UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de São José do Rio Preto

Danusy Lopes Santos

Variação morfológica em girinos bentônicos e nectônicos: influência de fatores espaciais ou ambientais?

São José do Rio Preto

2023

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Tese apresentada como parte dos requisitos para obtenção do título de Doutor em Biologia Animal, junto ao Programa de Pós-Graduação em Biologia Animal, Área de Concentração – Sistemática e Evolução, do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de São José do Rio Preto.

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Coorientador: Prof. Dr. Fausto Nomura

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2023

Ao meu pai João Natal dos Santos (in memorian),

que partiu antes que esse momento chegasse

Saudades

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RESUMO

A morfologia é um dos principais componentes do fenótipo de um organismo. Os girinos apresentam grande diversidade morfológica e comportamental, e ocupam uma ampla gama de habitats aquáticos e terrestres. Essa diversidade tem disso atribuída às características físicas do ambiente e à presença a predadores e competidores. Apesar dos girinos serem excelentes modelos para estudos morfológicos e de variação fenotípica, existe uma carência em estudos envolvendo variação morfológica, suas origens e distribuição no espaço e no tempo. Outra carência envolvendo o estudo de girinos está na relação entre as estruturas anatômicas e suas respectivas funções, e variações morfológicas apresentadas por essas estruturas. Considerando a ausência de estudos com ampla abrangência geográfica acerca das variações morfológicas, assim como sobre a organização e funcionamento de órgãos e sistemas, e se eles sofrem modificações de acordo com diferenças no uso do ambiente, nossos objetivos gerais foram: (1) determinar a variação em caracteres morfológicos externos em girinos de diferentes guildas ecomorfológicas, verificando a existência de padrõesassociados a distância espacial entre populações ao longo da distribuição geográfica das espécies alvo; (2) caracterizar o sistema nervoso de girinos de diferentes guildas ecomorfológicas, buscando correlacionar a morfologia do sistema nervoso ao ambiente utilizado. Utilizando técnicas de morfometria geométrica, além de imagens tridimensionais, nós analisamos a variação da morfologia externa. Por meio de modelos tridimensionais analisamos as variações morfológicas no cérebro de girinos de três diferentes guildas ecomorfológicas e verificamos se as variações morfológicas observadas podem estar associadas a diferenças no uso do habitat. Nossos resultados demonstram que a morfologia externa dos girinos é correlacionada com a distância espacial entre as populações de todas as espécies analisadas, com exceção de *Rhinella diptycha*. A morfologia do cérebro variou acima do esperado, principalmente as regiões do cerebelo, optic tectum e diencéfalo. Observamos também que essas variações estão associadas ao uso da coluna d'água e também ao tamanho do cérebro. Girinos nectônicos de *D.* minutus, que usam o ambiente em três dimensões, apresentaram cérebro com maior tamanho e cerebelo mais volumoso

Palavras-chave: Girinos. Morfologia. Microtomografia computadorizada. Guildas ecomorfológicas. Padrões espaciais.

ABSTRACT

Morphology is one of the main components of an organism's phenotype. Thus questions and hypotheses related to the origin of morphological variation and its distribution in space and time are common within biology. Tadpoles occupy a wide range of aquatic and terrestrial habitats and include a great diversity of ecomorphotypes, with diverse morphological and behavioral adaptations, differences in developmental time, size at metamorphosis and in physiological aspects, both in response to the physical characteristics of the environment and/or in response to the presence of predators and competitors, thus being excellent models for morphological and phenotypic variation studies. However, there is a lack of studies involving morphological variation, its origins and distribution in space and time using tadpoles. Another deficiency involving the study of tadpoles is the relationship between anatomical structures and their respective functions, and morphological variations presented by these structures. Considering the absence of basic information and studies with a wide geographical distribution about morphological variations, as well as about the organization and functioning of organs and systems, and how they are modified to meet the demands of different types of environment our general objective was: (1) to determine the variation in larval morphological characters in tadpoles from different ecomorphological guilds, verifying the existence of patterns, associated with distance between populations or environmental variables along the geographic distribution of the target species , (2) to characterize the nervous system of tadpoles from different ecomorphological guilds, seeking to correlate the morphology of the nervous system to the environment used. Using geometric morphometric techniques, in addition to three-dimensional images, we analyzed the variation in external morphology found in tadpole populations from different ecomorphological guilds and whether this variation is associated with the spatial distance between populations along their geographic distribution. In addition, through three-dimensional models, we analyzed the morphological variations presented by the brain of tadpoles from three different guilds and whether the observed morphological variations could be associated with these guilds. Our results indicate that the external morphology of tadpoles presents a spatial correlation for all species analyzed with the exception of *Rhinella diptycha*.

As for the brains, we observed a variation above the expected, mainly for the regions of the cerebellum, optic tectum and diencephalon. We also observed that these variations are associated with guilds and brain size.

Key words: Tadpoles. Morphology. Micro computed tomography (µCT). Ecomorphological guilds. Spatial patterns.

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Sumário

1. INTRODUÇAO

As análises de dados morfológicos têm sido cruciais para o avanço da biologia comparativa, melhorando a compreensão sobre uma infinidade de processos ecológicos e evolutivos (Ricklefs & Miles, 1994). A morfologia é, sem dúvida, um dos principais componentes do fenótipo de um organismo (Adams et al., 2004). Assim, os traços morfológicos sempre estiveram no centro das atenções da pesquisa biológica, constituindo importantes evidências para uma ampla variedade de campos de investigação. Dessa forma da taxonomia tradicional à escola moderna de sistemática, passando pela fisiologia, desenvolvimento, ecologia, biogeografia e até a biologia evolutiva moderna, praticamente todos os campos biológicos incluem questões e hipóteses relacionadas a como a variação morfológica surge e como ela é distribuída no tempo e na escala espacial (Kaliontzopoulou, 2011).

Ainda não está claro como e por que a variação fenotípica é estruturada geograficamente em espécies amplamente distribuídas, especialmente em regiões tropicais (Monteiro et al., 2003; Alvarado-Serrano et al., 2013, Maestri et al. 2016). Uma compreensão da variação morfológica e sua estrutura espacial é de importância crucial para questões fundamentais em biologia evolutiva, e pode ajudar em questões sistemáticas e decisões de conservação (Thomassen et al., 2010; Zamudio et al., 2016; Wood et al., 2017; Cadotte & Tucker, 2018; Morales et al., 2021). O padrão espacial de distribuição de um taxon é definido pelo arranjo de entidades individuais no espaço e as relações geográficas entre elas. A capacidade de avaliar padrões espaciais é um pré-requisito para entender os complicados processos espaciais subjacentes à distribuição de um fenômeno (Chou, 2005).

Dessa forma, nos últimos anos, tem-se reconhecido que a variação morfológica e genética entre populações também deve ser investigada em um contexto espacial (Diniz-Filho

et al., 2000). Isso porque, ao longo de sua distribuição geográfica, uma espécie pode enfrentar ambientes distintos e pressões seletivas, o que pode gerar descontinuidades nas características genotípicas e fenotípicas entre diferentes populações (Trevisan et al. 2014), levando a diferentes estruturas de variação morfológica (Berthouly-Salazar et al., 2012). Assim, mesmo espécies intimamente relacionadas e com estratégias de história de vida semelhantes podem variar em grau e estrutura geográfica de variação morfológica e diferir em padrões de distribuição geográfica (Hopkins &Thurman, 2010; Lezcanon et al., 2012).

A variação intraespecífica pode ser definida como a variação total de uma determinada característica morfológica (ou conjunto de características) expressa por indivíduos de uma mesma espécie (Albert et al., 2011). As causas da variação intraespecífica dependem de uma variedade de mecanismos, incluindo adaptação local, seleção artificial, condições parentais e plasticidade fenotípica (Violle et al, 2012; Des Roche et al, 2018). A plasticidade fenotípica é a capacidade de um organismo de alterar sua fisiologia e/ou morfologia como resultado de sua interação com o meio ambiente, sendo bastante comum em girinos (McIntyre et al., 2004). Então, quando gerada por mecanismos evolutivos, a variação de características intraespecíficas pode refletir adaptação microgeográfica, seleção divergente e até especiação incipiente (Mimura et al., 2017). Quando gerados pela plasticidade, os traços podem mudar rapidamente dentro de gerações e diferir drasticamente entre populações em habitats diferentes (Des Roche et al., 2018). Além disso, a ocorrência de variação nos caracteres morfológicos é relativamente comum em organismos amplamente distribuídos e, portanto, suas populações são geograficamente segregadas (West-Eberhard et al., 1989; Des Roches et al., 2013; Des Roches et al., 2018).

A variação geográfica entre populações de uma determinada espécie é um resultado inevitável da variação espacial no ambiente, impulsionada pela divergência genética e/ou plasticidade fenotípica (Mayr, 1977). Portanto, variações fenotípicas, refletindo as pressões

seletivas que atuam nas populações (Hopkins & Thurman, 2010), podem mostrar claramente padrões morfológicos criados por essas pressões na distribuição geográfica das espécies (Hopkins & Thurman, 2010; Lezcano et al., 2012; Trevisan et al., 2014; Hošková et al., 2020). Por outro lado, algumas espécies tendem a possuir padrões espaciais diferentes. Por exemplo, complexos de caracteres morfológicos usualmente distinguidos em abelhas tendem a apresentar diferentes padrões espaciais em grandes escalas. Assim, caracteres de tamanho são caracterizados por fortes padrões espaciais de longa distância, já os ângulos de pigmentação e venação das asas são caracterizados pela ausência desses padrões de grande escala, e os caracteres podem possuir autocorrelação local e regional mais forte, provavelmente refletindo os principais processos microevolutivos que provavelmente determinam sua variação no espaço (Diniz-Filho et al., 2000).

Dessa forma, a variação morfológica intraespecífica está no centro da teoria da evolução. Mais recentemente, tem grande importância também no âmbito de estudos interessados em variação neutra (Sokal et al., 1989; Barbujani, 2000; Relethford, 2008). Isso se deve ao fato de que a maioria dos processos evolutivos neutros ocorrem em um contexto espacial (Epperson, 2003), onde a variação genética originada por mutações aleatórias dentro de populações locais pode se dispersar por meio de fluxo gênico mediado geograficamente (Perez et al., 2010). Os padrões de variação morfológica entre populações fornecem material fundamental para a compreensão do papel da diferenciação genética e da seleção natural na especiação e adaptação dos organismos (Van Buskirk, 2017). Desta forma, a variação intraespecífica pode mediar a coexistência de espécies (Turcotte & Levine, 2016).

As primeiras tentativas de caracterizar geometricamente as diferenças entre formas biológicas foi realizada por Albrecht Durer (Vier Bucher von Menschlicher Proportion, 1524), responsável pela primeira caracterização geométrica da forma humana sobre uma grade sobreposta, inclusive antes da noção sobre as coordenadas retangulares propostas por

Reneé Descartes (Monteiro & Reis, 1999). Grandes pensadores como Galileu (1638) ilustraram as diferenças na forma de ossos de animais, e ao longo do tempo, grandes naturalistas como Darwin têm fundamentado suas teorias com base em observações nas diferenças das formas biológicas. No início do século 20, a biologia passou de um campo descritivo, para uma ciência quantitativa, e as análises morfológicas entraram na chamada "revolução quantitativa" (Bookstein, 1998). Durante muitos anos pesquisadores buscaram técnicas que permitissem quantificar estatisticamente a variação de forma de organismos e/ ou estruturas até que na década de 1980 houve o surgimento da Morfometria Geométrica (MG), técnica que permite analisar a forma dos organismos ou de determinada estrutura considerando o espaço de forma geométrica e usando métodos estatísticos multivariados (Souto et al., 2021).

A morfometria geométrica (GM) revolucionou a forma de medir, estudar e perceber a diversidade morfológica (Rohlf & Marcus, 1993; Adams et al., 2004). Ao preservar as propriedades geométricas das estruturas de interesse e estabelecer uma base matemática sólida para o estudo da forma, a morfometria geométrica fornece uma ferramenta poderosa para representar e estudar a variação morfológica de forma mais realista e integrada (Bookstein, 1991; Rohlf, 2000). Além disso, esse método captura diferenças de forma mais sutis que geralmente não são recuperadas em medições lineares (Ilić et al., 2019). Em função disso, a morfometria geométrica tem sido amplamente utilizada para estudar a variação intraespecífica em vários grupos taxonômicos, como morcegos (Schmieder et al., 2015), besouros (Benitez et al., 2020), caranguejos (Hampton et al., 2014), cobras (Huntley et al., 2021), lagartos (Kaliontzopoulou et al., 2010), além de estudos com plantas (Dalastra et al., 2021) e vários outros grupos.

Os anfíbios, sendo excelentes organismos modelo, têm sido objeto de extensa pesquisa morfológica, servindo muitas vezes como casos paradigmáticos no estudo da variação

fenotípica (Kaliontzopoulou, 2011; Baldo et al., 2014; Barrionuevo, 2020; Lopes et al., 2020; Quinzio & Goldberg, 2020). Paralelamente aos anuros adultos, os girinos anuros se diversificaram em uma ampla gama de habitats aquáticos e terrestres e incluem uma grande diversidade de ecomorfotipos (Ilić et al., 2019). Os girinos apresentam uma gama diversificada de adaptações morfológicas e comportamentais, diferenças no tempo de desenvolvimento, tamanho na metamorfose e nos aspectos fisiológicos, tanto em resposta às características físicas do ambiente (Eterovick & Barata, 2006; Eterovick et al., 2010) e/ou em resposta à presença de predadores e competidores (Releya, 2001; Michel, 2012).

Estudos abrangendo variação morfológica em girinos são escassos na literatura (e.g. Conte et al., 2007; Provete et al., 2013; Pezzuti et al., 2016), e podem ajudar a avançar a nossa compreensão sobre a influência do ambiente e da filogenia na variação morfológica e no desenvolvimento da fase larval. A escassez de informações acerca do grau de variação morfológica intra– e interpopulacional, é uma das grandes dificuldades encontradas em estudos de ecologia de comunidades de anuros (Verdade et al., 2012; Toledo et al., 2014). Jordani et al. (2018) apresentou a primeira quantificação da variação morfológica intraespecífica de girinos, com base em oito características funcionais de 22 espécies da Mata Atlântica e de 13 espécies da Floresta Amazônica . Apesar de algumas características apresentarem grande variação intraespecífica, considerando todas as características juntas, a variabilidade intraespecífica foi de cerca de 30% da variação total, menor que a interespecífica, tanto para girinos da Mata Atlântica quanto para os da Floresta Amazônica (Jordani et al., 2018). Vários estudos mostraram que essa porcentagem de variação morfológica intraespecífica pode ter um papel importante nos padrões de diversidade e na estrutura da comunidade (Jung et al., 2010; Albert et al., 2012). Além disso, a plasticidade fenotípica em girinos pode resultar em variação morfológica significativa entre populações de diferentes os ambientes e, consequentemente, aumentar a variabilidade morfológica entre as

populações de girinos (Marques & Nomura, 2015). Assim, diferentes fatores ambientais, como tamanho da área úmida, estresse por perda de água, densidade de girinos, disponibilidade de recursos alimentares, presença e densidade de predadores, entre outros, são capazes de induzir mudanças morfológicas que aumentam a variação intraespecífica (Van Buskirk, 2017; Touchon e Robertson, 2018).

Rossa-Feres et al. (2015) reconheceram que a compreensão dos fatores que controlam a variação morfológica é uma das lacunas de conhecimento mais importantes nos estudos sobre o estágio larval. Compreender quais fatores estão associados à plasticidade fenotípica e como eles influenciam a variação morfológica em sistemas naturais, além do grau de variação morfológica inter e intraespecífica em diferentes escalas espaciais, são necessários para formular melhores hipóteses ecológicas.

Também é uma lacuna de conhecimento a avaliação da relação entre as estruturas anatômicas das larvas e suas respectivas funções (Rossa- Feres et al., 2015). Muitas das informações disponíveis sobre o assunto são derivadas de estudos realizados principalmente com *Xenopus laevis*, e em alguns casos com girinos dos gêneros *Rana* e *Bufo*, e adotadas como padrão para a maioria das espécies (Vera Candioti, 2006).

Um girino é um mosaico de órgãos que são remodelados como o pâncreas exócrino e o trato digestivo, degenerados durante a metamorfose (e.g. tegumento), permanecem funcionalmente em repouso (e.g. partes do sistema excretor e reprodutivo), ou são funcionais tanto em girinos como nos adultos embora possam apresentar funções distintas nas diferentes fases (e.g. pulmão, Altig & McDiarmid, 1999). Em vários casos como o do sistema nervoso, as informações são escassas e fragmentadas (Quinzio & Fabrezi, 2018). O sistema nervoso, constituído pelo cérebro e seus nervos cranianos (Figura 1) e pela medula espinhal e seus nervos, está associado a todos os comportamentos dos vertebrados (Lanoo, 1999).

Um excelente resumo sobre a neurobiologia de anfíbios, incluindo estruturas adultas, é fornecido por Wilczynski (1992). Outros estudos abordaram o crescimento do cerebelo (e.g. Hauser et al. 1986, a,b). Uray et al. (1987) descreveu as mudanças do sistema nervoso durante o processo de metamorfose. Mais recentemente, pesquisadores têm mostrado que apesar de estudos associando genes a doenças serem realizados principalmente em camundongos, os girinos também podem fornecer evidências muito fortes sobre a função de genes em humanos. Sendo o processo de recriar algumas variantes de um gene em girinos bem mais simples e mais rápido (Macken et al., 2021).

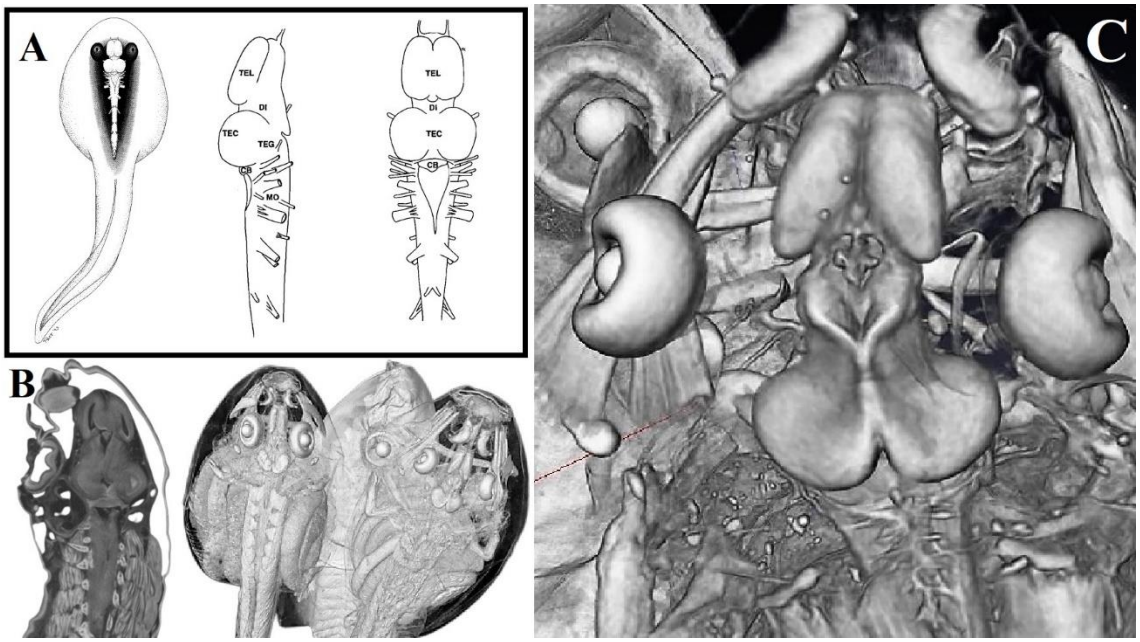

Figura 1*:* A. Vista dorsal do cérebro e da medula espinhal em um girino. Os nervos cranianos e espinhais são mostrados como se tivessem sido cortados (imagem de Lannoo em Altig & McDiarmid, 1999). B. Imagem segmentada e renderizada do sistema nervoso em girinos. C. Renderização da imagem com destaque para o cérebro. *CB= cerebellum; TEC= tectum; DI= diencephalon; TEL= telencephalon.

Além da escassez e fragmentação das informações sobre o sistema nervoso, o significado funcional e evolutivo das variações no cérebro dos girinos ainda não foi investigado (Altig & McDiarmid, 1999), talvez pelo fato do sistema nervoso ser considerado um sistema anatômico invariável (Quinzio & Fabrezi, 2018). Entretanto o cérebro dos girinos

das diferentes espécies não é igual, e estão disponíveis poucas informações básicas sobre a organização e funcionamento do sistema nervoso como, por exemplo, como se dá a integração do desenvolvimento com a diferenciação do sistema nervoso, e como ele é alterado para atender às demandas de diferentes ambientes (Quinzio & Fabrezi, 2018). Para outros grupos, como os Primatas, há evidências para uma associação entre o tamanho do cérebro e variáveis ecológicas, como às demandas de forrageamento do nicho ecológico de uma espécie (Powell et al., 2017).

A diversidade morfológica entre girinos de diferentes espécies é imensa, bem como os tipos de habitats aquáticos e semiaquáticos onde ocorre seu desenvolvimento. Uma relação ecomorfológica usualmente aceita é aquela entre a morfologia externa e a profundidade da coluna d'água ocupada pelos girinos. Assim, girinos bentônicos ocupam o fundo dos ambientes aquáticos, e apresentam corpo deprimido, olhos dorsais e nadadeiras baixas, girinos nectônicos ocupam o meio da coluna d'água e tem corpo comprimido, olhos laterais e nadadeiras altas, e girinos neustônicos se alimentam no filme d'água e se posicionam próximo à superfície, apresentam corpo comprimido, olhos laterais, nadadeira dorsal baixa e nadadeira ventral alta, além de disco oral terminal ou dorsalmente direcionado. Apesar de proposta há bastante tempo (Altig, 1989) e ser considerada como uma associação comprovada, a relação entre a morfologia externa e a profundidade da coluna d'água ocupada pelos girinos começou a ser testada apenas recentemente (Marques et al., 2019). A despeito da profundidade da coluna d'água ocupada requerer diferentes habilidades dos girinos, como flutuabilidade, percepção do habitat e de possíveis predadores em duas (girinos bentônicos) ou em três dimensões (girinos nectônicos), até hoje nenhum estudo avaliou a relação do uso diferencial do espaço com o sistema nervoso.

Considerando a ausência de informações básicas e estudos com ampla abrangência geográfica acerca das variações morfológicas, assim como sobre a organização e

funcionamento de órgãos e sistemas, e como eles são modificados para atender às demandas de diferentes tipos de ambiente e do uso diferencial da profundidade da coluna d'água, esta tese está estruturada em dois capítulos.

No primeiro capítulo buscamos determinar a variação em caracteres da morfologia externa em girinos de seis espécies com ampla distribuição geográfica, verificando se existem padrões associados à distância entre populações ao longo da distribuição geográfica das espécies alvo.

No segundo capítulo, nosso objetivo foi caracterizar o sistema nervoso central de girinos bentônicos e nectônicos, buscando verificar sua associação com o uso da coluna d'água.

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CAPÍTULO 1

Intraspecific morphological variation tadpoles along of the geographic distribution

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Abstract: Tadpoles are known for their high phenotypic plasticity and are therefore excellent tools for morphological and phenotypic variation studies. However, there is a lack of studies involving morphological variation, its origins and distribution in space and time using tadpoles. Using geometric morphometry, we investigated the morphological variation in six species from three ecomophological guilds and whether this variation presents any spatial pattern along the geographical distribution of the analyzed species. We identified clear morphological variation among the populations of the six species analyzed, five of them associated with geographical distance. Widely distributed species generally tend to present considerable variation within their ranges, which may have implications for the taxonomic history of the species, making it difficult to correctly identify the species and transforming intrapopulation variations into diagnostic differences.

Keywords: Larval morphology. Geometric morphometrics. Spatial pattern.

Resumo: Girinos são conhecidos por uma alta plasticidade fenotípica sendo assim excelentes ferramentas para estudos morfológicos e de variação fenotípica. Entretanto existe uma carência em estudos envolvendo variação morfológica, suas origens e distribuição no espaço e no tempo utilizando girinos. Utilizando a morfometria

geométrica, nós investigamos a variação morfológica em seis espécies de três guildas ecomofologicas e se essa variação apresenta algum padrão espacial ao longo da distribuição geográfica das espécies analisadas. Identificamos clara variação morfológica entre as populações das seis espécies analisadas, cinco delas associadas a distância geográfica. Espécies amplamente distribuídas geralmente tendem a apresentar considerável variação dentro de suas áreas de distribuição podendo ter implicações na história taxonômica das espécies, dificultando a identificação correta das espécies e transformando variações intrapopulacionais em diferenças diagnósticas.

Palavras-chave: Morfologia larval. Morfometria geométrica. Padrões espaciais.

1. Introduction

Analyses of morphological data have been crucial to the advancement of comparative biology, improving understanding about a multitude of ecological and evolutionary processes (Ricklefs & Miles, 1994). Morphology is undoubtedly one of the main components of an organism's phenotype (Adams et al., 2004). As such, morphological traits have always been at the center of attention of biological research, comprising important pieces of evidence for a wide variety of investigation fields. From traditional taxonomy to the modern school of systematics, through physiology, development, ecology, biogeography, and all the way to modern evolutionary biology, practically all biological fields include questions and hypotheses related to how morphological variation emerges and how it is distributed across temporal and spatial scales (Kaliontzopoulou, 2011).

Although geographic variation among the populations of a same species is an inevitable outcome of the spatial variation in the environment, driven by genetic divergence and/or phenotypic plasticity (Mayr, 1977), it remains unclear how much and why

morphological variation is geographically structured in widely distributed species, especially in tropical regions (Monteiro et al., 2003; Alvarado-Serrano et al., 2013, Maestri et al., 2016). An understanding of morphological variation and its spatial structure is of crucial importance to fundamental questions in evolutionary biology, and can help systematic questions and conservation decisions (Thomassen et al., 2010; Zamudio et al., 2016; Wood et al., 2017; Cadotte & Tucker, 2018; Morales et al., 2021). In this way, in recent years, it has been recognised that morphological and genetic variation among populations should also be investigated in a spatial context (Diniz-Filho et al., 2000). That's because along its geographical distribution, a species may face distinct environments and selective pressures, which might generate discontinuities on the genotypic and phenotypic traits between different populations (Trevisan et al., 2014), driving different structures of morphological variation (Berthouly-Salazar et al., 2012). Intraspecific morphological variation is in the center of theory of evolution, and the patterns of morphological variation between populations provide fundamental material for understanding the role of genetic differentiation and natural selection in the speciation and adaptation of organisms (Van Buskirk, 2017). In this way, intraspecific variation can mediate species coexistence (Turcotte & Levine, 2016).

Studies on intraspecific variation in animal assemblages are not so common as in plants, but some studies have evidenced a great intraspecific variation in fishes (Zhao et al., 2014) and in anuran tadpoles, as at genera level (Baldo et al. 2014) as in single species (McIntyre et al., 2004; Grosjean, 2005; Zhao et al., 2017). Parallel to adult frogs, anuran tadpoles diversified into a wide range of aquatic and terrestrial habitats and include a wide diversity of ecomorphotypes (Roeltlanst et al., 2011). Tadpoles present a diversified array of morphological and behavioral adaptations, differences in developmental time, size in metamorphosis and physiological aspects, both in response to the physical characteristics of the environment (Eterovick & Barata, 2006; Eterovick et al., 2010) and/or in response to the

presence of predators and competitors (Releya, 2001; Michel, 2012). Additionally, phenotypic plasticity in tadpoles can result in significant morphological variation among environments and, consequently, increasing morphological variability across tadpoles' populations (Marques & Nomura, 2015).

Jordani et al. (2018) presented the first quantification of tadpoles' intraspecific morphological variation, with basis on eight functional traits from tadpoles of 22 species of Atlantic Forest and 13 species from Amazon Forest. Considering all traits together, the intraspecific variability was around 30% of total variation as to tadpoles from Atlantic Forest as to those of Amazon Forest (Jordani et al., 2018), similar to intraspecific variation found in plants (e.g., Albert, 2010; Siefert et al., 2015). This intraspecific variability was related mostly to traits associated with swimming ability to escape from predators, especially with those of tail musculature and height of fins (Jordani et al., 2018). In fact, different environmental factors such as wetland-size, water-loss stress, tadpole density, availability of food resources, presence and density of predators, among others, are capable to induce morphological changes that enhance intraspecific variation (Van Buskirk, 2017; Touchon & Robertson, 2018). On the other hand, geographic patterns in the phenotypic variation may be much more associated with neutral processes such as genetic drift than environmental factors (Perez et al., 2009; Abreu et al., 2018).

Here we determined the morphological variation in tadpoles from six species, representing tadpoles of three ecomorphological guilds: benthic, nektonic and (*sensu* Altig & Johnston (1986), benthic-neustonic (*sensu* Rossa-Feres et al., 2004). Unlike previous studies that mostly analyze the phenotypic plasticity from an experimental approach (e.g., Touchon & Robertson, 2018; Burraco et al., 2022; Sergio et al., 2021), here we rely our analysis on the natural variation found in populations. Our aims were to evaluate if this variation is associated

with the spatial distance between populations along their geographic distribution, and if it differs according to the different tadpoles' ecomorphological guilds.

2. Material and methods

Specimens and morphological analyses

We analyze tadpoles (Table 1) deposited principally in the scientific collections Amphibia-Tadpoles (DZSJRP) and Zoological Collection of the Federal University of Goiás (ZUFG). In total, we analyzed 835 tadpoles in stages 34 to 40 (Gosner 1960) from 46 sites in 40 localities (Annex 1) in Brazil (Appendix 1).

Tadpoles were positioned in the lateral view over a layer of water-based gel, and photographed with a Sony Alpha 230 camera, secured on a tripod at a constant distance from the camera to the tadpole to 20 cm. From the photographs, 33 landmarks (modified from Navarro Acosta & Vera Candioti, 2017, Figure 1) were selected and digitized using the software TpsUtil (Rohlf, 2013a) and TpsDig2 (Rohlf, 2013b) programs. The criteria for landmark selection included their effectiveness to represent the entire geometric form. Thus, we decided to exclude the landmark referring to the tip of the tail, because in previous analysis, due to the difficulty to standardize the tail tip position, it result in increased morphological variation that was an artifact of positioning and not a morphological change.

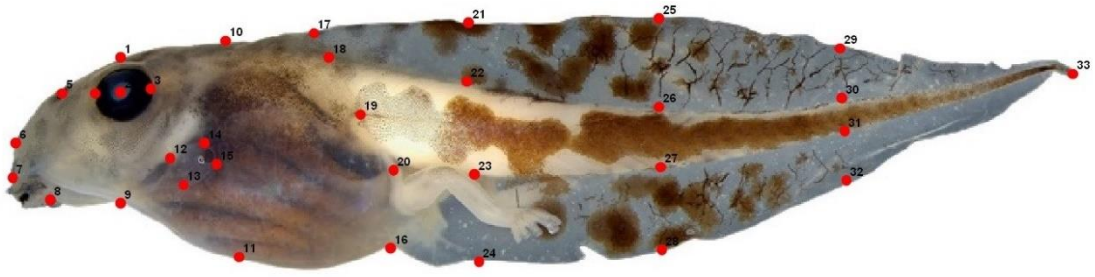

Figure 1. Graphical representation of a tadpole of *Scinax* genus showing the locations of 33 landmarks.

For all tadpoles, the shape information was extracted using a Procrustes superimposition analysis, which is a procedure that removes the information of size, position, and orientation to standardize each specimen according to centroid size (Rohlf, 1990), and obtain partial warp and uniform component scores (Rohlf and Bookstein, 1990; Bookstein, 1991; Zelditch et al., 2004).

Statistical analyses

After applying the transformation, we obtained the partial warp scores that represent the deformation in external morphology of tadpoles. Next, we performed a principal component analysis (PCA) and extracted the eigenvectors that represented at most of the total variation to summarize the morphological variation. To identify which external morphological traits accounts for the difference among populations we performed a canonical variate analysis (CVA), using shape coordinates as a response variable and populations as the explanatory variable (Klingenberg et al., 2011). The results were reported as Mahalanobis distances and the respective p-values for these distances, after permutation tests (10,000 iterations). Also, we used the Mantel test to see if there was a correlation between interpopulational variation and geographical distance. The Mantel test is a permutational correlation test used on two distance or similarity matrices (Mantel, 1967; Mantel & Valand, 1970). In our analyses, the spatial variables were extracted through the analysis of principal coordinates of neighbor matrices (PCNM) method, developed by Borcard & Legendre (2002).

A regression analysis was performed to examine the effect of size, using centroid size, on the shape variation shown in the Procrustes coordinates.

3. Results

For all species PCA showed that the majority of the shape variation was explained in the first three dimensions, accounting from 56 to 66% of morphological variation (Table 1).

Table 1. Principal Component Analysis (PCA) table, showing correlation coefficients for variables on the three first Principal Components.

Species	PC1	PC2	PC3	%variance explained
Boana albopunctata	34.81%	17.90%	13.77%	66.48%
Dendropsophus minutus	27.45%	23.69%	8.84%	60%
Physalaemus cuvieri	33%	12%	10%	55%
Rhinella diptycha	28.70%	17.60%	10.03%	56%
Scinax fuscovarius	34.34%	15.44%	11.34%	61%
Trachycephalus typhonius	28.48%	17.84%	11.87%	58%

The PCA scatter plot of overall body shape variation separated a few groups along the first axis (PC1). From the left to the right of this axis, tadpoles of *B. albopunctata* (Appendix 2) varied from larger to smaller body size, lower to higher fins, shorter to longer tail, spiracle more posterior to more anteriorly positioned on the body, and its opening from more posterodorsal to more posteriorly directed. The variation shown by tadpoles of *D. minutus* (Appendix 3) was from larger to smaller body, globular to triangular shape, and longer to shorter spiracle. Tadpoles of *P. cuvieri* (Appendix 4) varied, along PC1, from shorter to longer body, and from longer to shorter tail length; moreover the spiracle and eye position varied from medial to a more dorsal position along the vertical axis of body. The morphological variation in tadpoles of *S. fuscovarius* (Appendix 5) and of *R. diptycha*, (Appendix6) varied from larger to smaller body, longer to shorter tail, lower to higher fins, and those of *R. diptycha* also varied from higher to lower caudal musculature. The

morphological variation in tadpoles of *T. typhonius* (Appendix 7) varied from smaller to larger body, longer to shorter tail, and spiracle opening from more posterodorsal to more posteriorly directed.

Along PC 2, from bottom to up, tadpoles of *B. albopunctata* varied mostly on the spiracle that change from a more anterior to a more posterior position along the longitudinal axis of the body. Tadpoles of *D. minutus*, *T. typhonius* and *P. cuvieri* varied from higher to lower body and fins, and those of *T. typhonius* also varied from medial to more dorsal position of spiracle and eye. Tadpoles of *R. diptycha* and *S. fuscovarius* varied from smaller to larger body, those of *S. fuscovarius* also varied from higher to lower fins, and those of *R. diptycha* varied from in the spiracle position, from more dorsal to more ventrally along vertical axis of body and from posterodorsal to more posteriorly spiracle opening direction.

Two canonical vectors accounted for the most part of the total among-group variation, indicating from 48% to 67% of populational segregation [[or morphological differentiation]] (Table 2). The largest overlap in scatter plot was observe in *D. minutus* and *P. cuvieri*, with the separation according to the geographical origin of the sampled populations best seen to *B. albopunctata*, *R. diptycha*, *S. fuscovarius*, and *T. typhonius*.

Table 2. Percentage of variation explained by each canonical axis (CV).

Species	CV1	CV2
Boana albopunctata	34.81%	17.90%
Dendropsophus minutus	27.45%	23.69%
Physalaemus cuvieri	33%	12%
Rhinella diptycha	28.70%	17.60%
Scinax fuscovarius	34.34%	15.44%
Trachycephalus typhonius	28.48%	17.84%

The benthic species *B. albopunctata* and *P. cuvieri*, behaved differently on the scatter plots of canonical variate analysis. The populations of *B. albopunctata* were morphologically different, showing overlap only among the populations of Mineiros, GO and Icém, SP, localities that are not near one another. According to CVA, the Mahalanobis distance (Table 3) showed greater distance among Leopoldo de Bulhões, GO and Campo Grande, MS, while Mineiros and Icém showed the lowest value. The CV1 pointed out a slight increase in the fin, snout, and slightly larger spiracle.

Table 3. P-values from permutation tests (10,000 permutation rounds) for Mahalanobis distances among groups for *B. albopunctata*.

P-values from permutation tests (10000 permutation rounds) for Mahalanobis distances among groups:

	Bonfinópolis-GO	Campo Grande-MS	Icém-SP	Leopoldo de Bulhões-GO	Mineiros-GO	Nova Itapirema-SP	Portelândia-GO	Rio Verde-GO	Serranópolis-GO
Campo Grande-MS	0,1349								
Icém-SP	0,0885	0,0003							
Leopoldo de Bulhões-GO	<.0001	0,0475	0,0063						
Mineiros-GO	0,0102	0,0005	<.0001	0,0059					
Nova Itapirema-SP	0,0084	<.0001	<.0001	0,0003	<.0001				
Portelândia-GO	0,2503	0,0144	0,0003	0,1012	0,0004	0,0008			
Rio Verde-GO	0,0463	0,0001	<.0001	0,0011	<.0001	<.0001	0,0009		
Serranópolis-GO	0,0652	0,0013	<.0001	0,0273	<.0001	<.0001	0,004	<.0001	
Silvânia-GO	<.0001	0,0053	<.0001	0,033	0,0002	<.0001	0,006	<.0001	0,0018

On the other hand, *P. cuvieri* tadpoles showed a great morphological overlap among populations. Only the population of Dueré, TO, showed no morphological overlap with the other populations. The populations of Dueré and Corumbá (MS) showed great Mahalanobis distance (Table 4), while the lowest values were observed between Cocalzinho and Cavalcante, both municipalities of Goiás State. The CV1 showed a slight increase in the dorsal fin and in the ventral region.

Table 4. P-values from permutation tests (10,000 permutation rounds) for Mahalanobis distances among groups for *P. cuvieri*.

P-values from permutation tests (10000 permutation rounds) for Mahalanobis distances among groups:

	Barro Alto-GO	Bonito-MS	Cachoeira Alta-GO	Chapada dos Veadeiros National Park	Cocalzinho-GO	Corumbá-MS	Cavalcante-GO	Duêre-TO	General Carneiro-PR	Icém-SP	Nova Itapirema-SP	Palma-PR	Passos Maia-SC	Rio Verde-GO	Santa Cruz de Goiás-GO	Santa Fé do Sul-SF	São José do Ribamar-MA	Serranópolis-GO	Teresina de Goiás-GO
Bonito-MS	<.0001																		
Cachoeira Alta-GO	<.0001	<.0001																	
Chapada dos Veadeiros National Park	<.0001	<.0001	<.0001																
Cocalzinho-GO	<.0001	<.0001	<.0001	<.0001															
Corumbá-MS	<.0001	0,0001	<.0001	<.0001	<.0001														
Cavalcante-GO	<.0001	0,0002	<.0001	0,0023	0,0001	0,0013													
Duêre-TO	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001												
General Carneiro-PR	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001											
Icém-SP	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0,0001	<.0001	<.0001										
Nova Itapirema-SP	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0,0001	<.0001	<.0001	<.0001									
Palma-PR	<.0001	<.0001	<.0001	<.0001	<.0001	0,0001	0,0001	<.0001	<.0001	<.0001	<.0001								
Passos Maia-SC	<.0001	<.0001	<.0001	<.0001	<.0001	0,0006	0,0014	0,0001	<.0001	<.0001	<.0001	<.0001							
Rio Verde-GO	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001						
Santa Cruz de Goiás-GO	<.0001	0,0001	<.0001	<.0001	<.0001	<.0001	0,0006	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001					
Santa Fé do Sul-SF	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001				
São José do Ribamar-MA	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001			
Serranópolis-GO	<.0001	<.0001	<.0001	<.0001	<.0001	0,0001	0,0004	<.0001	<.0001	<.0001	<.0001	<.0001	0,0001	<.0001	<.0001	<.0001	<.0001		
Teresina de Goiás-GO	<.0001	0,0006	<.0001	0,0001	<.0001	0,0002	0,0024	0,0001	<.0001	0,0001	<.0001	<.0001	<.0001	0,0001	0,0005	<.0001	<.0001	0,0001	
Teodoro Sampaio-SP	<.0001	<.0001	<.0001	<.0001	<.0001	0,0001	0,0004	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001

For *R. diptycha*, only the populations of Teodoro Sampaio and Nova Itapirema, SP, show no overlap with the other populations. In relation to Mahalanobis distances (Table 5), the highest values were seen between Macaubal and Teodoro Sampaio, SP, and the smallest between Iporá, GO and Nova Granada, SP. The CV1 showed an increase in body height and in the ventral region.

Table 5. P-values from permutation tests (10,000 permutation rounds) for Mahalanobis distances among groups for *R. diptycha*.

P-values from permutation tests (10000 permutation rounds) for Mahalanobis distances among groups:

	Corumbá-MS	Iporá-Go	Macaubal-SP	Nova Granada-SP	Nova Itapirema-SP	Palmeiras de Goiás-GO	São Domingo-GO	Santa Fé do Sul-SP
Iporá-GO	<.0001							
Macaubal-SP	<.0001	<.0001						
Nova Granada-SP	0,0001	<.0001	<.0001					
Nova Itapirema-SP	<.0001	<.0001	<.0001	<.0001				
Palmeiras de Goiás-GO	0,0001	<.0001	<.0001	<.0001	<.0001			
São Domingos-SP	0,0004	<.0001	<.0001	<.0001	<.0001	0,0003		
Santa Fé do Sul-sp	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	
Teodoro de Sampaio-SP	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001

The populations of *D. minutus* collected in Mineiros and Jataí, GO, São José do Barreiro, SP and São José dos Pinhais, PR did not overlap with the other populations. In relation to Mahalanobis distances (Table 6), the highest values were seen between populations of Mineiros and São José dos Pinhais, and the smallest between populations of Bonfinópolis and Nova Itapirema. Considering the CV1, it is possible to notice an increase in the body and fin, and a more depressed snout.

Table 6. P-values from permutation tests (10,000 permutation rounds) for Mahalanobis distances among groups for *D. minutus*.

P-values from permutation tests (10000 permutation rounds) for Mahalanobis distances among groups:

	Bonfinópolis-GO	Botucatu-SP	Candoí-PR	General Carneiro-PR	Gália-SP	Icém-SP	Jataí-GO	Leopoldo de Bulhões-GO	Mineiros-GO	Nova Itapirema-SP	Passos Maia-SC	Ribeirão Grande-SP	São José do Barreiro-SP	São Jose dos Pinhais-PR
Botucatu-SP	0,0001													
Candoí-PR	<.0001	<.0001												
General Carneiro-PR	<.0001	<.0001	<.0001											
Gália-SP	<.0001	<.0001	<.0001	<.0001										
Icém-SP	<.0001	0,0001	<.0001	<.0001	<.0001									
Jataí-GO	0,0002	<.0001	<.0001	<.0001	<.0001	<.0001								
Leopoldo de Bulhões-GO	0,0042	0,0022	<.0001	<.0001	<.0001	0,0014	0,005							
Mineiros-GO	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0,0017						
Nova Itapirema-SP	0,0002	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0,002	<.0001					
Passos Maia-SC	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0,0005	<.0001	<.0001				
Ribeirão Grande-SP	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0,0001	<.0001	<.0001	<.0001	<.0001			
São José do Barreiro-SP	0,0001	<.0001	<.0001	<.0001	<.0001	<.0001	0,0003	0,0022	<.0001	<.0001	<.0001	<.0001		
São Jose dos Pinhais-PR	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0,0006	<.0001	<.0001	<.0001	<.0001	<.0001	
Vargem Bonita-SC	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0,0003	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001

The populations of *S. fuscovarius* collected in Botucatu, Ipiguá, Passos Maia, Vargem Bonita and São Miguel do Araguaia show no overlap with the other populations. In relation to Mahalanobis distances (Table 7), the highest values were seen between Passos Maia and São

Miguel de Araguaia, and the smallest between Mirassol and Nova Itapirema. The CV1 shows a slight increase in the dorsal fin.

Table 7. P-values from permutation tests (10,000 permutation rounds) for Mahalanobis distances among groups for *S. fuscovarius*.

P-values from permutation tests (10000 permutation rounds) for Mahalanobis distances among groups:

	Botucatu-SP	Gália-SP	Ipiguá-SP	Jataí-GO	Mirassol-SP	Nova Itapirema-SP	Passos Maia-RS	São Miguel do Araguaia-GO
Gália-SP	<.0001							
Ipiguá-SP	<.0001	<.0001						
Jataí-G0	<.0001	<.0001	<.0001					
Mirassol-SP	<.0001	<.0001	<.0001	<.0001				
Nova Itapirema	<.0001	<.0001	<.0001	<.0001	<.0001			
Passos Maia-RS	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		
São Miguel do Araguaia-GO	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	
Vargem Bonita-SC	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001

Already in *T. typhonius* all populations differed morphologically, without any overlap, with the smallest Mahalanobis distance (Table 8) being observed in the populations of Cidade de Goiás and Itapirapuã in the state of Goiás. The greatest distance was between Itapirapuã in the state of Goiás and Nova Itapirema in the state of São Paulo. The CV1 shows an increase in size at body height, lower fins and a shorter total length.

Table 8. P-values from permutation tests (10,000 permutation rounds) for Mahalanobis distances among groups for *T. typhonius*.

P-values from permutation tests (10000 permutation rounds) for Mahalanobis distances among groups:

	Cidade de Goiás-GO	Itapirapuã-GO	Mirassol-GO	Nova Itapirema-SP	São José do Rio Preto-SP
Itapirapuã-GO	<.0001				
Mirassol-GO	<.0001	<.0001			
Nova Itapirema-SP	<.0001	<.0001	<.0001		
São José do Rio Preto-SP	<.0001	<.0001	<.0001	<.0001	
São José do Rio Preto-SP	<.0001	<.0001	<.0001	<.0001	<.0001

With the exception of *R. diptycha* (R= -0.013 P>0.5892) the Mantel test indicated a correlation between interpopulational variation and geographical distance to tadpoles of *B. albopunctata* (R= 0.1933, p< 0.0017), *D. minutus* (R= 0.2395, p< 0.0001), *P. cuvieri* (R=

0.1721, p< 0.0001), *S. fuscovarius* (R= 0.1362, p< 0.0017), and *T. typhonius* (R= 0.2296, p< 0.0001).

The multivariate regression resulted in a low percentage, indicating there was no association between shape and size to *B. albopunctata* (16.52%, Appendix 1), *D. minutus* (8.16%, Appendix 2), *P. cuvieri* (4.37%, Appendix 3), *R. diptycha* (5.31%, Appendix 4), *S. fuscovarius* (7.34%, Appendix 5), *T. typhonius* (13.99%, Appendix 6). Only in *T. typhonius* was it possible to observe a geographic structure.

Finally, the multivariate regression showed a weak relationship (R2= 0.00093, p=0.95; Figure 2) between morphological variation and sample size indicating that the sample size was not directly related to the variation observed.

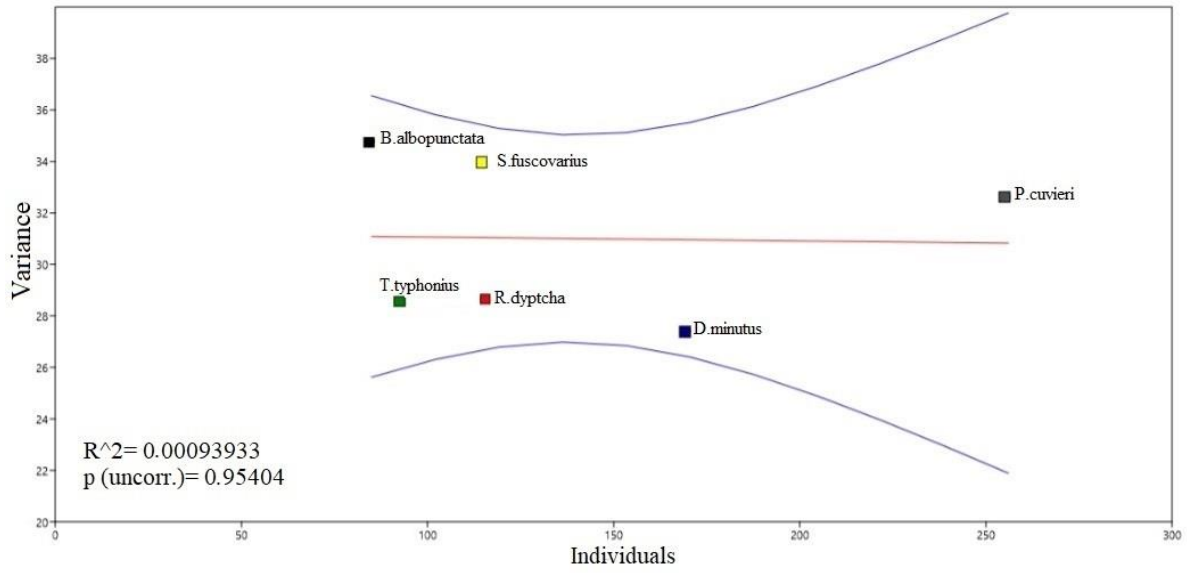

Figure 2. Multivariate regression between the sampled species.

4. Discussion

In order to understand the course of evolutionary change, studies on geographical variation analyze the various levels of morphological variation either at the intraspecific or

interpopulation level (Guill & Heins, 1996). Our data clearly show variation in the external morphology between the populations of the analyzed species, five of them (*Boana albopunctata*, *Dendropsophus minutus*, *Physalaemus cuvieri*, *Scinax fuscovarius*, and *Trachycephalus typhonius*) associated with geographical distance.

Species with larger geographic distributions (i.e. 'widespread') are more likely to encounter varied environmental conditions and barriers to gene flow than geographically-restricted species (Bilodeau et al., 2015). Variation among geographically separated populations is promoted if geographic barriers prevent frequent migration between populations (e.g. birds from the Purus–Madeira by Abreu & Anciães, 2018), if populations respond plastically to local environmental pressures (*S. fuscovarius* tadpoles Quinzio & Goldberg, 2020), or if selection acts locally to produce clinal variation or patchness (Hellberg et al., 2002; Blanckenhorn & Demont, 2004; Russell & Bauer, 2005). Morphological traits may vary among geographic distribution as a result of adaptation to local environments (Quinzio & Goldberg, 2020) or neutral drift (Lee et al., 2016), which are not mutually exclusive and may act together on phenotypic traits (Sun et al., 2013; Engen & Saether, 2016).

Although considered excellent models for the study of phenotypic variation (Miner et al., 2005), most studies of tadpole morphology have been experimental, and have tended to focus on intra-population phenotypic plasticity, reflecting a knowledge gap in terms of field data and inter-population comparisons (Van Buskirk, 2009; Rossa-Feres et al., 2015; Lopes et al., 2020).

Among the three nektonic species analyzed here, *D. minutus* and *S. fuscovarius* presented no relationship of intraspecific morphological variation and individual size with geographic distribution. Southern populations did not tend to cluster with any other

population. Both these species are widely distributed across Brazil and South America, and are considered species complexes (Gehara et al., 2014; Vaz Silva et al., 2020). Widely distributed species usually show considerable phenotypic variation within their ranges (Armbruster & Schwaegerle, 1996). It is unlikely that a single phenotype could promote the best performance for a species under all environmental conditions to which it is subjected (Pakkasmaa & Piironen, 2001). In this context, environmental factors are usually considered as important drivers of the geographic variation in the morphology of organisms, because several species manifest different phenotypes at sites with different environmental conditions such as biotic and abiotic factors (Van Buskirk et al., 1997; Verberk et al., 2021; Verberk et al., 2021; Boelter et al., 2022).

That way, variations in morphological characters between individuals of a population or between populations of a species are always expected (Travis, 1994). However, variation within species is generally considered to be lower than variation among species, although this assumption is rarely tested (Martins et al., 2020, Jordani et al., 2019). Jordani et al. (2019) points out that the 33% of trait variability was less than the interspecific variation, and was due to within-species variation, with the average varied widely among traits.

Populations may exhibit intraspecific variation even in the absence of selective forces due to the effects of random genetic drift alone, in accordance to pure neutral mechanisms. Random neutral genetic recombination accumulates through time, driving allelic frequencies to vary among localities. In addition, because of higher gene flow among geographically closer localities, more distant populations can be isolated by distance (Wright, 1943; Barbujani, 1987). Consequently, there will be subtle changes among neighboring populations and increasing differentiation as they get farther apart (Abreu et al., 2018).

Although geographic distance is a factor that can generate morphological variations, there are cases in which this factor does not influence the shape of organisms. Lopes et al. (2020) observed that morphometric differences in tadpoles of *Dendropsophus nanus* and *D. minutus* did not indicate any significant pattern of spatial autocorrelation. Quizio & Goldberg (2020) showed that Argentinean populations of *S. fuscovarius* tadpoles exhibited differences in the shape and size, result interpreted as a by-product of local geographical conditions, and possible associations with biotic and abiotic factors cues. Interestingly the authors noted the occurrence of two distinct tadpole morphs for the species, one a smaller and depressed form of the entire body plan in the most distant population, whereas other populations were larger, with taller bodies and shorter tails. The first faced higher temperatures, lower and scattered rainfall, plus the presence of other anuran tadpoles and aquatic invertebrate and vertebrates, which could act as environmental stressors affecting the growth of body and tail. A similar result was observed in tadpoles of *S. squalirostris* (Boelter et al., 2022).

Trachycephalus typhonius was the only nektonic species to present a geographic pattern in external morphology. Populations from a 45outhern distribution in our sample are larger in size than those at southern. Studies show that populations can be locally adapted to the predominant temperatures of their environments (Ståhlberg et al., 2001), with the tadpole growth rates are strongly affected by both abiotic and biotic factors (Alford, 1999).

Despite the strong relation of morphological variation with geographic distance (except in *R. diptycha*), some species such as *D. minutus* and *P. cuvieri*, showed great overlap between most localities sampled (Appendix 3-4). Only *T. typhonius*, the present the greatest Mahalanobis distance correspondente to the greatest distance between the sampling sites.

Already when we analyze the benthic species *B. albopunctata* and *P. cuvieri*, both of them did not show geographic patterns. However, the regression analysis found an association

between shape and size in *B. albopunctata* with 16.52% (p = 0.001) of shape variation predicted by regression on size. In *P. cuvieri*, although smaller populations are observed, this does not seem to be associated with the shape of the animals and no spatial pattern was observed.

Teasing apart the effects of local adaptation and gene flow may be a complex task, but is a key to improve our understanding of the drivers of intraspecific morphological variation. The combination of random and non-random factors, together with barriers in the distribution of the species with wide distribution may lead to morphological differences. Goldelber et al. (2018) demonstrated the presence of intraspecific variation during the development of *Boana riojana* tadpoles, over a period of two years. observing the presence of two different morphs in the series with a longer duration of the larval period. Furthermore the intraspecific heterochrony described for the larval development of *B. riojana* does not lead to phenotypic variation at the end of metamorphosis. The presence of morphological variation can have implications for the taxonomic history of the species, making it difficult to correctly identify the species, and transforming intrapopulation variations into diagnostic differences, as in the cases of *Bokermannohyla riojanus* and *B. nanuzae*.

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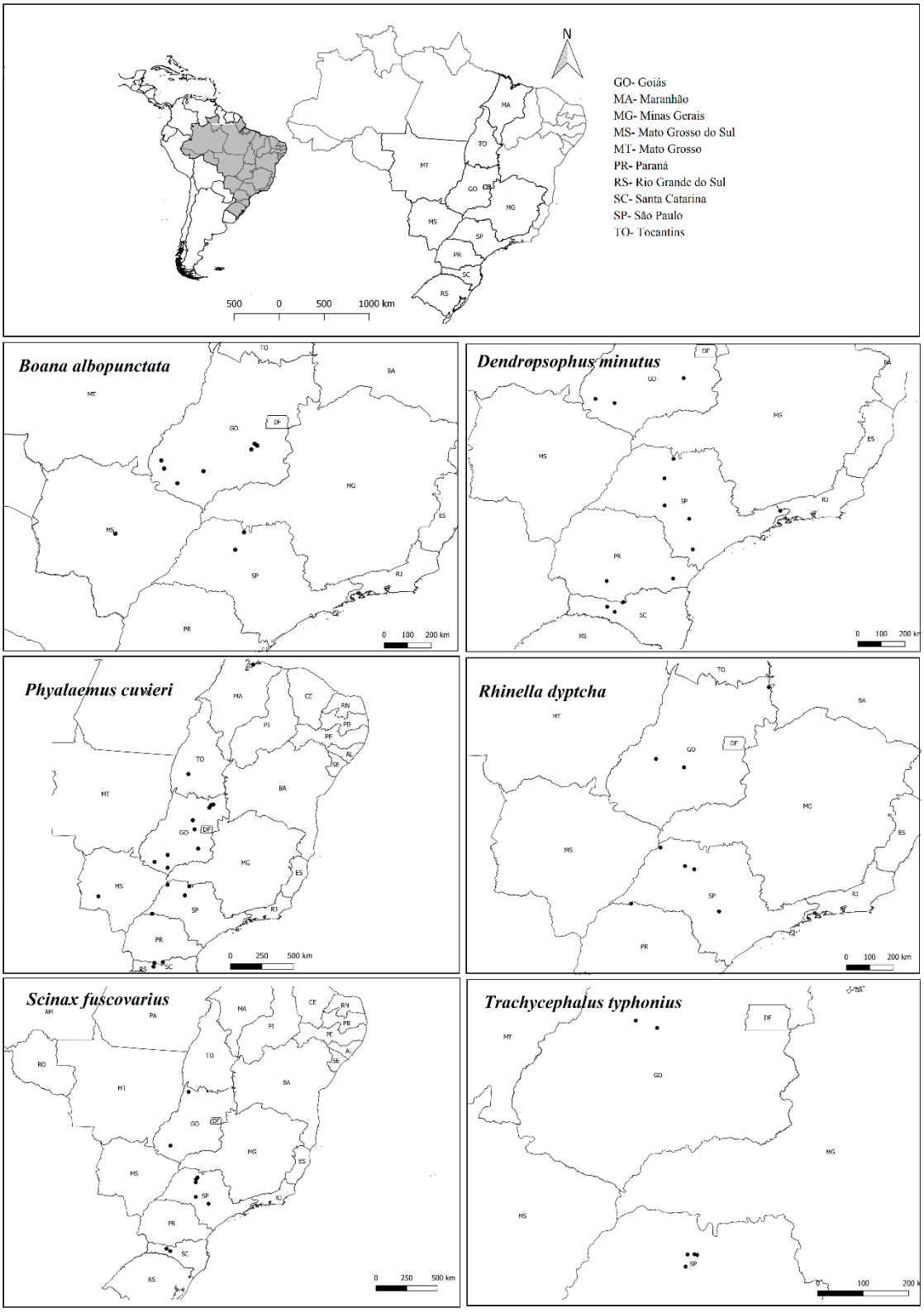
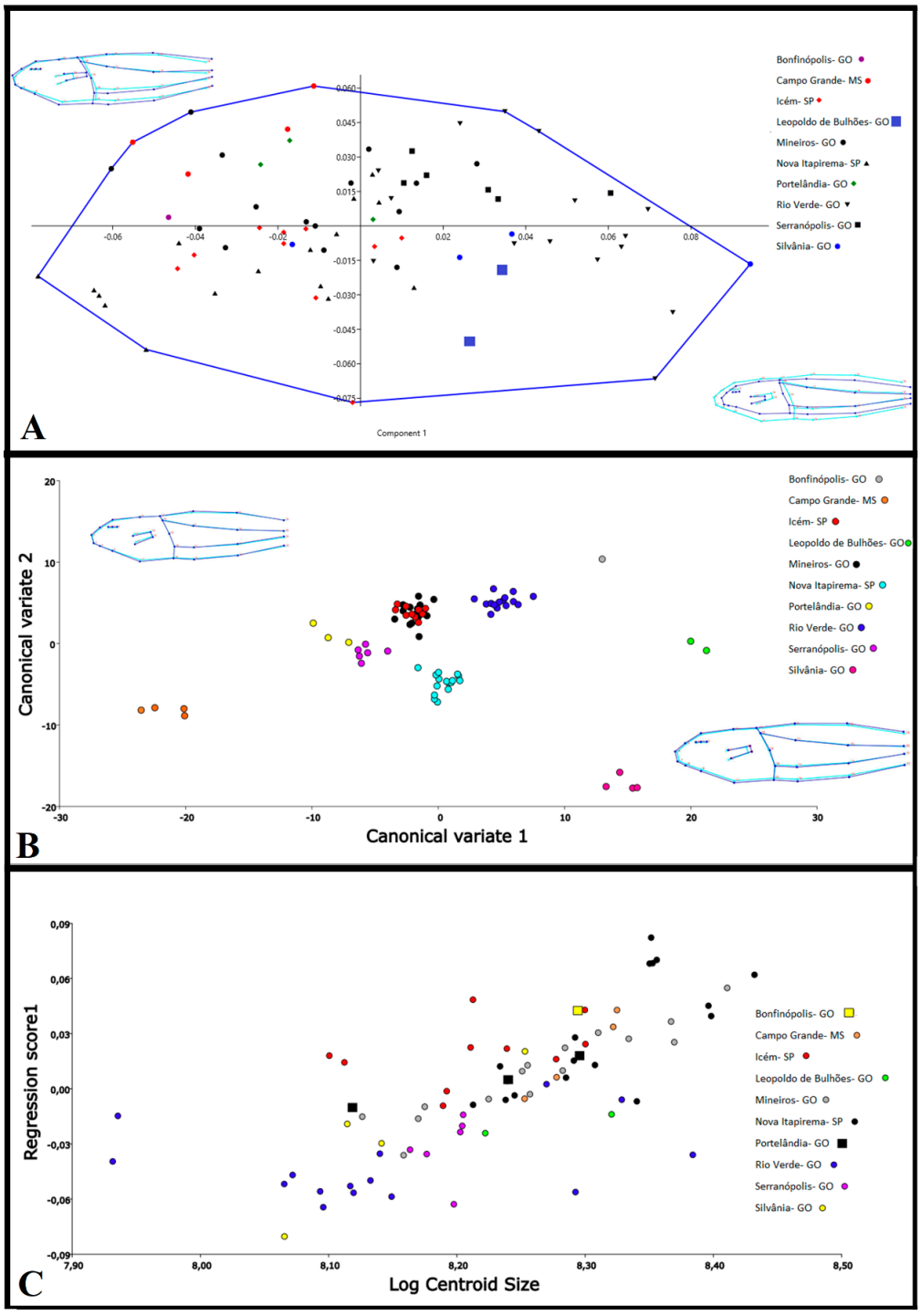
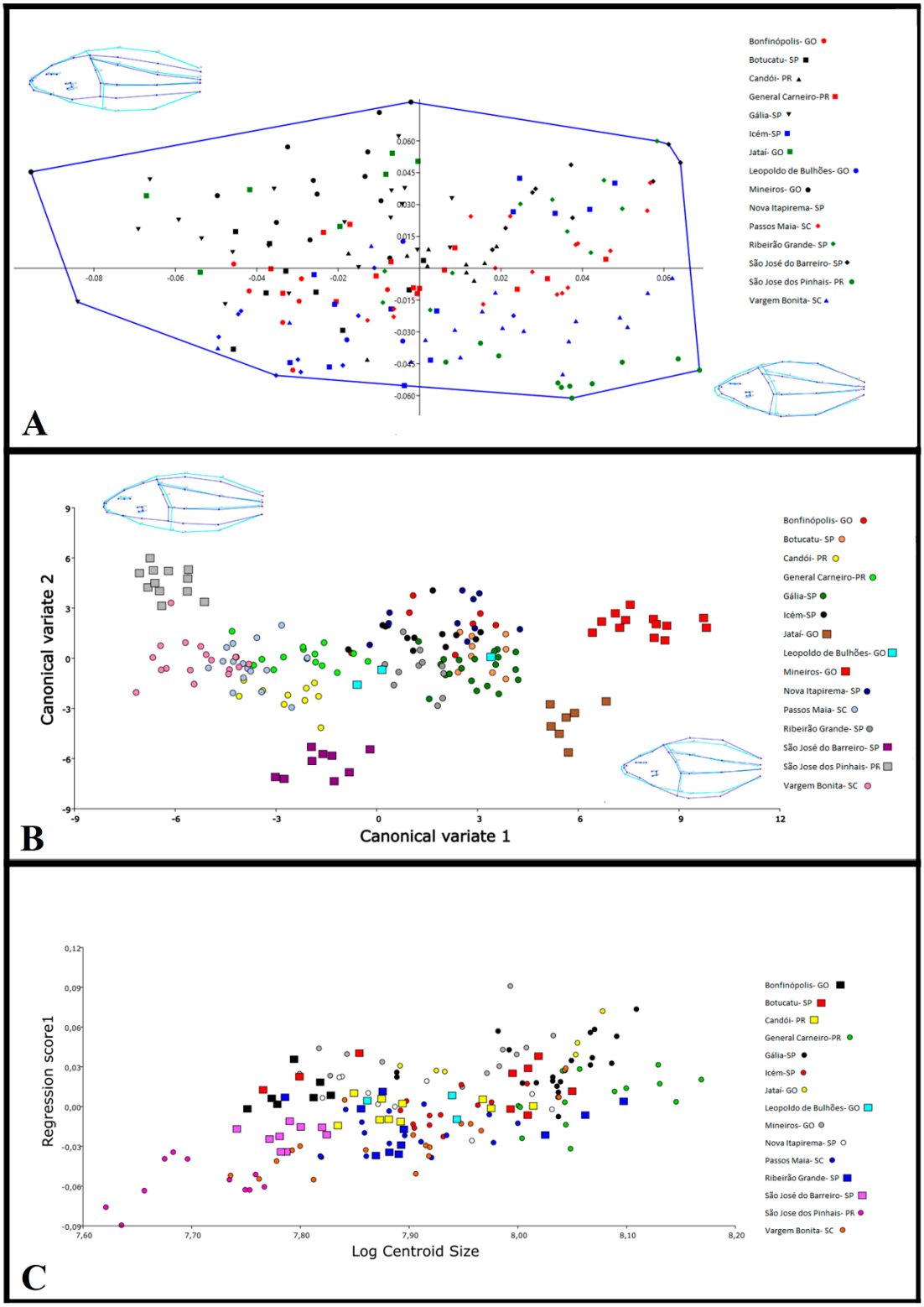
Appendix 1. Maps showing the locations of the populations sampled.



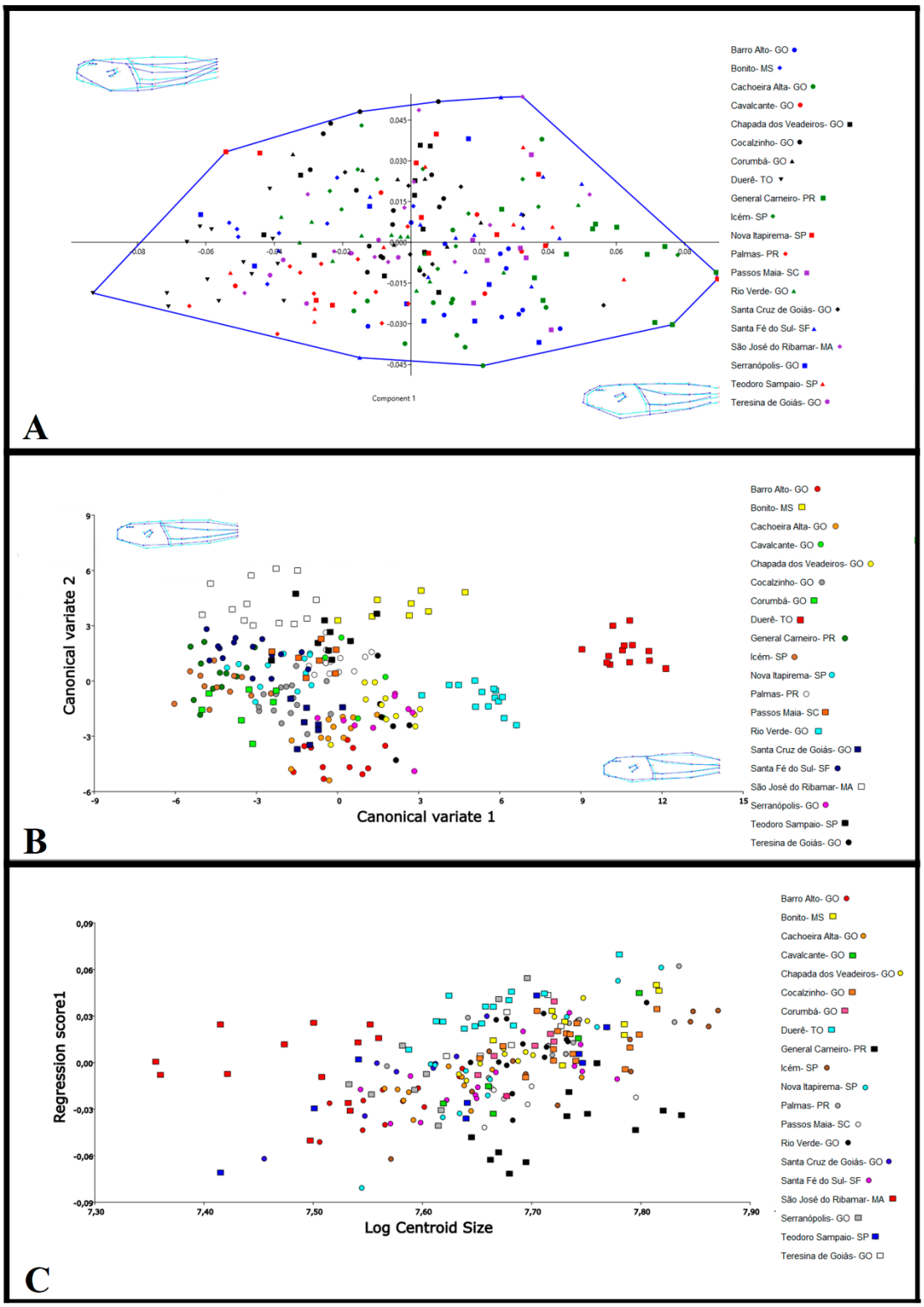
Appendix 2. *Boana albopunctata* analyzes: A). Principal component analysis (PCA). B). Canonical variate analysis between populations from different regions (CVA). C). Multivariate regression.



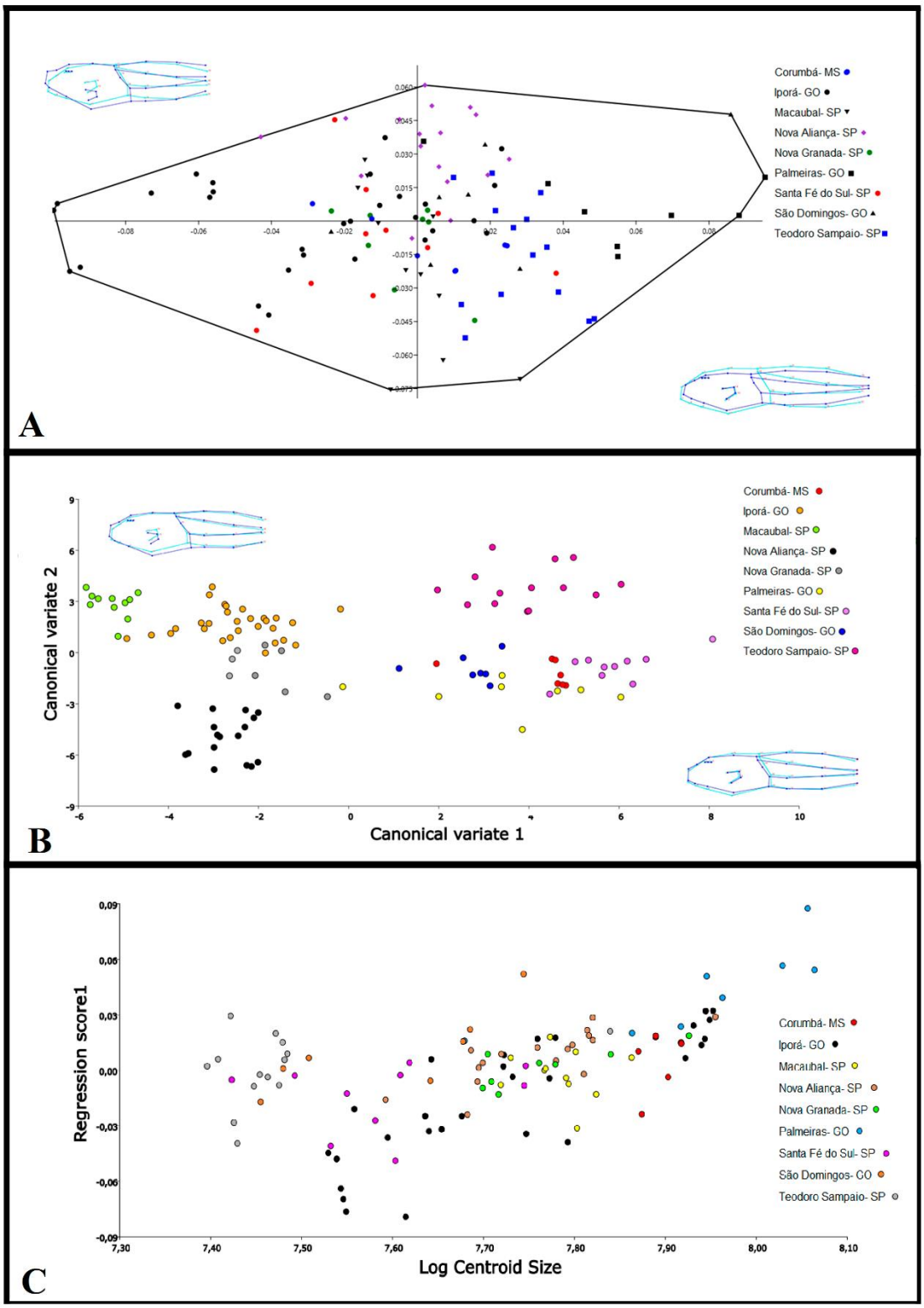
Appendix 3. *Dendropsophus minutus* analyzes: A). Principal component analysis (PCA). B). Canonical variate analysis between populations from different regions (CVA). C). Multivariate regression.



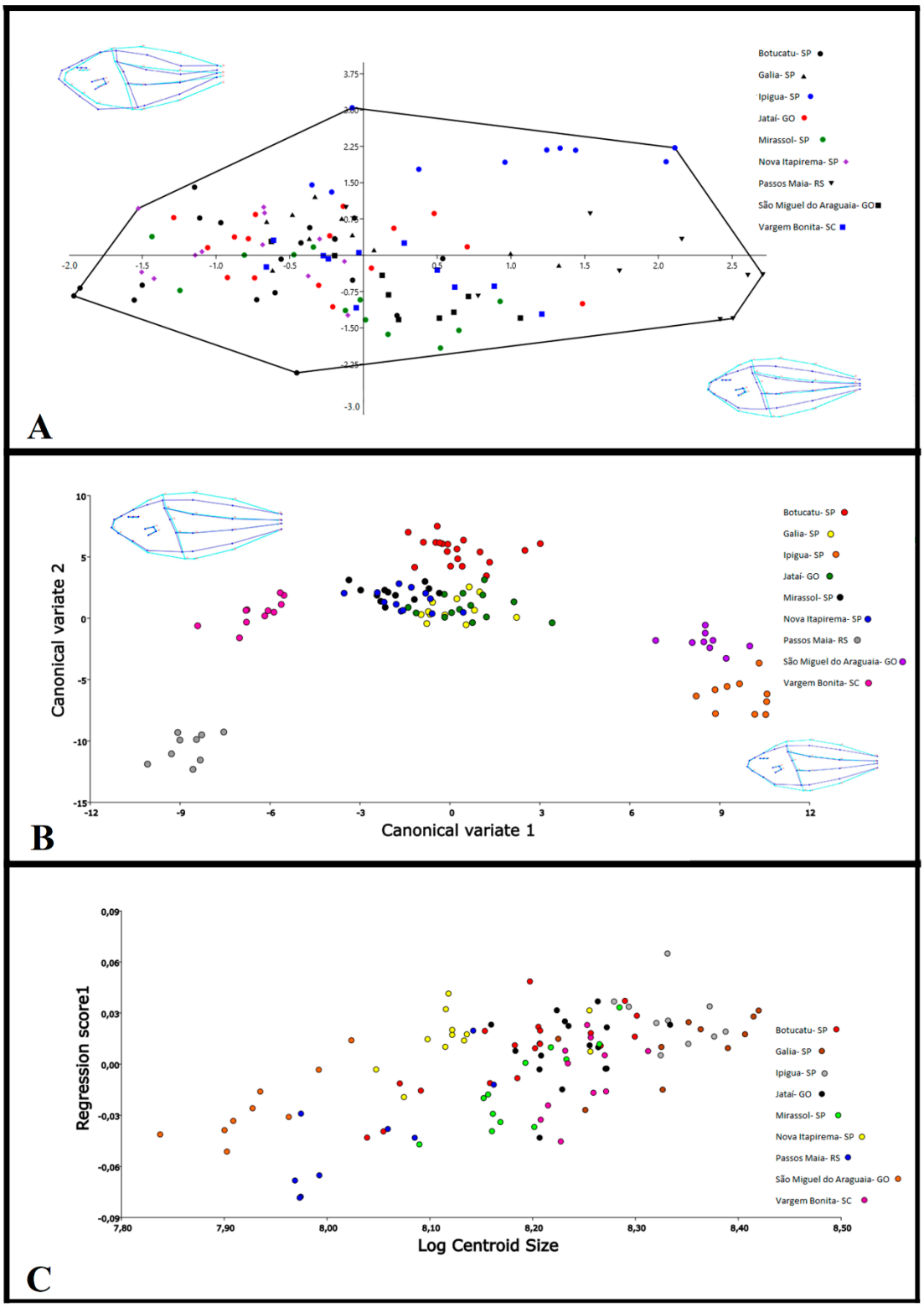
Appendix 4. *Physalaemus cuvieri* analyzes: A). Principal component analysis (PCA). B). Canonical variate analysis between populations from different regions (CVA). C). Multivariate regression.



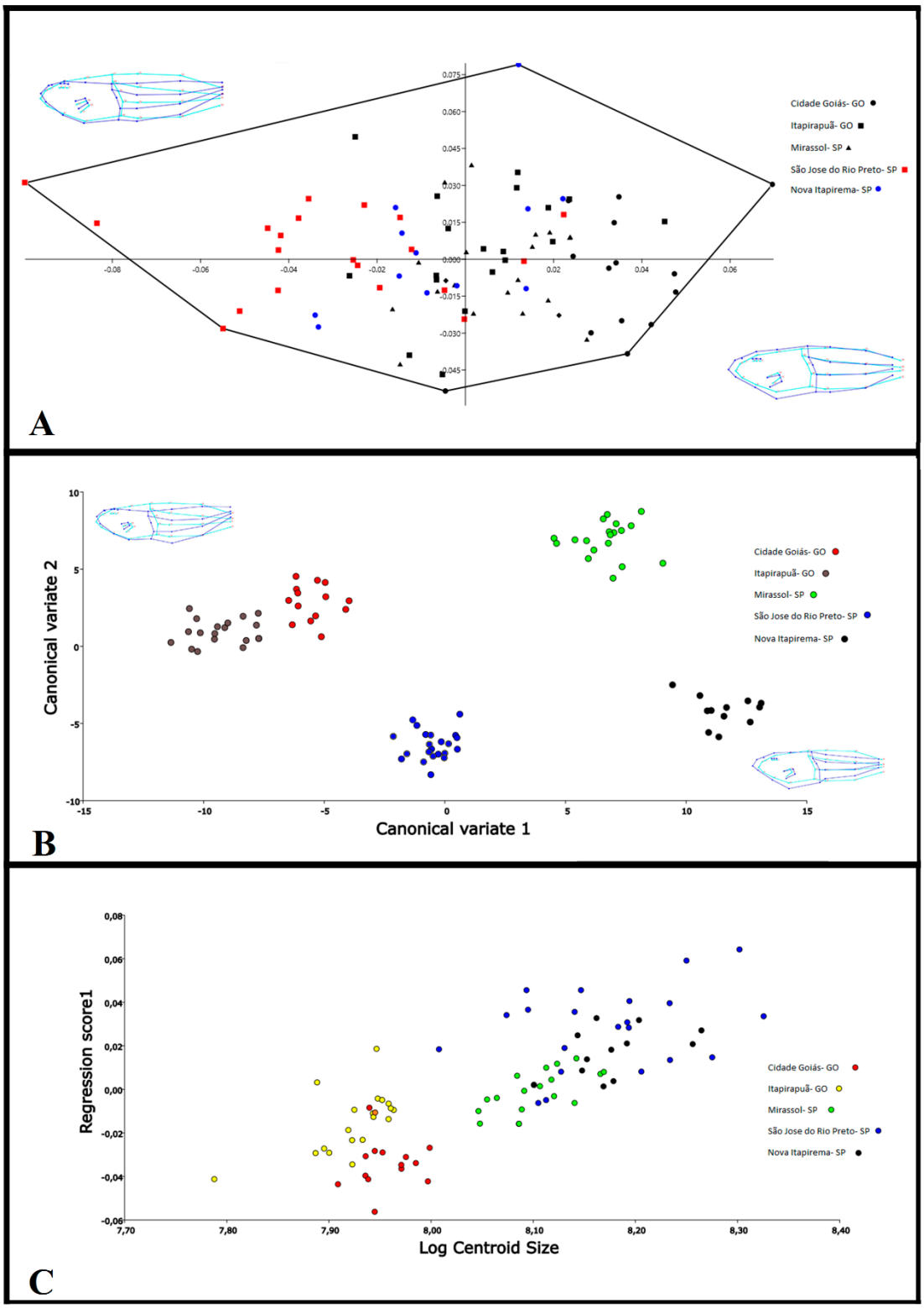
Appendix 5. *Rhinella diptycha analyzes: A). Principal component analysis (PCA). B). Canonical variate analysis between populations from different regions (CVA). C). Multivariate regression.*



Appendix 6. *Scinax fuscovarius analyzes: A). Principal component analysis (PCA). B). Canonical variate analysis between populations from different regions (CVA). C). Multivariate regression.*



Appendix 7. *Trachycephalus typhonius* analyzes: A). Principal component analysis (PCA). B). Canonical variate analysis between populations from different regions (CVA). C). Multivariate regression.



Annex 1: Specimens examined.

Species	Zoological Collection	Number	Locality
Boana albopunctata	ZUFG	1426	Bonfinopolis -GO
Boana albopunctata	ZUFG	1446	Bonfinopolis -GO
Boana albopunctata	ZUFG	1464	Bonfinopolis -GO
Boana albopunctata	ZUFG	1595	Bonfinopolis -GO
Boana albopunctata	ZUFG	2441	Bonfinopolis -GO
Boana albopunctata	ZUFG	124	Mineiros-GO
Boana albopunctata	ZUFG	235	Mineiros-GO
Boana albopunctata	ZUFG	266	Mineiros-GO
Boana albopunctata	ZUFG	1328	Mineiros-GO
Boana albopunctata	ZUFG	2767	Mineiros-GO
Boana albopunctata	ZUFG	55	Rio Verde- GO
Boana albopunctata	ZUFG	57	Rio Verde- GO
Boana albopunctata	ZUFG	76	Rio Verde- GO
Boana albopunctata	ZUFG	152	Rio Verde- GO
Boana albopunctata	ZUFG	760	Rio Verde- GO
Boana albopunctata	ZUFG	1375	Serranopolis-GO
Boana albopunctata	DZSJRP	465.1	Icém- SP
Boana albopunctata	DZSJRP	524.1	Icém- SP
Boana albopunctata	DZSJRP	592.1	Icém- SP
Boana albopunctata	DZSJRP	640.2	Icém- SP
Boana albopunctata	DZSJRP	211.2	Nova Itapirema- SP
Boana albopunctata	DZSJRP	228.1	Nova Itapirema- SP

Boana albopunctata	DZSJRP	234.1	Nova Itapirema- SP
Boana albopunctata	DZSJRP	328.1	Nova Itapirema- SP
Dendropsophus minutus	ZUFG	1602	Bonfinopolis -GO
Dendropsophus minutus	ZUFG	3078	Mineiros-GO (PNE)
Dendropsophus minutus	DZSJRP	3454.5	Botucatu-SP
Dendropsophus minutus	DZSJRP	1870.3	Candoí- PR
Dendropsophus minutus	DZSJRP	2022.2	Conde D'Eu-SP
Dendropsophus minutus	DZSJRP	2001.2	Conde D'Eu-SP
Dendropsophus minutus	DZSJRP	2647.1	Gália-SP
Dendropsophus minutus	DZSJRP	1670.3	General Carneiro-PR
Dendropsophus minutus	DZSJRP	538.1	Icém-SP
Dendropsophus minutus	DZSJRP	570.1	Icém-SP
Dendropsophus minutus	DZSJRP	2435.2	Icém-SP
Dendropsophus minutus	DZSJRP	2150.3	Jataí-GO
Dendropsophus minutus	DZSJRP	3325.1	Nova Itapirema-SP
Dendropsophus minutus	DZSJRP	1580.3	Passos Maia-SC
Dendropsophus minutus	DZSJRP	1863.3	Ribeirao Grande-SP
Dendropsophus minutus	DZSJRP	1019.1	São Jose dos Pinhais-PR
Dendropsophus minutus	DZSJRP	1677.2	Vargem Bonita-SC
Physalaemus cuvieri	ZUFG	808	Barro Alto-GO
Physalaemus cuvieri	ZUFG	855	Barro Alto-GO
Physalaemus cuvieri	ZUFG	889	Barro Alto-GO
Physalaemus cuvieri	ZUFG	1766	Cachoeira Alta-GO
Physalaemus cuvieri	ZUFG	1990	Cavalcante-GO

Physalaemus cuvieri	ZUFG	2001	Cavalcante-GO
Physalaemus cuvieri	ZUFG	2004	Cavalcante-GO
Physalaemus cuvieri	ZUFG	1894	Chapada dos Veadeiros-GO
Physalaemus cuvieri	ZUFG	2286	Chapada dos Veadeiros-GO
Physalaemus cuvieri	ZUFG	2288	Chapada dos Veadeiros-GO
Physalaemus cuvieri	ZUFG	39	Cocalzinho-GO
Physalaemus cuvieri	ZUFG	42	Cocalzinho-GO
Physalaemus cuvieri	ZUFG	175	Cocalzinho-GO
Physalaemus cuvieri	ZUFG	38	Corumbá-GO
Physalaemus cuvieri	ZUFG	48	Corumbá-GO
Physalaemus cuvieri	ZUFG	3135	Duerê-TO
Physalaemus cuvieri	ZUFG	1207	Pires do Rio-GO
Physalaemus cuvieri	ZUFG	1067	Serranopolis-GO
Physalaemus cuvieri	ZUFG	1071	Serranopolis-GO
Physalaemus cuvieri	DZSJRP	1608.1	General Carneiro-PR
Physalaemus cuvieri	DZSJRP	1620.4	General Carneiro-PR
Physalaemus cuvieri	DZSJRP	1631.4	General Carneiro-PR
Physalaemus cuvieri	DZSJRP	2852	Palmas-PR
Physalaemus cuvieri	DZSJRP	1583.4	Passos Maia-SC
Physalaemus cuvieri	DZSJRP	1726.9	Passos Maia-SC
Physalaemus cuvieri	MAP	4053	Bonito-MS
Physalaemus cuvieri	DZSJRP	635.1	Icém-SP
Physalaemus cuvieri	DZSJRP	645.1	Icém-SP
Physalaemus cuvieri	DZSJRP	166.3	Nova Itapirema-SP

Physalaemus cuvieri	DZSJRP	177.3	Nova Itapirema-SP
Physalaemus cuvieri	DZSJRP	433.3	Santa Fé do Sul-SP
Physalaemus cuvieri	DZSJRP	1273	Teodoro Sampaio-SP
Physalaemus cuvieri	DZSJRP	1277	Teodoro Sampaio-SP
Physalaemus cuvieri	DZSJRP	1282.2	Teodoro Sampaio-SP
Physalaemus cuvieri	DZSJRP	1310.6	Teodoro Sampaio-SP
Rhinella diptycha	UFG	2903	Iporá-GO
Rhinella diptycha	UFG	2258	Iporá-GO
Rhinella diptycha	UFG	2260	Iporá-GO
Rhinella diptycha	UFG	2261	Iporá-GO
Rhinella diptycha	UFG	2907	Palmeiras-GO
Rhinella diptycha	UFG	1771	São Domingos-GO
Rhinella diptycha	DZSJRP	377.1	Santa Fé do Sul-SP
Rhinella diptycha	DZSJRP	1296.1	Teodoro Sampaio-SP
Rhinella diptycha	DZSJRP	1325.1	Macaubal-SP
Rhinella diptycha	DZSJRP	3427.1	Nova Aliança-SP
Rhinella diptycha	MAP	1510	Corumbá-MS
Scinax fuscovarius	ZUFG	3083	São Miguel do Araguaia-GO
Scinax fuscovarius	DZSJRP	3446.2	Botucatu-SP
Scinax fuscovarius	DZSJRP	3448.2	Botucatu-SP
Scinax fuscovarius	DZSJRP	2647.5	Galia-SP
Scinax fuscovarius	DZSJRP	2648.4	Galia-SP
Scinax fuscovarius	DZSJRP	3363	Ipigua-SP
Scinax fuscovarius	DZSJRP	2144.2	Jataí-GO

Scinax fuscovarius	DZSJRP	2150.2	Jataí-GO
Scinax fuscovarius	DZSJRP	2311	Jataí-GO
Scinax fuscovarius	DZSJRP	3369.1	Mirassol-SP
Scinax fuscovarius	DZSJRP	144.1	Nova Itapirema-SP
Scinax fuscovarius	DZSJRP	271.5	Nova Itapirema-SP
Scinax fuscovarius	DZSJRP	301.9	Nova Itapirema-SP
Scinax fuscovarius	DZSJRP	1575.6	Passos Maia-RS
Scinax fuscovarius	DZSJRP	1575.5	Vargem Bonita-SC
Trachycephalus typhonius	UFG	1916	Cidade Goiás-GO
Trachycephalus typhonius	UFG	2632	Jussara-GO
Trachycephalus typhonius	DZSJRP	3353.2	Mirassol-SP
Trachycephalus typhonius	DZSJRP	1336.2	São Jose do Rio Preto-SP
Trachycephalus typhonius	DZSJRP	3346.2	São Jose do Rio Preto-SP
Trachycephalus typhonius	DZSJRP	2420.1	Nova Itapirema-SP

CAPÍTULO 2

Brain variation in tadpoles of different ecomorphological guilds

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Abstract: The relationship between the anatomical structures of tadpoles and their respective functions still have large gaps in knowledge, in addition to being considered an invariable anatomical system. Using computed tomography images and geometric morphometric analysis we analyze the brain of tadpoles of three tadpoles from different ecomorphological guilds. We present here the first effort to understand the central nervous system of neotropical tadpoles. Brain morphology clearly differed among the analyzed species and also presented intraspecific variations. Factors such as the differential use of the depth of the water column were related to variation in brain anatomy, being nektonic tadpoles that use the habitat in three dimensional way, presented the greater brain and more voluminous cerebellum.

Keywords: Ecomorphology. Larval morphology. Nervous system.

Resumo: A relação entre as estruturas anatômicas dos girinos e suas respectivas funções ainda possuem grandes lacunas de conhecimento, além de ser considerado um sistema anatômico invariável. Utilizando imagens de tomografia computadorizada e análise morfométrica geométrica analisamos o cérebro de girinos de três girinos de diferentes guildas ecomorfológicas. Apresentamos aqui os primeiros esforços para entender o sistema nervoso de girinos neotropicais. Observando que a morfologia cerebral difere claramente entre as espécies analisadas além de apresentar variações intraespecíficas, e que fatores como o uso do ambiente e o uso diferencial da profundidade da coluna d'água estão diretamente relacionados à variação na anatomia cerebral.

Palavras-chave: Ecomorfologia. Morfologia larval. Sistema nervoso.

1. INTRODUCTION

Although all vertebrates share a common basic brain organization, different species exhibit wide variability in terms of volume~~s~~ and proportional size~~s~~ of different subdivisions and neurais systems (Butler and Hodos, 2005). The variation in the size and composition of the brain often correlates with diverse sensory, behavioral, social, and cognitive skills (Butler and Hodos, 2005). The transition from fish to amphibians marks the beginning of the origin of tetrapods and the major adaptive radiation in vertebrate evolution, reflecting directly into changes in brain morphology (Northcutt & Royce, 1975; Ten Donkelaar, 1998). Most amphibians, and particularly anurans, are characterized by a larval period and a metamorphosis that changes many of the body parts, allowing them to survive in a terrestrial environment (Altig & McDiarmid, 1999).

The general structure and functional organization of the amphibian brain includes several apparently primitive features (such as a small size) as well as the standard major brain areas and basic connectional patterns found in all vertebrates (Wilczynski, 2019). Studies regarding anurans' nervous system increased in the last years, and anurans have played a prominent role as experimental animals in brain research for comparative studies (Gonzalez et al. 2020). However, most of the studies have focused on few some species of anurans, as *Xenopus laevis* and *Rana temporaria* (e.g. Eagleson et al., 1990; Munõz et al., 2014). Moreover, studies on the functional morphology of the central nervous system in frogs have focused mainly on the prey-capture visual system (Deban et al., 2001; Gonda et al., 2010; Trokovic et al., 2011), and the vocalization and auditory systems (Edwards & Kelley, 2001).

Almost no variation in the gross anatomy of the brain has been reported in anurans (Manzano et al., 2017). Taylor et al. (1995) indicated that despite the overall similarity of anuran brains, some differences and adaptations to fossorial and arboreal modes of life do appear to exist. Similar results were observed by Liao et al. (2015) that have tested the effects of phylogeny and ecology on the evolution of brain size in frogs. The authors concluded that whereas ecology did not impact overall brain size, habitat and diet types did impact the size of the telencephalon.

Brain development shows high plasticity in response to environmental heterogeneity (Tooled et al., 2021). However, it is unknown how environmental variation during development may affect brain architecture across life history in species with complex life cycles (Trokovic et al., 2011). Brain size and brain morphology (*i.e.*, proportional relationships among brain regions) are important traits that shape cognitive ability and behavior and have been linked to variation in ecological performance (Axelrod

et al., 2021). The brain size explains a large portion of the variation in brain features across vertebrate taxa (Axelrod et al., 2018). Furthermore, brain size is often related to habitat use, both interspecifically (Lecchini et al., 2014; Fischer et al., 2015;) and intraspecifically (Ahmed et al., 2017; Axelrod et al., 2018).

When compared with adults, studies with the brains of anuran tadpoles are even more restricted. As they are important model organisms, studies involving the brain of tadpoles are largely associated with regeneration (Cairns et al., 2018), genetics (Helbinga et al., 2006; Ledón-Rettig, 2021), toxicology (Yost et al,. 2016; Rutkoski et al., 2021; Spirhanzlova et al., 2022; McClelland et al., 2022) and on responses to density (Gonda et al., 2010). Lanoo (1999) detailed the organization of the tadpole brain and the organization and function of the spinal cord, with a discussion of how the various sensory systems of tadpoles are related to the nervous system morphology. Moreover, Lanoo (1999) summarized the organization of the tadpole nervous system with a brief consideration of the changes that it undergoes during metamorphosis. Research conducted with species of *Rana*, *Xenopus*, *Rhinella* (previously in the genus *Bufo*) and *Boana* (previously in the genus *Hyla*) genus are, in many cases, the only source of information for tadpole brain anatomy, being a starting point for understanding the brain of tadpoles.

There are two significant generalizations that can be drawn from a review of the nervous and sensory systems of anuran tadpoles: few taxa have been examined, but there are many variations exhibited even among this small number (Altig & McDiarmid, 1999). Although the brain has been shown to be affected by environmental conditions in basal vertebrates (*e.g.*, in fish: Kihslinger & Nevitt, 2006; Gonda et al., 2009), to our knowledge only one study investigated the effect of different environmental factors on brain development in amphibians (Gonda et al., 2010). The majority of articles that report large

variations in brain size or other structure in the nervous system (Kotrschal et al., 1998; Day et al., 2005; Gonda et al., 2010) demonstrated mostly an interspecific correlation among brain architecture and various ecological factors (e.g., diet and foraging behavior) and/or behavioural and life-history traits (e.g., foraging strategy and migratory behavior; Garamszegi & Eens, 2004; Sol et al., 2008; Trokovic et al., 2011).

Despite many studies of tadpoles' adaptive developmental plasticity related to morphological and behavioral phenotypes have been published, there are fewer examples related to the nervous system (Woodley et al., 2015). Furthermore, none of the available information is related to tadpoles of neotropical species. Therefore, in this study we explore the relationship between brain morphology and habitat use in tadpoles of three different ecomorphological guilds. The ecomorphological guilds of tadpoles were proposed with basis on habitat and feeding preferences, morphological adaptations and life history traits (Altig & Johnston, 1989; Altig & McDiarmid, 1999b; Orton, 1953; Van Dijk, 1972). Herein, to characterize the guilds we consider tadpoles' general morphology and the use of water column. Tadpoles of nektonic guild present a triangular shaped body, lateral eyes and high fins, and feed and forage in the water column (Altig & Johnston, 1989; Altig & McDiarmid, 1999b). Tadpoles of benthic guild present a globular/depressed body, dorsal eyes and low fins, and feed and forage at the bottom of aquatic habitat (Altig & Johnston, 1989; Altig & McDiarmid, 1999b). Tadpoles benthic/neustonic, despite present a morphology of typical bottom dwellers and feed and forage at the bottom during the day, displace to and forage at water surface at night (Rossa-Feres et al., 2004; Rossa-Feres & Nomura, 2006).

Specifically, we analyze the brain of tadpoles of *Dendropsophus minutus* (nektonic guild, *sensu* Altig & Johnston, 1989), *Physalaemus cuvieri* (benthic guild, *sensu* Altig &

Johnston, 1989), and *Rhinella diptycha* (benthic/neustonic guild, as *Bufo schneideri* in Rossa-Feres & Nomura, 2006). We hypothesized that tadpoles of the nektonic and benthic/neustonic guilds have more developed nervous systems than benthic tadpoles, as a nektonic organisms, they use the space in three dimensions, while benthic tadpoles, due to be restricted to the bottom of water bodies, use the environment in two dimensions, which requires less complexity of nervous system.

2. MATERIAL AND METHODS

2.1 Sample preparation

We analyzed tadpoles from the Zoological Collection of the Federal University of Goiás (ZUFG). In total, we used 10 tadpoles between Gosner' stages 34 and 38, of each one of three species of different guilds: nektonic tadpoles of *Dendropsophus minutus*, benthic tadpoles of *Physalaemus cuvieri*, and benthic-neustonic tadpoles *Rhinella diptycha*.

To gather morphological data, we photographed all tadpoles with a Sony Alpha 230 camera, positioned with a tripod at 20 cm from the tadpoles, and measured their total length before taking them to tomographic scanning. Morphometric measurements were made using the software ImageJ (1.51i).

In preparation for the tomographic scanning, we stained the specimens using 0.3% PTA in 70% EtOH (Metscher 2009). First, we maintained the tadpoles in 70% EtOH three times, for about 15 minutes each. Then, we transferred the tadpoles to the staining solution of PTA 5% for about 48 hours. Subsequently, we washed the tadpole again with 70% EtOH for at least 1 hour. We changed the 70% EtOH solution one time after 30min in this step. At the end of this process, we transferred the tadpole to an eppendorf tube containing

absolute ethanol. The acquisition of the tomographic images were held in a micro-CT scanner from General Electric Phoenix using a high-power nanofocus X-ray tube. Pixel/voxel resolution are possible down to 2 μm depending on sample size.

From the radiographs acquisition, we obtained a 3D volume of the brains and cerebellum following Fidalgo et al. (2020) and we rendered these images to obtain the total length of the brain. To perform the brain rendering, we segmented it manually into 3D voxel models, using the magic wand tool in the ImageTools software, and Avizo v.2019.1 (https://www.thermofisher.com/avizo) to obtain all measurements.

2.2. Geometric morphometric analysis

The rendered images were used for morphometric analyses. We identified twenty-five landmarks (Figure 1A) from the brain images and we digitized them using the software TpsUtil (Rohlf, 2013a) and TpsDig2 (Rohlf, 2013b) programs. We extracted the shape information for all individuals using the Procrustes superimposition method, to remove the effects of size, position, and orientation from each coordinate set and to standardize each specimen according to its centroid size (Zelditch et al. 2004). After applying the Procrustes superimposition method, we extracted the partial warp scores that represent the deformation in the brain of tadpoles (Rohlf, 1990, Rohlf and Bookstein 1990; Bookstein 1991; Zelditch et al. 2004).

We computed the average shape of all brain images to visualize the variation in brain shape (Zelditch et al. 2004). For that, we performed a Principal Component Analysis (PCA) using the covariance matrices of shape variation and the average shape variation among species (Zelditch et al., 2004). We also performed a Canonical Variate Analysis (CVA) to obtain a better graphical representation of the data and to discriminate groups based on brain shape variation (Klingenberg, 2011). We reported the results as

Mahalanobis distances and the respective p-values for these distances, after a permutation tests (10,000 iterations) (Klingenberg & Monteiro, 2005).

A regression analysis was performed to examine the effect of size, using centroid size, on the shape variation shown in the Procrustes coordinates. Analyses were performed using MorphoJ v1.07a.

2.3. Volume and linear morphometric data

We registered the following measurements for each brain and cerebellum image: brain size, brain volume, cerebellum volume (Figure 1B). We used the Avizo 8.0 software to calculate the brain size, brain volume, and cerebellum volume, while we used the ImageJ (1.5li) to obtain the total length of the tadpoles.

Relationships between brain volume, brain size, and cerebellum volume were evaluated with the General Linear Model (GLM). To control for size differences, we also registered tadpole's total length (mm). Total length was estimated from standard length (TL) and used as an independent variable. Ecomorphological guilds were used as a factorial variable. Analyzes were performed using the R program for statistical computing (version 3.5.2).

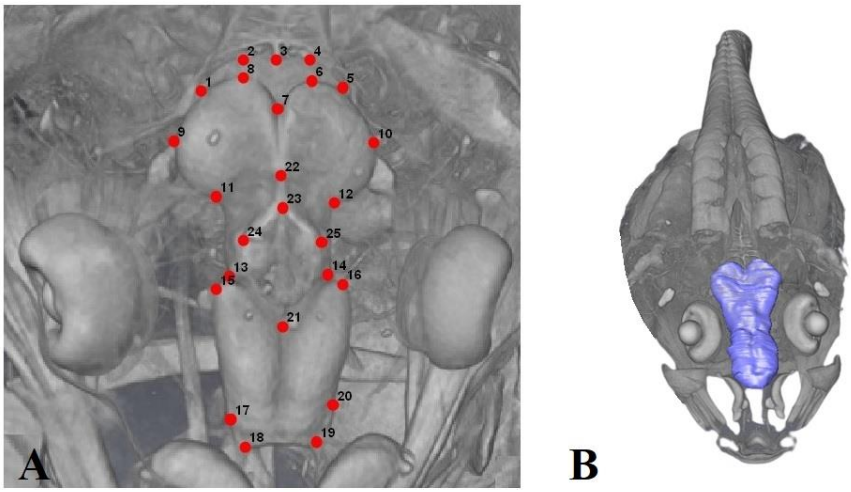

Figure 1A- Rendered image of the brain and cerebellum with the distribution of the 33 landmarks used (landmarks 1-8 represent the cerebellum). 1B- 3D model representing cerebrum and cerebellum.

3. RESULTS

3.1 Brains shape

The first three components of the PCA accounted for 64.01% of the total variation of the brain shape among species (PC1: 38.34%, PC2: 16.78%, PC3: 8.88%). Half of the shape variance of all analyzed individuals was represented by the first two dimensions of the shape space (Figure 2). We found that the three species have consistent morphological differences in brain shape, with larger intraspecific variation in *Physalaemus cuvieri*. Overall, tadpoles of *D. minutus* have more robust and wider brains, while *R. diptycha* have slender brains.

Considering the first axis (PC1), from the left to the right, the brain of the *P. cuvieri* showed an increase in the telencephalon length (associated to changes in three landmarks 14-5, and 18-19), a narrowing of the optic tectum and diencephalon, and an increase at the superior medial part of the optic tectum (associated to the landmark 7). *Dendropsophus minutus* brains had a widening at the coronal plane of the brain, a lateral increase of the cerebellum (landmarks 5 and 8) and a shorter diencephalon (landmark 21). *Rhinella diptycha* brains have a narrowing of the cerebellum (landmarks 2, 3 and 4) and a narrowing in the telencephalon (landmarks 17, 18, 19 and 20).

Along the second axis (PC2), we noted an increasing in the length of the cerebellum (landmarks 2, 3, 4 and 5), an increasing in the superior medial part of the optic tectum (represented by landmark 7), a shortening of the diencephalon (landmark 21), and a narrowing in the telencephalon in *P. cuvieri*. For *D. minutus*, we observed an asymmetrical decreasing, with the right side of the brain and the superior medial part of the optic tectum

with a larger decrease than the left side (landmark 7). We observed the same asymmetrical variation on the right side of the brain in *R. diptycha*, with a narrowing and a deepening in the telencephalon on the left side (landmark 21). Moreover, the shape variation among the brain of the species could be resumed by the first two canonical vectors, indicating from 44% to 55% of morphological differentiation.

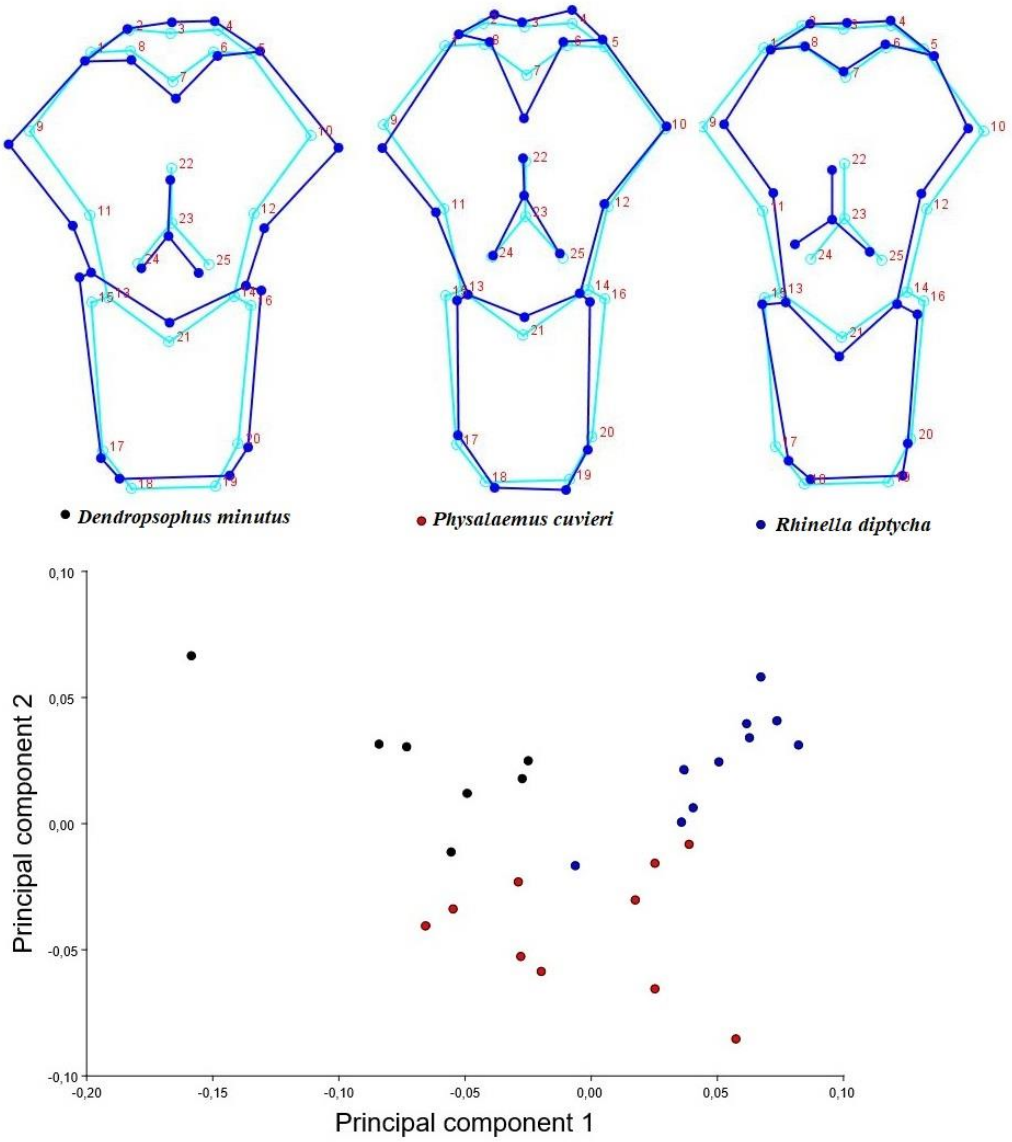

Figure 2. Principal component analysis (PCA). The figures show the wireframe visualization of the average shape for the species.

The CVA (Figure 3) presents well separated groups, with CV1 pointing to the shape of the cerebellum as an important feature in the differentiation between species, while CV2 indicates the width of the optic tectum as an important feature in the differentiation of species.

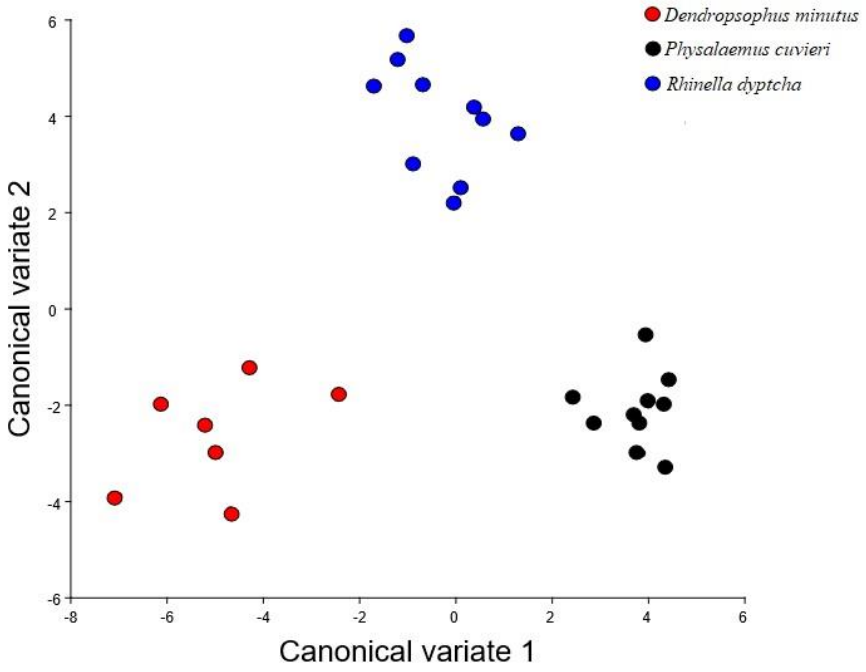

Figure 3. Canonical variates analyses (CVA).

The multivariate regression showed that the influence of size on the different traits evaluated was little noticeable for each species, with the shape variation showed a greater influence of the allometry only for *D. minutus.*

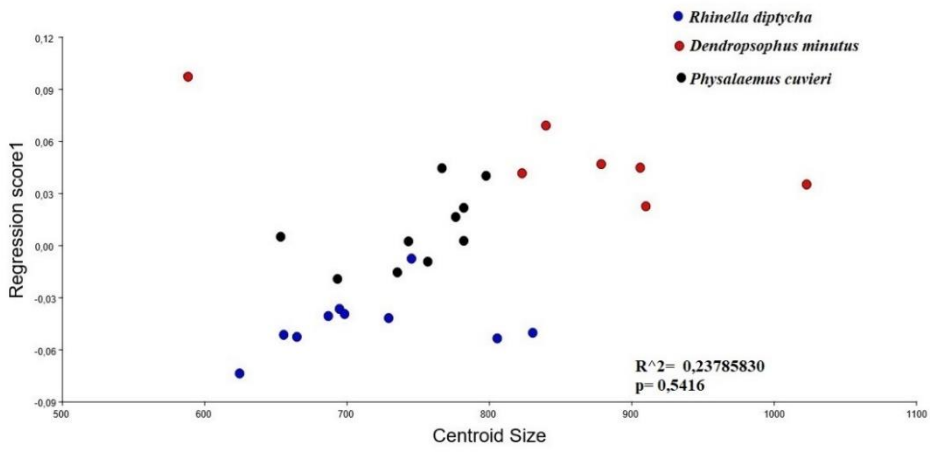

Figure 4. Multivariate regression between the sampled species.

3.2 Brain size variation

When we relate brain size and total length of the analyzed tadpoles, we observe that *D. minutus* have the larger brains, while *P. cuvieri* and *R. diptycha* have the smaller ones. Morevoer, size of the brain variation is not related to the total length of the tadpole among the species herein considered (Figure 5).

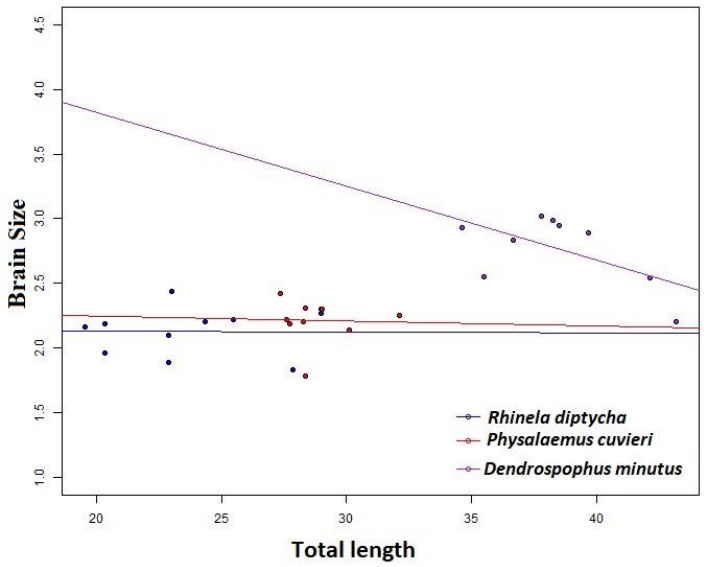

Figure 5. Relationship between brain size and total length.

Moreover when we analyzed the relationship between cerebellum volume and brain size, no effect of brain size on cerebellum volume is observed, indicating that cerebellum volume varies in function of guild, with nektonic tadpoles having a cerebellum of greater volume. The tadpoles of *R. diptycha* considered benthic/neusctonic present an intermediate cerebellum volume between the tadpoles of *D. minutus* and *P. cuvieri*. It is possible to notice that the individuals of *R. diptycha* still present a larger intraspecific variation in the volume of the cerebellum (Figure 6).

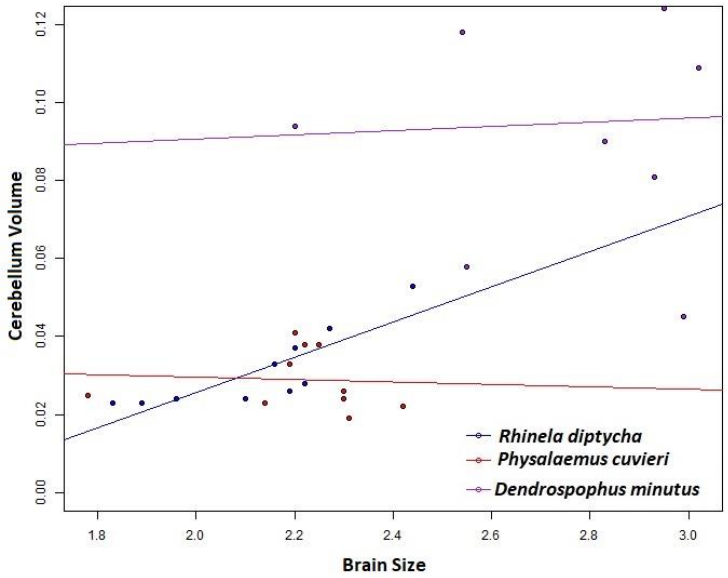

Figure 6. Relationship between cerebellum volume and brain size.

On the other hand, when analyzing brain volume, our data show an interaction between brain size and guild, with nektonic tadpoles of *D. minutus* having larger and more voluminous brain. For these tadpoles, brain volume increases with increasing brain size. For the other guilds there is no difference in the effect of brain size on the brain volume (Figure 7).

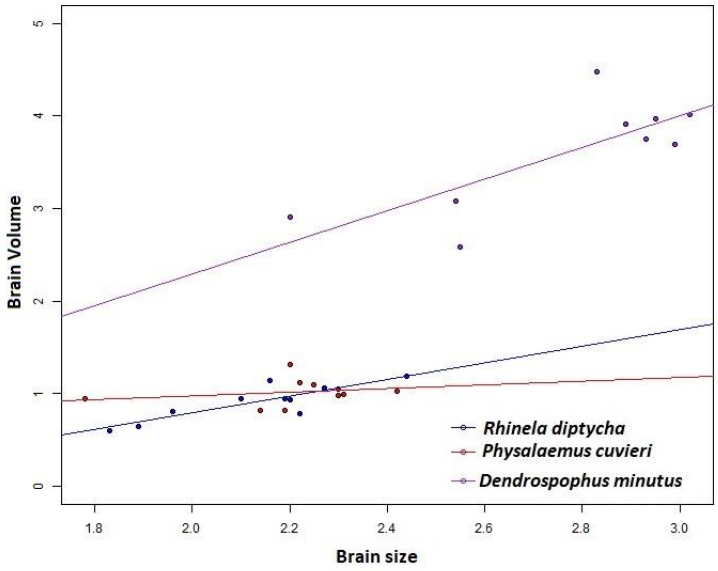

Figure 7. Relationship between brain volume and brain size.

5 DISCUSSION

Despite the long-standing interest in the evolution of the brain, relatively little is known about variation in brain anatomy in frogs. The morphogeometric analysis demonstrates that there is much variation both at intraspecific and species level, being interspecific variation greater than the intraspecific variation.

For tadpoles of the three studied species much of the intraspecific variation can be observed in the shape of the cerebellum (except in *R. diptycha*) and dimensions of the optic tectum. Specifically, brain architecture and behavior are strongly correlated (Burns & Rodd, 2008; Wilson & McLaughlin, 2010), and since behavior is plastic (e.g., Lima & Dill, 1990; Sousa et al., 2014), it is expected brain architecture to be also plastic.

The midbrain (optic tectum and motor tegument integrates first-order (primary) visual inputs with higher order inputs from the lateral line, auditory, and vestibular systems"

(Lannoo, 1999). Thus, the observed morphological variation may be associated with adaptations to different conditions, from visual detection of predators and refuge areas to balance and positioning in the water column. The cerebellum mediates motor learning and coordination. It receives “primary inputs from all of the sensory systems except the terminal nerve and olfaction, including vision, acousticolateral, gustatory, and general sensations, and sends motor commands to the muscles and glands of the head, cervical region, and viscera. The reticular formation of the brain stem is involved with integrating sensory inputs and with overall arousal” (Lannoo, 1999).

In our analyzes the cerebellum presented a large intraspecific variation in *D. minutus* and *P. cuvieri*, when compared to *R. diptycha*. This may indicate that behavioral variation (mainly to escape predators) may be lower in *R. diptycha*, since tadpoles of the species have a tegument with unpalatable substances. However, tadpoles of this species present a daily migration (they forage at the bottom during the day and on the surface, with the ventral/belly region up, at night) which could suggest a greater variation in the cerebellum, which was not confirmed by our analyses.

We also detected an asymmetry on the left - right side of the brain and in the superior part of the optic tectum, with a larger decrease in the left side in *D. minutus* and *P. cuvieri*, and in the right side of the brain in *R. diptycha*, with a narrowing on the left side and a deepening in the telencephalon (landmark 21). Proshchina & Savel’ev (1998) showed that in tadpoles of *R. temporaria* the right brain hemisphere had a larger volume of gray matter in in early stages developmental, but this asymmetry disappears between the stages 27–36. The asymmetry we observed can either common in the samples, restricted to some developmental stages (as found by Proshchina and Savel’ev, 1998) or even can be an artifact caused by the images. New studies with larger samples must be carried out to corroborate the asymmetry hypothesis.

It was also possible to observe that the brain morphology clearly differs among the analyzed species (as noted for the adult stage by Manzamo et al., 2017). The shape of the cerebellum, optic tectum and telecephalon is more complex and more variable than typically assumed. Ecomorphological guilds can explain the interspecific variation in brain shape. Nektonic tadpoles, which explore the water column, can have larger brain volume, as observed here to *D. minutus*. Tadpoles of the *D. minutus* have larger and more voluminous brain in addition to a cerebellum with greater volume, than the other two species. The most voluminous cerebellum in *D. minutus* may be associated with the utilization of the space in three dimensions, since the cerebellum is responsible for the functions in motor learning and coordination (Freeman, 1965; Lannoo, 1999). Structurally, brain size/volume is an important measure once it correlates with cognitive/behavioral abilities (Rushton & Ankney, 2009; Witelson et al., 2006), which are needed to displace in the water column, in a three dimension habitat. Benthic tadpoles of *P. cuvieri* are known to forage predominantly near to the bottom, present smaller and less variable cerebellum. These results corroborate our hypothesis that tadpoles of the nektonic guild have more developed nervous systems than benthic tadpoles. The tadpoles of *R. diptycha* considered benthic/neustonic present an intermediate condition between the tadpoles of *D.minutus* and *P.cuvieri* about these characteristics.

Other factors, like diet and the degree of predation risk, were important to predict the variation in total brain size and the size of different brain parts of tadpoles (Gonda et al., 2011; Trokovic et al., 2011). Trokovich et al. (2011) show that tadpoles grown at high density presented large optic tecta at metamorphosis, whereas tadpoles grown under predation risk presented small diencephalon, similar to our observations in unpalatable tadpoles of *R. diptycha* in comparison to the diencephalon size of palatable *D. minutus* and *P. cuvieri* tadpoles.

Manzamo et al. (2017) propose that brain morphology in the adult stage may be related to the locomotor mode. Acordance with our results, previous comparative studies of brain evolution revealed that interspecific differences in the relative volumes of brain parts across taxa are related to several ecological factors in primates (diet; Yopak et al., 2010), cichlids (feeding habis, turbidity and water depth; Huber et al., 1997), bats (habitat complexity; Safi & Dechmann, 2005), and in birds (migration and general latitude; West, 2014). Particularly, habitat complexity seems to be a good predictor of the size variation in forebrain and telencephalon in fishes (Huber et al., 1997; Pollen et al., 2007; Mai et al. 2016; Axelrod et al., 2018; Axelrod et al., 2020). In fact, Pollen et al. (2007) found that environmental and social factors differentially affect the brain, but environmental factors show a broader effect on a range of brain structures compared to social factors in cichlid fish. Again, this explains the larger and more voluminous brain and cerebellum of *D. minutus* tadpoles, which are nektonic and by using the habitat in three dimensions, are exposed to stimulus from all directions in space.

Moreover, our study brings new data about the debate on the relationship of brain parts to overall brain size and total size of tadpoles (e.g., Stephan et al., 1981; Lefebvre et al., 2004).

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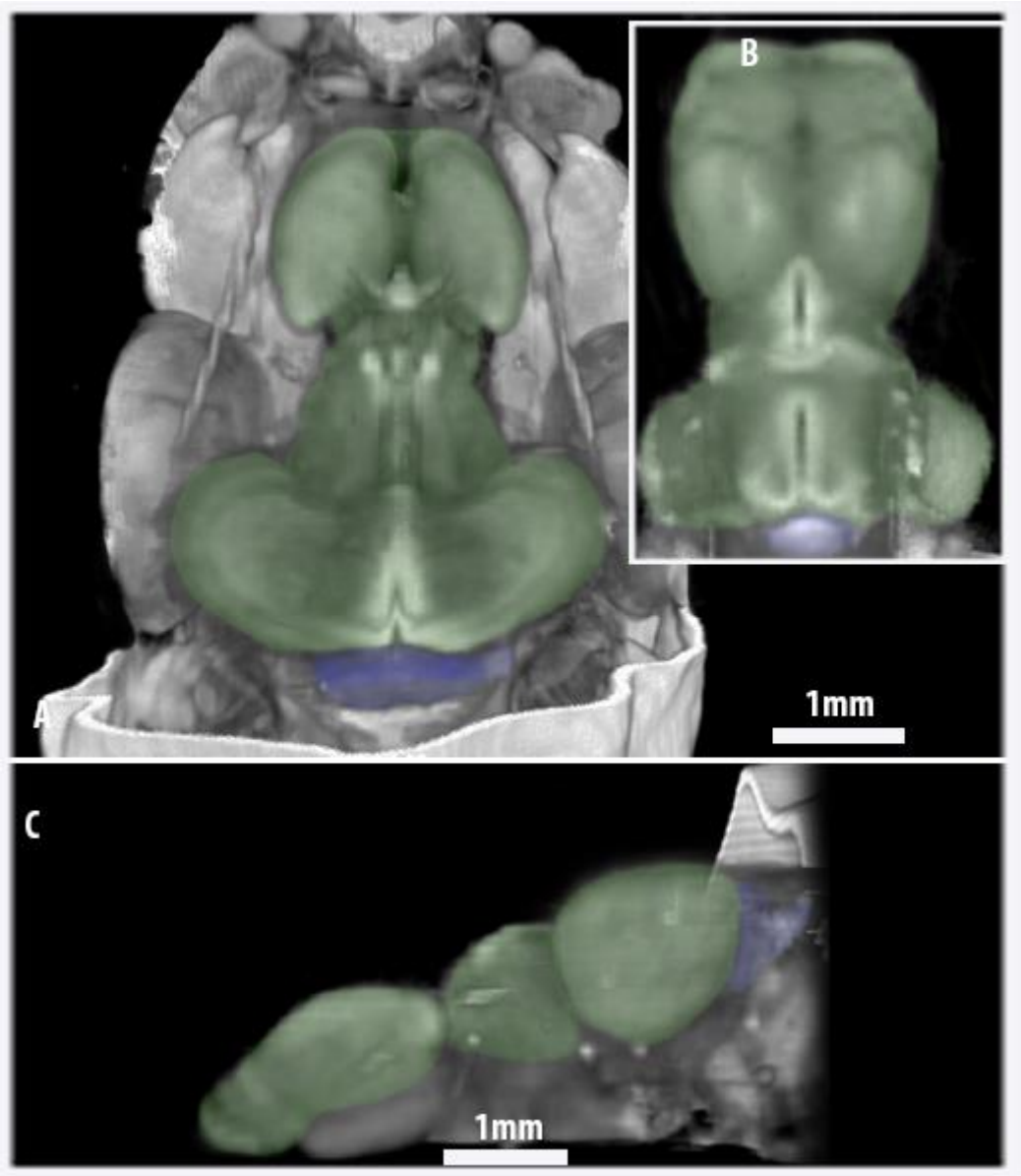
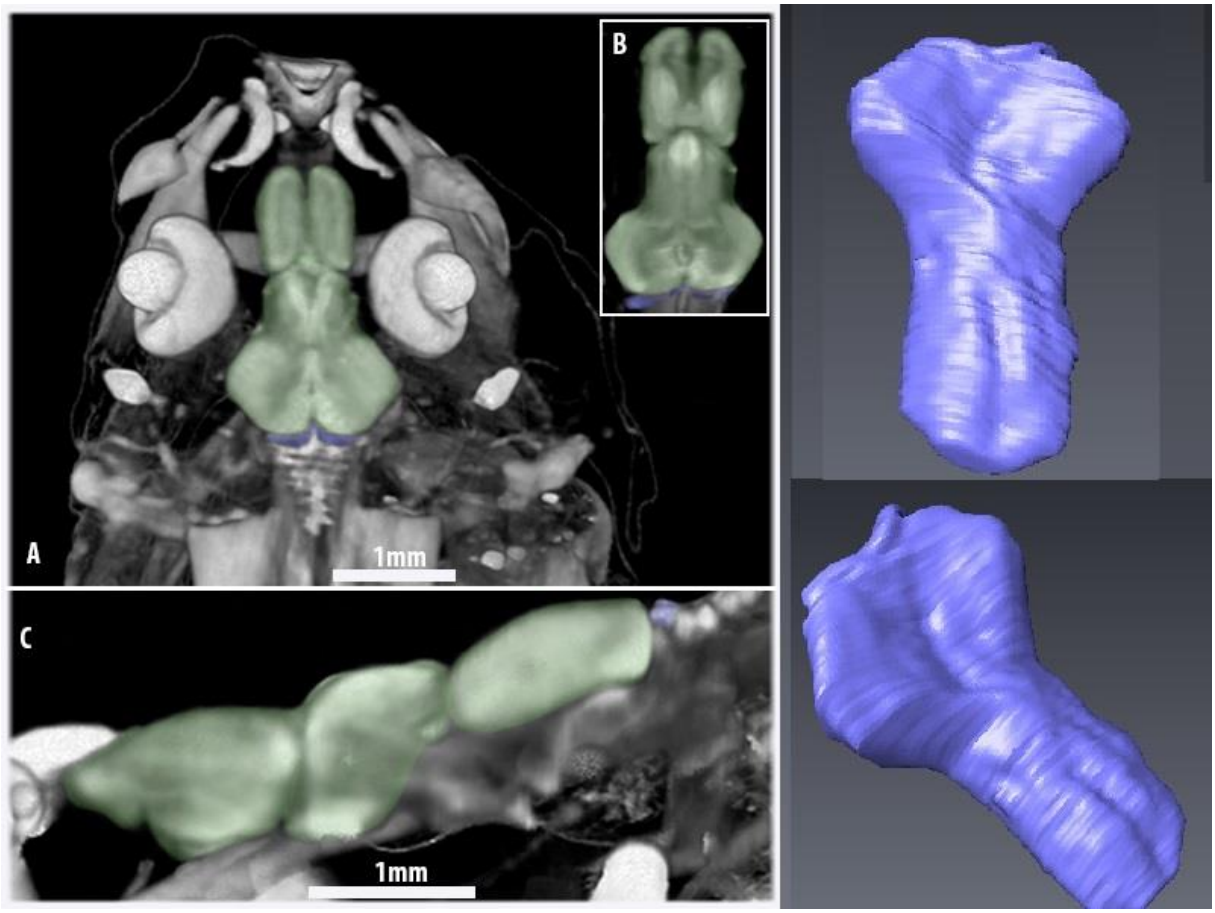
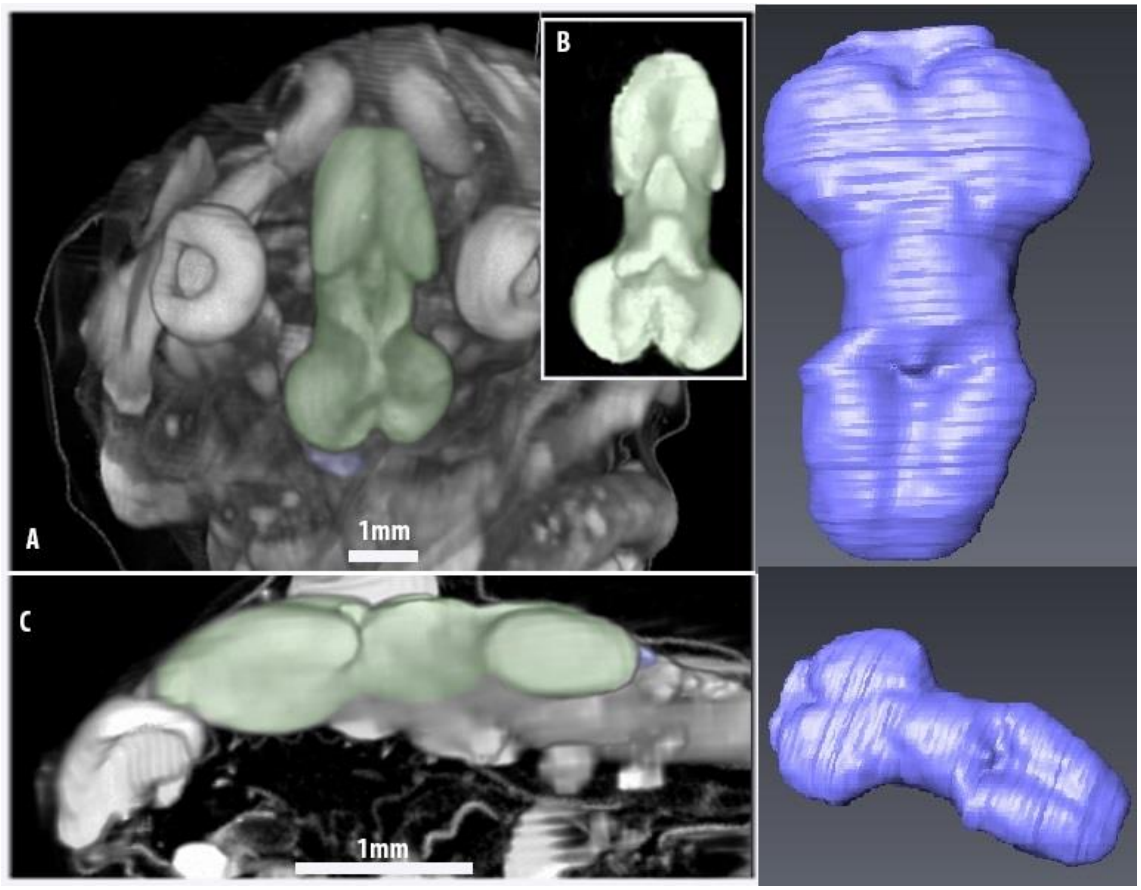
Anexo 1. 3D Micro-CT scan of an tadpole of *Dendropsophus minutus* in dorsal and lateral views.



Anexo 2. 3D Micro-CT and 3D volume scan of an tadpole of *Physalaemus cuvieri* in dorsal and lateral views.



Anexo 3. 3D Micro-CT and 3D volume scan of an tadpole of *Rhynella diptycha* in dorsal and lateral views.



7. CONCLUSÃO GERAL

A variação morfológica sempre despertou a atenção de pesquisadores de várias áreas da biologia. Sua compreensão e de sua estrutura espacial é de importância crucial para questões fundamentais em biologia evolutiva, e pode ajudar em questões sistemáticas e decisões de conservação. Por utilizarem uma ampla gama de habitats girinos apresentam uma ampla diversidade morfológica, o que pode ocasionar uma gama diferente de soluções adaptativas, sendo assim excelentes modelos para estudos morfológicos e de variação fenotípica. Entretanto existe uma carência em estudos envolvendo variação morfológica, suas origens e distribuição no espaço e no tempo utilizando girinos.

Em nosso primeiro capítulo, nossos dados mostram clara variação na morfologia externa entre as populações das espécies analisadas, cinco (*Boana albopunctata*, *Dendropsophus minutus*, *Physalaemus cuvieri*, *Scinax fuscovarius*, e *Trachycephalus typhonius*) delas associadas a distância geográfica. *Rhinella diptycha* foi a única espécie analisada que não apresentou correlação entre o grau de variação morfológica e a distância geográfica. Espécies amplamente distribuídas geralmente tendem a apresentar considerável variação dentro de suas áreas de distribuição, o que pode ter implicações na história taxonômica das espécies, dificultando a identificação correta das espécies e transformando variações intrapopulacionais em diferenças diagnósticas.

Algumas espécies como *Physalaemus cuvieri* apresentaram grande sobreposição entre as populações amostradas. Outras como *Trachycephalus typhonius* apresentaram segregações claras entre as populações.

Nosso segundo capítulo trouxe informações sobre a morfologia e a variação observada no cérebro e cerebelo das espécies *Dendropsophus minutus, Physalaemus*

cuvieri e *Rhinella diptycha*. Essa análise é inédita para girinos no Brasil. Com isso podemos observar diferenças morfológicas entre as espécies, demonstrando que o cérebro dos girinos não são todos iguais e, também, que apresentam variações intraespecificas. Nossos dados mostraram também que os girinos de *Dendropsophus minutus* apresentaram cérebros maiores que as outras espécies analisadas, e que o tamanho do cérebro não está correlacionada com o comprimento total dos indivíduos.

Não observamos relação entre o volume do cerebelo com o tamanho do cérebro, indicando que a variação nessa característica pode estar associada as guildas ecomorfológicas as quais as espécies pertencem. Novamente, girinos de *Dendropsophus minutus*, representantes da guilda nectônica, apresentaram maior volume do cerebelo.

Já quando analisamos o volume do cérebro observamos uma interação entre o tamanho do cérebro e as guildas ecomorfológicas, com o volume do cérebro de *Dendropsophus minutus* aumentando com o tamanho do cérebro. Entretanto essa relação foi observada apenas para essa espécie.

Outra observação interessante foi a indicação de assimetria no cérebro das espécies analisadas. Entretanto ressaltamos que novas análises precisam ser realizadas para corroborar essa observação.

Trouxemos aqui os primeiros esforços para uma melhor compreensão do sistema nervoso dos girinos brasileiros e sua relação com outras variáveis. Ressaltamos, entretanto, que novos estudos precisam ser realizados para um melhor conhecimento da diversidade morfológica, suas relações entre forma e função e associação com uso de ambiente e fatores ambientais.