesso em: 27 jun. 2023.

MCCALLUM, M. L.; MCCALLUM, J. L. Publication trends of natural history and field studies in herpetology. **Herpetol. Conserv. Biol.**, v. 1, p. 62–67. Disponível em: https://www.herpconbio.org/Volume_1/Issue_1/McCallum_McCallum_2006.pdf. Acesso em: 27 jun. 2023.

MCCARTHY, J. F.; SHUGART, L. R. Biological Markers of Environmental Contamination. **Biomarkers Environ. Contam.**, p. 3–14, 2018. Disponível em:

https://doi.org/10.1201/9781351070263-2. Acesso em: 27 jun. 2023.

MCMURRY, S. T. et al. Influence of Land Use on Body Size and Splenic Cellularity in Wetland Breeding Spea spp. **J. Herpetol.**, v. 43, p. 421–430, 2009. Disponível em: https://doi.org/10.1670/07-126r2.1. Acesso em: 27 jun. 2023.

MENG, Y. et al. Pathological and microbiological findings from mortality of the Chinese giant salamander (Andrias davidianus). **Arch. Virol.**, v. 159, p. 1403–1412, 2014. Disponível em: https://doi.org/10.1007/S00705-013-1962-6. Acesso em: 27 jun. 2023.

MORALES, H. D.; ROBERT, J. Characterization of Primary and Memory CD8 T-Cell Responses against Ranavirus (FV3) in Xenopus laevis. **J. Virol.**, v. 81, p. 2240–2248, 2007. Disponível em: https://doi.org/https://doi.org/10.1128/JVI.01104-06. Acesso em: 27 jun. 2023

PAETOW, L. J. et al. Effects of herbicides and the chytrid fungus Batrachochytrium dendrobatidis on the health of post-metamorphic northern leopard frogs (Lithobates pipiens). **Ecotoxicology and Environmental Safety**, v. 80, p. 372–380, 2012. Disponível em: https://doi.org/10.1016/j.ecoenv.2012.04.006. Acesso em: 27 jun. 2023.

PHILLIPS, J. R. et al. The mechanics of air breathing in African clawed frog tadpoles, Xenopus laevis (Anura: Pipidae). **Journal of Experimental Biology**, v. 225, 2022. Disponível em: https://doi.org/10.1242/jeb.243102. Acesso em: 27 jun. 2023.

PHILLIPS, K. Where have all the frogs and toads gone? A recent workshop described an apparent decline worldwide of amphibian populations. **Bioscience**, v. 40, p. 422–424, 1990. Disponível em: https://doi.org/10.2307/1311385. Acesso em: 27 jun. 2023.

PAPERNA, I.; LAINSON, R. Alloglugea bufonis nov. gen., nov. sp. (Microsporea: Glugeidae), a microsporidian of Bufo marinus tadpoles and metamorphosing toads (Amphibia: Anura) from Amazonian Brazil. **Diseases of Aquatic Organisms**, v. 23, p. 7–16, 1995. Disponível em: https://doi.org/10.3354/dao023007. Acesso em: 27 jun. 2023.

PELTZER, P. M. et al. Biotoxicity of diclofenac on two larval amphibians: Assessment of development, growth, cardiac function and rhythm, behavior and antioxidant system. **Science of The Total Environment**, v. 683, p. 624–637, 2019. Disponível em: https://doi.org/10.1016/j.scitotenv.2019.05.275. Acesso em: 27 jun. 2023.

PRIYADARSHANI, S. et al. Heavy metal mediated immunomodulation of the Indian green frog, Euphlyctis hexadactylus (Anura: Ranidae) in urban wetlands. **Ecotoxicology and Environmental Safety**, v. 116, p. 40–49, 2015. Disponível em: https://doi.org/10.1016/j.ecoenv.2015.02.037. Acesso em: 27 jun. 2023.

PYASTOLOVA, O. A. et al. Utilization of Amphibians in Bioindication Research. Russian **Journal of Ecology**, v. 27, n. 5, p. 361-365, 1996.

REBOUÇAS, R. et al. Sexual maturity and growth of male toads (Rhinella ornata): A comparison between insular and mainland populations. **Zoological Anzeiger**, v. 283, p. 12–19, 2019. Disponível em: https://doi.org/10.1016/j.jcz.2019.07.002. Acesso em: 27 jun. 2023.

REDMOND, C. When Is an Alternative Not an Alternative? Supporting Progress for Absolute Replacement of Animals in Science. **Human-Animal Studies**, v. 22, p. 654–672, 2019. Disponível em: https://doi.org/10.1163/9789004391192_028. Acesso em: 27 jun. 2023.

RIBAS, L. et al. Expression Profiling the Temperature-Dependent Amphibian Response to Infection by Batrachochytrium dendrobatidis. **PLoS One**, v. 4, p. e8408, 2009. Disponível em: https://doi.org/10.1371/journal.pone.0008408. Acesso em: 27 jun. 2023.

ROLLINS-SMITH, L. A. et al. Involvement of glucocorticoids in the reorganization of the amphibian immune system at metamorphosis. **Developmental Immunology**, v. 5, p. 145–152, 1997. Disponível em: https://doi.org/10.1155/1997/84841. Acesso em: 27 jun. 2023.

ROLLINS-SMITH, L. A. et al. An amphibian model to test the effects of xenobiotic chemicals on development of the hematopoietic system. **Environmental Toxicology and Chemistry**, v. 23, p. 2863–2867, 2004. Disponível em: https://doi.org/10.1897/03-530.1. Acesso em: 27 jun. 2023.

SALLA, R. F. et al. Novel findings on the impact of chytridiomycosis on the cardiac function of anurans: Sensitive vs. tolerant species. **PeerJ**, v. 6, p. e5891, 2018. Disponível em: https://doi.org/10.7717/peerj.5891. Acesso em: 27 jun. 2023.

SALLA, R. F. et al. Differential liver histopathological responses to amphibian chytrid infection. **Diseases of Aquatic Organisms**, v. 142, p. 177–187, 2020. Disponível em: https://doi.org/10.3354/dao03541. Acesso em: 27 jun. 2023.

SANTANA, E. et al. Morphophysiological traits of an amphibian exposed to historical industrial pollution in a Brazilian biodiversity hotspot. **Amphibia-Reptilia**, v. 79, p. 1–13, 2021. Disponível em: https://doi.org/10.1163/15685381-bja10050. Acesso em: 27 jun. 2023.

SCALIA, M. et al. The Spleen Pigment Cells in Some Amphibia. **Pigment Cell Research**, v. 17, p. 119–127, 2004. Disponível em: https://doi.org/10.1046/j.1600-0749.2003.00115.x. Acesso em: 27 jun. 2023.

SCARAMELLA, N. et al. Short-term responses of Rana arvalis tadpoles to pH and predator stress: adaptive divergence in behavioural and physiological plasticity? **Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology**, v. 192, p. 669–682, 2022. Disponível em: https://doi.org/10.1007/S00360-022-01449-2/TABLES/3. Acesso em: 27 jun. 2023.

SCHEELE, B. C. et al. Amphibian fungal panzootic causes catastrophic and ongoing loss of biodiversity. **Science**, v. 363, n. 6434, p. 1459-1463, 2019. DOI: 10.1126/science.aav0379. PMID: 30923224.

SICHEL, G. et al. The Amphibian Kupffer Cells Build and Demolish Melanosomes: An Ultrastructural Point of View. **Pigment Cell Research**, v. 10, p. 271–287, 1997. Disponível em: https://doi.org/10.1111/j.1600-0749.1997.tb00687.x. Acesso em: 27 jun. 2023.

SILVA, M. B. da et al. Leukocyte Profiles in Odontophrynus carvalhoi (Amphibia: Odontophrynidae) Tadpoles Exposed to Organophosphate Chlorpyrifos Pesticides. **Water, Air, & Soil Pollution**, v. 231, 2020. Disponível em:

https://doi.org/10.1007/s11270-020-04726-4. Acesso em: 27 jun. 2023.

SIMON, M. P. et al. Effects of Acid Exposure on Natural Resistance and Mortality of Adult Rana pipiens. **Environmental Toxicology and Chemistry**, v. 36, p. 697–699, 2002. Disponível em: https://doi.org/10.1670/0022-1511(2002)036[0697:EOAEON]2.0.CO;2. Acesso em: 27 jun. 2023.

SPEARE, R. et al. Pathology of Mucormycosis of Cane Toads in Australia. **Journal of Wildlife Diseases**, v. 33, p. 105–111, 1997. Disponível em: https://doi.org/10.7589/0090-3558-33.1.105. Acesso em: 27 jun. 2023.

STUART, S. N. et al. Status and trends of amphibian declines and extinctions worldwide. **Science**, v. 306, p. 1783–1786, 2004. Disponível em: https://doi.org/10.1126/SCIENCE.1103538. Acesso em: 27 jun. 2023.

SUTTIE, A. W. Histopathology of the Spleen. **Toxicologic Pathology**, 2006. Disponível em: https://doi.org/10.1080/01926230600867750. Acesso em: 27 jun. 2023.

TAMORI, Y.; WAKAHARA, M. Conversion of red blood cells (RBCs) from the larval to the adult type during metamorphosis in Xenopus: specific removal of mature larval-type RBCs by apoptosis. **International Journal of Developmental Biology**, v. 44, p. 373–380, 2000. Disponível em: https://doi.org/10.1387/IJDB.10949046. Acesso em: 27 jun. 2023.

TARIGO, J. et al. Reluctant to dive: coelomic effusion in a frog. **Veterinary Clinical Pathology**, v. 35, p. 341–344, 2006. Disponível em: https://doi.org/10.1111/J.1939-165X.2006.TB00145.X. Acesso em: 27 jun. 2023.

TURNER, R. J.; MANNING, M. J. Response of the toad, Xenopus laevis, to circulating antigens. I. Cellular changes in the spleen. **Journal of Experimental Zoology**, v. 183, p. 21–33, 1973. Disponível em: https://doi.org/10.1002/JEZ.1401830104. Acesso em: 27 jun. 2023.

UDROIU, I.; SGURA, A. The phylogeny of the spleen. **Quarterly Review of Biology**, v. 92, p. 411–443, 2017. Disponível em: https://doi.org/10.1086/695327. Acesso em: 27 jun. 2023.
VAN METER, R. J. et al. Induced Hepatic Glutathione and Metabolomic Alterations Following Mixed Pesticide and Fertilizer Exposures in Juvenile Leopard Frogs (Lithobates sphenocephala). **Environmental Toxicology and Chemistry**, v. 41, p. 122–133, 2022. Disponível em: https://doi.org/10.1002/etc.5245. Acesso em: 27 jun. 2023.

VASCONCELOS-TEIXEIRA, R. et al. Stress Response, Immunity, and Organ Mass in Toads (Rhinella diptycha) Living in Metal-Contaminated Areas. **Biological Trace Element Research**, v. 200, p. 800–811, 2022. Disponível em: https://doi.org/10.1007/s12011-021-02699-x. Acesso em: 27 jun. 2023.

VELASCO, J. A. et al. Synergistic impacts of global warming and thermohaline circulation collapse on amphibians. **Communications Biology**, v. 4, 2021. Disponível em: https://doi.org/10.1038/s42003-021-01665-6. Acesso em: 27 jun. 2023.

VIRIATO, C. et al. Evaluation of the potential teratogenic and toxic effect of the herbicide 2,4-D (DMA® 806) in bullfrog embryos and tadpoles (Lithobates catesbeianus).

Chemosphere, v. 266, 2021. Disponível em: https://doi.org/10.1016/j.chemosphere.2020.129018. Acesso em: 27 jun. 2023.

VOSS, S. R. et al. Ambystoma mexicanum, the axolotl: a versatile amphibian model for regeneration, development, and evolution studies. **Cold Spring Harbor Protocols**, 2009. Disponível em: https://doi.org/10.1101/PDB.EMO128. Acesso em: 27 jun. 2023.

WAKE, D. B.; KOO, M. S. Amphibians. **Current Biology**, v. 28, p. R1237–R1241, 2018. Disponível em: https://doi.org/10.1016/J.CUB.2018.09.028. Acesso em: 27 jun. 2023.

WOMACK, M. C. et al. State of the Amphibia 2020: A Review of Five Years of Amphibian Research and Existing Resources. **Herpetological Review**, v. 110, p. 638–661, 2022. Disponível em: https://doi.org/10.1643/H2022005. Acesso em: 27 jun. 2023.

ZUASTI, A. et al. The melanogenic system of Xenopus laevis. **Archives of Histology and Cytology**, v. 61, p. 305–316, 1998. Disponível em: https://doi.org/10.1679/AOHC.61.305. Acesso em: 27 jun. 2023.

3. ARTIGO 2:

The amphibian´s kidneys as a source of Biomarkers: a Mini-Systematic Review

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Abstract

Amphibians are the most threatened vertebrates on the planet. With their biphasic life cycle and thin integument, they are vulnerable to many environmental stressors. The amphibian kidney is an organ that, in addition to other functions, plays an essential role in the electrolyte and osmotic balance to compensate for the large fluctuations that can occur due to the thickness of the integument. Another function that is indispensable in the face of the great contamination of many ecosystems is the elimination of toxic compounds through urine. Alterations in kidney histomorphology are associated with most deaths of these animals in captivity and these analyses provide biomarkers with great potential for environmental monitoring. A systematic review of amphibian kidney histology and its applications to ecotoxicology was carried out, as a result, a historical analysis was performed, discussing the advances of studies over the years and the most current trends of the most recent works in the area. In addition, the species and other characteristics of the studies were compared as the country of origin of the publication. The main renal biomarkers in response to pathogens, pollution and contamination and those used for monitoring and conservation of ex situ populations were also listed and discussed. It was found that despite the vast and complete reference literature, renal biomarkers are underutilized in research for environmental monitoring and toxicology.

Keywords:

3.1 Introduction

Amphibians are the most endangered vertebrates on the planet (IUCN, 2023). This class is composed of frogs, toads, salamanders, and caecilians and possess a dual lifestyle, spending their lives both in water and on land (VENCES; KOHLER, 2008; LIEDTKE et al., 2022; AMPHIBIAWEB, 2023; FROST, 2023). These animals inhabit almost all the continents, with the exception of the poles. However, in recent decades, a concerning trend has emerged, as amphibian populations worldwide have experienced alarming declines (KACOLIRIS et al., 2022; AMPHIBIAWEB, 2023; FROST, 2023). Since the 1970s, amphibians have faced a myriad of threats, including habitat loss, pollution, climate change, disease outbreaks, and the introduction of invasive species (GRANT et al., 2020; AMPHIBIAWEB, 2023).

Due to the high sensitivity of these organisms to environmental changes, amphibians are considered as good bioindicators of environmental health (LANGLOIS, 2021; MANI et al., 2021). In fact, several studies have been using amphibians as experimental models in ecotoxicology (SLABY et al., 2019; AZIZISHIRAZI et al., 2021; LANGLOIS, 2021; BURGOS-ACEVES et al., 2022), as well as for the study of emerging diseases (FISHER et al., 2021; GROGAN et al., 2023).

In these studies, the main tools for assessing the impacts of contaminants and/or pathogens on amphibians consist of biomarkers. Biomarkers, also known as biological markers, are experimental measurements of biological samples that can indicate the occurrence of a normal function of an organism, or a pathological/toxicological response to any strange pathogens or toxicants (DEPLEDGE, 2020). To evaluate morphological biomarkers, several organs and systems can be used as sources to obtain biological measurements in amphibians, such as the heart (JONES-COSTA et al., 2018; PELTZER et al., 2022), blood (DE GREGORIO et al., 2021; ROBINSON et al., 2021; DAVIS et al., 2023), gonads (Slaby et al., 2019; LENT et al., 2021), liver (DE OLIVEIRA et al., 2016; Salla et al., 2020; BURRACO et al., 2023), among others.

Among the intricate systems of amphibians, the kidneys play a pivotal role in maintaining the delicate balance between internal conditions and the surrounding environment by regulating water, balancing electrolytes, excreting waste products, and controlling acid-base homeostasis (SCHMIDT-NIELSEN, 2002; PESSIER, 2009). These functions are particularly significant for amphibians, as they have evolved to inhabit diverse habitats, ranging from arid deserts to freshwater ecosystems (AMPHIBIAWEB, 2023; FROST, 2023). Additionally, the amphibian’s kidneys may participate as one of the main routes of toxicant elimination (HUANG; KARASOV, 2000), so this organ presents great relevance for ecotoxicological studies.

Thus, this mini-systematic review aimed to explore the histology of amphibian kidneys, comparing the main features among the species, and compiling how different environmental stressors and anthropogenic disturbances may impact the histomorphology of these organs. By gathering these data, we intend to identify knowledge gaps, as well as to highlight the potential of renal biomarkers to expand its use in further environmental monitoring studies. We also emphasize the importance of searching for less invasive methodologies, in order to comply with the principles of the "3Rs" (Replacement, Reduction and Refinement) in scientific research.

3.2 Methodology

The searches were conducted using the databases “Scopus”, “Web of Science”, “PubMed”, and “ScienceDirect”. The keywords were combined with the Boolean operator "OR", and the operator "AND" between the term "kidney" and other amphibian-related terms, including “amphibia”, “amphibian”, “newt”, “caudata”, “gymnophiona”, “anura”, “toad”, “frog”, “tadpole”; AND “histology”, “histopathology”, “optic microscopy”, “morphology”, “histomorphology”. A total of 433 scientific manuscripts were later sorted for eligibility purposes, including only studies associated with the amphibian kidney histomorphology that were published until 2023 (beginning with the oldest paper found). Other eligible studies cited within the selected papers were also included. The exclusion criteria consisted of non-English papers, non-peer-reviewed literature, duplicated manuscripts, bibliographic reviews, technical documents, unpublished methodologies, and other studies that did not attend to the research aims. Finally, 74 manuscripts, published from 1975 to 2023, were selected for the review (Fig. 1). These papers were fully read to extract relevant data, such as: publication year, country, taxonomical informations and main results including the used biomarkers. Graphic illustrations were produced in Adobe Illustrator software, and the map of global distribution of the studies was plotted in Excel software.

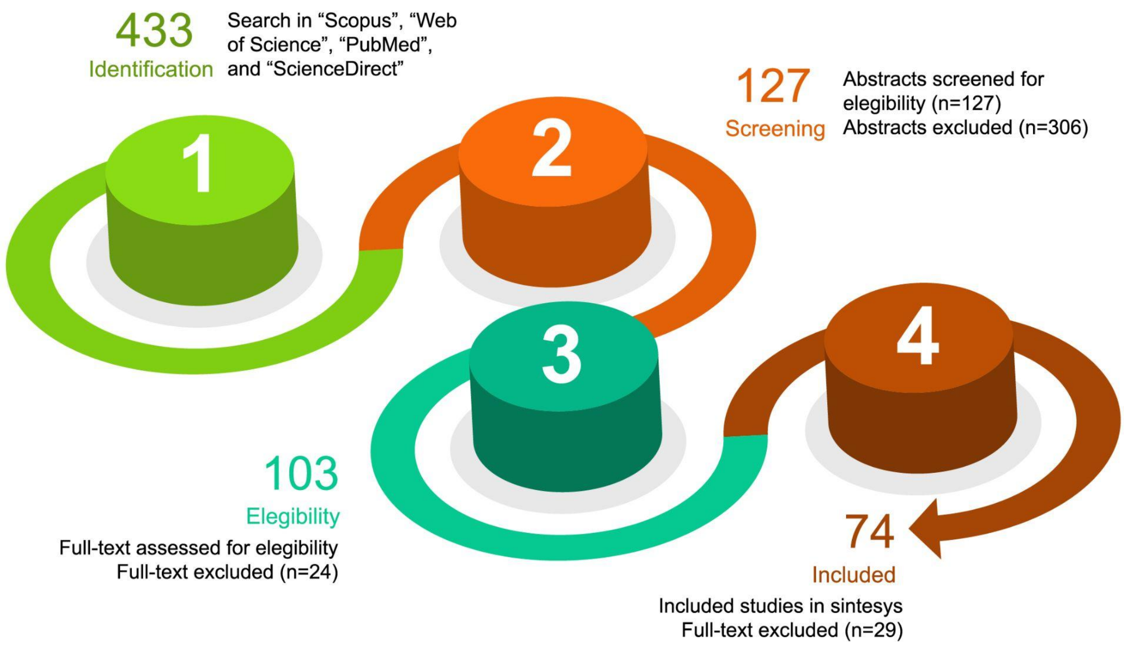

Figure 1. Methodological screening and selection of the studies concerning the amphibian kidneys morphology and histology as a source of biomarkers.

3.3 Results and Discussion

3.3.1 Scientometric data: chronology and global distribution

The first record considered in this paper dates back to the 1970s with the characterization of the innervation in the urogenital system of males of three anuran species, including *Xenopus laevis*, *Rana temporaria*, and *Bufo bufo* (UNSICKER et al., 1975) (Fig. 2). Later, Pons et al. (1982) evaluated the renal anatomy and histomorphology of the Common Tree Frog (*Hyla arborea*) across seasons in Europe. A few years later, efforts to describe the renal tissue of female *Typhlonectes compressicaudus*, were conducted in Japan (SAKAI et al., 1986;1988). One of the few amphibian species that can inhabit high salinity environments, *Rana cancrivora*, also had its renal parenchyma evaluated concerning its specializations to the physiological challenges of estuaries and mangroves (UCHIYAMA et al., 1990). Still in the 90's, a North American research group was dedicated to studying the histomorphology and the renal innervation of *Amblystoma mexicanum* (DITRICH; SPLECHTNA, 1992), while researchers from this same group were also the first to use renal biomarkers of axolotls to evaluate the genetic variable effects of a pathology (EGAR; JARIAL, 1991).

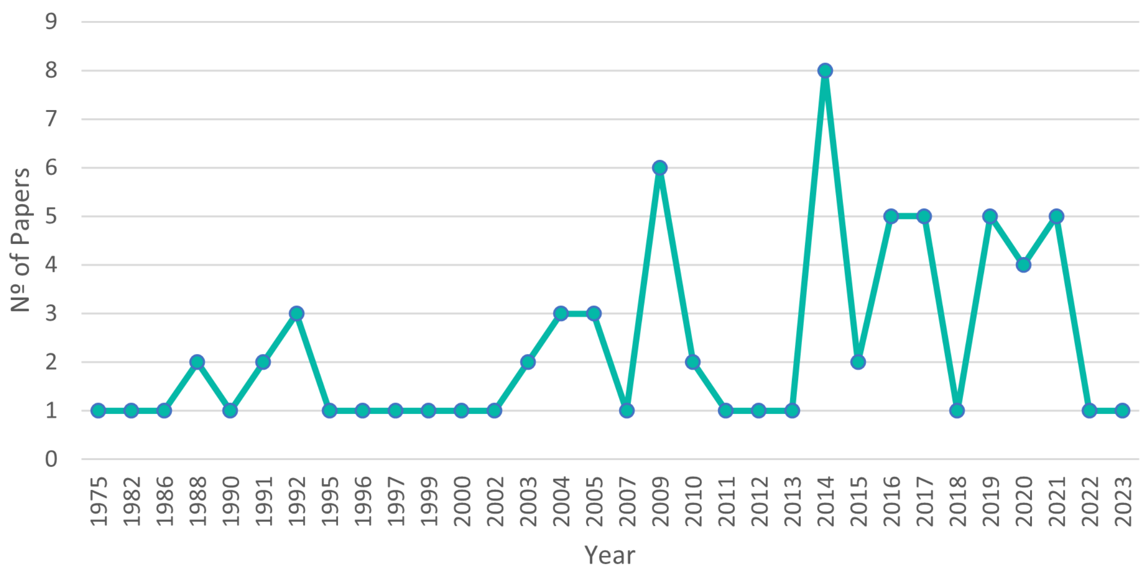

Figure 2. Number of studies on amphibian kidney histomorphology published per year.

The first paper that reported histomorphological biomarkers of the kidney in environmental studies have evaluated the effects of lead nitrate in *Rana ridibunda*

(LOUMBOURDIS, 2003). The following year also followed the same trend, with Arrieta et al. (2004) evaluating the effects of lead in another species, *Bufo arenarum*. Thus, studies in the areas of pathology and ecotoxicology have increased over time, however studies dedicated to renal characterizations, mainly with caudata, have continued to be the focus of some research groups (e.g. NICHOLSON; SIEGEL, 2014 HARTLEY et al., 2019; YARTSEV; EVSEEVA, 2021).

The origin of the 74 studies were distributed among 22 countries. Most studies were concentrated in the United States (27.03%), followed by Brazil (10.81%), Germany, Austria, Italy and Japan (6.76% each) (Fig. 3). The predominance of North American studies on amphibians is documented, however the low participation of some European countries and Australia contradicts the trend of research in other areas of herpetology (HOULAHAN et al., 2000).

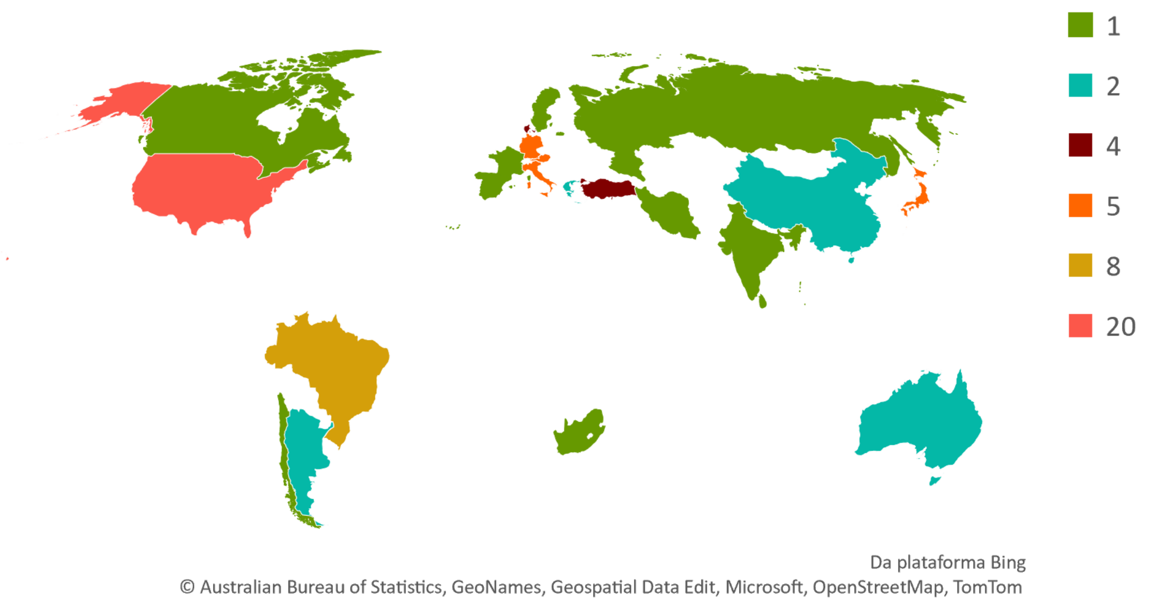

Figure 3. Global map of countries and the number of published papers concerning amphibian renal morphology.

3.3.2 Taxonomy and Sex

As expected, studies with anurans comprise more than 60% of the research, followed at a distance by the other taxonomic orders (Fig. 4A). A greater number of studies with anuran amphibians was already expected due to the fact that this is the most numerous order (FROST, 2023), therefore, mostly known and studied among herpetologists. Despite this dominance, a greater than expected number of studies with salamanders and cecilia have also been documented, which may be attributed to the morphological and functional diversity of the kidneys among the taxa.

Overall, more than eighty species of amphibians were evaluated in the articles of this survey, however more than 25% of the articles were devoted to the study of model species such as *Lithobates catesbeianus* (12.05%) *Xenopus laevis* (10.84%) *Ambystoma mexicanum* (7.23%), or to species considered as invasive, such as *Rhinella marina* (4.82%). Indeed, *L. catesbeinus* and *X. laevis* are widely known as model-species for many scientific areas, including pathology (RAMSEY et al., 2010; URBINA et al., 2018), ecotoxicology (FREITAS et al., 2019; CAROTENUTO et al., 2023), molecular biology (CAROTENUTO et al., 2023), and others. *Ambystoma mexicanum* have also been used as a frequent and useful model in molecular areas, developmental studies, and especially in regeneration tests (VOSS et al., 2009), which also explains its greater application among studies. Research with Neotropical species, as in all areas of herpetology, needs more encouragement, not only for improving knowledge about native fauna, but also because more data are still needed so that relative sensitivity and risk assessment studies of amphibians can progress (DAAM et al., 2019).

More than 75% of the studies either used both genders or did not inform the sex of the individuals. Studies using only males represent about 20%, while those considering only females account for less than 10% of the studies (Fig. 4B). The higher occurrence of studies with males is the recurrent in review are probably related to the fact that they are easier to locate through vocalization in the wild (REBOUÇAS et al., 2019). Also, such dominance can be correlated with the modified kidney structures that are part of the reproductive system in males (MØBJERG et al., 1998), while these structures are not observed in females or have a greatly reduced role because there is no tissue connection between the kidneys and the gonads in females (MØBJERG et al., 1998).

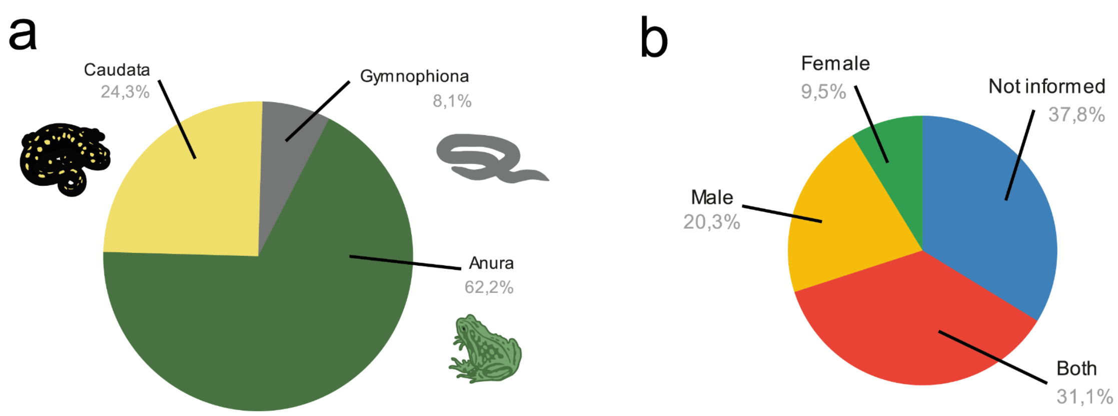

Figure 4: (a) Percentage of studies with each amphibian taxon. **(b)** Percentage of studies separated by sex.

3.3.3. The Amphibian Kidney: Function, Anatomy and Histomorphology

Osmoregulation and electrolyte balance in amphibians depend on several organs: the kidneys are responsible for filtering and reabsorbing important molecules; the urinary bladder that serves as a store of water and electrolytes; the skin, which acts in the water uptake; and the lymph hearts, which are structures located near the cloaca that redirect the excess of lymphatic fluids back into the circulation through the portal-renal system (FORZÁN et al., 2017).

Vertebrate kidneys' work on the principle of filtration-reabsorption. Urine is formed by glomerular filtration, which prevents the passage of large molecules such as proteins, and the tubular reabsorption process ensures that important substances such as glucose and vitamins are not lost (SCHMIDT-NIELSEN, 2002). The kidneys of amphibians are unable to concentrate their urine with osmolality above that of plasma, so it is critical that these organisms have tolerance to large fluctuations in plasma and electrolyte composition, as a physiological strategy to conserve water in the body (FORZÁN et al., 2017). Most amphibians live in freshwater, so the organism has a higher osmotic potential than the surrounding environment. Due to their thin and permeable skin, there is an influx of water that causes amphibians to excrete extremely dilute urine (SCHMIDT-NIELSEN, 2002). The excreta produced by anurans change with ontogeny, but it is also influenced by the species' mode of life. Larvae and adults that present exclusively aquatic life-cycles excrete ammonia through the kidneys, skin, and gills; while terrestrial adults convert the ammonia to urea for excretion. A few arboreal and/or water-sparing species may excrete uric acid (FÓRZAN et al., 2017). Throughout the metamorphosis the vascular system develops and begins to function like that of the adults (DITRICH; LAMETSCHWANDTNER, 1992). In addition, hematopoietic activity that was concentrated in the liver and kidneys in tadpoles shifts to be detected mainly in the bone marrow, but also in the spleen and liver of adult individuals (ARIKAN; ÇIÇEK, 2014).

In anurans the kidney is flattened dorso-ventrally and elongated like a ribbon (UNSICKER et al., 1975; PONS et al., 1982; UCHIYAMA et al., 1990; DITRICH & LAMETSCHWANDTNER, 1992; RICHTER, 1995; MØBJERG et al., 1998; MØBJERG et al., 2000; FINKLER et al., 2014; RHEUBERT et al., 2017; LAMETSCHWANDTNER; MINNICH, 2020; ALNOAIMI et al., 2021; REGUEIRA et al., 2022), while in cecilians the organ is more elongated and thinner, following the body structure of the taxon (SAKAI et al., 1986; 1988; MASOOD-PARVEEZ et al., 1992; MØBJERG et al., 2004; BLEYZAC et al., 2005).

In salamanders the kidney, which is also dorso-ventrally flattened, has two distinct parts: the genital and the pelvic kidney (NICHOLSON; SIEGEL, 2014; PARTO et al., 2014; SIEGEL; RABE, 2016; HARTLEY et al., 2019).The genital portion presents alterations that indicate that its function might be related to sperm transport and not to urine formation, the glomeruli are smaller and the cellular structures of tubules are distinct (NICHOLSON; SIEGEL, 2014; PARTO et al., 2014; SIEGEL; RABE, 2016; HARTLEY et al., 2019). As a consequence, the genital region of the kidney in this class of amphibians is thinner than the pelvic region (NICHOLSON; SIEGEL, 2014; PARTO et al., 2014; SIEGEL & RABE, 2016; HARTLEY et al., 2019). There are differences among amphibian species with respect to the shape, distribution, and size of the glomeruli and tubules (KON et al., 1988), however, these structures also have many similarities for identification and evaluation under the microscope.

With regard to the main tissue components of the amphibian kidney, the renal corpuscle is composed of the Bowman's capsule and the glomerulus. The Bowman's capsule consists of two tissue layers: the visceral and the parietal layers, and the space between these two layers is called the Bowman's space, which is responsible for receiving the glomerular filtrate. The visceral or inner layer surrounds the glomerular capillaries. The proximal tubule is formed by cubic epithelial cells with an apical brush border. The distal tubule is also composed of cubic epithelial cells, but without a brush border and a lighter cytoplasm. The collecting duct is formed by cubic or columnar epithelium (PONS et al., 1982; UCHIYAMA et al., 1990; DITRICH E LAMETSCHWANDTNER, 1992; MØBJERG et al., 1998; 2000; RHEUBERT et al., 2017; AKAT, 2019).

3.3.4. Renal biomarkers in response to pathogens

Brannelly et al. (2016) evaluated the effects of the chytrid fungus on three species and found quite specific responses from each of them. While *Litoria verreauxii alpina* showed no effect on the renal biomarkers, *Rhinella marina* and *Pseudophryne corroboree* proved to be more sensitive to the fungus, with reduced cell density of the hematopoietic tissue (Brannelly et al., 2016). Also, the renal parenchyma of *Pyxicephalus adspersus* infected by another fungus, *Cladosporium allicinum*, showed granulomas, multifocal nodular to coalescing and diffuse lesions (Grassi et al., 2023).

Duncan et al. (2004) analyzed individuals of *Megophrys nasuta* infected by the Myxozoa parasite (Cnidaria) and recorded various degrees of renal tubular dilatation and necrosis, as well as mild to severe non-suppurative tubulointerstitial nephritis associated with vegetative stages of the parasite. Tadpoles of more than five species of anurans with parasite-associated death (Perkinsea) showed various macroscopic effects such as increased kidney size (nephromegaly), discoloration, and tissue lesions, including necrosis of epithelial cells and intracytoplasmic invasion by the infectious agent. In addition, parasite infestation has been detected in the intravascular and perivascular interstitial space, and in glomerular tufts, in most cases replacing more than 60% of renal tubules and glomeruli (ISIDORO-AYZA et al., 2019).

Anurans are very susceptible to worm infestations and other parasites, in particular for their voracious feeding habit (BOWER et al., 2019), but the effects of the infestations may vary among species. Moretti et al. (2017) found a positive association among the relative masses of the heart, intestine, and kidneys of *Rhinella icterica* with parasite intensities in these frogs. A positive association between the organ mass and parasite intensity generally indicates the morphological plasticity of the host to parasite damage (HOLMES; ZOHAR, 1990). However no morphological alterations were found in the kidneys, heart, alimentary tract, exocrine pancreas or skeletal muscle tissue of *Anaxyrus terrestri*s infected by *Ichthyophonus spp* (KIRYU; LANDSBERG, 2015). The kidneys can also be a site of parasitic infestation by *Hyaloklossia labbé*, as documented in *Pelophylax porosus porosus* (TOKIWA et al., 2021). In *Xenopus laevis*, the parasite *Protopolystoma xenopodis* was also found in the kidneys in the subadult stage of the hosts, then the parasite migrates to other parts of the body to complete its development (THEUNISSEN et al., 2014).

Necrosis, hemorrhage, and the infestation of viral particles could be observed in the kidneys of Iridovirus-infected *Andrias davidianus* (Meng et al., 2014). Tissues of *X. laevis* individuals infected with ranavirus also exhibited considerable changes in the kidney, especially in the renal proximal tubular epithelium. Necrosis was mainly observed in the proximal tubules, areas of leukocytes, and glomeruli, with expanded mesangial spaces and necrotic cellular debris. In contrast, distal tubules showed little damage (no extensive necrosis), although eosin positive material indicative of the presence of protein was observed (ROBERT et al., 2005). Also, in *L. catesbeianus*, ranavirus was associated with glomerulotubular degeneration, hemorrhage , necrosis, and increased melanomycrophages in the renal parenchyma (NEVES et al., 2016).

Overall, the above studies demonstrate the great potential of kidney analyzes as sources of relevant information for pathological areas of study, elucidating the role of different pathogens in target organs through use of morphological and tissue biomarkers.

3.3.5. Renal biomarkers in response to pollution

Due to the filtering role of the kidneys, these organs can directly act as a toxicokinetic route for the elimination of various toxicants (KIM, 2017). Thus, this section will highlight the main renal biomarkers used in environmental monitoring studies with amphibians.

One of the main sources of contamination of aquatic ecosystems is sewage and drainage from suburban regions (WEAR et al., 2021). Sewage residues reach several water bodies as their final destination (WEAR et al., 2021), which represent some of the main reproductive habitats for amphibians (KNUTSON et al., 2004). Studies like those of Anzaldua & Goldberg (2020) have assessed internal and external morphological abnormalities in response to suburban drainage ditches in *Osteopilus septentrionalis*, *Rhinella marina*, *Lithobates sphenocephala, Anaxyrus terrestris*, and *Anaxyrus americanus*. The researchers found renal tissue lesions and a high occurrence of anatomical kidney abnormalities such as disintegrating organs and surface darkening. The few normal individuals were females or young males.

The Triclosan is a widely used biocide with antimicrobial activity that is frequently added to health-care products (YAZDANKHAH et al., 2006). These bacteriostatic products also reach water bodies, and have been shown to cause pronounced histopathological responses in the kidneys of *Xenopus laevis*, including mineralization of the kidney´s posterior portion, tubular regeneration, interstitial fibrosis, granulomatous inflammation, tubule epithelial necrosis, tubular vacuolization, intratubular proteinic fluid, and atrophy (FORT et al., 2017). Residues of the flame retardant (BDE-47) also have changed the morphological proportions of the kidneys of *Xenopus laevis* males (LI et al., 2020).

Several drugs also seem to act through renal toxicokinetic pathways in amphibians. In *Xenopus tropicali*s the synthetic hormone EE2 and and the fungicide clotrimazole, did not cause significant differences in kidney morphology or histology, however there were biochemical changes and all individuals exposed to EE2 developed ovaries, which indicates the potential endocrine disruptor action of these compounds (GYLLENHAMMAR et al., 2009). In *Xenopus laevis* exposed to other endocrine disruptors, the xenoestrogen (4-tert-octylphenol) and the xenoandrogen (17β-trenbolone), the prevalence and severity of kidney lesions were similar between treatments and control in most cases. However, there was tubule mineralization and dilation in the kidneys of juvenile individuals (HASELMAN et al., 2016).

Potentially toxic metals are also known to alter the histomorphology of the kidneys, which appears to be a target organ for bioaccumulation (ARRIETA et al., 2004; CAPALDO et al., 2016). In studies with *Euphlyctis hexadactylus* exposed to metals, authors reported tubular degeneration, tubular necrosis, progressive chronic nephropathy, mineralization, renal infiltrate inflammation, and Bowman's space dilation (JAYAWARDENA et al., 2017). The effect of hexavalent chromium was also documented for the kidneys of *L. catesbeianus* tadpoles, which showed areas with inflammatory infiltrates, multiple granulomas, tubular and glomerular hypertrophy, and tubular necrosis (MONTEIRO et al., 2018). Lead accumulation sites were evaluated in *Bufo arenarum*, and renal tissue was identified as one of the accumulation targets for this metal. Additionally, increased Pb concentrations in the tissues and dry mass of the organ were observed (ARRIETA et al., 2004). Light microscopy of the kidneys revealed morphological alterations resulting from the exposure to lead nitrate in *Rana ridibunda*, particularly in the proximal tubule cells. The most severe changes, such as vacuolization, infiltration, brush border destruction, and proximal tubule injury, were detected in animals exposed for a period of 10 and 30 days (LOUMBOURDIS, 2003). Cd exposures in of *Rana ridibunda* also caused renal alterations, including an increase in the number of apoptotic bodies and hyaline globules (LOUMBOURDIS, 2005). The salamander *Triturus carnifex* also presented Cd bioaccumulation, as well as increased apoptosis, glomerular expansion, reduction of Bowman's space, dilated renal tubules, degeneration in epithelial cells, and necrosis (CAPALDO et al., 2016).

Agrochemicals are also considered as potential contributors to the worldwide decline of amphibian populations (AGOSTINI et al., 2020), and kidney biomarkers can be of great value for ecotoxicity studies of their components. Many effects of agriculture have been reported in *Pelophylax ridibundu*s, which showed renal degeneration, congestion, dilatation, and necrosis in the proximal and distal tubules; as well as expansions of the Bowman's space, lymphocyte infiltrations, and glomerulonephritis (SISMAN et al., 2019). Whyle for *Ambystoma tigrinum*, deltamethrin did not significantly alter the relative mass of the kidney nor caused histopathological changes (FROESE et al., 2009), *Pelophylax ridibundus* proved to be more sensitive to the pesticide, presenting glomerular shrinkage, hemorrhage, degeneration, infiltration, increased Bowman's distance and eosinophilic tubular lumens in the kidneys (ALNOAIMI et al., 2021). Similar alterations have been reported to *Hyla intermedia* when exposed to pyrimethanil, showing macrophage infiltration, hemorrhage, destruction of the brush border of the proximal tubules, widening of the bowman space, reduction in capillary caliber, cytoplasmic vacuolization, swelling, tubular dilatation, cytoplasm reduction, and the presence of proteinaceous fluid (BERNABÒ et al., 2017).

The effects of carbaryl on the kidney tissue of *Bufotes variabilis* were also investigated and highlighted the presence of mononuclear cell infiltration, hypertrophied Bowman's capsule cells, deformation, vacuolization, karyolysis and necrosis of the renal tubule epithelium, brush border destruction, glomerular shrinkage, hemorrhage and fibrosis (Çakıcı, 2015). In *L. catesbeianus,* the pesticide 2,4-D (DMA® 806) also caused tissue disorganization and inflammatory infiltrates with a predominance of mononuclear cells (VIRIATO et al., 2021), which indicates that these compounds may act through similar pathways.

These studies show that the analysis of renal biomarkers and the comparison of endpoints on different species can help in future studies for the determination of OAPs (adverse outcome pathways), as well as for environmental risk assessment research.

3.3.6 Renal biomarkers in ex-situ and conservation studies

In 2014, North American researchers conducted a large study by performing necropsy and histopathology of the organs of thirteen species of anurans that died for various reasons, while kept in captivity. Although they could not specify the cause of these deaths, they were mostly associated with kidney diseases, specifically polycystic nephropathy (PESSIER et al., 2014).

Kidney analyzes can also be useful in monitoring the health of amphibians of frog farms. In a case study, captive *Lithobates catesbeianus* started to show some clinical signs of disease, so renal histopathology helped the researchers to elucidate the case, revealing granulomas in different stages, increased Bowman's space, glomerular hemorrhage, tubules with fibrous material, and the renal parenchyma showed dystrophic calcification (SEIXAS et al., 2019). Similarly, the renal histology of *Lepidobatrachus laevis* individuals with lethargy and other indications of disease showed varying combinations of tubular epithelial binucleation, karyomegaly and cytoplasmic vacuolization, polycystic kidney disease, and renal carcinoma (WOMBLE et al., 2020).

Renal analysis may even be relevant for improving the food quality of captive animals. A quality feeding test with different protein contents had been conducted with bullfrogs in commercial breeding facilities (SEIXAS FILHO et al., 2009) and the results indicate that all formulations were harmful not only to the kidneys, but also to the liver and the heart of *Lithobates catesbeianus*. All the tested feed were associated with renal alterations, including areas of tubulonephrosis and calcification of renal tubules, which indicates that the animals were fed with proteins of low biological value. These studies showed that low quality food can compromise the performance and health of the animals and may reduce the efficiency of the immune system (SEIXAS FILHO et al., 2009).

Another method that has also been widely used in amphibian conservation studies is the use of Visible Implant Elastomers (VIE) and Passive integrated transponders (PIT), which are used for tagging, identifying, and monitoring individuals. Cabot et al. (2021) have compared the effects of these techniques on *Rhinella marina* individuals. Despite the efficiency of these methods for marking individuals, after receiving the VIE, in several cases the implant migrated to the kidneys, and caused many renal alterations, such as small vessel dilatation, edema, and granulomatous nephritis, that severely progressed over time. The PIT, on the other hand, remained retained in its optimal location, causing minimal histological changes, therefore was recommended by the authors as a better method for identifying adult anurans (CABOT et al., 2021). Comparative studies like this should be highly encouraged to ensure more ethical and efficient research, minimizing to the maximum the possible harms on the organisms. In fact, the creation of less invasive and more efficient methodologies is an urgent demand so that we can actually monitor and protect amphibian communities as well as contributing to the development of ecotoxicology as a whole.

3.4 Conclusions and Knowledge Gaps

The histomorphology of the amphibian kidneys is well studied, and herpetologists have already performed a careful description of several species. Due to its indispensable function for excretion, toxin elimination, and electrolyte balance, the kidney is a key organ for maintaining the animal's homeostasis in the face of environmental disturbances. Size fluctuations in the glomeruli and Bowman's capsule may indicate changes in glomerular filtration efficiency, while effects caused in the proximal and distal tubules may compromise the reabsorption of important and valuable compounds for maintaining homeostasis of the individual such as proteins and other electrolytes.

The kidneys can act as targets of several pathologies, so many renal biomarkers have already been described (see supplementary material). From all the papers that applyed renal biomarkers, more than 90% reported responses after the exposure of the animals to the stressors, which indicates that the renal function of amphibians is a sensitive source for the analysis of biomarkers, responding to a wide range of contaminants. Thus, the amphibian kidneys can be used as a versatile source of biomarkers, responding to pathogens, pollutants and environmental changes. In addition, histopathological evaluations can help contain disease outbreaks, identify parasites and diseases of amphibians that are kept in laboratories, zoos and commercial breeders, so these tests can help not only natural populations, but also those living in captivity. Despite so much data being available, renal biomarkers are still underrated in environmental assessment and monitoring. Thus, we encourage scientists to employ such biomarkers, always looking for methodological alternatives and adaptations that can minimize invasive techniques, such as the use of cell cultures, *in silico* analysis, or even the reuse of samples from museums.

REFERENCES

AGOSTINI, M. G.; ROESLER, I.; BONETTO, C.; RONCO, A. E.; BILENCA, D. Pesticides in the real world: The consequences of GMO-based intensive agriculture on native amphibians. **Biological Conservation**, v. 241, 108355, 2020.

AKAT, Esra (2019). "Histological and histochemical study on the mesonephric kidney of Pelophylax bedriagae (Anura: Ranidae)." **Turkish Journal of Zoology**, Vol. 43, No. 2, Article 8.

ALNOAIMI, F., DANE, H. & ŞİŞMAN, T. (2021). "Histopathologic and genotoxic effects of deltamethrin on marsh frog, Pelophylax ridibundus (Anura: Ranidae)." **Environmental Science and Pollution Research**, 28, 3331–3343. https://doi.org/10.1007/s11356-020-10711-5

ANZALDUA, S. P., & GOLDBERG, J. (2020). "Hotspot of Adult Cuban Treefrog and Cane Toad Multi-organ Abnormality in Suburban South-west Florida." **Asian Herpetological Research**, 11, 139-154. 10.16373/j.cnki.ahr.190045.

ARRIETA, ., BRUZZONE, ., APARTÍN, . et al. (2004). "Biosensors of Inorganic Lead Exposure and Effect in an Adult Amphibian." **Arch Environ Contam Toxicol**, 46, 224–230. https://doi.org/10.1007/s00244-003-2221-8

AZIZISHIRAZI, A.; KLEMISH, J. L.; PYLE, G. G. Sensitivity of Amphibians to Copper. **Environmental Toxicology and Chemistry**, v. 40, n. 7, p. 1810-1821, jul. 2021. doi:

10.1002/etc.5049. Epub 2021 May 6. PMID: 33749926.

BERNABÒ, I., GUARDIA, A., MACIRELLA, R., TRIPEPI, S., & BRUNELLI, E. (2017). "Chronic exposures to fungicide pyrimethanil: multi-organ effects on Italian tree frog (Hyla intermedia)." **Scientific Reports**, 7(1), 6869. https://doi.org/10.1038/s41598-017-07367-6. PMID: 28761072; PMCID: PMC5537256.

BLEYZAC, P., CORDIER, G., & EXBRAYAT, J. M. (2005). "Morphological Description of Embryonic Development of Immune Organs in Typhlonectes compressicauda (Amphibia, Gymnophiona)." **Journal of Herpetology**, 39(1), 57-65. (1 March 2005)

BOWER, D. S.; BRANNELLY, L. A.; MCDONALD, C. A.; WEBB, R. J.; GREENSPAN, S. E.; VICKERS, M.; ...; GREENLEES, M. J. A review of the role of parasites in the ecology of reptiles and amphibians. **Austral Ecology**, v. 44, n. 3, p. 433-448, 2019.

BRANNELLY, L., WEBB, R., SKERRATT, L., & BERGER, L. (2016). "Effects of chytridiomycosis on hematopoietic tissue in the spleen, kidney and bone marrow in three diverse amphibian species." **Pathogens and Disease**, Volume 74, Issue 7, October 2016, ftw069. https://doi.org/10.1093/femspd/ftw069

BURRACO, P.; SALLA, R. F.; ORIZAOLA, G. Exposure to ionizing radiation and liver histopathology in the tree frogs of Chornobyl (Ukraine). **Chemosphere**, v. 315, p. 137753, fev. 2023. doi: 10.1016/j.chemosphere.2023.137753. Epub 3 jan. 2023. PMID: 36608893.

BURGOS-ACEVES, M. A.; FAGGIO, C.; BETANCOURT-LOZANO, M.; GONZÁLEZ-MILLE, D. J.; ILIZALITURRI-HERNÁNDEZ, C. A. Ecotoxicological perspectives of microplastic pollution in amphibians. **Journal of Toxicology and Environmental Health B, Critical Reviews**, v. 25, n. 8, p. 405-421, nov. 2022. doi: 10.1080/10937404.2022.2140372. Epub 2022 Nov 9. PMID: 36351281.

CABOT, M. L., TROAN, B. V., ANGE-VAN HEUGTEN, K., SCHNELLBACHER, R. W., SMITH, D., RIDGLEY, F., MINTER, L. J. (2021). "Migration and Histologic Effects of Visible Implant Elastomer (VIE) and Passive Integrated Transponder (PIT) Tags in the Marine Toad (Rhinella marina)." **Animals**, 11, 3255. https://doi.org/10.3390/ani11113255

ÇAKICI, Ö. (2015). "Histopathologic changes in liver and kidney tissues induced by carbaryl in Bufotes variabilis (Anura: Bufonidae)." **Experimental and Toxicologic Pathology**, 67(3), 237-243. https://doi.org/10.1016/j.etp.2014.12.003. Epub 2015 Jan 5. PMID: 25573057.

CAPALDO, A., GAY, F., SCUDIERO, R., TRINCHELLA, F., CAPUTO, I., LEPRETTI, M., MARABOTTI, A., ESPOSITO, C., & LAFORGIA, V. (2016). "Histological changes, apoptosis and metallothionein levels in Triturus carnifex (Amphibia, Urodela) exposed to environmental cadmium concentrations." **Aquatic Toxicology**, 173, 63-73. https://doi.org/10.1016/j.aquatox.2016.01.009. Epub 2016 Jan 27. PMID: 26851569.

CAROTENUTO, R.; PALLOTTA, M. M.; TUSSELLINO, M.; FOGLIANO, C. Xenopus laevis (Daudin, 1802) as a Model Organism for Bioscience: A Historic Review and Perspective. **Biology (Basel)**, v. 12, n. 6, p. 890, 20 jun. 2023. DOI: 10.3390/biology12060890. PMID: 37372174; PMCID: PMC10295250.

CARVALHO, E. T., & JUNQUEIRA, L. C. (1999). "Histology of the kidney and urinary bladder of Siphonops annulatus (Amphibia-Gymnophiona)." **Archives of Histology and Cytology**, 62(1), 39-45. https://doi.org/10.1679/aohc.62.39. PMID: 10223741.

DAAM, M. A.; MOUTINHO, M. F.; ESPÍNDOLA, E. L. G.; SCHIESARI, L. Lethal toxicity of the herbicides acetochlor, ametryn, glyphosate and metribuzin to tropical frog larvae. **Ecotoxicology**, v. 28, n. 6, p. 707-715, ago. 2019. DOI: 10.1007/s10646-019-02067-5. PMID: 31250286.

DANIELS, O., FABBRO, L., & MAKIELA, S. (2014). "The effects of the toxic cyanobacterium Limnothrix (strain AC0243) on Bufo marinus larvae." **Toxins** (Basel), 6(3), 1021-1035. https://doi.org/10.3390/toxins6031021. PMID: 24662524; PMCID: PMC3968374.

DAVIS AK, MAERZ JC. Assessing Leukocyte Profiles of Salamanders and Other Amphibians: A Herpetologists' Guide. **Methods Mol Biol.** 2023;2562:443-458. doi: 10.1007/978-1-0716-2659-7_29. PMID: 36272093.

DE ABREU MANSO, P. P., DE BRITO-GITIRANA, L., & PELAJO-MACHADO, M. (2009). "Localization of hematopoietic cells in the bullfrog (Lithobates catesbeianus)." **Cell and Tissue Research**, 337(2), 301-312. https://doi.org/10.1007/s00441-009-0803-0. Epub 2009 May 16. PMID: 19449034.

DE GREGORIO, L. S.; FRANCO-BELUSSI, L.; DE OLIVEIRA, C. (2021). Leukocyte profile of tadpoles and juveniles of Lithobates catesbeianus Shaw, 1802 (Anura) and the effects of nonylphenol and cyproterone acetate. **South American Journal of Herpetology**, 20(1), 75-83.

DE OLIVEIRA, C. R.; FRACETO, L. F.; RIZZI, G. M.; SALLA, R. F.; ABDALLA, F. C.; COSTA, M. J.; SILVA-ZACARIN, E. C. Hepatic effects of the clomazone herbicide in both its free form and associated with chitosan-alginate nanoparticles in bullfrog tadpoles. **Chemosphere**, v. 149, p. 304-313, abr. 2016. doi: 10.1016/j.chemosphere.2016.01.076. Epub 10 fev. 2016. PMID: 26874058.

DEPLEDGE, M. H. (2020). The rational basis for the use of biomarkers as ecotoxicological tools. In: **Nondestructive biomarkers in vertebrates**, pp. 271-295. CRC Press.

DITRICH, H., & SPLECHTNA, H. (1991). "Endothelial nuclear bulging: Morphological evidence for an intraglomerular perfusion regulating mechanism in the axolotl." **Tissue and Cell**, 23(3), 325-329. https://doi.org/10.1016/0040-8166(91)90050-4. PMID: 18621164.

DITRICH, H., & SPLECHTNA, H. (1992). "The vascular structure of the kidney of the neotenous axolotl and its metamorphosed counterpart." **Journal of Morphology**, 211(1), 55-62. https://doi.org/10.1002/jmor.1052110107. PMID: 29865583.

DITRICH, H., & LAMETSCHWANDTNER, A. (1992). "Glomerular development and growth of the renal blood vascular system in Xenopus laevis (Amphibia: Anura: Pipidae) during metamorphic climax." **Journal of Morphology**, 213(3), 335-340. https://doi.org/10.1002/jmor.1052130306. PMID: 1404405.

DUNCAN, A. E., GARNER, M. M., BARTHOLOMEW, J. L., REICHARD, T. A., & NORDHAUSEN, R. W. (2004). "Renal myxosporidiasis in Asian horned frogs (Megophrys nasuta)." **Journal of Zoo and Wildlife Medicine**, 35(3), 381-386. https://doi.org/10.1638/03-048. PMID: 15526894.

EGAR, M. W., & JARIAL, M. S. (1991). "Structural changes in the proximal tubule of the short-toes axolotl mutant." **Tissue and Cell**, 23(5), 631-639. https://doi.org/10.1016/0040-8166(91)90019-p. PMID: 18621179.

FINKLER, Michael S.; HAYES, Christopher J.; RIFAI, Lina. "Sexual Dimorphisms In Metabolism, Organ Mass, and Reproductive Energetics In Pre-breeding American Toads (Anaxyrus americanus)." **Copeia**, v. 2014, n. 3, pp. 447-453. doi: 10.1643/CP-13-167

FISHER, M. C.; PASMANS, F.; MARTEL, A. Virulence and Pathogenicity of Chytrid Fungi Causing Amphibian Extinctions. **Annual Review of Microbiology**, v. 75, p. 673-693, 8 out. 2021. doi: 10.1146/annurev-micro-052621-124212. Epub 5 ago. 2021. PMID: 34351790.

FORT, DJ; MATHIS, MB; PAWLOWSKI, S; WOLF, JC; PETER, R; CHAMP, S. "Effect of triclosan on anuran development and growth in a larval amphibian growth and development assay." **Journal of Applied Toxicology**, 37(10), 1182-1194. doi: 10.1002/jat.3474. Epub 2017 Apr 24. PMID: 28436085.

FROESE, JM; SMITS, JE; FORSYTH, DJ; WICKSTROM, ML. "Toxicity and immune system effects of dietary deltamethrin exposure in tiger salamanders (Ambystoma tigrinum)." **Journal of Toxicology and Environmental Health A**, 72(8), 518-526. doi: 10.1080/15287390802706363. PMID: 19267312.

FREITAS, J. S.; GIROTTO, L.; GOULART, B. V.; ALHO, L. O. G.; GEBARA, R. C.; MONTAGNER, C. C.; SCHIESARI, L.; ESPÍNDOLA, E. L. G. Effects of 2,4-D-based herbicide (DMA® 806) on sensitivity, respiration rates, energy reserves and behavior of tadpoles. **Ecotoxicol Environ Saf**, v. 182, 109446, 30 out. 2019. DOI: 10.1016/j.ecoenv.2019.109446. PMID: 31323523.

GRASSI, A.; GAMBINI, M.; PANTOLI, M.; TOSCANO, S.; ALBERTETTI, A.; DEL FRASSINO, DM.; UGOCHUKWU, ICI.; ROMEO, O.; OTRANTO, D.; CAFARCHIA, C. "A Lethal Case of Disseminated Cladosporium allicinum Infection in a Captive African Bullfrog." **Journal of Fungi**, 9(2), 191. https://doi.org/10.3390/jof9020191

GROGAN, L. F.; MANGAN, M. J.; McCALLUM, H. I. Amphibian infection tolerance to chytridiomycosis. **Philosophical Transactions of the Royal Society B: Biological Sciences**, v. 378, n. 1882, p. 20220133, 31 jul. 2023. doi: 10.1098/rstb.2022.0133. Epub 12 jun. 2023. PMID: 37305912; PMCID: PMC10258672.

GROSS, ML; HANKE, W.; KOCH, A.; ZIEBART, H.; AMANN, K.; RITZ, E. "Intraperitoneal protein injection in the axolotl: the amphibian kidney as a novel model to study tubulointerstitial activation." **Kidney International**, 62(1), 51-59. doi: 10.1046/j.1523-1755.2002.00402.x. PMID: 12081563.

GROSS, ML; PIECHA, G.; BIERHAUS, A.; HANKE, W.; HENLE, T.; SCHIRMACHER, P.;

RITZ, E. "Glycated and carbamylated albumin are more 'nephrotoxic' than unmodified albumin in the amphibian kidney." **American Journal of Physiology. Renal Physiology**, 301(3), F476-F485. doi: 10.1152/ajprenal.00342.2010. Epub 2011 Mar 2. PMID: 21367923.

GYLLENHAMMAR, I.; ERIKSSON, H.; SÖDERQVIST, A.; LINDBERG, RH.; FICK, J.; BERG, C. "Clotrimazole exposure modulates aromatase activity in gonads and brain during gonadal differentiation in Xenopus tropicalis frogs." **Aquatic Toxicology**, 91(2), 102-109. doi: 10.1016/j.aquatox.2008.10.005. Epub 2008 Nov 1. PMID: 19036460.

HARTLEY, J.; GRIBBINS, KM.; SIEGEL, DS. "Modification of genital kidney nephrons for sperm transport in a plethodontid salamander, Eurycea longicauda." **Journal of Morphology**, 280, 1452-1461. https://doi.org/10.1002/jmor.21041

HASELMAN, JT.; KOSIAN, PA.; KORTE, JJ.; OLMDSTEAD, AW.; IGUCHI, T.; JOHNSON, RD.; DEGITZ, SJ. "Development of the Larval Amphibian Growth and Development Assay: effects of chronic 4-tert-octylphenol or 17β-trenbolone exposure in Xenopus laevis from embryo to juvenile." **Journal of Applied Toxicology**, 36(12), 1639-1650. https://doi.org/10.1002/jat.3330. Epub 2016 May 3. PMID: 27143402.

HAUGAN, BM.; HALBERG, KA.; JESPERSEN, A.; PREHN, LR.; MØBJERG, N. "Functional characterization of the vertebrate primary ureter: structure and ion transport mechanisms of the pronephric duct in axolotl larvae (Amphibia)." **BMC Developmental Biology**, 10, 56. https://doi.org/10.1186/1471-213X-10-56. PMID: 20507566; PMCID: PMC2891660.

HUANG, Y. W.; KARASOV, W. H. (2000). Oral bioavailability and toxicokinetics of 3, 3', 4, 4', 5‐pentachlorobiphenyl in northern leopard frogs, Rana pipiens. Environmental Toxicology and Chemistry: An International Journal, v. 19, n. 7, p. 1788-1794.

ICHIMURA, K.; KURIHARA, H.; SAKAI, T. "Actin filament organization of foot processes in vertebrate glomerular podocytes." **Cell and Tissue Research**, 329(3), 541-557. https://doi.org/10.1007/s00441-007-0440-4. Epub 2007 Jun 29. PMID: 17605050.

ICHIMURA, K.; KURIHARA, H.; SAKAI, T. "Beta-cytoplasmic actin localization in vertebrate glomerular podocytes." **Archives of Histology and Cytology**, 72(3), 165-174. https://doi.org/10.1679/aohc.72.165. PMID: 20513979.

ISIDORO-AYZA, M.; GREAR, DA.; CHAMBOUVET, A. "Pathology and Case Definition of Severe Perkinsea Infection of Frogs." **Veterinary Pathology**, 56(1), 133-142. https://doi.org/10.1177/0300985818798132. Epub 2018 Sep 20. PMID: 30236039.

JAYAWARDENA, UA.; ANGUNAWELA, P.; WICKRAMASINGHE, DD.; RATNASOORIYA, WD.; UDAGAMA, PV. "Heavy metal-induced toxicity in the Indian green frog: Biochemical and histopathological alterations." **Environmental Toxicology and Chemistry**, 36(10), 2855-2867. https://doi.org/10.1002/etc.3848. Epub 2017 Jun 19. PMID: 28474750.

JONES-COSTA, M.; FRANCO-BELUSSI, L.; VIDAL, F. A. P.; GONGORA, N. P.; CASTANHO, L. M.; DOS SANTOS CARVALHO, C.; SILVA-ZACARIN, E. C. M.; ABDALLA, F. C.; DUARTE, I. C. S.; OLIVEIRA, C.; DE OLIVEIRA, C. R.; SALLA, R. F.

Cardiac biomarkers as sensitive tools to evaluate the impact of xenobiotics on amphibians: the effects of anionic surfactant linear alkylbenzene sulfonate (LAS). **Ecotoxicology and Environmental Safety**, v. 151, p. 184-190, 30 abr. 2018. doi: 10.1016/j.ecoenv.2018.01.022. Epub 30 jan. 2018. PMID: 29351853.

KACOLIRIS, F. P. et al. Current threats faced by amphibian populations in the southern cone of South America. **Journal for Nature Conservation**, v. 69, p. 126254, 2022. DOI: 10.1016/j.jnc.2022.126254.

KIM, K. B. Toxicokinetics of xenobiotics. In: Lu's **Basic Toxicology: Fundamentals, Target Organs, and Risk Assessment**. 2017.

KIRYU, Y.; LANDSBERG, JH. "Neurotropic mesomycetozoean-like infection in larvae of the southern toad Anaxyrus terrestris in Florida, USA." **Diseases of Aquatic Organisms**, 113(2), 157-162. https://doi.org/10.3354/dao02836. PMID: 25751858.

KNUTSON, M. G.; RICHARDSON, W. B.; REINEKE, D. M.; GRAY, B. R.; PARMELEE, J. R.; WEICK, S. E. Agricultural ponds support amphibian populations. **Ecological Applications**, v. 14, n. 3, p. 669-684, 2004.

KON, Y.; HASHIMOTO, Y.; KITAGAWA, H.; KUDO, N. "Distribution and morphology of the renin-containing cells in the amphibian kidneys." **Nihon Juigaku Zasshi**, 50(2), 325-332. https://doi.org/10.1292/jvms1939.50.325. PMID: 3290561.

LAMETSCHWANDTNER, A.; MINNICH, B. "Renal microvasculature in the adult pipid frog, Xenopus laevis: A scanning electron microscope study of vascular corrosion casts." **Journal of Morphology**, v. 281, n. 7, p. 725-736, jul. 2020. https://doi.org/10.1002/jmor.21132. Epub 2020 May 6. PMID: 32374496; PMCID: PMC7383921.

LANGLOIS, V. S. Amphibian Toxicology: A Rich But Underappreciated Model for Ecotoxicology **Research. Archives of Environmental Contamination and Toxicology**, v. 80, n. 4, p. 661-662, maio 2021. doi: 10.1007/s00244-021-00844-0. Epub 2021 Apr 11. PMID: 33839894.

LENT, E. M.; BABBITT, K. J.; PINKNEY, A. E. Effects of Environmental Contaminants at Great Bay National Wildlife Refuge on Anuran Development, Gonadal Histology, and Reproductive Steroidogenesis: A Comparison of In Situ and Laboratory Exposures. **Archives of Environmental Contamination and Toxicology**, v. 80, n. 4, p. 663-679, maio 2021. doi: 10.1007/s00244-020-00741-y. Epub 22 maio 2020. PMID: 32444957.

LI, JB.; LI, YY.; SHEN, YP.; ZHU, M.; LI, XH.; QIN, ZF. "2,2',4,4'-tetrabromodipheny ether (BDE-47) disrupts gonadal development of the Africa clawed frog (Xenopus laevis)." **Aquatic Toxicology**, v. 221, 105441, apr. 2020. https://doi.org/10.1016/j.aquatox.2020.105441. Epub 2020 Feb 5. PMID: 32045789.

LOUMBOURDIS, NS. "Nephrotoxic effects of lead nitrate in Rana ridibunda." **Archives of Toxicology**, v. 77, n. 9, p. 527-532, sep. 2003. https://doi.org/10.1007/s00204-003-0487-2. Epub 2003 Jul 15. PMID: 12879213.

LOUMBOURDIS, NS. "Hepatotoxic and nephrotoxic effects of cadmium in the frog Rana ridibunda." **Archives of Toxicology**, v. 79, n. 8, p. 434-440, aug. 2005. https://doi.org/10.1007/s00204-005-0652-x. Epub 2005 Jun 7. PMID: 15940473.

MANI, M., ALTUNIŞIK, A., GEDIK, K. Bioaccumulation of Trace Elements and Health Risk Predictions in Edible Tissues of the Marsh Frog. **Biological Trace Element Research**, v. 200, n. 10, p. 4493-4504, out. 2022. doi: 10.1007/s12011-021-03017-1. Epub 2021 Nov 12. PMID: 34766248.

MASOOD-PARVEEZ, U.; BHATTA, GK.; NADKARNI, VB. "Interrenal of a female gymnophione amphibian, Ichthyophis beddomei, during the annual reproductive cycle." **Journal of Morphology**, v. 211, n. 2, p. 201-206, feb. 1992. https://doi.org/10.1002/jmor.1052110208. PMID: 29865567.

MENG, Y.; MA, J.; JIANG, N.; ZENG, LB.; XIAO, HB. "Pathological and microbiological findings from mortality of the Chinese giant salamander (Andrias davidianus)." **Archives of Virology**, v. 159, n. 6, p. 1403-1412, jun. 2014. https://doi.org/10.1007/s00705-013-1962-6. Epub 2014 Jan 3. PMID: 24385158.

MESEGUER, J.; GARCÍA-AYALA, A.; LÓPEZ-RUIZ, A.; ESTEBAN, MA. "Structure of the amphibian mesonephric tubule during ontogenesis in Rana ridibunda L. tadpoles: early ontogenetic stages, renal corpuscle formation, neck segment and peritoneal funnels." **Anatomical Embryology (Berl)**, v. 193, n. 4, p. 397-406, apr. 1996. https://doi.org/10.1007/BF00186696. PMID: 8694275.

MØBJERG, N.; LARSEN, EH.; JESPERSEN, Å. "Morphology of the Nephron in the Mesonephros of Bufo bufo (Amphibia, Anura, Bufonidae)." **Acta Zoologica**, v. 79, p. 31-50, 1998. https://doi.org/10.1111/j.1463-6395.1998.tb01140.x

MØBJERG, N.; LARSEN, EH.; JESPERSEN, A. "Morphology of the kidney in larvae of Bufo viridis (Amphibia, Anura, Bufonidae)." **Journal of Morphology**, v. 245, n. 3, p. 177-195, sep. 2000. https://doi.org/10.1002/1097-4687(200009)245:3<177::AID-JMOR1>3.0.CO;2-F. PMID: 10972968.

MØBJERG, N.; JESPERSEN, A.; WILKINSON, M. "Morphology of the kidney in the West African caecilian, Geotrypetes seraphini (Amphibia, Gymnophiona, Caeciliidae)." **Journal of Morphology**, v. 262, n. 2, p. 583-607, nov. 2004. https://doi.org/10.1002/jmor.10244. PMID: 15376276.

MONTEIRO, JADN.; CUNHA, LAD.; COSTA, MHPD.; REIS, HSD.; AGUIAR, ACDS.; OLIVEIRA-BAHIA, VRL.; BURBANO, RMR.; ROCHA, CAMD. "Mutagenic and histopathological effects of hexavalent chromium in tadpoles of Lithobates catesbeianus (Shaw, 1802) (Anura, Ranidae)." **Ecotoxicology and Environmental Safety**, v. 163, p. 400-407, nov. 2018. https://doi.org/10.1016/j.ecoenv.2018.07.083. Epub 2018 Jul 29. PMID: 30064085.

MORETTI, EH.; MAZON, AF.; DOS SANTOS, TE.; SANTOS, LRA.; CARVALHO, JFG.; TAVARES, DC.; ANTONIAZZI, MM. "Behavioral, physiological and morphological correlates of parasite intensity in the wild Cururu toad (Rhinella icterica)." **International**

Journal for Parasitology-Parasites and Wildlife, v. 6, n. 3, p. 146-154, 2017. Disponível em: http://hdl.handle.net/11449/163779.

NAYA, DE.; VELOSO, C.; SABAT, P.; BOZINOVIC, F. "The effect of short- and long-term fasting on digestive and metabolic flexibility in the Andean toad, Bufo spinulosus." **Journal of Experimental Biology**, v. 212, Pt 14, p. 2167-2175, jul. 2009. https://doi.org/10.1242/jeb.030650. PMID: 19561206.

NEVES, P.; CATROXO, M.H.; HIPOLITO, M.; OLIVEIRA, C.; MOSTERIO, C. "Use of polyclonal antibodies, electron microscopy and histopathology to detect iridovirus-like particles in bullfrogs." **Boletim do Instituto de Pesca**, v. 42, p. 523-531, 2016. https://doi.org/10.20950/1678-2305.2016v42n3p523.

NICHOLSON, AE.; SIEGEL, DS. "Modifications of the genital kidney proximal and distal tubules for sperm transport in Notophthalmus viridescens (Amphibia, Urodela, Salamandridae)." **Journal of Morphology**, v. 275, n. 8, p. 914-922, aug. 2014. https://doi.org/10.1002/jmor.20268. Epub 2014 Mar 19. PMID: 24643856.

PARTO, P.; VAISSI, S.; SHARIFI, M. "Structure Organization of Urinary System in the Yellow Spotted Mountain Newts (Salamandridae: Neurergus microspilotus)." **Asian Herpetological Research**, v. 10, p. 60, 2014. https://doi.org/10.3724/SP.J.1245.2014.00060.

PELTZER, P. M.; CUZZIOL BOCCIONI, A. P.; ATTADEMO, A. M.; MARTINUZZI, C. S.; COLUSSI, C. L.; LAJMANOVICH, R. C. (2022). Risk of chlorine dioxide as an emerging contaminant during SARS-CoV-2 pandemic: enzyme, cardiac, and behavior effects on amphibian tadpoles. **Toxicology and Environmental Health Sciences**, 1-11.

PESSIER, AP.; BAITCHMAN, EJ.; CRUMP, P.; WILSON, B.; GRIFFITH, E.; ROSS, H. "Causes of mortality in anuran amphibians from an ex situ survival assurance colony in Panama." **Zoo Biology**, v. 33, n. 6, p. 516-526, nov-dec 2014. https://doi.org/10.1002/zoo.21166. Epub 2014 Sep 25. PMID: 25255888.

PONS, G.; GUARDABASSI, A.; PATTONO, P. "The kidney of Hyla arborea (L.) (Amphibia Hylidae) in autumn, winter and spring: histological and ultrastructural observations." **Monitore Zoologico Italiano - Italian Journal of Zoology**, v. 16, n. 3, p. 261-281, 1982. https://doi.org/10.1080/00269786.1982.10736404

RAMSEY, J. P.; REINERT, L. K.; HARPER, L. K.; WOODHAMS, D. C.; ROLLINS-SMITH, L. A. Immune defenses against Batrachochytrium dendrobatidis, a fungus linked to global amphibian declines, in the South African clawed frog, Xenopus laevis. **Infect Immun**, v. 78, n. 9, p. 3981-92, set. 2010. DOI: 10.1128/IAI.00402-10. PMID: 20584973; PMCID: PMC2937463.

REGUEIRA, E.; O'DONOHOE, MEA.; PAVÓN NOVARIN, M.; MICHOUD ETCHEVERRÍA, GC.; TROPEA, C.; HERMIDA, GN. "Integrating morphology and physiology of the key endocrine organ during tadpole development: The interrenal gland." **Journal of Anatomy**, v. 241, n. 6, p. 1357-1370, dec. 2022. https://doi.org/10.1111/joa.13759. Epub 2022 Sep 2. PMID: 36056596; PMCID: PMC9644952.

RHEUBERT, JL.; COOK, HE.; SIEGEL, DS.; TRAUTH, SE. "Histology of the Urogenital System in the American Bullfrog (Rana catesbeiana), with Emphasis on Male Reproductive Morphology." **Zoological Science**, v. 34, n. 5, p. 445-451, oct 2017. https://doi.org/10.2108/zs170060. PMID: 28990475.

RICHTER, S. "The opisthonephros of Rana esculenta (Anura). I. Nephron development." **Journal of Morphology**, v. 226, n. 2, p. 173-187, nov. 1995. https://doi.org/10.1002/jmor.1052260206. PMID: 29865351.

ROBERT, J.; MORALES, H.; BUCK, W.; COHEN, N.; MARR, S.; GANTRESS, J. "Adaptive immunity and histopathology in frog virus 3-infected Xenopus." **Virology**, v. 332, n. 2, p. 667-675, feb. 2005. https://doi.org/10.1016/j.virol.2004.12.012. PMID: 15680432.

ROBINSON, S. A.; CHLEBAK, R. J.; YOUNG, S. D.; DALTON, R. L.; GAVEL, M. J.; PROSSER, R. S.; BARTLETT, A. J.; DE SOLLA, S. R. Clothianidin alters leukocyte profiles and elevates measures of oxidative stress in tadpoles of the amphibian, Rana pipiens. **Environmental Pollution**, v. 284, 117149, 1 set. 2021. doi: 10.1016/j.envpol.2021.117149. Epub 15 abr. 2021. PMID: 33894534.

SAKAI, T.; BILLO, R.; KRIZ, W. "The structural organization of the kidney of Typhlonectes compressicaudus (Amphibia, Gymnophiona)." **Anatomia Embryologia**, v. 174, n. 2, p. 243-252, 1986. https://doi.org/10.1007/BF00824340. PMID: 3740458.

SAKAI, T.; BILLO, R.; NOBILING, R.; GORGAS, K.; KRIZ, W. "Ultrastructure of the kidney of a South American caecilian, Typhlonectes compressicaudus (Amphibia, Gymnophiona). I. Renal corpuscle, neck segment, proximal tubule and intermediate segment." **Cell and Tissue Research**, v. 252, n. 3, p. 589-600, jun. 1988. https://doi.org/10.1007/BF00216646. PMID: 3396057.

SEIXAS, J. T.; SANTANA, A. C.; MARTINS, A. C. P. F.; MESQUITA, E. F. M.; MELLO, S. C. R. P.; HIPOLITO, M. "Presence of ascites in bullfrog breeders reared and kept on a frog farm." **Arquivo Brasileiro de Medicina Veterinária e Zootecnia**, v. 71, n. 6, p. 1955-1960, 2019. https://doi.org/10.1590/1678-4162-11143

SEIXAS FILHO, J. T.; HIPOLITO, M.; MARTINS, A. M. C. R. P. da F.; RODRIGUES, E.; CASTAGNA, A. A.; MELLO, S. C. R. P. "Histopathological alterations in bullfrog juveniles fed commercial rations of different crude protein levels." **Revista Brasileira de Zootecnia**, v. 38, n. 12, p. 2306-2310, 2009. https://doi.org/10.1590/S1516-35982009001200003

SIEGEL, D. S.; RABE, B. "Genital and Pelvic Kidney Renal Corpuscles of the Red-spotted Newt, Notophthalmus viridescens (Amphibia, Urodela, Salamandridae)." **Copeia**, v. 104, n. 2, p. 469-474, 9 Jun. 2016.

SIEGEL, D. S.; SEVER, D. M.; ALDRIDGE, R. D. "The pelvic kidney of male Ambystoma maculatum (Amphibia, Urodela, Ambystomatidae) with special reference to the sexual collecting ducts." **Journal of Morphology**, v. 271, n. 12, p. 1422-1439, dec. 2010. https://doi.org/10.1002/jmor.10883. PMID: 20872840.

SIEGEL, D. S.; ALDRIDGE, R. D.; RHEUBERT, J. L.; GRIBBINS, K. M.; SEVER, D. M.; TRAUTH, S. E. "The testicular sperm ducts and genital kidney of male Ambystoma maculatum (Amphibia, Urodela, Ambystomatidae)." **Journal of Morphology**, v. 274, n. 3, p. 344-360, mar. 2013. https://doi.org/10.1002/jmor.20100. Epub 2012 Nov 29. PMID: 23192852.

SIEGEL, D. S.; ALVINO, S.; TRAUTH, S. E.; SEVER, D. M.; GRIBBINS, K. M. "Spermatogenic cycle of a plethodontid salamander, Eurycea longicauda (Amphibia, Urodela)." **Spermatogenesis**, v. 4, n. 3, p. e982420, dec. 2014. https://doi.org/10.4161/21565562.2014.982420. PMID: 26413402; PMCID: PMC4581059.

SISMAN, T.; KESKIN, M.; DANE, H.; ADIL, Ş.; GEYIKOGLU, F.; COLAK, S.; CANPOLAT, E. "Marsh Frog (Pelophylax ridibundus) as a Bioindicator to Assess Pollution in an Agricultural Area." **Pakistan Journal of Zoology**, v. 53, 10.17582/journal.pjz/20190103130130, 2020.

SLABY, S.; MARIN, M.; MARCHAND, G.; LEMIERE, S. Exposures to chemical contaminants: What can we learn from reproduction and development endpoints in the amphibian toxicology literature? **Environmental Pollution**, v. 248, p. 478-495, maio 2019. doi: 10.1016/j.envpol.2019.02.014. Epub 2019 Feb 4. PMID: 30831345.

STURLA, M.; PRATO, P.; MASINI, M. A.; UVA, B. M. "Ion transport proteins and aquaporin water channels in the kidney of amphibians from different habitats." **Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology**, v. 136, n. 1, p. 1-7, sep. 2003. https://doi.org/10.1016/s1532-0456(03)00141-8. PMID: 14522594.

THEUNISSEN, M.; TIEDT, L.; DU PREEZ, L. H. "The morphology and attachment of Protopolystoma xenopodis (Monogenea: Polystomatidae) infecting the African clawed frog Xenopus laevis." **Parasite**, v. 21, p. 20, 2014. https://doi.org/10.1051/parasite/2014020. Epub 2014 May 14. PMID: 24823278; PMCID: PMC4018937.

TOKIWA, T.; CHOU, S.; TOCHIGI, Y.; KATAYAMA, K.; DUSZYNSKI, D. W. "Hyaloklossia Labbé, 1896 (Alveolata: Apicomplexa) in frogs: Description of a new species and proposing a new subfamily to accommodate these enigmatic parasites." International **Journal for Parasitology: Parasites and Wildlife**, v. 15, p. 199-207, 2021. https://doi.org/10.1016/j.ijppaw.2021.05.005. PMID: 34141567; PMCID: PMC8187853.

UCHIYAMA, M.; MURAKAMI, T.; YOSHIZAWA, H.; WAKASUGI, C. "Structure of the kidney in the crab-eating frog, Rana cancrivora." **Journal of Morphology**, v. 204, n. 2, p. 147-156, May 1990. https://doi.org/10.1002/jmor.1052040204. PMID: 2348460.

UNSICKER, K.; AXELSSON, S.; OWMAN, C.; SVENSSON, K. G. "Innervation of the male genital tract and kidney in the amphibia, Xenopus laevis Daudin, Rana temporaria L., and Bufo bufo L." **Cell and Tissue Research**, v. 160, n. 4, p. 453-484, Jul. 1975. https://doi.org/10.1007/BF00225764. PMID: 807332.

URBINA, J.; BREDEWEG, E. M.; GARCIA, T. S.; BLAUSTEIN, A. R. Host–pathogen dynamics among the invasive American bullfrog (Lithobates catesbeianus) and chytrid fungus (Batrachochytrium dendrobatidis). **Hydrobiologia**, v. 817, p. 267-277, 2018.

VIRIATO, C.; FRANÇA, F. M.; SANTOS, D. S.; MARCANTONIO, A. S.; BADARÓ-PEDROSO, C.; FERREIRA, C. M. "Evaluation of the potential teratogenic and toxic effect of the herbicide 2,4-D (DMA® 806) in bullfrog embryos and tadpoles (Lithobates catesbeianus)." **Chemosphere**, v. 266, p. 129018, Mar. 2021. https://doi.org/10.1016/j.chemosphere.2020.129018. Epub 2020 Nov 19. PMID: 33250224.

VOSS, S. R.; EPPERLEIN, H. H.; TANAKA, E. M. Ambystoma mexicanum, the axolotl: a versatile amphibian model for regeneration, development, and evolution studies. **Cold Spring Harb Protoc**, v. 2009, n. 8, pdb.emo128, ago. 2009. DOI: 10.1101/pdb.emo128. PMID: 20147230.

WEAR, S. L.; ACUÑA, V.; MCDONALD, R.; FONT, C. Sewage pollution, declining ecosystem health, and cross-sector collaboration. **Biological Conservation**, v. 255, 109010, 2021.

WOMBLE, M. A.; LEWBART, G. A.; SHIVE, H. R. "Pathologic Lesions of the Budgett Frog (Lepidobatrachus laevis), an Emerging Laboratory Animal Model." **Comparative Medicine**, v. 70, n. 3, p. 239-247, Jun. 2020. https://doi.org/10.30802/AALAS-CM-19-000071. Epub 2020 Mar 31. PMID: 32234112; PMCID: PMC7287390.

YARTSEV, V.; EVSEYEVA, S. "The Male Urogenital System of a Salamander Ranodon sibiricus (Amphibia, Caudata)." **Current Herpetology**, v. 40, p. 10, 2021. https://doi.org/10.5358/hsj.40.10.

4. ARTIGO 3

Histological description of the spleen and kidneys of the Yellow Cane Toad (*Rhinella icterica*)

Abstract

Studying the morphology of amphibians can provide important information about the physiology and the characteristics that shaped different species along the evolution. In this context, this study aimed to characterize the histological components of the spleen and the kidneys of *Rhinella icterica*, establishing a comparison among other amphibians already described in the literature. For this, six specimens (adult males and females) were collected in an area of Atlantic Forest in the municipality of Tapiraí, state of São Paulo, Brazil. The specimens were transported to the Laboratory of Physiology Conservation (LaFisC) research laboratory (UFSCar-Sorocaba), where they remained under acclimatization. Specimens were euthanized and their spleen and kidneys were dissected and prepared for histomorphological analysis. The microscopic analysis showed that the spleen and kidneys of *R. icterica* have their histomorphology similar to that of other anurans. We observed that the spleen does not have well-defined boundaries between the white and red pulps, as they are diffusely distributed throughout the splenic parenchyma. Another notable feature is the spleen's crucial role in the immune system, with distinct regions for defense cells and erythrocytes. In the kidneys, the tubules and glomeruli are distributed in three regions of the renal parenchyma, and the presence of renal corpuscles (glomeruli and Bowman's capsules) plays an important role in blood filtration. The present study highlighted the importance of expanding research to monitor the health of anuran populations, enabling its conservation and management of these species.
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4.1 Introduction

The neotropical species *Rhinella icterica* (Spix, 1824), popularly known as the “yellow cururu frog”, belongs to the Bufonidae family, (FROST, 2023), presenting a wide distribution from the Atlantic Forest in Brazil to the east of Paraguay and some provinces of Argentina (MANEYRO; KWET, 2008). Predominantly inhabiting forests and semi-arid regions, the genus *Rhinella* presents anatomo-physiological adaptations to face the challenges of each habitat (MAILHO-FONTANA et al., 2021). Yellow cane toads are robust individuals with well-marked cephalic crests and parotid glands close to the eyes. Warty patterns occur especially on the yellow-green back of males, while females have a yellowish back with regular patterns of black and brown spots (AMPHIBIAWEB, 2023).

Furthermore, in terms of thermoregulation, the species has a minimum thermal tolerance ranging from 1.9°C to 5.1°C, and the maximum tolerance is around 39°C. The preferred activity temperature is between 20°C and 27°C, although these parameters may vary with seasons and air humidity (ANDERSON et al., 2018). Regarding their diet, they primarily feed on arthropods such as beetles, ants, dragonflies, opiliones, and spiders. On the other hand, studies investigating their diets have also found plant matter, soil, and even traces of their own skin in their stomach contents (SABAGH et al., 2012). These anurans are highly voracious and capable of preying on poisonous and dangerous species, such as the yellow scorpion (*Tityus serrulatus*). Remarkably, they have a high resistance to scorpion venom, tolerating five times the lethal dose described for rats, which represents the equivalent of the venom of ten scorpions (JARED et al., 2020).

In this study, we chose *Rhinella icterica* as the model its widespread distribution (IUCN, 2023), and its ecological importance as a predator of species harmful to humans for example (JARED et al., 2020). Additionally, its robustness, as well as ease identification and capture make them ideal candidates as environmental bioindicators. Another relevant factor is the fact that there are no records of severely fragmented populations, with the species being classified as "Least Concern" on the International Union for Conservation of Nature (IUCN) Red List (IUCN, 2023). Therefore, the specimens collected for this study did not threaten the stability and resilience of its natural populations.

Histopathological studies using internal organs of amphibians as biomarkers have contributed to many scientific advances, especially regarding morphological and cellular alterations. The kidneys are vital organs in amphibians, as they are responsible for regulating water balance, excreting metabolic waste products, and maintaining ion homeostasis. Thus, it is a very important organ in the study of histopathology (PESSIER, 2009). Investigating the histology of the kidneys of *R. icterica* allows a deeper understanding of the physiological processes related to excretion and reabsorption in a neotropical species. In a study conducted by Pessier (2014), they received anurans that came to die in facilities targeted for ex situ conservation in Panama and the United States. After necropsy and histopathological analyses, they found that the deaths were mostly accompanied by kidney injuries and disease. Demonstrating how the kidney is a source of biomarkers related to the most diverse pathologies and also with the quality of life and habitat of these animals, including not only the monitoring of physical parameters, but also other factors such as the quality of the food offered (SEIXAS FILHO et al., 2009).

The spleen, in turn, is an important lymphoid organ in the immune system of amphibians (UDROIU; SGURA, 2017). Histomorphological analysis of the spleen in this species can provide insights into the immune health, potential effects of environmental (VASCONCELOS-TEIXEIRA et al., 2022) and physical stress (FRANCHINI et al., 2016), or in response to pathogens (HUANG et al., 2019).

Thus, in this article, we explore for the first time the histomorphology of the kidneys and spleen of the neotropical species *Rhinella icterica*, addressing tissue organization, structural characteristics and components, and compared these features with the histomorphology of other amphibian species. Understanding the histomorphology of these organs is crucial for expanding our knowledge of amphibian physiology and adaptation, as well as providing relevant information for conservation and management strategies. This data can provide a technical foundation to future research in histology, herpetology, veterinary histopathology and ecotoxicology.

4.2 Methodology

4.2.1. Collection of individuals

For this study, six healthy adult individuals of *R. icterica* (including males and females) were used for the splenic and renal histologic characterization. The specimens were collected from a fragment of Atlantic Forest in the municipality of Tapiraí, SP (DMS: Tapiraí - 23.918155; -120 47.470454/ SisBio Authorization 61551-1). After the collection, the specimens were immediately transported in aerated plastic boxes to the Laboratory of Physiology Conservation (LaFisC) at the Federal University of São Carlos (Sorocaba campus, Brazil). In the laboratory, animals were individually maintained in plastic containers (37 x 27 x 32 cm) with 50 mL of dechlorinated water. The containers were kept at a 15º inclination so that they could freely move between a wet/immersed area and a dry portion of the container. During the acclimatization period (17 days), the frogs were fed with crickets and beetle larvae, offered twice a day. The animals were kept at 20 ºC, on a 12:12 hour day/ night cycle until euthanasia. Animals were euthanized by spinal cord and brain destruction (Stevens, 2011; AVMA, 2020). Throughout this period, water was monitored daily to ensure that physical and chemical parameters were maintained at acceptable levels (pH 7.5, ammonia levels <1 mg / L - CONAMA, 2011; ASTM, 2004). All procedures were previously authorized by Unicamp's Ethics and Animal Experimentation Committee (CEUA Protocol 5032-1/2018).

4.2.2. Routine Morphological Analysis

The spleen and kidneys of each individual were removed and weighed. Portions were sectioned and fixed at room temperature in buffered paraformaldehyde (4%) for 24h, followed by washing in phosphate buffer (48h). Afterwards, they were dehydrated in increasing series of alcohol solutions: 15%, 30%, 50%, 70%, 80%, 85%, 90%, 95% and 100% for 60 minutes each bath. The last concentration was performed twice in a row. After this period, the tissues were embedded in historesin (Leica®) and histological sections of 3 µm (resin) were obtained through microtome (Leica® - RM 2255 microtome), in collaboration with the Laboratory of Structural and Functional Biology (LABEF) of the UFSCar, Sorocaba *campus*. The sections were stained in hematoxylin and eosin (H.E) for histological description under light microscopy and sealed with ERV-Mount. The analysis and photomicrographs were taken with a Leica® Microscope (model - DM1000) and Leica Application Suite V3.8 (LAS V3.8) software.

4.3 Results and Discussion

4.3.1 Histomorphology Description of the Spleen

The spleen of *Rhinella icterica* can be found associated with the small intestine in the peritoneal cavity, and exhibits a rounded shape, with a reddish-brown coloration. A similar histomorphological organization has been reported for various other anuran species (JORDAN; SEIDEL, 1923; DIENER; NOSSAL, 1966; TURNER; MANNING, 1973; BALDWIN; COHEN, 1981; BARRUTIA et al., 1983; HADJI-AZIMI et al., 1987; ALVAREZ, 1990; DULAK, 1990; DULAK et al., 1993; KINNEY et al., 1996; BRICKER et al., 2012; FRANCO BELUSSI; OLIVEIRA, 2016; LAMETSCHWANDTNER et al., 2016; AKAT, 2018). Another interesting fact is that this organ is covered by a connective tissue capsule with no trabecular system. Therefore, the splenic artery penetrates directly into the parenchyma through a thinner part of the capsule called the hilum (UDROIU; SGURA, 2017) (Figure 13 and 14F). This characteristic has already been described for *Lithobates catesbeianus* (JORDAN; SEIDEL 1923; BRICKER et al., 2012), *Bufo marinus* (DIENER;

NOSSAL, 1966), *Xenopus laevis* (TURNER; MANNING, 1973; BALDWIN; COHEN, 1981; HADJI-AZIMI et al., 1987; KINNEY et al., 1996; BRICKER et al., 2012; LAMETSCHWANDTNER et al., 2016), *Bufo calamita* (BARRUTIA et al., 1983), *Rana perezi* (ALVAREZ, 1990), *Physalaemus nattereri* (FRANCO BELUSSI; OLIVEIRA, 2016), and *Pelophylax bedriagae* (AKAT, 2018), which also lack a trabecular system. In birds and mammals, the splenic capsule also consists of a dense irregular connective tissue but it penetrates the splenic parenchyma, forming the trabecular system (UDROIU; SGURA, 2017). In amphibians, a trabecular structure was only observed in *Ambystoma mexicanum* (DEMIRCAN et al., 2016), *Bombina variegata* (DULAK, 1990), and *Bombina orientalis* (DULAK et al., 1993), therefore, different evolutionary selective pressures may have led to these differentiations. The two functional regions of the *R. icterica* spleen do not have easily distinguished boundaries. The spleen cytoarchitecture of *R. icterica* is named diffuse; therefore, the white and red pulps do not have well-defined boundaries and are diffusely distributed throughout the splenic parenchyma (Figures 1 and 2A-E).

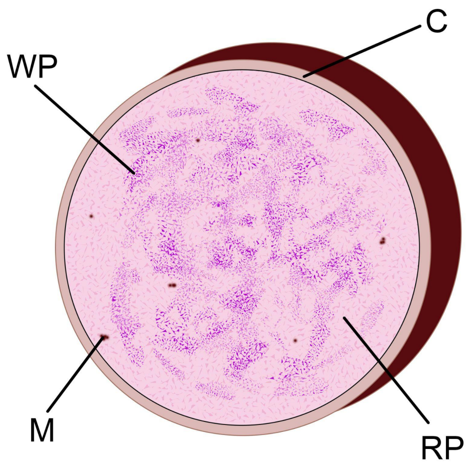

Figure 1: Schematic representation of the diffuse organization of *Rhinella icterica* splenic tissue. The white pulp can be distinguished by the profusion of lymphoid cell nuclei, while the red pulp appears lighter due to the erythrocytes cytoplasm that fill the splenic sinuses. In the red pulp it is also possible to observe some dark brown spots, the melanomacrophages. (**C**: capsule; **M**: melanomacrophage; **RP**: red pulp; **WP**: white pulp)

The splenic cytoarchitecture of amphibians is related to taxonomy. In general, basal groups present diffuse architecture of the spleen, while derived groups present nodular architecture (MANNING, 1991; FRANCO-BELUSSI; OLIVEIRA, 2016). The diffuse pattern can be found in amphibians such as the bullfrog (*Lithobates catesbeianus*) (JORDAN; SEIDEL 1923; BRICKER et al., 2012), the green frog (*Rana perezi*) (ALVAREZ, 1990), *Physalaemus nattereri* (FRANCO BELUSSI; OLIVEIRA, 2016), the levant water frog (*Pelophylax bedriagae*) (AKAT, 2018), and the axolotl (*Ambystoma Mexicanum*) (DEMIRCAN et al., 2016). Other species such as *Bufo marinus* (DIENER; NOSSAL, 1966), *Bufo calamita* (BARRUTIA et al., 1983), which are part of the same family as *R. icterica* (Bufonidae), and other anurans like *Bombina orientalis* (DULAK et al., 1993), *Bombina variegata* (DULAK, 1990) and *Xenopus laevis* (TURNER; MANNING, 1973; BALDWIN; COHEN, 1981; HADJI-AZIMI et al., 1987; KINNEY et al., 1996; BRICKER et al., 2012; LAMETSCHWANDTNER et al., 2016) present nodular cytoarchitecture.

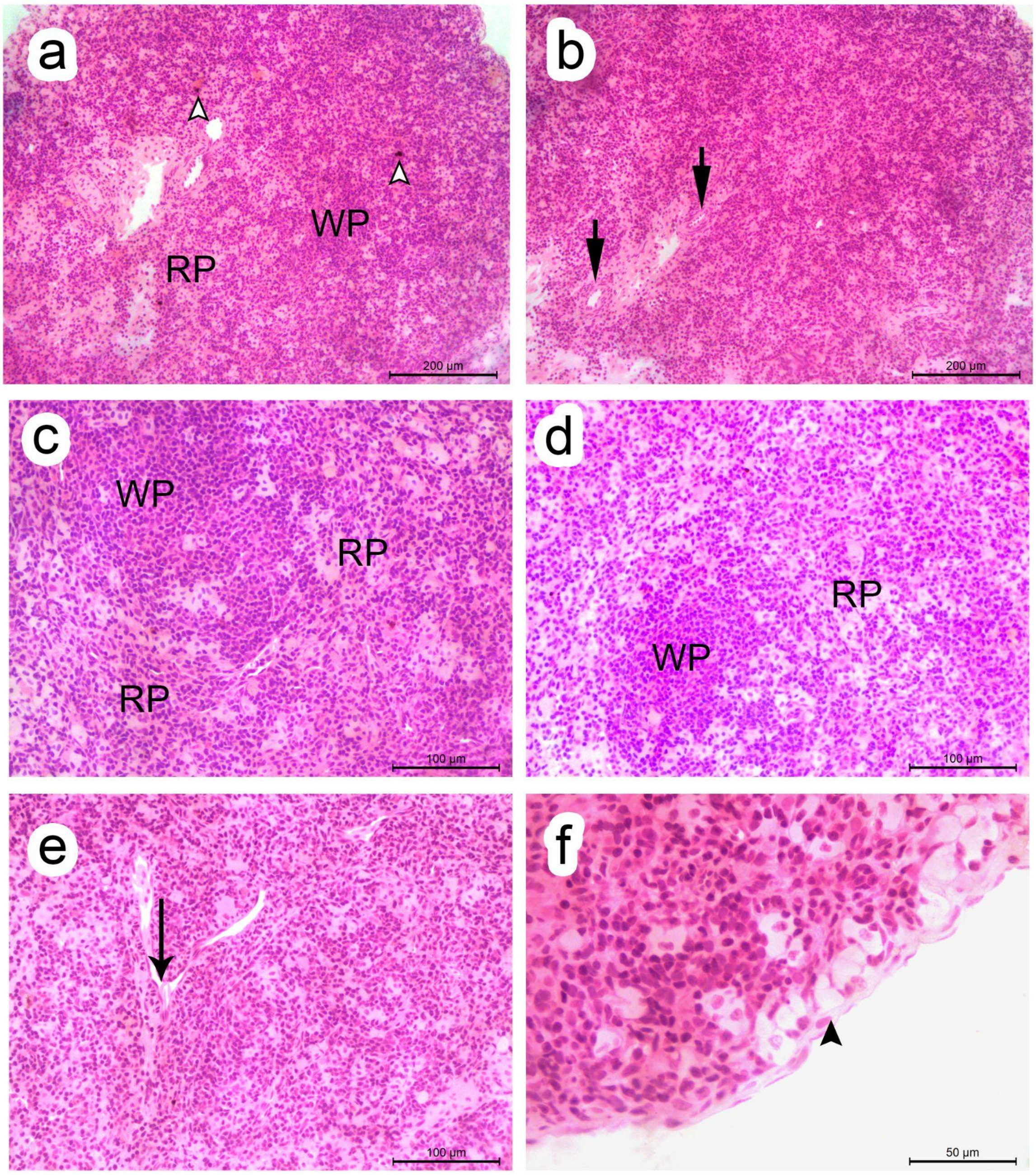

Figure 2: Histological sections of *Rhinella icterica's* spleen (HE). a-e) General organization of splenic tissue. f) Connective tissue capsule that surrounds the organ. (**RP**: red pulp; **WP**: white pulp; **white arrow head**: melanomacrophages; **short arrow**: splenic arterioles; **long arrow**: splenic vein; **black arrow head**: capsule)

The red pulp is composed of splenic cords through which blood flows, containing many nucleated erythrocytes, macrophages and lymphocytes. This portion also contains a few round dark colored structures, known as melanomacrophages, which can be isolated or forming clusters (Figure 3) . The white pulp is a lymphoid region, containing different types of white blood cells. Exogenous organisms in the circulating blood in the red pulp are captured by macrophages and can reach the white pulp to initiate the individual's immune response (DEMIRCAN et al., 2016; UDROIU; SGURA, 2017).

It is possible to observe a diverse population of immune cells that includes lymphocytes and macrophages (Figure. 3). Lymphocytes are responsible for adaptive immune responses, playing a vital role in the recognition and elimination of pathogens, and participating in cell-mediated immunity, including the recognition and destruction of infected or abnormal cells (UDROIU; SGURA, 2017). Macrophages are another important cell type found in the *R. icterica* spleen (Fig. 3). They function as phagocytes, engulfing and digesting foreign particles, dead cells, and other debris. Macrophages also play a critical role in presenting antigens to lymphocytes, initiating and regulating immune responses (DEMIRCAN et al., 2016; UDROIU; SGURA, 2017). Granulocytes, including neutrophils and eosinophils, are also present (Figure 3) and contribute to the immune response by releasing antimicrobial substances and participating in inflammation. The presence of these diverse cell types in the amphibian spleen highlights its critical role in the immune system (FRANCO BELUSSI; OLIVEIRA, 2016; DEMIRCAN et al., 2016; UDROIU; SGURA, 2017).

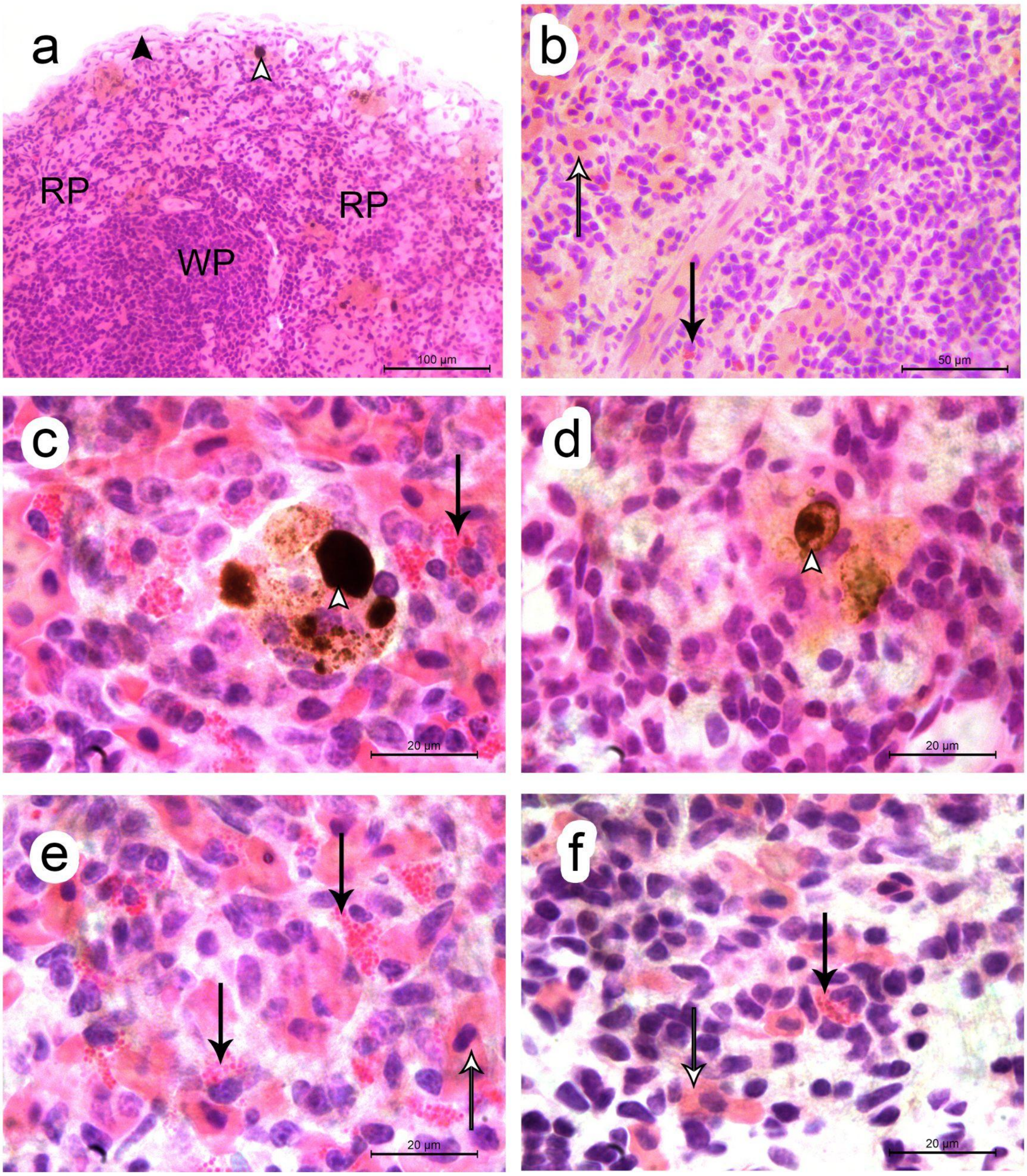

Figure 3: Histological sections of Rhinella icterica spleen (HE). a) General organization of splenic tissue. b) Red pulp region with erythrocytes, macrophages and lymphocytes. c-d) Melanomacrophagic center. e-f) Splenic tissue macrophages and lymphocytes (**RP**: red pulp; **WP**: white pulp; **black arrow** head: capsule; **white arrow head**: melanomacrophages; **black arrow**: leukocyte; **white arrow**: macrophage)

The histopathology of the spleen can be an interesting tool to obtain effect biomarkers for ecotoxicology and pathology studies. Knowing the main structures and organization of the tissue in healthy individuals is essential in order to accurately detect abnormalities. The increased frequency of melanomacrophage centers, as an example, may indicate that the body is initiating immune response, while their reduction may point to an immunosuppressive situation (BÓKONY et al., 2020). Such a sensitive organ can provide a wide range of biomarkers that respond differently depending on the sensitivity of each species (BÓKONY et al., 2020) or the toxicokinetic routes of each toxicant (AWAAD, 2020). The presence of hemorrhages, necrosis, granulomas and fluctuations in cell density can also indicate disease, intoxication, or other physiological stress (DULAK & PLYTYCZ, 1989; BRODKIN et al., 1992; CULLEN et al., 1995; BERGER et al., 1997; SPEARE et al., 1997; LINZEY et al., 2003; TARIGO et al., 2006; MCMURRY et al., 2009; FREMONT-RAHL et al., 2010; CHENG et al., 2014; HARIDY et al., 2014; MENG et al., 2014; FORZAN et al., 2015; PRIYADARSHANI et al., 2015; LI et al., 2017; ÇAKIC, 2018; HUANG et al., 2019).

4.3.2 Histomorphology Description of the Kidneys

The kidneys of *Rhinella icterica* are dorsoventrally flattened (ribbon shape), as also observed in other anuran adults such as *Bufo Bufo* (UNSICKER et al., 1975; MØBJERG et al., 1998), *Xenopus laevis* (UNSICKER et al., 1975; LAMETSCHWANDTNER & MINNICH, 2020), *Rana temporaria* (UNSICKER et al., 1975), *Hyla arborea* (PONS et al., 1982), *Rana cancrivora* (UCHIYAMA et al., 1990), *Rana esculenta* (RICHTER, 1995), *Anaxyrus americanus* (FINKLER et al., 2014), *Lithobates catesbeianus* (RHEUBERT et al., 2017), *Pelophylax bedriagae* (AKAT, 2019), and *Pelophylax ridibundus* (ALNOAIMI et al., 2021).

A healthy specimen has two kidneys, located in the peritoneum, one on each side of the body. The kidneys are brown in color, and present a lobed dorsal surface and a smooth ventral surface (Fig. 4). It can be transversally divided into two regions: dorsolateral region, where the convoluted tubules are found in greater profusion, especially the proximal tubules; and the ventral region, which presents a more spaced distribution of the distal tubules and collecting ducts (Fig. 4). Furthermore, it is notable that the most fully developed glomeruli are found between these two regions, as also described in *Rhinella diptycha* (previously named *Rhinella schneideri*) (SANTANA et al., 2011) (same genus), and in other anuran species (DITRICH & LAMETSCHWANDTNER, 1992; MØBJERG et al., 1998; 2000; FARIAS et al., 1998; BERNABÒ et al., 2017; AKAT, 2019).

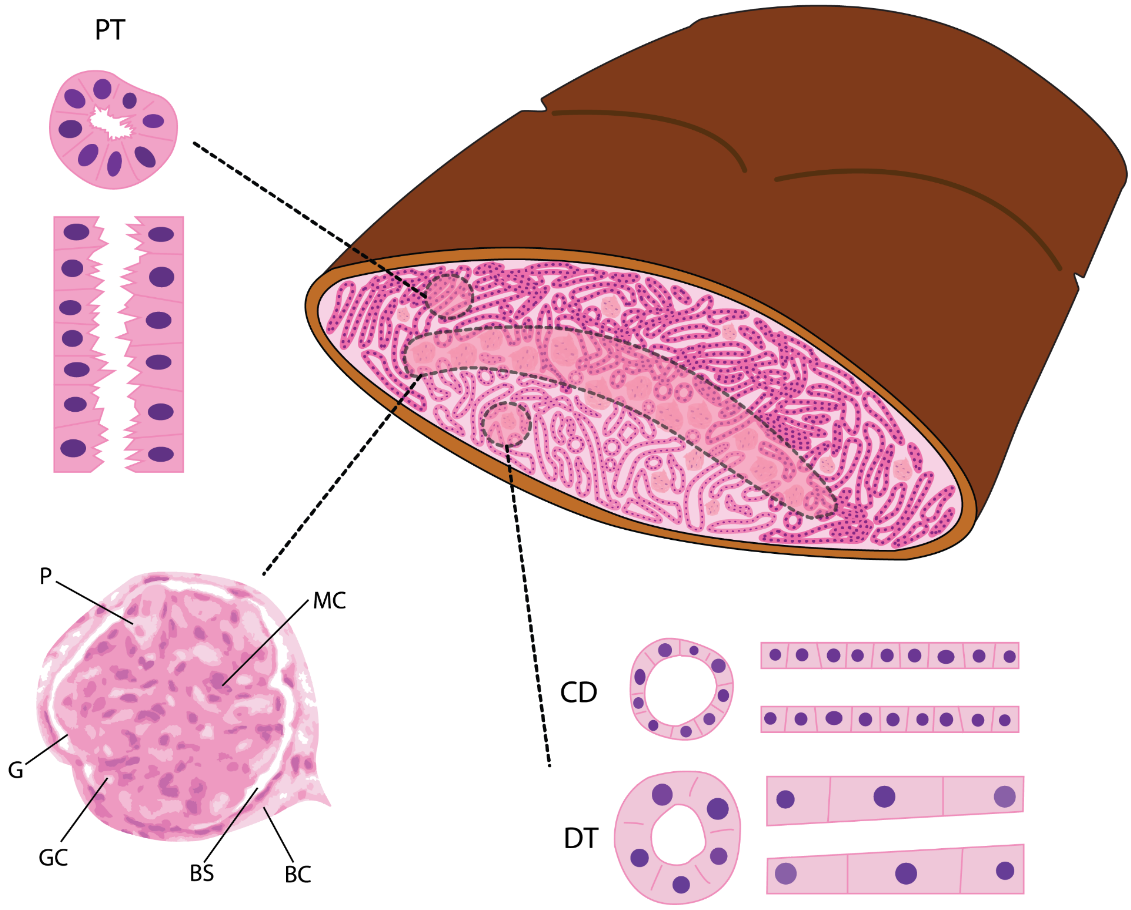

Figure 4: Schematic cross section representation of *Rhinella icterica* main renal structures and general organization. Note the distribution of tubules and glomeruli. The parenchyma can be divided into three main regions: the dorso-lateral region comprises renal tubules in greater profusion, especially proximal tubules. The ventral region presents distal tubules and collecting ducts. Among these regions, the central band is where the largest number of glomeruli and the most developed ones are. (**G**:glomerulus; **GC**: glomerular capillary; **BS**: Bowman's space; **BC**: Bowman's capsule; **MC**: mesangial cell; **P**: podocyte; **CD**: collecting duct; **DT**: distal tubule; **PT**: proximal tubule).

Although the shape and tissue organization of the amphibian kidneys are different from those of mammals and lacking a Henle loop, and because of that amphibians are unable to produce urine that is more concentrated than plasma (SCHMIDT-NIELSEN, 2002), the renal corpuscle structure is quite similar (Fig. 4). The renal corpuscles, comprising the glomerulus and Bowman's capsule, are responsible for the initial filtration of the blood and the formation of the primary urine. The glomerulus is a tuft of capillaries surrounded by specialized cells called podocytes, while the Bowman's capsule acts as a cup-like structure that encloses the glomerulus and collects the filtrate, like in other anuran adults (UNSICKER et al., 1975; PONS et al., 1982; UCHIYAMA et al., 1990; RICHTER, 1995 MØBJERG et al., 1998; FINKLER et al., 2014; RHEUBERT et al., 2017; AKAT, 2019; LAMETSCHWANDTNER; MINNICH, 2020; ALNOAIMI et al., 2021). also, next to the renal corpuscle, it is possible to observe the glomerular artery (Fig. 5 and 6).

Amphibians possess different types of renal tubules, including proximal tubules, distal tubules, and collecting ducts (UNSICKER et al., 1975; PONS et al., 1982; UCHIYAMA et al., 1990; RICHTER, 1995 MØBJERG et al., 1998; FINKLER et al., 2014; RHEUBERT et al., 2017; AKAT, 2019; LAMETSCHWANDTNER; MINNICH, 2020; ALNOAIMI et al., 2021). In *Rhinella icterica* the proximal tubules have cells with the cytoplasm slightly more stained than the others tubule´s cells and a brush border in the lumen. The nuclei of these cells usually follows the columnar format of the cells, assuming a slightly oval shape (Fig. 4; 5 and 6). The distal tubules and collecting ducts have less intense staining and round nuclei, and can be differentiated by the positioning, number, and size of the cells that compose them. The distal tubules have comparatively larger cytoplasm, wider walls, and more widely spaced nuclei. The collecting ducts, therefore, have as comparatively more cells, with smaller cytoplasm and nuclei closer together (Figures. 4; 5 and 6).

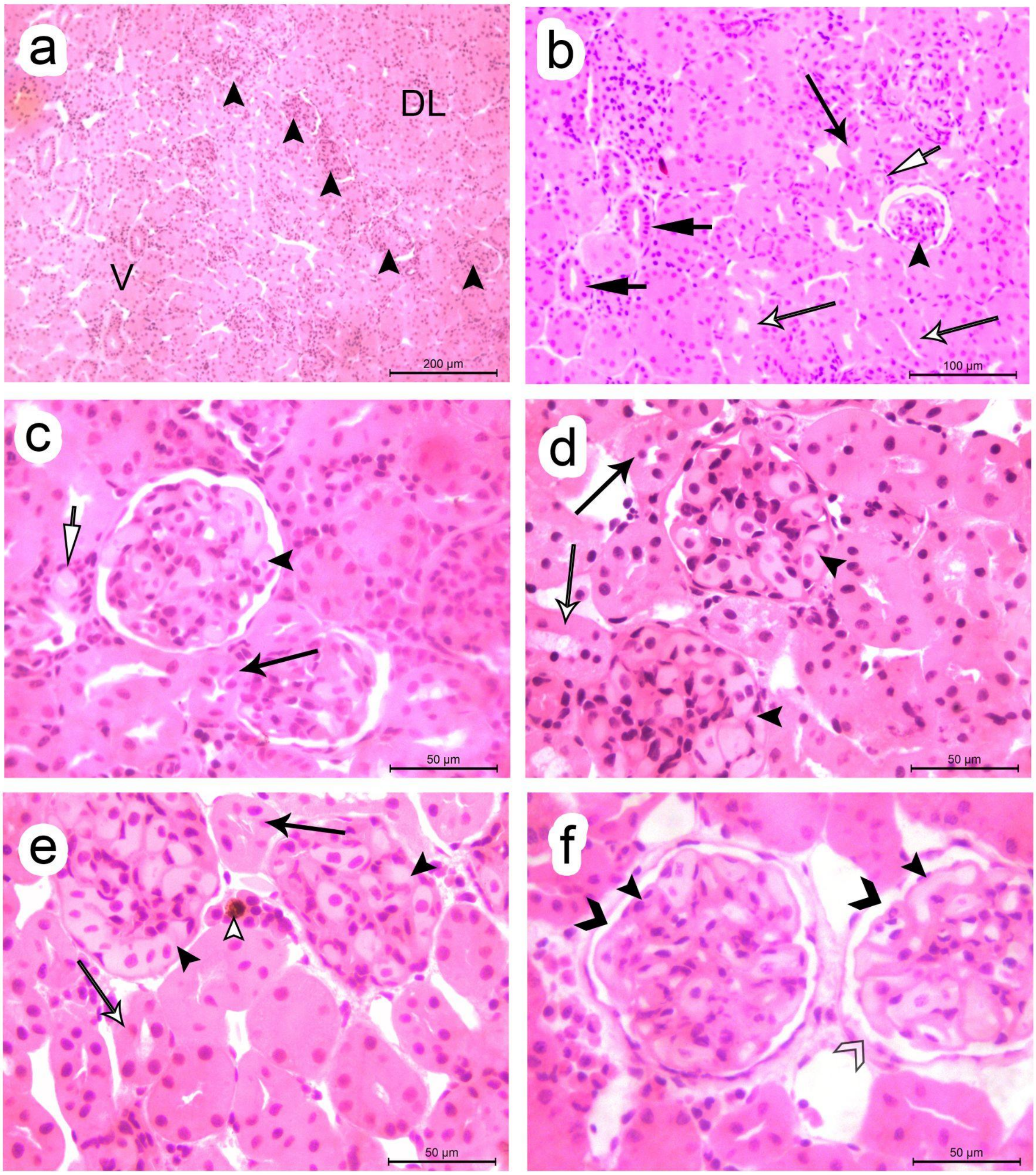

Figure 5: Histological sections of Rhinella icterica kidney (HE). a) General organization of kidney tissue, most glomeruli are between the ventral and dorsolateral regions. b-d) Close to the glomerulus are the glomerular artery, convoluted tubules, and collecting ducts. The proximal convoluted tubules can be distinguished from the others by two main characteristics: their cytoplasm is slightly darker than the others and the cells have an apical brush border (facing the lumen). The distal tubules and collecting ducts have the palest cytoplasm, to differentiate them, in addition to the positioning in the tissue, it is necessary to consider the size of the cytoplasm. Even if the divisions are not clear, it is possible to differentiate them by the space between the nuclei, the collecting ducts have cells with a larger nucleus and smaller cytoplasm than the cells of the distal tubules.e) It is possible to observe melanomacrophages in some points of the renal parenchyma. f) Glomeruli are isolated from other structures by Bowman's capsule and Bowman's space. (**DL**: dorsolateral region; **V**:

ventral region; **black arrowhead**: glomerulus; **white arrowhead**: melanomacrophages; **short black arrow**: collecting duct; **short white arrow**: glomerular artery; **long black arrow**: proximal tubule; **long white arrow**: distal tubule; **black V shape**: Bowman's space; **white V shape**: Bowman's capsule).

In salamanders, male's kidneys are part of the sperm transport complex. These genital nephrons present modifications in their structure for the transport of spermatozoa (SIEGEL et al., 2014; NICHOLSON; SIEGEL, 2014; PARTO et al., 2014; SIEGEL; RABE, 2016; HARTLEY et al., 2019). In bullfrogs the renal division into genital and pelvic portions has also been documented (RHEUBERT et al., 2017). In *Rhinella icterica* males of the present study, it was possible to find some modified tubules containing spermatozoa in the lumen (Fig. 6F). The presence of such modified nephrons involved in sperm transport has been documented in other anurans, including *X. laevis* (UNSICKER et al., 1975) and *L. catesbeianus* (RHEUBERT et al., 2017).

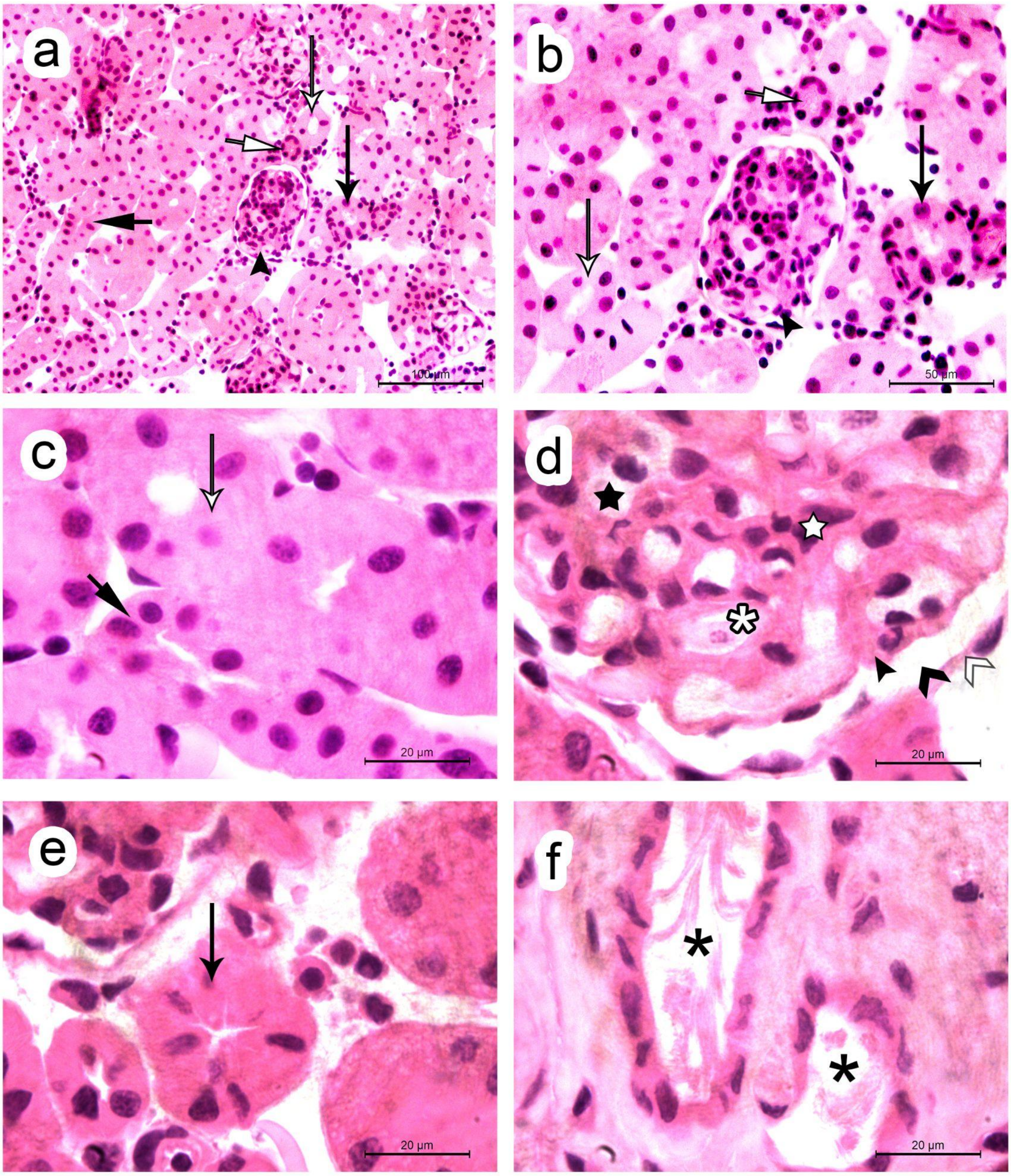

Figure 6: Histological sections of Rhinella icterica kidney (HE). a-b) Renal tissue structures.c) Distal tubule and collecting duct d) Glomerulus detail. e) Proximal tubules. f) Modified nephrons containing sperm can be found in male kidney tissue as there is communication between the kidney and gonadal systems. (**black arrowhead**: glomerulus; **white arrowhead**: melanomacrophages; **short black arro**w: collecting duct; **short white arrow**: glomerular artery; **long black arrow**: proximal tubule; **long white arrow**: distal tubule; **black V shape**: Bowman's space; **white V shape**: Bowman's capsule; **black star**: podocyte; **white star**: mesangial cell; **white asterisk**: erythrocyte; **black asterisk**: modified nephron)

4.4 CONCLUSIONS

The histomorphology of the spleen of *R. icterica* resembles those made for other anurans of nearby taxa, with diffuse cytoarchitecture and parenchyma divided into two functional compartments: the white and red pulps. In these regions, defense cells, erythrocytes and melanomacrophage centers, which in summary constitute the main lymphoid organ in anurans. The kidneys of the yellow cane toad did not show many striking differences from other anurans, which demonstrates that this function was evolutionarily well maintained. The tubules and glomeruli were distributed in the renal parenchyma into three regions. In males it was also possible to find modified nephrons for the transport of spermatozoa, as it also occurs in other amphibian groups due to the connection of the urinary and genital systems.

REFERENCES

AKAT, E. Histological characteristics of the pancreas and spleen of Pelophylax bedriagae (Anura: Ranidae). **Biharean Biol**, v. 12, p. 102–105, 2018.

AKAT, E. Histological and histochemical study on the mesonephric kidney of Pelophylax bedriagae (Anura: Ranidae). **Turkish Journal of Zoology**, v. 43, n. 2, Article 8, 2019. Disponível em: https://doi.org/10.3906/zoo-1807-24. Acesso em: 30/05/2023.

ALNOAIMI, F.; DANE, H.; ŞİŞMAN, T. Histopathologic and genotoxic effects of deltamethrin on marsh frog, Pelophylax ridibundus (Anura: Ranidae). **Environmental Science and Pollution Research**, v. 28, p. 3331–3343, 2021. https://doi.org/10.1007/s11356-020-10711-5.

ALVAREZ, R. An ultrastructural study of the spleen of the ranid frog Rana perezi. Journal of Morphology, v. 204, p. 25–32, 1990. https://doi.org/10.1002/jmor.1052040104.

ALVES-FERREIRA, G. et al. Unraveling global impacts of climate change on amphibians distributions: A life-history and biogeographic-based approach. **Frontiers in Ecology and Evolution**, v. 10, p. 987237, 2022.

AMPHIBIAWEB. Database acessível em: https://amphibiaweb.org. **University of California**, Berkeley, CA, USA. Acesso em: 30/05/2023.

ANDERSON, R. C. O.; BOVO, R. P.; ANDRADE, D. V. Seasonal variation in the thermal biology of a terrestrial toad, Rhinella icterica (Bufonidae), from the Brazilian Atlantic Forest. **Journal of Thermal Biology**, v. 74, p. 77–83, 2018. https://doi.org/10.1016/j.jtherbio.2018.03.011.

AWAAD, A. The biodistribution of melanomacrophages and reactivity of PEG or amine-functionalized iron oxide nanoclusters in the liver and spleen of Egyptian toad after intraperitoneal or oral injections: Histochemical study. **Acta Histochemica**, v. 122, p. 151629, 2020. https://doi.org/10.1016/j.acthis.2020.151629.

BALDWIN III, W. M.; COHEN, N. A giant cell with dendritic cell properties in spleens of the anuran amphibian Xenopus laevis. **Developmental & Comparative Immunology**, v. 5, n. 3, p. 461–473, 1981.

BARRUTIA, M. S. G. et al. Non-lymphoid cells of the anuran spleen: An ultrastructural study in the natterjack, Bufo calamita. **American Journal of Anatomy**, v. 167, p. 83–94, 1983. https://doi.org/10.1002/aja.1001670108.

BERGER, L.; SPEARE, R.; HUMPHREY, J. Mucormycosis in a Free-ranging Green Tree Frog from Australia. **Journal of Wildlife Diseases**, v. 33, p. 903-907, 1997. Disponível em: https://doi.org/10.7589/0090-3558-33.4.903. Acesso em: 30 jun. 2023.

BERNABÒ, I. et al. Chronic exposures to fungicide pyrimethanil: multi-organ effects on Italian tree frog (Hyla intermedia). **Scientific Reports**, v. 7, n. 1, p. 6869, 2017. Disponível em: https://doi.org/10.1038/s41598-017-07367-6. PMID: 28761072; PMCID: PMC5537256.

BÓKONY, V. et al. Effects of two little-studied environmental pollutants on early development in anurans. **Environmental Pollution**, v. 260, p. 114078, 2020. Disponível em: https://doi.org/10.1016/j.envpol.2020.114078.

BRICKER, N. K.; RASKIN, R. E.; DENSMORE, C. L. Cytochemical and immunocytochemical characterization of blood cells and immunohistochemical analysis of spleen cells from 2 species of frog, Rana (Aquarana) catesbeiana and Xenopus laevis. **Veterinary Clinical Pathology**, v. 41, p. 353-361, 2012. Disponível em: https://doi.org/10.1111/j.1939-165X.2012.00452.x.

BRODKIN, M. A. et al. Response of Rana pipiens to Graded Doses of the Bacterium Pseudomonas aeruginosa. **Journal of Herpetology**, v. 26, p. 490-495, 1992. Disponível em: https://doi.org/10.2307/1565128.

BUSKIRK, J. V. Natural variation in morphology of larval amphibians: phenotypic plasticity in nature? **Ecological monographs**, v. 79, n. 4, p. 681-705, 2009.

ÇAKICI, Ö. Histopathological analysis of carbaryl-induced toxicity in the spleen of Levantine frog, Pelophylax bedriagae (Anura: Ranidae). **Environmental Science and Pollution Research**, v. 25, p. 24917-24922, 2018. Disponível em: https://doi.org/10.1007/S11356-018-2538-5/TABLES/1.

CHENG, K. et al. Isolation of a Bohle-like iridovirus from boreal toads housed within a cosmopolitan aquarium collection. **Diseases of Aquatic Organisms**, v. 111, p. 139-152, 2014. Disponível em: https://doi.org/10.3354/DAO02770.

CULLEN, B. R. et al. Experimental infection of Australian anurans (Limnodynastes terraereginae and Litoria latopalmata) with Bohle iridovirus. Diseases of Aquatic Organisms, v. 23, p. 83-92, 1995. Disponível em: https://doi.org/10.3354/DAO023083.

DEMIRCAN, T. et al. A histological atlas of the tissues and organs of neotenic and metamorphosed axolotl. **Acta Histochemica**, v. 118, p. 746-759, 2016. Disponível em: https://doi.org/10.1016/j.acthis.2016.07.006.

DIENER, E.; NOSSAL, G. J. V. Phylogenetic Studies on the Immune Response I. Localization of Antigens and Immune Response in the Toad, Bufo marinus. **Immunology**, v. 10, p. 535, 1966.

DITRICH, H.; LAMETSCHWANDTNER, A. Glomerular development and growth of the renal blood vascular system in Xenopus laevis (Amphibia: Anura: Pipidae) during metamorphic climax. **Journal of Morphology**, v. 213, n. 3, p. 335-340, 1992. Disponível em: https://doi.org/10.1002/jmor.1052130306.

DULAK, J.; PŁYTYCZ, B. The effect of laboratory environment on the morphology of the spleen and the thymus in the yellow-bellied toad, Bombina variegata (L.). **Developmental and Comparative Immunology**, v. 13, p. 49-55, 1989. Disponível em: https://doi.org/10.1016/0145-305X(89)90016-5.

DULAK, J. The architecture of the spleen of the yellow-bellied toad, Bombina variegata. **Zoological Society of London**, v. 37, p. 1990-1991, 1990.

DULAK, J. et al. Immune-complex-trapping cells in the spleen of the oriental fire-bellied toad, Bombina orientalis. **Cell and Tissue Research**, v. 271, p. 155-158, 1993. Disponível em: https://doi.org/10.1007/BF00297553.

FARÍAS, A. et al. Structure of the Bufo arenarum kidney: renal corpuscle, neck segment and proximal tubule. **Biocell**, v. 22, n. 3, p. 187-196, 1998. PMID: 10892449.

FINKLER, M. S. et al. Sexual Dimorphisms In Metabolism, Organ Mass, and Reproductive Energetics In Pre-breeding American Toads (anaxyrus Americanus). **Copeia**, v. 2014, n. 3, p. 447-453, 2014. Disponível em: https://doi.org/10.1643/CP-13-167.

FORZÁN, M. J. et al. Clinical signs, pathology and dose-dependent survival of adult wood frogs, Rana sylvatica, inoculated orally with frog virus 3 (Ranavirus sp., iridoviridae). **Journal of General Virology**, v. 96, p. 1138-1149, 2015. Disponível em: https://doi.org/10.1099/vir.0.000043.

FRANCHINI, A.; DELLA ROCCA, A.; BERTOLOTTI, E. The spleen and skin wound healing in Xenopus adults. **Journal of Morphology**, v. 277, p. 888-895, 2016. Disponível em: https://doi.org/10.1002/jmor.20542.

FRANCO-BELUSSI, L.; DE OLIVEIRA, C. The spleen of Physalaemus nattereri (Amphibia: Anura): morphology, melanomacrophage pigment compounds and responses to α-melanocyte stimulating hormone. **Italian Journal of Zoology**, v. 83, p. 298-305, 2016. Disponível em: https://doi.org/10.1080/11250003.2016.1194488.

FREMONT-RAHL, J. J. et al. Mycobacterium liflandii outbreak in a research colony of Xenopus (Silurana) tropicalis frogs. **Veterinary Pathology**, v. 48, p. 856-867, 2011. Disponível em: https://doi.org/10.1177/0300985810388520.

FROST, D. R. Amphibian Species of the World: an Online Reference. Version 6.1. Electronic Database accessible at https://amphibiansoftheworld.amnh.org/index.php. **American Museum of Natural History**, New York, USA. doi.org/10.5531/db.vz.0001

GRANT, C, E. H.; MILLER, D. A.; MUTHS, E. A synthesis of evidence of drivers of amphibian declines. **Herpetologica**, v. 76, n. 2, p. 101-107, 2020.

HADJI-AZIMI, I.; COOSEMANS, V.; CANICATTI, C. Atlas of adult Xenopus laevis laevis hematology. **Developmental and Comparative Immunology**, v. 11, p. 807–874, 1987. doi.org/10.1016/0145-305X(87)90068-1

HARIDY, M. et al. Mycobacterium marinum infection in Japanese forest green tree frogs (Rhacophorus arboreus). **Journal of Comparative Pathology**, v. 151, p. 277–289, 2014. doi.org/10.1016/J.JCPA.2014.04.014

HARTLEY, J.; GRIBBINS, K. M.; SIEGEL, D. S. Modification of genital kidney nephrons for sperm transport in a plethodontid salamander, Eurycea longicauda. **Journal of Morphology**, v. 280, p. 1452–1461, 2019. doi.org/10.1002/jmor.21041

HUANG, X. et al. Candidate Animal Disease Model of Elizabethkingia Spp. Infection in Humans, Based on the Systematic Pathology and Oxidative Damage Caused by E. miricola in Pelophylax nigromaculatus. **Oxidative Medicine and Cellular Longevity**, 2019. doi.org/10.1155/2019/6407524

IUCN. **The IUCN Red List of Threatened Species**. Version 2022-2. https://www.iucnredlist.org ISSN 2307-8235

JARED, C. et al. Toads prey upon scorpions and are resistant to their venom: a biological and ecological approach to scorpionism. **Toxicon**, v. 178, p. 4-7, 2020. doi.org/10.1016/j.toxicon.2020.02.013

JORDAN, H. E.; SPEIDEL, C. C. Studies on lymphocytes. I. Effect of splenectomy, experimental hemorrhage and a hemolytic toxin in the frog. **American Journal of Anatomy**, v. 32, p. 155–187, 1923. doi.org/10.1002/AJA.1000320203

KINNEY, K. S.; FELTEN, S. Y.; COHEN, N. Sympathetic innervation of the amphibian spleen: developmental studies in Xenopus laevis. **Dev. Comp. Immunol.**, v. 20, n. 1, p. 51-59, jan.-fev. 1996. DOI: 10.1016/0145-305X(95)00036-S. PMID: 8738936.

LAMETSCHWANDTNER, A.; MINNICH, B. Renal microvasculature in the adult pipid frog, Xenopus laevis: A scanning electron microscope study of vascular corrosion casts. **Journal of Morphology**, v. 281, p. 725-736, 2020. doi.org/10.1002/jmor.21132

LI, W. T. et al. Mycolactone-producing Mycobacterium marinum infection in captive Hong Kong warty newts and pathological evidence of impaired host immune function. **Diseases of Aquatic Organisms**, v. 123, p. 239–249, 2017. doi.org/10.3354/DAO03092

LIEDTKE, H. C.; WIENS, J. J.; GOMEZ-MESTRE, I. The evolution of reproductive modes and life cycles in amphibians. **Nature Communications**, v. 13, n. 1, p. 7039, 2022.

LINZEY, D. W. et al. Role of environmental pollutants on immune functions, parasitic infections and limb malformations in marine toads and whistling frogs from Bermuda. **International Journal of Environmental Health Research**, v. 13, p. 125–148, 2003. doi.org/10.1080/0960312031000098053

MAILHO-FONTANA, P. L. et al. Skin and poison glands in toads (Rhinella) and their role in defence and water balance. **Acta Zoologica**, v. 103, p. 112–128, 2021. doi.org/10.1111/azo.12390

MANNING, M. J. Histological organization of the spleen: implications for immune functions in amphibians. **Research in Immunology**, v. 142, p. 355–359, 1991. doi.org/10.1016/0923-2494(91)90091-V

MCMURRY, S. T. et al. Influence of Land Use on Body Size and Splenic Cellularity in Wetland Breeding Spea spp. **Journal of Herpetology**, v. 43, p. 421–430, 2009. doi.org/10.1670/07-126r2.1

MENG, Y. et al. Pathological and microbiological findings from mortality of the Chinese giant salamander (Andrias davidianus). **Archives of Virology**, v. 159, p. 1403–1412, 2014. doi.org/10.1007/S00705-013-1962-6

MØBJERG, N.; LARSEN, E. H.; JESPERSEN, Å. Morphology of the Nephron in the Mesonephros of Bufo bufo (Amphibia, Anura, Bufonidae). **Acta Zoologica**, v. 79, p. 31-50, 1998. https://doi.org/10.1111/j.1463-6395.1998.tb01140.x

MØBJERG, N.; LARSEN, E. H.; JESPERSEN, A. Morphology of the kidney in larvae of Bufo viridis (Amphibia, Anura, Bufonidae). **Journal of Morphology**, v. 245, n. 3, p. 177-195, 2000. https://doi.org/10.1002/1097-4687(200009)245:3<177::AID-JMOR1>3.0.CO;2-F

NICHOLSON, A. E.; SIEGEL, D. S. Modifications of the genital kidney proximal and distal tubules for sperm transport in Notophthalmus viridescens (Amphibia, Urodela, Salamandridae). **Journal of Morphology**, v. 275, n. 8, p. 914-922, 2014. https://doi.org/10.1002/jmor.20268

O'DONOHOE, M. A. et al. Comparative skin histology of neotropical odontophrynid frogs. **Zoologischer Anzeiger**, v. 301, p. 127-144, 2022.

OLIVEIRA, B. F. et al. AmphiBIO, a global database for amphibian ecological traits. **Scientific data**, v. 4, n. 1, p. 1-7, 2017.

PARTO, P.; VAISSI, S.; SHARIFI, M. Structure Organization of Urinary System in the Yellow Spotted Mountain Newts (Salamandridae: Neurergus microspilotus). **Asian Herpetological Research**, v. 5, p. 60-67, 2014. https://doi.org/10.3724/SP.J.1245.2014.00060

PESSIER, A. P. Edematous Frogs, Urinary Tract Disease, and Disorders of Fluid Balance in Amphibians. **Journal of Exotic Pet Medicine**, v. 18, n. 1, p. 4-13, 2009. https://doi.org/10.1053/j.jepm.2008.10.003

PESSIER, A. P. et al. Causes of mortality in anuran amphibians from an ex situ survival assurance colony in Panama. **Zoo Biology**, v. 33, n. 6, p. 516-526, 2014. https://doi.org/10.1002/zoo.21166

PONS, G.; GUARDABASSI, A.; PATTONO, P. The Kidney of Hyla arborea (L.) (Amphibia Hylidae) in Autumn, Winter and Spring: Histological and Ultrastructural Observations. **Monitore Zoologico Italiano - Italian Journal of Zoology**, v. 16, n. 3, p. 261-281, 1982. https://doi.org/10.1080/00269786.1982.10736404

PRIYADARSHANI, S. et al. Heavy metal mediated immunomodulation of the Indian green frog, Euphlyctis hexadactylus (Anura: Ranidae) in urban wetlands. **Ecotoxicology and Environmental Safety**, v. 116, p. 40-49, 2015. https://doi.org/10.1016/j.ecoenv.2015.02.037

REGIS-ALVES, E. et al. Structural cutaneous adaptations for defense in toad (Rhinella icterica) parotoid macroglands. **Toxicon**, v. 137, p. 128-134, 2017.

RHEUBERT, J. L. et al. Histology of the Urogenital System in the American Bullfrog (Rana catesbeiana), with Emphasis on Male Reproductive Morphology. **Zoological Science**, v. 34, n. 5, p. 445-451, 2017. https://doi.org/10.2108/zs170060

RICHTER, S. The opisthonephros of Rana esculenta (Anura). I. Nephron development. **Journal of Morphology**, v. 226, n. 2, p. 173-187, 1995. https://doi.org/10.1002/jmor.1052260206

RUDH, A.; QVARNSTRÖM, A. Adaptive colouration in amphibians. **Seminars in cell & developmental biology**, v. 24, n. 6-7, p. 553-561, jun. 2013.

SABAGH, L. T.; CARVALHO-E-SILVA, A. M. P. T.; ROCHA, C. F. D. Diet of the toad Rhinella icterica (Anura: Bufonidae) from Atlantic Forest Highlands of southeastern Brazil. **Biota Neotropica**, v. 12, n. 4, 2012. https://doi.org/10.1590/S1676-06032012000400027

SANTANA, K. L.; SILVA, T. B.; FERNANDES, M. S. Aspectos morfo-histológicos e funcionais do sistema urinário do sapo, Rhinella schneideri (Werner, 1894) em estresse hídrico. **Nucleus**, v. 8, n. 1, 2011. https://doi.org/10.3738/nucleus.v8i1.526

SIEGEL, D. S.; RABE, B. Genital and Pelvic Kidney Renal Corpuscles of the Red-spotted Newt, Notophthalmus viridescens (Amphibia, Urodela, Salamandridae). **Copeia**, v. 104, n. 2, p. 469-474, 2016.

SIEGEL, D. S. et al. Spermatogenic cycle of a plethodontid salamander, Eurycea longicauda (Amphibia, Urodela). **Spermatogenesis**, v. 4, n. 3, p. e982420, 2014. https://doi.org/10.4161/21565562.2014.982420

SPEARE, R. et al. Pathology of mucormycosis of cane toads in Australia. **Journal of Wildlife Diseases**, v. 33, p. 105-111, 1997. https://doi.org/10.7589/0090-3558-33.1.105

TARIGO, J. et al. Reluctant to dive: coelomic effusion in a frog. **Veterinary Clinical Pathology**, v. 35, p. 341-344, 2006. https://doi.org/10.1111/j.1939-165X.2006.tb00145.x

TURNER, R. J.; MANNING, M. J. Response of the toad, Xenopus laevis, to circulating antigens. I. Cellular changes in the spleen. **Journal of Experimental Zoology**, v. 183, p.

21-33, 1973. https://doi.org/10.1002/jez.1401830104

UCHIYAMA, M. et al. Structure of the kidney in the crab-eating frog, Rana cancrivora. **Journal of Morphology**, v. 204, n. 2, p. 147-156, 1990. https://doi.org/10.1002/jmor.1052040204

UDROIU, I.; SGURA, A. The Phylogeny of the Spleen. **The Quarterly Review of Biology**, v. 92, p. 411-443, 2017. https://doi.org/10.1086/695327

UNSICKER, K. et al. Innervation of the male genital tract and kidney in the amphibia, Xenopus laevis Daudin, Rana temporaria L., and Bufo bufo L. **Cell and Tissue Research**, v. 160, n. 4, p. 453-484, 1975. https://doi.org/10.1007/BF00225764

VASCONCELOS-TEIXEIRA, R. et al. Stress Response, Immunity, and Organ Mass in Toads (Rhinella diptycha) Living in Metal-Contaminated Areas. **Biological Trace Element Research**, v. 200, p. 800-811, 2022. https://doi.org/10.1007/s12011-021-02699-x

5 Conclusões

Os biomarcadores fornecidos pelo rim dos anfíbios são pouco explorados, dada a importância deste órgão nos processos de eliminação de toxicantes e a quantidade de trabalhos com descrições ricas em detalhamento para consulta e comparação. Já a histomorfologia do baço dos anfíbios é pouco estudada, mesmo que este órgão seja o principal responsável pelas respostas imunológicas desses animais. A falta de estudos descritivos e que utilizam biomarcadores esplênicos indica que nos trabalhos em que são utilizados outros órgãos como fonte de biomarcadores (e.g. fígado, pele), o baço e, em menor escala, o rim são dispensados sem análises. Isso faz com que mais animais precisem ser eutanasiados para "fins estatísticos". Utilizar abordagens integrativas com outras áreas do conhecimento, utilizando múltiplos biomarcadores pode otimizar os trabalhos de exposição em ensaios ecotoxicológicos e para o monitoramento ambiental. Ainda que a histologia seja uma ferramenta poderosa na avaliação de efeitos subletais de diversos estressores, métodos não invasivos e não destrutivos devem ser incentivados, principalmente aqueles que não afetam a sobrevivência do animal, em espécies nativas e/ou em populações com grau de vulnerabilidade.

A histomorfologia do baço de *R. icterica* assemelha-se a outros anuros de táxons próximos, com a citoarquitetura difusa, o parênquima esplênico é dividido em dois compartimentos funcionais: as polpas branca e vermelha. Nessas regiões, células de defesa, eritrócitos e centros melanomacrófagos que compõem o principal órgão linfoide dos anuros. Os rins do sapo da cana-amarela não apresentaram diferenças muito marcantes em relação a outros anuros. Os túbulos e glomérulos estão distribuídos no parênquima renal em três regiões. Nos machos é possível encontrar néfrons modificados para o transporte de espermatozóides, como também ocorre em outros grupos de anfíbios devido à conexão dos sistemas urinário e genital.

REFERÊNCIAS

AMPHIBIAWEB. Database accessible at https://amphibiaweb.org. **University of California, Berkeley**, CA, USA. Accessed 17 May 2023.

AZEVEDO CALDERON, L. D.; SILVA, A. D.; CIANCAGLINI, P.; STÁBELI, R. G. Antimicrobial peptides from Phyllomedusa frogs: from biomolecular diversity to potential nanotechnologic medical applications. **Amino acids**, jan. 2011, p. 29-49.

BRATTSTROM, B. H. Amphibian temperature regulation studies in the field and laboratory. **American Zoologist**, v. 19, n. 1, p. 345-356, fev. 1979. DOI: 10.1093/icb/19.1.345.

BORGIOLI, G.; FRANGIONI, G. Blood and splenic respiratory compensation in larval newts. **Italian Journal of Zoology**, v. 64, p. 221–226, 1997. DOI: 10.1080/11250009709356200.

CALDERON, M. R. et al. Anthropogenic impacts on rivers: use of multiple indicators to assess environmental quality status. **Hydrobiologia**, 2022, p. 1-19. DOI: 10.1007/s10750-022-05090-6.

GREEN, D. M.; LANNOO, M. J.; LESBARRÈRES, D.; MUTHS, E. Amphibian population declines: 30 years of progress in confronting a complex problem. **Herpetologica**, v. 76, n. 2, p. 97-100, 2020.

ELGUERO, J.; CAMPILLO, N.; PÁEZ, J. A. Analgésicos no convencionales: Epibatidina, un potente analgésico nicotínico. In: **Anales de la Real Academia de Farmacia**, v. 62, n. 3, p. 303-321, 1996.

DOBROVOLJC, K., FALNOGA, I., ŽNIDARIČ, M.T., MAZEJ, D., ŠČANČAR, J., BULOG, B. Cd, Cu, Zn, Se, and metallothioneins in two amphibians, Necturus maculosus (Amphibia, Caudata) and Bufo bufo (Amphibia, Anura). **Biol. Trace Elem. Res.** 150, 178–194. https://doi.org/10.1007/s12011-012-9461-2

FISHER, M.C., PASMANS, F., MARTEL, A. Virulence and Pathogenicity of Chytrid Fungi Causing Amphibian Extinctions. **Annu. Rev. Microbiol**. 75, 673–693, 2021. https://doi.org/10.1146/annurev-micro-052621-124212

FORZÁN, M.J., JONES, K.M., VANDERSTICHEL, R.V., WOOD, J., KIBENGE, F.S.B., KUIKEN, T., WIRTH, W., ARIEL, E., DAOUST, P.Y. Clinical signs, pathology and dose-dependent survival of adult wood frogs, Rana sylvatica, inoculated orally with frog virus 3 (Ranavirus sp., iridoviridae). **J. Gen. Virol.** 96, 1138–1149, 2015.

FROST, D. R. Amphibian Species of the World: an Online Reference. Version 6.1. Electronic Database accessible at https://amphibiansoftheworld.amnh.org/index.php. **American Museum of Natural History**, New York, USA. doi.org/10.5531/db.vz.0001

GANIVET, E. Growth in human population and consumption both need to be addressed to reach an ecologically sustainable future. **Environment, Development and Sustainability**, v. 22, n. 6, p. 4979-4998, 2020.

GRAYFER, L., EDHOLM, E.S., ROBERT, J. Mechanisms of amphibian macrophage development: Characterization of the Xenopus laevis colony-stimulating factor-1 receptor. **Int. J. Dev. Biol**. 58, 757–766, 2014. https://doi.org/10.1387/ijdb.140271jr

HASLAM, I. S.; ROUBOS, E. W.; MANGONI, M. L.; YOSHIZATO, K.; VAUDRY, H.; KLOEPPER, J. E.; PATTWELL, D. M.; MADERSON, P. F.; PAUS, R. From frog integument to human skin: dermatological perspectives from frog skin biology. **Biol Rev Camb Philos Soc.** v. 89, n. 3, p. 618-55, ago. 2014. DOI: 10.1111/brv.12072. PMID: 24299058.

HAYES, T.B. Histological examination of the effects of corticosterone in larvae of the western toad, Bufo boreas (Anura: Bufonidae), and the oriental fire-bellied toad, Bombina orientalis (Anura: Discoglossidae). **J. Morphol**. 226, 297–307, 1995. https://doi.org/10.1002/JMOR.1052260306

HOCKING, D. J.; BABBITT, K. J. Amphibian contributions to ecosystem services. **Herpetological conservation and biology**, 2014.

HOFFMANN, F.; KLOAS, W. The antiestrogens tamoxifen and fulvestrant abolish estrogenic impacts of 17α-ethinylestradiol on male calling behavior of Xenopus laevis. **PLoS One**, v. 7, n. 9, e44715, 2012. DOI: 10.1371/journal.pone.0044715. PMID: 23028589; PMCID: PMC3445530.

KACOLIRIS, F. P. et al. Current threats faced by amphibian populations in the southern cone of South America. **Journal for Nature Conservation**, v. 69, p. 126254, 2022. DOI: 10.1016/j.jnc.2022.126254.

LIEDTKE, H. C.; WIENS, J. J.; GOMEZ-MESTRE, I. The evolution of reproductive modes and life cycles in amphibians. **Nature Communications**, v. 13, n. 1, p. 7039, 2022.

LILLYWHITE, Harvey B. Water relations of tetrapod integument. **J Exp Biol.** v. 209, Pt 2, p. 202-26, jan. 2006. DOI: 10.1242/jeb.02007. PMID: 16391344.

MAHMOOD, Tariq et al. Metal concentrations in common skittering frog (Euphlyctis cyanophlyctis) inhabiting Korang River, Islamabad, Pakistan. **Basic and Applied Herpetology**, v. 30, 2016. DOI: 10.11160/bah.16001.

MORALES, H. D.; ROBERT, J. Characterization of Primary and Memory CD8 T-Cell Responses against Ranavirus (FV3) in Xenopus laevis. **J. Virol.**, v. 81, p. 2240–2248, 2007. Disponível em: https://doi.org/https://doi.org/10.1128/JVI.01104-06. Acesso em: 27 jun. 2023

RIBAS, L. et al. Expression Profiling the Temperature-Dependent Amphibian Response to Infection by Batrachochytrium dendrobatidis. **PLoS One**, v. 4, p. e8408, 2009. Disponível em: https://doi.org/10.1371/journal.pone.0008408. Acesso em: 27 jun. 2023.

SCHMIDT-NIELSEN, Knut. Fisiologia animal: adaptação e meio ambiente. 5ª ed. São Paulo: Santos, 2002. 611 p. ISBN 8572880429, 9788572880428.

STUART, S. N. et al. Status and trends of amphibian declines and extinctions worldwide. **Science**, v. 306, p. 1783–1786, 2004. Disponível em: https://doi.org/10.1126/SCIENCE.1103538. Acesso em: 27 jun. 2023.

TSENG, A. S.; BEANE, W. S.; LEMIRE, J. M.; MASI, A.; LEVIN, M. Induction of vertebrate regeneration by a transient sodium current. **J Neurosci**. v. 30, n. 39, p. 13192-200, 29 set. 2010. DOI: 10.1523/JNEUROSCI.3315-10.2010. PMID: 20881138; PMCID: PMC2965411.

UDROIU, I.; SGURA, A. The phylogeny of the spleen. **Quarterly Review of Biology**, v. 92, p. 411–443, 2017. Disponível em: https://doi.org/10.1086/695327. Acesso em: 27 jun. 2023.

VENCES, M.; KÖHLER, J. Global diversity of amphibians (Amphibia) in freshwater. In: **FRESHWATER ANIMAL DIVERSITY ASSESSMENT**. p. 569-580, 2008. DOI: 10.1007/978-1-4020-8259-7_54.

APÊNDICE - A

Supplementary material : Table 2 - Pathogenic stressors and their effects

Reference	***Species***	**Stress Factor**	**Effects on the spleen**
Huang et al., 2019	*Pelophylax nigromaculatus*	*Elizabethkingia miricola*	Reduction of interstitial cells, reticular system hyperplasia and necrosis
Fort et al., 2016	*Rhinella marina*	Lake sediment and *Mycobacterium chelonae*	Tissue injuries, white pulp reduction
Fremont-Rahl et al., 2011	*Xenopus tropicalis*	*Mycobacterium liflandii*	Increase in organ size, granulomas with associated bacteria, increase in melanomacrophages
Li et al., 2017	*Paramesotriton hongkongensis*	*Mycobacterium marinum*	Necrotic lesions surrounded by melanomacrophages with bacterial clusters
Haridy et al., 2014	*Rhacophorus arboreus*	*Mycobacterium marinum*	Increase in organ size, macroscopic white spots, granulomas, increase in melanomacrophages and necrosis
Tarigo et al., 2006	*Xenopus laevis*	*Mycobacterium sp.*	Granulomas
Brodkin et al., 1992	*Rana pipiens*	*Pseudomonas aeruginosa*	Increase in organ size and reduction in spleen cells count
Forzan et al., 2015	*Lithobates sylvaticus (Rana sylvatica)*	Ranavírus	Increase in organ size, hematopoietic tissue hyperplasia, hemorrhage, necrosis and melanomacrophages reduction

Continuation: **Table 2** : Pathogenic stressors and their effects

Reference	*Species*	Stress Factor	Effects on the spleen
Cheng et al., 2014	*Anaxyrus boreas boreas*	Ranavírus	Abscesses and necrosis
Morales & Robert, 2007	*Xenopus laevis*	Ranavírus	Activation of immune response and memory
Cullen et al., 1995	*Limnodynastes terraereginae & Litoria latopalmata*	Bohle iridovirus	Punctual pynosis, necrosis in the white pulp, splenic pulp melanizationand erythrocytes with pyknotic nuclei
Meng et al., 2014	*Andrias davidianus*	Iridovirus	Haemorrhage, necrosis, vacuolar degeneration, infestation of viral particles
Paperna & Lainson, 1995	*Bufo marinus*	Alloglugea bufonis nov. gen., nov. sp.	Increase in organ size, macroscopic white spots
Branelly et al., 2016	*Pseudophryne corroboree, Litoria verreauxii alpina e Rhinella marina*	Batrachochytrium dendrobatidis	No effects for the evaluated biomarkers
Paetow et al., 2012	*Lithobates pipiens*	Batrachochytrium dendrobatidis	No effects for the evaluated biomarkers
Speare et al., 1997	*Bufo marinus*	M. amphibiorum	Increase in organ size, pallor of the organ and granulomas
Berger et al., 1997	*Litoria caerulea*	M. amphibiorum	Increased organ size, 2 to 3 mm nodules, granulomas

* Pathogens tested in amphibians and their main effects on splenic anatomy, histomorphology and function.

Table 3 : Chemical stressors and their effects.

Reference	***Species***	**Stress Factor**	**Effects on the spleen**
Turner & Manning, 1973	*Xenopus laevis*	Sheep erythrocytes and human gamma globulin	Fluctuations in spleen size, increase in pyroninophilic cells, vacuolation of red pulp cell cytoplasm
Bokony et al., 2020	*Bufo bufo*	Carbamazepine and Terbuthylazine	Pigmentation reduction
Bokony et al., 2020	*Rana dalmatina*	Carbamazepine and Terbuthylazine	Reduction in organ size and increased pigmentation
Marques et al., 2009	*Rana perezi Seoane*	Deactivated uranium mine site	No effects for the evaluated biomarkers
McMurry et al., 2009	*Spea bombifrons and Spea multiplicata*	Land use	Reduction in organ size and spleen cells count
Romanova et al., 2021	*Pelophylax ridibundus and Pelophylax lessonae*	Mn, Cu, Cr, Al, Fe, Zn, and Sr	Chromium and iron accumulation
Priyadarshani et al., 2015	*Euphlyctis hexadactylus*	Metal contamination	Reduction in organ size and defense cell count
Santana et al., 2021	*Rhinella ornata*	Industrial contamination	Increase in organ size
Linzey et al., 2003	*Bufo marinus e Eleutherodactylus johnstonei*	Pesticide and metal contamination	White pulp and immune response reduction

Toxicants tested in amphibians and their main effects on splenic anatomy, histomorphology and function

Continuation: **Table 3** - Chemical stressors and their effects.

Reference	***Species***	**Stress Factor**	**Effects on the spleen**
Fort et al., 2016	*Rhinella marina*	Lake sediment	Tissue injuries, white pulp reduction
Vasconcelos-Teixeira et al., 2022	*Rhinella diptycha (Rhinella schneideri)*	Metal contamination	Reduction in organ size and imune response
Paetow et al., 2012	*Lithobates pipiens*	Atrazine and glyphosate	No effects for the evaluated biomarkers
Hayes, 1995	*Bufo boreas and Bombina orientalis*	Corticosterone	Total organ involution
Franco Belussi & Oliveira, 2016	*Physalaemus nattereri*	α-melanocyte stimulating hormone	Increase in catabolic pigments and pigmentation
Arrieta et al., 2004	*Bufo arenarum*	Pb	Lead accumulation
Dobrovoljc et al., 2012	*Bufo bufo*	Cd, Cu, Zn, Se and Metallothioneins	No effects for the evaluated biomarkers
Dobrovoljc et al., 2012	*Necturus maculosus*	Cd, Cu, Zn, Se and Metallothioneins	Cadmium accumulation
Awaad, 2020	*Bufo regularis reuss*	Iron oxide nanoclusters	Reduction of melanomacrophages (intraperitoneal injection), increase in melanomacrophages (oral injection)

Toxicants tested in amphibians and their main effects on splenic anatomy, histomorphology and function

Continuation: **Table 3** - Chemical stressors and their effects.

Reference	***Species***	**Stress Factor**	**Effects on the spleen**
Froese et al., 2009	*Ambystoma tigrinum*	Deltamethrin	No effects for the evaluated biomarkers
Çakıc, 2018	*Pelophylax bedriagae*	Carbaryl	Hemorrhage, increased sinusoid width, sinusoidal congestion in dilated sinusoids, increase in melanomacrophages, fibrosis, separation in capsule, hypertrophic plasma cell
Viriato et al., 2021	*Lithobates catesbeianus*	2,4-D (DMA® 806)	Reduction in organ size
Rollins-Smith et al 2004	*Xenopus laevis*	Diazinon	Altered ability of transplanted stem cells to contribute to hematopoiesis

Toxicants tested in amphibians and their main effects on splenic anatomy, histomorphology and function

Table 4 : Physical stressors and their effects.

Reference	*Species*	Stress Factor	Effects on the spleen
Vasconcelos-Teixeira et al., 2022	*Rhinella diptycha (Rhinella schneideri)*	Physical restraint	increased stress biomarkers and reduced immune response
Frangioni et al., 1997	*Hydromantes genei*	Temperature	6°C: increase in organ size (splenic congestion) | 18° and 24°C: reduction in organ size
Frangioni & Borgioli, 1996	*Triturus carnifex*	Oxygenation	Reduction in organ size (as oxygen decreases)
Malvin et al., 1995	*Bufo woodhousii*	Oxygenation	No effects for the evaluated biomarkers
Frangioni & Borgioli, 1991	*Triturus cristatus carnifex*	Temperature	Reduction in organ size (as temperature increases)
Frangioni & Borgioli, 1991b	*Triturus cristatus carnifex*	Oxygenation	Reduction in organ size (as oxygen decreases)
Dulak & Plytycz, 1989	*Bombina variegata*	Laboratory conditions	Decreased organ size, increased melanomacrophages, increased connective tissue portion, and reduced white pulp lymphocytes
Franchini et al., 2016	*Xenopus laevis*	Wound Healing	Dilation of central arteries associated with follicular cells and melanomacrophages, mitotic cells and apoptotic cells

Conditions tested in amphibians and their main effects on splenic anatomy, histomorphology and function.

Continuation: **Table 4** : Physical stressors and their effects

Reference	***Species***	**Stress Factor**	**Effects on the spleen**
Brodkin et al., 2003	*Rana pipiens*	pH and Temperature	Increased susceptibility to bacterial infections
Simon et al., 2002	*Rana pipiens*	pH and Temperature	Reduction in spleen cells count and increased susceptibility to bacterial infections
Bagwill et al., 2016	*Spea multiplicata*	Water loss	No effects for the evaluated biomarkers
Frangioni & Borgioli, 1989	*Triturus cristatus carnifex*	Oxygenation	Reduction in organ size (as oxygen decreases)
Frangioni & Borgioli, 1993	*Triturus cristatus carnifex*	Oxygenation	Reduction in organ size (as oxygen decreases)
Borgioli & Frangione, 1997	*Triturus carnifex*	Temperature	Reduction in organ size (>27°C)

Conditions tested in amphibians and their main effects on splenic anatomy, histomorphology and function.

Table 5 - Main renal biomarkers in response to stressors. (**N/S**: not specified; **NRTB**: renal biomarkers tested showed no response; **RED**: reduction; **INC**: increase; **PT**: proximal tubule).

Reference	Stressor	Main Renal Biomarkers
Brannelly et al., 2016	Bd	RED. hematopoietic tissue RED. celular density
Grassi et al., 2023	*Cladosporium allicinum*	Granuloma Tissue lesions
Duncan et al., 2004	Myxozoa (Cnidaria)	Tubular dilatation Necrosis Nephritis
Isidoro-Ayza et al., 2019	Perkinsea	Nephromegaly Necrosis Infestation RED. Pigmentation
Kiryu & Landsberg, 2015	*Ichthyophonus* spp	NRTB
Moretti et al., 2017	N/S	Nephromegaly
Theunissen et al., 2014	*Protopolystoma xenopodis*	Infestation
Tokiwa et al., 2021	Hyaloklossia Labbé	Infestation

Continuation: **Table 5 -** Main renal biomarkers in response to stressors. (**N/S**: not specified; **NRTB**: renal biomarkers tested showed no response; **RED**: reduction; **INC**: increase; **PT**: proximal tubule).

Reference	Stressor	Main Renal Biomarkers
Meng et al., 2014	Iridovirus	Necrosis Hemorrhage Infestation
Neves et al., 2016	Ravavírus	Glomerulotubular degeneration Hemorrhage Necrosis INC. Melanomacrophages
Robert et al. 2005	Ravavírus	Necrosis INC. Mesangial spaces
Seixas et al., 2019	Clinical signs	Granuloma Necrosis INC. Bowmans Space Hemorrhage Calcification Fibrosis
Seixas Filho et al., 2009	Food quality	Calcificatio Nephrosis

Continuation: **Table 5 -** Main renal biomarkers in response to stressors. (**N/S**: not specified; **NRTB**: renal biomarkers tested showed no response; **RED**: reduction; **INC**: increase; **PT**: proximal tubule).

Reference	Stressor	Main Renal Biomarkers
Womble et al., 2020	Clinical signs	Cariomegaly Cytoplasmic vacuolization Carcinoma Polycystic disease
Cabot et al., 2021	Tag	Vessel dilatation Edema Nephritis
Anzaldua and Goldberg, 2020	N/S	Anatomical abnormatilies Tissue lesions INC. Pigmentation
Arrieta et al., 2004	Pb	Accumulation
Capaldo et al., 2016	Cd	Accumulation INC. Apoptosis INC. Glomerular Size RED. Bowmans Space Tubular dilatation Cell degeneration Necrosis

Continuation: **Table 5 -** Main renal biomarkers in response to stressors. (**N/S**: not specified; **NRTB**: renal biomarkers tested showed no response; **RED**: reduction; **INC**: increase; **PT**: proximal tubule).

Reference	Stressor	Main Renal Biomarkers
Jayawardena et al., 2017	Cd, Cr, Cu, Pb, and Zn	Tubular degeneration Necrosis Mineralization Inflamation INC. Bowmans Space
Loumbourdis, 2003	Pb	Vacuolization Brush border destruction Inflamation PT lesion
Loumbourdis, 2005	Cd	INC. Apoptosis
Monteiro et al., 2018	Hexavalent Cr	Inflamation Granuloma Necrosis Glomerulotubular Hypertrophy
Alnoaimi et al., 2021	Deltamethrin	RED. glomeruli size Hemorragia Tubular degeneration INC. Bowmans Space

Continuation: **Table 5 -** Main renal biomarkers in response to stressors. (**N/S**: not specified; **NRTB**: renal biomarkers tested showed no response; **RED**: reduction; **INC**: increase; **PT**: proximal tubule).

Reference	Stressor	Main Renal Biomarkers
Bernabò et al., 2017	Pyrimethanil	INC. Melanomacrophage Hemorragia Brush border destruction INC. Bowmans Space Vacuolization Edema Tubular dilatation
Çakıcı, 2015	Carbaryl	Inflamation Bowman´s Capsule deformation Vacuolization Necrosis RED. glomeruli size Brush border destruction Hemorragia
Froese et al., 2009	Deltamethrin	NRTB
Sisman et al., 2019	N/S	Tubular degeneration Tubular congestion Tubular dilatation Necrosis INC. Bowmans Space Inflamation Glomerulonephritis

Continuation: **Table 5 -** Main renal biomarkers in response to stressors. (**N/S**: not specified; **NRTB**: renal biomarkers tested showed no response; **RED**: reduction; **INC**: increase; **PT**: proximal tubule).

Reference	Stressor	Main Renal Biomarkers
Viriato et al., 2021	2,4-D (DMA® 806)	Tissue lesion Inflamation
Gyllenhammar et al., 2009	EE2 Clotrimazol	NRTB
Fort et al., 2017	Triclosan	Mineralization Inflamation Granuloma Fibrosis Vacuolization Tubular atrophy
Haselman et al., 2016	4-tert-octylphenol 17β-trenbolone	Mineralization Tubular dilatation
Li et al., 2020	BDE-47	Anatomical abnormatilies