Jessyca Michele Citadini

Coevolução do comportamento antipredatório, desempenho locomotor e morfologia de anuros da Floresta Atlântica.

Coevolution of antipredator behavior, locomotor performance and morphology of anurans of the Atlantic Forest.

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RESUMO GERAL

Anfíbios anuros representam um grupo de vertebrados cujo plano corpóreo apresenta uma série de modificações associadas ao desempenho locomotor através de saltos, sendo estas especializações muito antigas e conservadas filogeneticamente. Embora estudos comparativos venham demonstrando associações entre desempenho de salto e diversidade de habitat e história de vida para esse grupo filogenético, as relações entre o desempenho locomotor e o comportamento antipredatório dentro do contexto da diversificação do uso do micro-habitat permanecem inexploradas. Primeiramente, nós testamos modelos adaptativos de evolução morfológica associados com a diversidade do uso do micro-habitat (aquático, arborícola, fossorial, reofílico e terrestre) em espécies de anuros e foi examinada a relação da distância máxima do salto como uma função dos componentes das variáveis morfológicas e do uso do micro-habitat. Nós também investigamos, tanto em nível intra quanto interespecífico a influência da complexidade do microambiente (arena vazia, arena com folhiços ou arena com folhiço e arbustos) e do tipo de estímulo (aproximação *versus* toque) na manifestação do comportamento antipredatório em anuros. Adicionalmente, nós investigamos o efeito do uso de diferentes tipos de refúgio pelos anuros quando sujeitos aos testes de simulação predatória no laboratório. Nossos resultados demonstram a existência de múltiplos ótimos adaptativos para os comprimentos dos membros associados aos diferentes usos do micro-habitat, com uma tendência de aumento dos membros posteriores em espécies reofílicas, arborícolas e aquáticas quando comparadas com espécies terrestres e fossoriais, as quais evoluíram em direção ao ótimo adaptativo com membros posteriores mais curtos. Além disso, espécies reofílicas, arborícolas e aquáticas apresentaram maior desempenho para o salto e membros posteriores mais longos quando comparadas com

espécies terrestres e fossoriais. Em seguida foi abordada a influencia da complexidade do ambiente no comportamento antipredatório e nossas análises intraspecíficas mostraram que o número de respostas ativas (salto) e passivas é fortemente dependente do tipo de estímulo e varia de acordo com a complexidade estrutural do ambiente. Simultaneamente, nossas análises comparativas interespecíficas mostraram que anuros modulam a distância saltada em resposta ao toque do predador de acordo com a complexidade ambiental, e nós ressaltamos uma variação interespecífica associada ao uso de micro-habitat. Por fim, foi investigado o uso de refúgios pelos anuros expostos a testes de simulação predatória. Nossos resultados mostram que os anuros usam a vegetação arbustiva, o folhiço e a água como areas de refúgio quando sujeitadas a simulação predatória. Além disso, ocorreu variação interespecífica na escolha de refúgios potencialmente associados à diversificação do uso de microhábitat.

Palavras-chave: anfíbios, anuros, ecomorfologia, desempenho locomotor, comportamento antipredatório, complexidade do ambiente, métodos filogenéticos comparativos.

ABSTRACT

Anuran amphibians represent a group of vertebrates whose body plan presents a series of changes associated with jumping locomotor performance; these specializations are very old and phylogenetically conserved. Although comparative studies have shown associations among jumping performance, habitat diversity and life history for this phylogenetic group, the relationship between locomotor performance and antipredator behavior remains unexplored within the context of diversification of microhabitat use and habitat. First, we tested adaptive models of morphological evolution associated with the diversity of microhabitat use (aquatic, arboreal, fossorial, torrent and terrestrial) in species of anurans and examined the relation of the maximum distance jumped as a function of components of morphological variables and microhabitat use. We also investigated, both at intra and interspecific levels, the influence of the complexity of microenvironment (empty arena, with leaf litter or bushes) and stimulus type (approach versus touch) on the manifestation of antipredator behavior in anurans. In addition, we investigated the effect of the use of different types of refuges by anurans when subjected to simulated predator tests in the laboratory. Our results demonstrate the existence of multiple optima of limb lengths associated to different microhabitats, with a trend of increasing hindlimbs in torrent, arboreal, aquatic species whereas fossorial and terrestrial species evolve toward optima with shorter hindlimbs. Moreover, arboreal, aquatic and torrent anurans have higher jumping performance and longer hindlimbs, when compared to terrestrial and fossorial species. Then, was addressed the influence of the complexity of environment on antipredator behavior and our intraspecific analyzes showed that the number of active responses (jump) and passive responses is strongly dependent on stimulus type and varies according to the structural complexity of the

environment. At the same time, our interspecific comparative analyzes showed that anurans modulate the distance jumped in response to the predator's touch according to environmental complexity, and we highlight an interspecific variation associated with the use of microhabitat. Lastly, was investigated the use of refuges by anurans exposed to simulated predation events. Our results show that anurans use bushes, leaf litter and water as refuge areas when subjected to simulated predator. Moreover, there is interspecific variation in the choice of refuges potentially associated to diversification of microhabitat use.

Keywords: amphibian, anurans, ecomorphology, locomotor performance, antipredator behavior, complexity of environment, phylogenetic comparative method.

INTRODUÇÃO GERAL

O paradigma ecomorfológico: morfologia, desempenho e *fitness*

A fisiologia tem como proposta o estudo integrado de características biológicas em diferentes níveis de organização, promovendo a síntese do conhecimento das funções dos organismos (Schmidt-Nielsen, 1972). Dentre as subáreas da fisiologia, a fisiologia comparada se propõe a explorar a diversidade da complexidade funcional dentro do contexto de sua relevância ecológica e evolutiva, acrescentando diversos níveis de organização biológica a serem integrados (Prosser, 1991). Entretanto, o estudo da integração de características fenotípicas complexas e organizadas em diferentes níveis, sua história evolutiva e relevância ecológica representa um enorme desafio para as ciências biológicas (Arnold, 1989; Wagner & Altenberg, 1996; Schlichting, 2004).

Em 1983, Arnold sugeriu um arcabouço conceitual para auxiliar a organização e planejamento do estudo da organização fenotípica. Segundo este autor, a seleção atuaria diretamente sobre características de desempenho em nível de organismo (velocidade máxima de corrida, capacidade aeróbia, resistência ao jejum, produção de leite, entre outras), e que a variação individual nestas características seria explicada, por sua vez, pela variação em características morfológicas, fisiológicas e comportamentais subjacentes (comprimento das patas, composição de fibras musculares, motivação para atividade física, quantidade de tecido adiposo estocado, entre outras). Em 1994, Garland & Losos argumentaram que o comportamento deveria estar em um nível de organização diferenciado de outras características consideradas por Arnold (1983) como subjacentes ao desempenho. Segundo Garland & Losos (1994), o comportamento atuaria como um filtro entre a seleção e o desempenho, já que quando defrontado com qualquer desafio

ambiental, um animal pode optar entre diversos padrões motores que podem empregar uma gama variada de intensidades do desempenho máximo.

O papel do comportamento em estudos ecomorfológicos

De acordo com Garland (1994a), comportamento pode ser definido como o padrão motor expresso pelo indivíduo, face às diversas opções possíveis frente a um determinado contexto relevante para o valor adaptativo, tais como, sucesso nas disputas por obtenção de recursos energéticos, uso de diferentes padrões de defesa antipredatória, entre outras. O papel do comportamento no paradigma ecomorfológico não é simples (Garland & Losos, 1994, Huey et al., 2003). Populações ou espécies associadas à ocupação de diferentes habitats podem apresentar comportamentos distintos que, por sua vez, levam à seleção de fenótipos que maximizam a eficácia desses comportamentos nesses ambientes (Mayr, 1963). Características morfológicas e fisiológicas limitam o desempenho de um indivíduo, que por sua vez pode influenciar os comportamentos, sendo o comportamento o nível de organização biológica em que as pressões seletivas atuam diretamente, afetando em última instância o *fitness* ou valor adaptativo (Garland & Losos, 1994). Paralelamente, o desempenho locomotor pode representar um fator limitante para muitos padrões comportamentais expressos em diversos contextos ecologicamente relevantes, tais como, a obtenção de alimentos, o escape de predadores, a busca por parceiros reprodutivos e defesa de território (Dickinson et al., 2000). Entretanto, com algumas exceções (Martín & Lopez, 1995; Irschick & Losos, 1998; Van Damme, et al., 1998), os estudos sobre o desempenho locomotor têm-se concentrado nas capacidades funcionais dos animais, ignorando o contexto comportamental em que a locomoção é usada. Por exemplo, apesar de numerosos estudos abordarem as diferenças na capacidade máxima de corrida entre

espécies de lagartos (Garland & Losos, 1994; Miles 1994; Bauwens et al., 1995), pouca informação está disponível sobre quantas vezes, ou em que contextos comportamentais, os lagartos usam suas capacidades locomotoras na natureza (Irschick, 2000). Adicionalmente, Irschick & Losos (1998) investigaram como oito espécies de lagartos do gênero *Anolis* usam suas capacidades de corrida e de salto durante as atividades comportamentais (escapando de um predador, locomovendo em um ambiente sem perturbações e durante a alimentação). Atividades de corrida e salto foram utilizadas em diferentes intensidades durante os distintos comportamentos, e todas as espécies de lagartos variaram drasticamente tanto na capacidade de desempenho quanto na magnitude das respostas na natureza. No entanto, o estudo não examinou se há variação intraespecífica na forma como as capacidades locomotoras são utilizadas durante os diferentes comportamentos.

Estudos que relacionam morfologia, desempenho e diversidade ecológica são abundantes e têm concentrado em funções relevantes para o valor adaptativo, tais como, a locomoção, o evitar predadores e a alimentação (por exemplo, Losos, 1990, Sinervo & Losos, 1991, Herrel et al., 2005). De modo contrário, estudos ecomorfológicos que integram medidas de comportamento ainda são escassos na literatura (Gomes et al, 2002; López et al., 2005; Barros et al, 2016), embora o papel central do comportamento no paradigma ecomorfológico tenha sido reconhecido há muito tempo (Garland & Losos, 1994). Ao modificar o seu comportamento, os indivíduos podem ser capazes de melhorar a eficiência no uso de recursos. Em um contexto mais amplo, a relação entre comportamento e desempenho é crucial para a plena compreensão dos processos adaptativos (Santana & Dumont, 2009). Diante do apresentado, novos estudos comparativos devem contribuir para observar como as diferenças intra e interespecíficas de comportamentos poderiam estar associadas com as diferenças em morfologia,

capacidade de desempenho e/ou uso do habitat. Adicionalmente, soma-se ausência de estudos ecomorfológicos englobando o comportamento, principalmente para os anfíbios anuros da região Neotropical.

Anfíbios anuros como modelo para estudos em morfologia ecológica

Anfíbios anuros representam um grupo de vertebrados cujo plano corpóreo apresenta uma série de modificações associadas ao desempenho locomotor através de saltos, tais como membros pélvicos alongados, tíbia e fíbula fundidas, pelve poderosa fortemente ligada a uma coluna vertebral curta, formada por cinco a nove vértebras pré-sacrais reforçadas por zigapófises que restringem a flexão lateral, e vértebras mais caudais fundidas em um bastão sólido chamado uróstilo (Zug 1972; Emerson 1978). O uróstilo ajuda a enrijecer a parte inferior do dorso, sendo que essa rigidez é necessária para a transmissão eficaz da força produzida pelos membros posteriores para o corpo durante o salto (Storer et al, 2003). A cintura pélvica é uma estrutura estreita, rígida em forma de V, e na locomoção, transmite o impulso dos membros posteriores ao corpo. As especializações do corpo para o salto é a característica esquelética mais conspícua dos anuros (Storer et al, 2003). Essas especializações do plano corpóreo em anuros são muito antigas e conservadas filogeneticamente, estando presentes em seus aspectos mais básicos em fósseis do Jurássico (Shubin & Jenkins, 1995). Praticamente toda a força gerada para o salto é provida através do movimento simultâneo membros posteriores (Calow & Alexander, 1973; Marsh, 1994), enquanto os membros anteriores e a cintura peitoral absorvem o impacto da aterrissagem (Emerson, 1983). Dado que o trabalho mecânico exercido pela musculatura dos membros posteriores deve ser realizado ao longo do curto período em que os membros mantêm contato com o substrato, essa musculatura é capaz de exercer uma grande potência (Lutz & Rome, 1994). De fato, a

potência média de salto em muitas espécies equivale ou mesmo excede a potência máxima derivada da contração da musculatura dos membros posteriores, estimada a partir de medidas de contração isotônica, evidenciando também a contribuição de energia elástica estocada em fibras musculares e tendões (Marsh, 1994). Estudos têm associado à origem e manutenção das adaptações ao desempenho para o salto em anuros com pressões seletivas associadas à captura de alimentos e fuga de predadores (Gans & Parsons, 1966, Zug, 1972, Emerson, 1978, Gomes et al., 2009).

Apesar dos aspectos básicos constituintes do plano corpóreo dos anuros serem bastante conservados, o grupo apresenta grande diversidade na ocupação de micro-habitats, podendo ser encontrados na serrapilheira, dentro de cisternas de bromélias terrícolas e arborícolas, em câmaras subterrâneas, dentro de ocos e sob troncos de árvores, em fissuras e em cavidades de afloramentos rochosos, nas bordas de charcos, açudes, riachos e rios (Bertoluci & Rodrigues, 2002; Haddad et al., 2008). A diversidade com relação ao uso do micro-habitat encontra-se associada à evolução do desempenho locomotor e morfologia dos membros posteriores e da cintura pélvica. Através de amplos estudos comparativos realizados na década de 1970, Zug observou uma ampla diversidade no comprimento dos membros posteriores, claramente associada à variação em desempenho locomotor, e sugeriu uma relação com hábito. De acordo com estes estudos, anuros terrícolas e fossoriais tendiam a apresentar menor desempenho de salto e menor comprimento das patas traseiras quando comparados a anuros semi-aquáticos e arbóreos (Zug, 1972; 1978). Posteriormente, Gomes e colaboradores (2009) reanalisaram os dados de Zug (1978) através de métodos estatísticos corrigidos para as relações filogenéticas e confirmaram que espécies fossoriais apresentam menor desempenho de salto e membros posteriores mais curtos que as outras espécies. Esses resultados sugerem a existência de um compromisso entre

eficiência de escavação e desempenho de salto, resultando em especializações morfológicas (Gomes et al., 2009).

A locomoção está também estritamente ligada aos diversos fatores ambientais, tanto bióticos como abióticos que compõem o ecossistema no qual o indivíduo está inserido (Bennett & Licht, 1974; Bennett, 1985; Navas, 1996, Gomes et al., 2009, Titon Jr et al., 2010). Fatores extrínsecos, tais como a temperatura e o nível de hidratação, afetam o desempenho locomotor em diferentes linhagens de tetrápodes ectotérmicos, incluindo desempenho de salto em anuros (Rome et al., 1992; Gatten, 1987, Navas et al., 1999, Gomes et al., 2002). Adicionalmente, padrões de variação interespecífica da sensibilidade do salto a essas variáveis ambientais são condizentes com a hipótese de evolução adaptativa do desempenho de salto em anuros (John-Alder, et al., 1989; Navas, 1996, Wilson, 2001; Titon et al., 2010, Titon & Gomes, 2016). Entretanto, anfíbios anuros apresentam dezenas de padrões de comportamento antipredatório alternativos à fuga através de saltos, que são utilizadas em contextos comportamentais variados (Williams et al., 2000, Toledo, 2005; Toledo et al., 2010, 2011). É particularmente interessante observar que padrões motores antipredatórios mais passivos (tanatose e imobilidade, por exemplo), são mais frequentes quando as temperaturas corpóreas dos animais são mais baixas, ao passo que a fuga por meio dos saltos é mais frequentemente empregada a temperaturas mais altas (Gomes et al., 2002). Tais observações apontam para a evolução adaptativa da expressão de padrões motores antipredatórios, associada às restrições impostas pelas baixas temperaturas sobre o desempenho locomotor (Gomes et al., 2002).

Dado que variáveis ambientais também afetam o risco de detecção da presa por parte do predador, modelos teóricos e evidências empíricas sugerem que as presas não

fogem imediatamente após a detecção por parte de um predador, mas ajustam sua resposta de escape minimizando os custos de fuga (Ydenberg & Dill, 1986, Martín et al., 2005). Desta forma, um fator extrínseco adicional importante para a tomada de decisão do padrão motor antipredatório expresso, bem como da intensidade do salto empregado em relação ao desempenho máximo, seria a complexidade do microambiente ocupado pelo indivíduo. Em *Rana perezi*, tanto a distância de aproximação permitida ao predador, quanto à distância saltada em resposta à aproximação, são positivamente correlacionadas com a distância entre o indivíduo e o corpo de água mais próximo (Martín et al., 2005). A presença de cobertura vegetal na margem também reduziu a distância de aproximação permitida ao predador em *R. perezi*, principalmente em indivíduos maiores (Martín et al., 2005). Gomes e colaboradores (2002) observaram, para a perereca *Scinax hiemalis*, saltos muito maiores em resposta à simulação de predação em laboratório, com os indivíduos testados no chão aberto, que em seu ambiente natural.

Como descrito até então, uma série de estudos comparativos vêm sendo realizados nas últimas décadas, dedicados à evolução do desempenho de salto em anfíbios anuros, suas correlações com habitat e história de vida, bem como de seus fundamentos morfológicos e fisiológicos (Gans & Parsons, 1966, Zug, 1972, Emerson, 1978, Marsh, 1994, Gomes et al., 2009, Moen et al, 2013, Vidal-Garcia et al, 2014). Entretanto, as relações entre o desempenho locomotor e o comportamento antipredatório dentro do contexto da diversificação de habitats e uso do micro-habitat permanecem inexploradas para este grupo filogenético.

CAPÍTULO I

1. CAPÍTULO 1: Evolution of morphology and locomotor performance in anurans: Relationships with microhabitat diversification

**Evolution of morphology and locomotor performance in anurans:
Relationships with microhabitat diversification.**

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Abstract

The relations between morphology, performance, behavior and ecology provide evidence for multiple and complex phenotypic adaptations. The anuran body plan, for example, is evolutionarily conserved and shows clear specializations to jumping performance back at least to the early Jurassic. However, there are instances of more recent adaptation to habit diversity in the post-cranial skeleton, including relative limb length. The present study tested adaptive models of morphological evolution in anurans associated with the diversity of microhabitat use (aquatic, arboreal, fossorial, torrent, and terrestrial) in species of anuran amphibians from Brazil and Australia. We use phylogenetic comparative methods to determine which evolutionary models, including

Brownian motion (BM) and Ornstein-Uhlenbeck (OU) are consistent with morphological variation observed across anuran species. Furthermore, this study investigated the relationship of maximum distance jumped as a function of components of morphological variables and microhabitat use. We found there are multiple optima of limb lengths associated to different microhabitats with a trend of increasing hindlimbs in torrent, arboreal, aquatic, whereas fossorial and terrestrial species evolve toward optima with shorter hindlimbs. Moreover, arboreal, aquatic and torrent anurans have higher jumping performance and longer hindlimbs, when compared to terrestrial and fossorial species. We corroborate the hypothesis that evolutionary modifications of overall limb morphology have been important in the diversification of locomotor performance along the anuran phylogeny. Such evolutionary changes converged in different phylogenetic groups adapted to similar microhabitat use in two different zoogeographical regions.

Keywords: amphibian, frogs, ecomorphology, phylogenetic comparative method, jumping performance

1.1. Introduction

The relations between morphology, behavior and ecology have been studied in several phylogenetic groups in order to understand how different selective pressures have molded the evolution of morphological patterns (Irshchick and Losos, 1999). Locomotion and its morphological correlates have received a great deal of attention (Zug, 1972, 1978; Emerson, 1979; Losos, 1990b; Gomes et al., 2009; Jorgensen and

Reilly, 2013; Moen et al, 2013) because many ecologically relevant activities, such as escaping from predators, foraging and searching for reproductive partners, depend on locomotion (Meylan and Clobert, 2004; Husak et al., 2006). Therefore, locomotor performance has been widely regarded as probably associated with fitness (Christian and Tracy, 1981; Arnold, 1983; Garland, 1999).

An interesting group to address the relations between morphological specializations and locomotor performance are the Anurans. Anuran body shape presents specializations associated with jumping performance that are old and phylogenetically conserved, being present in their most basic aspects in fossils from the Jurassic period (Shubin and Jenkins, 1995). The origin and maintenance of adaptations to jumping performance in anuran amphibians are probably related to selective pressures associated with prey capture and escape from predators (Gans and Parsons, 1966; Zug, 1972; Emerson, 1978; Gomes et al., 2002). Despite the constituent basic aspects of body shape in anurans being highly conserved, this group displays a wide variety of microhabitat occupation, including leaf litter, underground chambers, edges of ponds, reservoirs, streams and rivers, inside phytotelmata such as bromeliads, and on bush vegetation and tree branches (Bertoluci and Rodrigues, 2002; Haddad et al., 2008).

The diversity in microhabitat use is linked to the evolution of locomotor performance and hindlimb morphology. Terrestrial and fossorial species tend to show lower jumping performance and shorter hindlimbs when compared with aquatic and arboreal species (Zug, 1972; 1978; Gomes et al., 2009, Enriquez-Urzelai et al., 2015). Also distinctive morphology and performance is associated with the microhabitat diversity, regardless of clade position or geographic precedence (Moen et al. 2013). Despite ecomorphological comparative investigations on anurans have been conducted

on large phylogenetic scales (Zug, 1972, 1978, Emerson, 1979, Marsh, 1994, Gomes et al, 2009, Moen et al, 2013; Enriquez-Urzelai et al, 2015), studies that incorporate measurements of locomotor performance are still scarce in the literature (Zug, 1972, 1978; Gomes et al., 2009). It is also noteworthy that these studies have been particularly biased in terms of zoogeographical areas, with poor representation of Neotropical and Ethiopic fauna, for example. Furthermore, the relations between morphology, locomotion performance and microhabitat use are rarely considered into an integrated evolutionary context.

Here, we use phylogenetic comparative methods to determine which evolutionary models, including Brownian motion and Ornstein-Uhlenbeck, are consistent with morphological variation observed across anuran species from Brazilian and Australian faunas. Specifically, we hypothesize that current variation in morphology across anuran species is consistent with stabilizing selection related to distinct phenotypic optima in different categories of microhabitat use. We predict that fossorial and terrestrial species have shorter hindlimbs than aquatic, arboreal and torrent species. To test this hypothesis, we compared the fit of species' morphology estimates to models that approximate evolution according to either Brownian motion or Ornstein-Uhlenbeck (OU) processes. Better fit of Brownian motion models would indicate that contemporary variation in morphology is consistent with the absence of consistent selection associated to microhabitat use. Alternatively, better fit of OU models would incorporate selection pressures that lead the adaptations towards different evolutionary optima across selective regimes (e.g. microhabitat use). Furthermore, we hypothesize that interspecific variation in anuran locomotor performance is associated with variation in morphology and microhabitat use. Particularly, we predict that fossorial and

terrestrial species have shorter hindlimbs and lower maximum jumping distance when compared to aquatic, arboreal and torrent species. Accordingly, we employed phylogenetic regressions to investigate the relationship of maximum distance jumped as a function of components of morphological variables and microhabitat use.

1.2. Materials and methods

1.2.1. Morphological data

1.2.1.1. Species, measurements in museum collections

This study was conducted using 64 anuran species (44 species from Brazil and 20 species from Australia). The Brazilian and Australian measured specimens were available, respectively, at the Museum of Zoology from the University of São Paulo (MZUSP) and the South Australian Museum in Adelaide (SAM). The choice of species was carried out in order to cover the maximum morphological diversity and occupation of structural niches within each phylogenetic group. With this sampling approach we intended to obtain the maximum number of evolutionary transitions along the phylogenetic tree, increasing the power to detect patterns of morphological evolution. We also considered the specimens availability in the museum collections. The number of individuals measured from each species ranged from three to 10 and all measurements were performed by JMC using digital calipers to the nearest 0.01 mm. Only adult males were measured, and sex was identified by external morphological cues, such as presence of vocal sacs, inguinal spots, nuptial pads, spines on fingers, darkened throats and differences in color generally present in males. In the absence of

external cues, sex determination occurred through the presence of vocal slits in adult males (Pombal Jr. & Izecksohn, 2011).

1.2.1.2. Morphological variables

Eight morphometric traits were obtained for each individual: humerus length (HUM, measured from tip of elbow to posterior insertion point of forelimb at the body wall); radioulnar length (RAD, measured from distal edge of outer palmar tubercle to tip of elbow); hand length (HAND, measured from distal edge of outer palmar tubercle to distal tip of third finger); femur length (FEM, measured from posterior tip of ischium to knee); tibiofibular length (TBFB, measured from tip of knee to tip of heel / proximal end of the tarsus); tarsus length (TAR, measured from tip of heel to proximal edge of inner metatarsal tubercle); foot length (FOOT, measured from proximal edge of inner metatarsal tubercle to tip of outstretched fourth toe); and the snout-to-vent length (SVL, measured from proximal edge of inner metatarsal tubercle to tip of outstretched fourth toe tip of snout to posterior end of the ischium). The same measurements were performed in animals used in the performance tests, and they were all included in the analysis.

1.2.1.3. Phylogenetic tree

A composite phylogenetic tree with 64 species was compiled (Figure 1-1), mainly based on topology and divergence times proposed by Pyron (2014), which is the most comprehensive current phylogenetic hypothesis for anurans. Phylogenetic information was available for 31 preserved Brazilian species and for all 20 preserved Australian

species in Pyron (2014). Some adjustments were necessary to include other 13 Brazilian species, as follows: For inclusion of *Rhinella ornata*, a species from the *R. crucifer* group of species (Baldissera et al., 2004), the topological position and divergence time for *Rhinella crucifer* in Pyron (2014) was assumed. For *Melanophryniscus moreirae*, the divergence time for the genus *Melanophryniscus* and the other clade formed by species of the family Bufonidae in Pyron (2014) was assumed. *Scinax rizibilis* were inserted in the topology at the base of the cladistic divergence between the clades *S. catharinae* and *S. ruber* according to Faivovich (2002). *Pseudis platensis* was inserted diverging from *P. paradoxa* and considering the divergence time for the genus *Pseudis* (Pyron, 2014). *Hylodes asper* was inserted diverging from *H. phyllodes* and considering the divergence time for the genus *Hylodes* (Pyron, 2014). *Physalaemus olfersii* was inserted diverging from *P. cuvieri* and considering the divergence time for the genus *Physalaemus* (Pyron, 2014). To include *Leptodactylus fuscus*, the topological position of *L. longirostris* was assumed (Pyron, 2014), given that *Leptodactylus fuscus* is a sister species of *L. longirostris* according by Pyron & Wiens (2011). To include *Leptodactylus latrans*, the topological position of *L. leptodactyloides* was assumed (Pyron, 2014), given that *Leptodactylus latrans* (former *L. ocellatus*) is a sister group of *L. leptodactyloides* by Pyron & Wiens (2011). *Chiasmocleis leucosticta* and *C. carvalhoi* were inserted in the phylogeny considering the maximum time of divergence of species from this genus in Pyron (2014). *Elachistocleis ovalis* was inserted in place of *E. bicolor* (Pyron, 2014). *Odontophrynus americanus* was inserted in the phylogeny occupying the topological position of *O. cultripes*, given that they are considered sister groups by Pyron and Wiens (2011). Given that information about the topological position and divergence time is unavailable for *Cycloramphus dubius*, this species was

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depicted in a polytomy at the base of the *Cycloramphus* clade (Pyron, 2014). The systematic nomenclature followed Frost (2015).

Another phylogenetic tree with 19 species for locomotor performance data was compiled (Figure 1-2), based on topology and divergence times proposed by Pyron (2014). Phylogenetic information was available for all Brazilian and Australian species in Pyron (2014).

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Figure 1-1: Composite phylogenetic tree for the 64 anuran species from Brazil and Australia included in the present morphological study, with topology and divergence times estimates (Mya) based on the literature. Each selective regime is represented by a different color: aquatic= red, arboreal= blue, fossorial= green, terrestrial= yellow, torrent= black.

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Figure 1-2: Phylogenetic tree for the 19 anuran species from Brazil and Australia included in the present locomotor performance study, with topology and divergence times estimates (Mya) based on the literature.

1.2.1.4. Species and collection localities for the study of locomotor performance

Males from 19 species of anurans were collected in several localities from Brazil (15 species) and Australia (four species) and are represented in Table 1-1.

Table 1-1. Species, localities and collect dates.

Species	Localities	Dates
Brazil		
<i>Leptodactylus labyrinthicus</i> (N= 5)	Luiz Antônio, SP (21°33'05"S; 47°39'16"W)	November 2013
<i>Hypsiboas faber</i> (N= 9)	Luiz Antônio, SP (21°33'05"S; 47°39'16"W)	November 2013
<i>Physalaemus cuvieri</i> (N= 3)	Luiz Antônio, SP (21°33'05"S; 47°39'16"W)	November 2013
<i>Physalaemus cuvieri</i> (N= 8)	Estação Biológica de Boracéia, Salesópolis, SP (23°39'13"S; 45°53'22"W)	January 2014
<i>Physalaemus olfersii</i> (N= 5)	Botanical Garden of São Paulo, São Paulo, SP (23°32'51" S; 46°38'10" W)	February 2014
<i>Aplastodiscus leucopygius</i> (N= 8)	Botanical Garden of São Paulo, São Paulo, SP (23°32'51" S; 46°38'10" W)	February 2014
<i>Scinax crospedospillus</i> (N= 8)	Botanical Garden of São Paulo, São Paulo, SP (23°32'51" S; 46°38'10" W)	February 2014
<i>Thoropa taophora</i> (N= 6)	Ubatuba, SP (23°26'02"S; 45°04'16"W)	March 2014
<i>Proceratophrys boiei</i> (N=5)	Parque Estadual Intervales, Ribeirão Grande, SP (24°16'24.55"S; 48°25'2.27"W)	October 2015
<i>Leptodactylus fuscus</i> (N=5)	University of São Paulo, São Paulo, SP (23°33'51"S; 46°43'48"W)	November 2015
<i>Leptodactylus podicipinus</i> (N= 6)	Selvíria, MS (20°22'02" S; 51°25'08" W)	November 2015
<i>Dermatonotes muelleri</i> (N= 5)	Selvíria, MS (20°22'02" S; 51°25'08" W)	November 2015
<i>Pseudis platensis</i> (N=8),	Selvíria, MS (20°22'02" S; 51°25'08" W)	November 2015
<i>Rhinella schneideri</i> (N=3)	Selvíria, MS (20°22'02" S; 51°25'08" W)	November 2015
<i>Hylodes asper</i> (N=5)	Ubatuba, SP (23°26'02"S; 45°04'16"W)	December 2015.
<i>Cycloramphus boraceiensis</i> (N=6)	Ubatuba, SP (23°26'02"S; 45°04'16"W)	December 2015.
Australia		
<i>Litoria ewingii</i> (N=6)	Adelaide, SA (34°55'43"S; 138°35'55"E)	November 2014
<i>Crinia signifera</i> (N=8)	Adelaide, SA (34°55'43"S; 138°35'55"E)	November 2014
<i>Limnodynastes tasmaniensis</i> (N=9)	Adelaide, SA (34°55'43"S; 138°35'55"E)	December 2014
<i>Litoria caerulea</i> (n=4)	Flinders Ranges, SA (32°28'22"S; 137°59'25"E)	January 2015

1.2.1.5. Animal maintenance in the laboratory

Brazilian species were placed in plastic boxes and transported to the laboratory in the University of São Paulo (Brazil), where they were housed individually, in terraria of 13 x 30 x 28 cm for the smaller species and in terraria of 27 x 45 x 32 cm for species larger than 80 mm SVL. Each terrarium contained fragments of vegetation, some stones and fresh water permanently available, and animals were fed weekly with small live cockroaches. The terraria were maintained in a climate-controlled room at 25°C ($\pm 1^\circ\text{C}$) and 12h: 12h dark: light cycle, with the darkness starting at 7:40 pm. The collections were performed under approved permission of Ministério do Meio Ambiente, ICMBio, SISBio (License number: 49250-1) and laboratory procedures were performed under the approval of the Comissão de Ética no Uso de Animais (CEUA), Instituto de Biociências da Universidade de São Paulo (Protocol number: 192/2013). Field work at Parque Estadual Intervales was conducted under authorization of the “Coordenadoria de informações técnicas, documentação e pesquisa ambiental” (COTEC, process number 611/2015), Instituto Florestal, Secretaria do Meio Ambiente. For the other localities, no specific authorizations were required.

Australian species were placed in cloth bags and transported to the laboratory to the University of South Australia (Australia), where they were housed individually, in terraria of 13 x 13 x 16 cm for the small species (*L. ewingii*, *L. tasmaniensis* and *C. signifera*) and in terraria of 28 x 19 x 36 cm for the larger species (*L. caerulea*). Each terrarium contained a sandy substrate, some stones and fragments of vegetation, fresh water permanently available, and animals were fed weekly with small live crickets. The

terraria were maintained in a climate-controlled room at 25°C ($\pm$ 1°C) and 12:12 dark:light cycle, with the darkness starting at 12 midnight, this adjustment was done in order to collect the data during the day, and the individuals were acclimatized to these conditions for over 1 week prior to the experiments. The inverse light-dark cycle had no discernible effects on any measure of behavior or physiological variables. Animals were collected, housed and studied under a permit from the University of South Australia Animal Ethics Committee (protocol n° 33636). For these localities, no specific authorizations were required. Although terrariums had different dimensions in Brazil and Australia, they were sufficient to allocate the frogs comfortably in both locations. In the same way, frogs were fed on cockroaches and crickets in Brazil and Australia, respectively, but the insects were equally consumed and frogs maintained body condition equally well in both locations. In this way, we are confident that the differences in maintenance of frogs between locations did not affect the results.

1.2.1.6. Locomotor Performance tests in the laboratory

Individuals were maintained in plastic containers in a climate-controlled room at 25°C for one hour before locomotor tests. Locomotor tests were performed on the floor (120 cm width and 285 cm length) at constant temperature room (25°C). The animals were stimulated to jump on a substrate with a good grip (Ethylene Vinyl Acetate sheets (EVA) by tapping gently in the posterior region of the individuals for six consecutive times. Starting and landing points were marked on the floor, and later, the distance between marks was measured. The longest jump of the series was used as the best estimate of maximum jumping performance. To conduct the data collection, the

observer used a headlamp with red light (500 Lumens), and data collection was performed within two weeks of the animals being captured. All data were collected during darkness, when frogs are fully active. In the laboratory this condition was between 08:00 pm and 00:00 am in Brazil and between 08:00 am and 12:00 am in Australia.

1.2.1.7. Comparative Analysis

Seven morphometric variables, based on forelimb and hindlimb, were used in the statistical analyses: humerus length, radioulnar length, hand length, femur length, tibiofibular length, tarsus length, foot length. All mean values were log₁₀-transformed and we performed descriptive statistics for all morphological variables per species. We regressed phylogenetically all variables against body size and used the residuals as size free variables (Revell, 2009). The size free variables were summarized with a phylogenetic Principal Component Analyses (pPCA) in which components with eigenvalues larger than 1.0 were saved for posterior analyses (Revell, 2009). We considered any absolute values higher than 0.5 as a high load. We also used phylogenetic regression to investigate the relationship between the maximum jumping distance and body size (Revell, 2009), and saved the residuals for posterior analyses. To test the hypothesis that interspecific variation in anuran locomotor performance is associated with morphological variation and microhabitat use, we implemented multiple phylogenetic linear models with the residuals of locomotor performance on body size (size free jump performance) as the dependent variable, the scores from morphological components of morphological variables as independent variables, and microhabitat use as a factor. We fitted Phylogenetic Generalized Least Squares (PGLS) and performed

model selection by sequentially removing variables explaining the least variation on each model tested (Purvis et al. [2000] and Stuart-Fox & Ord [2004]). We always started by fitting a model that included the interaction between the scores from morphological components and microhabitat use. We always considered these interactions as our first removal from the models if not significant (Engqvist, 2005). All variables included in the best-fitting models had a significance level of $P < 0.05$ via partial-F tests. We reported results from all models tested together with Akaike Information Criterion corrected for small sample size (AICc), and considered as the best-fit model the one showing higher AICc weight, according to Burnham & Anderson (2002). We conducted procedures for evolutionary models and PGLS with the software R version 3.0.2. Evolutionary analyses of morphology were conducted within a phylogenetic context using Brownian motion (BM) and Hansen's (1997) multiple optimum Ornstein-Uhlenbeck (OU) models (more information in Martins & Hansen 1997; Butler & King 2004). We used BM model here to represent stochastic evolution with no assumptions about adaptation with respect to the selective pressures associated with microhabitat use. The adaptive models of evolution were constructed by assigning adaptive regimes to the terminal branches of the phylogeny based on independent ecological data or descriptions for the extant species (Table 1-1) with ancestral regimes reconstructed based on linear parsimony using Mesquite version 2.75 (build 564) (Maddison & Maddison, 2011). Our adaptive model is based on microhabitat use and contains five optima: arboreal, aquatic, terrestrial, fossorial and torrent. We considered that (1) aquatic species live predominantly in or near water; (2) arboreal species live above the ground on vegetation; (3) burrowing species live predominantly amid leaf litter or in burrows; (4) terrestrial species live on the ground; and (5) torrent species live

closely associated with rocky, fast-flowing streams. We assigned species into habitat regimes (Figure 1-1) based on published descriptions of microhabitat use (Haddad et al. 2008; Tyler and Walker 2011) and personal observations from specialists: Prof. Miguel T. Rodrigues (University of São Paulo) and Prof. Michael Tyler (The University of Adelaide).

We used OUwie software package (Beaulieu *et al.* 2012) in the R statistical computing environment (R Development Core Team 2010) to fit each morphological aspect considered and the selective regime into an evolutionary model, assuming our phylogeny. For this, we used four evolutionary models: 1) BM1= single-rate Brownian motion; 2) BMS= Brownian motion with different rate parameters for each state on a tree; 3) OU1= Ornstein-Uhlenbeck model with a single optimum for all species and 4) OUM= Ornstein-Uhlenbeck model with different optimum associated to distinct selective regimes. The OUwie package offers a set of unique models allowing the trait optimum, rate of stochastic motion and strength of selection to vary across selective regimes. We compared the fits of each model using the Akaike information criterion corrected for small sample size (AICc, Burnham and Anderson 2002; Butler and King 2004). Best models present lowest AICc with differences larger than 2.

1.3. Results

1.3.1. Relations between morphological variables and microhabitat use

Descriptive statistics of morphological variables from each species are presented in Table 1-2 as the mean $\pm$ standard deviation. The phylogenetic Principal Component

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Analysis performed on morphometric traits retained two components. Component 1 of the morphological data set for 64 species (PC1 64) explained 57.75% of the total variance and was related to a positive association between measurements taken from hindlimb (femur, tibiofibular, tarsus and foot) and hand, which are the largest contributors, besides the humerus and radioulnar. Thus, in PC1 64, all variables are represented and can be interpreted as overall limb size. The second component (PC2 64) explained 18.78 % of total variance and was related to forelimb measurements (humerus and radioulnar – Table 1-3).

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Table 1-2. Morphological traits (means $\pm$ standard deviations, all in mm) of 44 Brazilian species and 20 Australian species of anurans that differ in habits.

Species	Country	Habit	(n)	SVL	HUM	RAD	HAND	FEM	TBFB	TAR	FOOT
<i>Ischnocnema juipoca</i>	BR	FOSS	5	18.01 $\pm$ 0.95	1.33 $\pm$ 0.15	1.5 $\pm$ 0.22	3.33 $\pm$ 0.47	7.62 $\pm$ 0.76	8.13 $\pm$ 0.36	4.21 $\pm$ 0.44	7.95 $\pm$ 0.55
<i>Ischnocnema parva</i>	BR	FOSS	10	18.40 $\pm$ 1.72	2.67 $\pm$ 0.48	3.23 $\pm$ 0.51	4.02 $\pm$ 0.52	8.64 $\pm$ 0.51	9.06 $\pm$ 0.86	5.18 $\pm$ 0.57	8.32 $\pm$ 0.77
<i>Dendrophryniscus brevipollicatus</i>	BR	ARBO	10	17.48 $\pm$ 2.61	3.17 $\pm$ 0.57	3.69 $\pm$ 0.46	4.66 $\pm$ 0.45	7.72 $\pm$ 0.93	8.24 $\pm$ 1.23	4.53 $\pm$ 0.79	7.10 $\pm$ 1.18
<i>Melanophryniscus moreirae</i>	BR	TERR	10	24.63 $\pm$ 2.01	4.54 $\pm$ 0.52	5.19 $\pm$ 0.38	6.23 $\pm$ 0.38	8.79 $\pm$ 0.63	8.86 $\pm$ 0.43	5.96 $\pm$ 0.49	9.51 $\pm$ 0.48
<i>Rhinella icterica</i>	BR	TERR	10	117.38 $\pm$ 17.52	18.82 $\pm$ 3.50	28.21 $\pm$ 5.00	31.73 $\pm$ 4.43	49.84 $\pm$ 8.49	47.12 $\pm$ 6.64	27.35 $\pm$ 3.22	48.30 $\pm$ 7.09
<i>Rhinella ornata</i>	BR	TERR	8	60.21 $\pm$ 7.25	10.79 $\pm$ 1.55	14.67 $\pm$ 1.50	16.29 $\pm$ 1.10	27.70 $\pm$ 2.89	28.58 $\pm$ 2.49	17.92 $\pm$ 1.63	27.59 $\pm$ 2.26
<i>Rhinella schneideri</i>	BR	TERR	13	113.54 $\pm$ 17.66	18.44 $\pm$ 3.73	25.65 $\pm$ 3.71	29.09 $\pm$ 3.46	50.24 $\pm$ 9.37	45.52 $\pm$ 6.33	30.26 $\pm$ 4.74	45.74 $\pm$ 5.42
<i>Cycloramphus acangatan</i>	BR	FOSS	10	35.08 $\pm$ 1.42	3.56 $\pm$ 0.56	5.86 $\pm$ 0.53	8.16 $\pm$ 0.43	12.95 $\pm$ 0.87	11.11 $\pm$ 0.59	7.79 $\pm$ 0.68	13.01 $\pm$ 0.65
<i>Cycloramphus boraceiensis</i>	BR	TORR	12	42.4 $\pm$ 5.74	7.29 $\pm$ 1.40	8.98 $\pm$ 1.55	12.37 $\pm$ 1.96	22.81 $\pm$ 3.35	21.63 $\pm$ 3.28	12.86 $\pm$ 1.56	21.53 $\pm$ 3.09
<i>Cycloramphus dubius</i>	BR	TORR	10	46.28 $\pm$ 2.16	7.25 $\pm$ 0.24	9.24 $\pm$ 0.66	13.56 $\pm$ 0.99	23.53 $\pm$ 1.48	22.72 $\pm$ 1.35	12.82 $\pm$ 1.25	22.97 $\pm$ 1.17
<i>Thoropa miliaris</i>	BR	TORR	10	82.87 $\pm$ 8.27	20.88 $\pm$ 3.57	15.89 $\pm$ 1.42	23.41 $\pm$ 2.09	43.82 $\pm$ 3.77	46.06 $\pm$ 3.89	25.16 $\pm$ 2.31	45.09 $\pm$ 3.91
<i>Thoropa taophora</i>	BR	TORR	6	64.64 $\pm$ 5.95	13.77 $\pm$ 2.21	13.82 $\pm$ 1.13	17.95 $\pm$ 2.02	34.14 $\pm$ 4.68	36.53 $\pm$ 3.80	20.24 $\pm$ 2.40	33.35 $\pm$ 3.60
<i>Aplastodiscus albosignatus</i>	BR	ARBO	10	39.50 $\pm$ 2.14	4.71 $\pm$ 0.58	7.34 $\pm$ 0.49	12.04 $\pm$ 0.57	19.94 $\pm$ 1.03	20.25 $\pm$ 0.65	14.37 $\pm$ 0.57	17.72 $\pm$ 0.40
<i>Aplastodiscus callipygius</i>	BR	ARBO	5	43.26 $\pm$ 1.87	4.68 $\pm$ 0.28	7.58 $\pm$ 0.29	12.77 $\pm$ 0.42	20.60 $\pm$ 1.07	20.33 $\pm$ 0.42	14.13 $\pm$ 0.24	18.59 $\pm$ 0.60
<i>Aplastodiscus leucopygius</i>	BR	ARBO	15	39.23 $\pm$ 1.90	5.01 $\pm$ 0.60	7.27 $\pm$ 0.44	11.63 $\pm$ 0.55	19.21 $\pm$ 1.22	19.56 $\pm$ 1.01	13.43 $\pm$ 0.78	16.85 $\pm$ 0.74
<i>Hypsiboas albopunctatus</i>	BR	ARBO	10	52.08 $\pm$ 3.88	7.09 $\pm$ 1.19	10.98 $\pm$ 0.97	14.28 $\pm$ 1.26	29.55 $\pm$ 2.22	30.86 $\pm$ 2.18	20.73 $\pm$ 1.31	25.19 $\pm$ 1.82
<i>Hypsiboas faber</i>	BR	ARBO	19	91.49 $\pm$ 4.84	12.35 $\pm$ 2.60	18.07 $\pm$ 1.83	29.58 $\pm$ 2.65	50.46 $\pm$ 3.79	51.23 $\pm$ 2.98	32.56 $\pm$ 2.02	41.11 $\pm$ 2.27
<i>Hypsiboas pardalis</i>	BR	ARBO	10	61.08 $\pm$ 4.43	9.05 $\pm$ 1.43	12.27 $\pm$ 1.25	17.98 $\pm$ 1.45	31.57 $\pm$ 2.32	32.92 $\pm$ 1.86	20.31 $\pm$ 1.20	26.00 $\pm$ 2.06
<i>Hypsiboas polytaenius</i>	BR	ARBO	8	28.33 $\pm$ 1.38	4.58 $\pm$ 0.35	4.94 $\pm$ 0.22	8.77 $\pm$ 0.65	14.23 $\pm$ 0.75	14.96 $\pm$ 0.88	10.24 $\pm$ 0.55	12.30 $\pm$ 0.81
<i>Hypsiboas latistratus</i>	BR	ARBO	7	32.87 $\pm$ 1.36	5.35 $\pm$ 0.39	5.72 $\pm$ 0.37	9.78 $\pm$ 0.78	15.64 $\pm$ 0.91	15.95 $\pm$ 1.02	10.69 $\pm$ 0.66	13.88 $\pm$ 0.65
<i>Scinax crospedospilus</i>	BR	ARBO	6	29.60 $\pm$ 1.01	4.63 $\pm$ 0.29	5.42 $\pm$ 0.68	8.73 $\pm$ 0.56	13.47 $\pm$ 1.38	15.41 $\pm$ 0.82	9.38 $\pm$ 0.38	13.09 $\pm$ 0.75
<i>Scinax perpusillus</i>	BR	ARBO	10	19.28 $\pm$ 1.20	2.59 $\pm$ 0.31	3.27 $\pm$ 0.58	5.35 $\pm$ 0.55	9.15 $\pm$ 0.89	9.98 $\pm$ 0.99	5.80 $\pm$ 0.69	7.52 $\pm$ 0.88
<i>Scinax rizibilis</i>	BR	ARBO	9	25.69 $\pm$ 1.31	3.35 $\pm$ 0.34	4.50 $\pm$ 0.36	7.05 $\pm$ 0.47	11.82 $\pm$ 0.24	12.83 $\pm$ 0.43	8.36 $\pm$ 0.36	10.24 $\pm$ 0.58
<i>Pseudis paradoxa</i>	BR	AQUA	10	51.70 $\pm$ 4.50	6.13 $\pm$ 0.68	9.37 $\pm$ 0.76	15.51 $\pm$ 1.27	30.45 $\pm$ 2.62	28.10 $\pm$ 2.15	15.39 $\pm$ 1.10	29.21 $\pm$ 2.61

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<i>Pseudis platensis</i>	BR	AQUA	8	44.43 ± 4.71	6.41 ± 1.11	8.25 ± 0.83	13.92 ± 1.73	27.19 ± 2.72	25.24 ± 2.85	13.91 ± 1.23	25.96 ± 3.10
<i>Hylodes phyllodes</i>	BR	TORR	10	31.38 ± 2.85	4.93 ± 1.01	6.46 ± 0.93	9.46 ± 1.09	15.27 ± 1.75	16.75 ± 2.13	9.23 ± 1.08	15.79 ± 1.91
<i>Hylodes asper</i>	BR	TORR	11	39.98 ± 1.79	7.7 ± 1.20	7.92 ± 0.87	13.17 ± 0.61	21.38 ± 1.22	22.2 ± 1.61	11.92 ± 0.79	19.79 ± 1.48
<i>Crossodactylus caramaschii</i>	BR	TORR	9	24.70 ± 0.91	4.40 ± 0.61	4.36 ± 0.47	7.25 ± 0.31	11.52 ± 0.66	12.65 ± 0.75	7.12 ± 0.28	12.58 ± 1.07
<i>Physalaemus cuvieri</i>	BR	TERR	18	29.2 ± 3.45	4.15 ± 0.91	5.51 ± 0.65	7.62 ± 0.81	11.92 ± 0.94	13.29 ± 0.80	7.64 ± 0.83	14.45 ± 1.22
<i>Physalaemus olfersii</i>	BR	FOSS	14	34.57 ± 1.59	5.22 ± 0.79	6.81 ± 0.82	9.42 ± 0.66	14.87 ± 0.89	16.6 ± 1.02	9.26 ± 0.44	17.63 ± 1.16
<i>Leptodactylus fuscus</i>	BR	TERR	15	44.79 ± 2.51	4.21 ± 1.30	7.11 ± 1.26	9.6 ± 0.66	19.44 ± 1.78	24.04 ± 1.11	15.04 ± 0.94	23.08 ± 1.59
<i>Leptodactylus labyrinthicus</i>	BR	TERR	5	149.33 ± 6.72	28.11 ± 5.22	29.24 ± 3.18	35.4 ± 1.82	62.35 ± 7.77	70.09 ± 6.91	43.9 ± 2.34	69.78 ± 6.00
<i>Leptodactylus latrans</i>	BR	TERR	10	109.73 ± 7.00	25.37 ± 6.52	20.15 ± 3.11	26.05 ± 2.29	47.05 ± 5.73	53.19 ± 3.55	32.67 ± 2.59	57.30 ± 3.48
<i>Leptodactylus notoaktites</i>	BR	FOSS	9	49.43 ± 1.97	5.85 ± 0.49	9.07 ± 0.71	11.05 ± 0.84	22.55 ± 1.48	26.70 ± 1.20	15.94 ± 0.70	28.95 ± 1.91
<i>Leptodactylus podicipinus</i>	BR	TERR	5	33.65 ± 2.04	4.46 ± 0.68	5.70 ± 0.28	8.58 ± 0.99	14.61 ± 0.77	14.50 ± 0.96	8.99 ± 0.82	17.05 ± 0.86
<i>Chiasmocleis carvalhoi</i>	BR	FOSS	7	26.47 ± 1.91	3.74 ± 0.25	4.87 ± 0.41	6.33 ± 0.30	10.40 ± 0.37	10.80 ± 0.26	6.99 ± 0.39	10.70 ± 0.34
<i>Chiasmocleis leucosticta</i>	BR	FOSS	7	20.41 ± 1.04	3.30 ± 0.31	3.47 ± 0.36	5.06 ± 0.33	7.66 ± 0.89	8.14 ± 0.36	5.30 ± 0.19	8.81 ± 0.76
<i>Dermatonotus muelleri</i>	BR	FOSS	5	55.03 ± 1.74	6.35 ± 0.80	11.76 ± 1.07	14.46 ± 1.16	21.91 ± 1.34	17.21 ± 1.24	12.12 ± 1.08	21.22 ± 1.33
<i>Elachistocleis ovalis</i>	BR	FOSS	10	28.49 ± 3.78	2.24 ± 0.64	3.78 ± 0.67	5.95 ± 0.72	9.89 ± 1.67	9.59 ± 1.28	6.65 ± 0.99	11.40 ± 1.39
<i>Odontophrynus americanus</i>	BR	FOSS	10	45.46 ± 1.63	6.02 ± 0.47	9.72 ± 0.50	14.45 ± 0.55	18.04 ± 1.01	15.69 ± 0.69	10.19 ± 0.49	22.45 ± 1.05
<i>Macrogenioglottus alipioi</i>	BR	FOSS	8	94.33 ± 4.78	14.64 ± 1.94	24.75 ± 1.69	29.83 ± 3.13	40.95 ± 3.03	33.32 ± 2.34	21.72 ± 1.65	38.19 ± 2.74
<i>Proceratophrys boiei</i>	BR	FOSS	15	50.55 ± 5.74	7.58 ± 1.84	10.4 ± 1.72	13.66 ± 1.59	19.97 ± 2.61	17.89 ± 2.04	10.78 ± 1.77	19.99 ± 2.91
<i>Proceratophrys melanopogon</i>	BR	FOSS	10	44.98 ± 3.19	3.97 ± 1.35	8.80 ± 1.29	12.26 ± 1.26	17.27 ± 3.74	16.72 ± 2.86	9.18 ± 1.43	18.27 ± 2.56
<i>Pipa carvalhoi</i>	BR	AQUA	10	35.12 ± 2.22	4.69 ± 0.40	5.25 ± 0.66	6.43 ± 0.71	16.22 ± 1.20	14.81 ± 1.22	9.23 ± 0.64	13.25 ± 1.48
<i>Litoria caerulea</i>	AU	ARBO	14	66.35 ± 5.68	9.65 ± 1.20	12.33 ± 1.46	20.16 ± 1.39	29.21 ± 2.17	28.78 ± 2.93	19.95 ± 2.97	26.17 ± 4.47
<i>Litoria ewingi</i>	AU	ARBO	16	33.45 ± 2.83	5.29 ± 1.00	6.29 ± 0.66	10.1 ± 1.17	15.25 ± 2.27	15.78 ± 1.66	10.26 ± 0.94	14.69 ± 1.41
<i>Litoria latopalmata</i>	AU	ARBO	3	35.98 ± 1.28	4.93 ± 0.60	6.74 ± 0.43	10.04 ± 0.37	20.19 ± 1.27	21.99 ± 1.50	12.39 ± 1.29	18.16 ± 1.36
<i>Litoria peronii</i>	AU	ARBO	8	51.93 ± 1.95	7.17 ± 1.09	9.58 ± 0.46	14.91 ± 0.88	23.21 ± 1.64	25.84 ± 0.88	15.21 ± 0.56	21.93 ± 1.04
<i>Litoria raniformis</i>	AU	TERR	5	62.59 ± 1.99	8.75 ± 0.71	10.64 ± 0.58	16.11 ± 0.37	29.45 ± 1.79	29.75 ± 0.70	20.47 ± 0.67	29.23 ± 0.76
<i>Litoria cultripes</i>	AU	FOSS	4	43.88 ± 4.04	6.60 ± 0.57	8.73 ± 0.77	10.84 ± 0.65	18.67 ± 1.37	17.17 ± 0.82	10.96 ± 0.80	17.70 ± 1.19
<i>Litoria maini</i>	AU	FOSS	4	40.65 ± 3.77	5.58 ± 0.66	7.43 ± 0.74	9.78 ± 0.81	18.1 ± 2.64	15.54 ± 1.13	9.77 ± 1.32	16.18 ± 1.39

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<i>Litoria platycephala</i>	AU	FOSS	6	56.5 ± 5.36	6.59 ± 0.72	10.39 ± 1.28	14.89 ± 0.93	22.63 ± 2.11	21.19 ± 0.93	12.86 ± 1.36	23.56 ± 1.66
<i>Crinia signifera</i>	AU	TERR	18	21.60 ± 1.57	3.90 ± 0.62	4.42 ± 0.68	5.78 ± 0.64	9.50 ± 1.02	9.78 ± 0.67	6.18 ± 0.51	11.81 ± 1.26
<i>Crinia parinsignifera</i>	AU	TERR	5	20.87 ± 1.38	3.02 ± 0.20	3.21 ± 0.48	4.84 ± 0.60	8.25 ± 0.78	8.90 ± 0.25	5.4 ± 0.40	10.38 ± 0.60
<i>Crinia riparia</i>	AU	TERR	3	19.72 ± 1.43	3.37 ± 0.41	4.23 ± 0.65	5.43 ± 0.41	8.27 ± 0.60	9.93 ± 0.84	6.63 ± 1.42	12.04 ± 1.21
<i>Crinia deserticola</i>	AU	TERR	6	17.07 ± 0.98	2.89 ± 0.28	2.73 ± 0.26	4.05 ± 0.40	7.08 ± 0.63	8.44 ± 0.39	4.95 ± 0.23	9.60 ± 1.10
<i>Limnodynastes dumerilii</i>	AU	FOSS	5	54.26 ± 2.30	8.36 ± 1.66	10.04 ± 0.67	14.7 ± 0.96	23.46 ± 2.22	21.91 ± 1.57	13.84 ± 0.66	27.42 ± 1.79
<i>Limnodynastes peronii</i>	AU	TERR	5	52.45 ± 4.18	8.58 ± 1.36	10.8 ± 1.30	13.03 ± 0.70	20.47 ± 5.05	22.18 ± 1.04	14.48 ± 0.73	25.25 ± 0.54
<i>Limnodynastes fletcheri</i>	AU	TERR	3	42.01 ± 1.82	6.19 ± 1.08	7.75 ± 1.94	11.01 ± 1.75	17.48 ± 1.40	17.35 ± 1.32	10.78 ± 1.05	19.64 ± 3.34
<i>Limnodynastes tasmaniensis</i>	AU	TERR	19	37.54 ± 5.21	5.67 ± 1.23	6.67 ± 1.14	9.71 ± 1.23	15.75 ± 2.75	16.14 ± 2.20	10.16 ± 1.37	18.52 ± 2.45
<i>Neobatrachus sudelli</i>	AU	FOSS	6	48.93 ± 2.66	5.73 ± 1.00	9.69 ± 1.14	11.72 ± 0.73	17.33 ± 1.48	15.32 ± 0.74	10.56 ± 0.84	19.35 ± 0.74
<i>Neobatrachus pictus</i>	AU	FOSS	5	47.46 ± 2.89	6.94 ± 1.16	10.05 ± 0.91	13.21 ± 2.02	19.45 ± 1.55	16.46 ± 1.09	11.69 ± 0.85	21.21 ± 1.36
<i>Pseudophryne briboni</i>	AU	TERR	6	26.29 ± 1.07	3.7 ± 0.57	4.88 ± 0.52	5.94 ± 0.23	8.08 ± 0.91	7.87 ± 0.45	6.04 ± 0.21	9.17 ± 0.67
<i>Uperolia capitulata</i>	AU	TERR	2	29.1 ± 1.16	3.1 ± 0.30	4.4 ± 0.37	6.1 ± 0.59	9.8 ± 0.38	9.8 ± 0.74	6.3 ± 0.57	11.6 ± 0.97

BR=Brazil, AU= Australia, AQUA= aquatic; ARBO= arboreal; FOSS= fossorial; TORR= torrent and TERR= terrestrial. SVL= snout-vent-length; HUM= humerus length, RAD= radioulnar length; FEM= femur length; TBFB= tibiofibular length; TAR= tarsus length.

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Table 1-3. Component loadings from the phylogenetic Principal Component Analysis (pPCA) performed on morphological variables for 64 anuran species from Brazil and Australia. Values with higher loadings in each component are highlighted in asterisk.

Component	1	2
Eigenvalues (λ)	4.04	1.31
Total variance explained (%)	57.75	18.78
Cumulative variance (%)	57.75	76.53
Humerus length	-0.580*	0.547
Radioulnar length	-0.503	0.755*
Hand length	-0.736*	0.346
Femur length	-0.878*	-0.223
Tibiofibular length	-0.890*	-0.352
Tarsus length	-0.863*	-0.231
Foot length	-0.776*	-0.313

The results of maximum likelihood estimation, as shown in Table 1-4, suggest that OUM is the best evolutionary model among the four candidates for PC1 64 (morphological component related to overall limb lengths), implying that there are multiple optima of limb lengths associated to different microhabitats. The evolutionary optima estimated for this model reveal a trend of increasing hindlimbs in torrent, arboreal, aquatic, whereas fossorial and terrestrial species evolve toward optima with shorter hindlimbs (Table 1-4). For PC2 64 (humerus and radioulnar), the best model is Brownian motion with different variance rates across microhabitats (BMS), implying that interspecific variation in this structure is not associated to selection related with microhabitat use.

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Table 1-4. AIC scores from models of morphological evolution. AICc = modified Akaike information criterion; BM1 = fits a single morphological variation rate across species; BMS= fits a model with different morphological variation rates for each microhabitat; OU1 = fits a single optimum for all species and OUM= fits different optimum for each microhabitat. PC1 64 and PC2 64 =Principal Component 1 and 2 respectively, of the morphological data set for 64 species. Model fit statistics for PC1 64 (hindlimb: femur, tibiofibular, tarsus, foot; and forelimb: hand), PC2 64 (radioulnar) and Body size (SVL 64). The best models are highlighted in bold.

	PC1 64	PC2 64	SVL 64
<i>Evolutionary models</i>	AICc	AICc	AICc
BM1	23.0733	6.86908	2.83938
BMS	26.1423	0	7.85394
OU1	17.9985	8.55756	0
OUM	0	16.0904	8.48335

Moreover, some microhabitats, such as fossorial and terrestrial, allow higher morphological variance in this morphological component than others (Table 1-5). Regarding body size, the OU1 models described stabilizing selection towards a single global optimum for all species ($\theta = 1.5461$) that is not associated with selective regimes of microhabitat use.

Table 1-5. Estimated parameters for the best fitting PC1 64 (hindlimb: femur, tibiofibular, tarsus, foot; and forelimb: hand) and PC2 64. The estimated optimal values (θ) and morphological diversification rate (σ) for each microhabitat.

	PC1 64(OUM)	PC2 64(BMS)
Microhabitat use	θ	σ
Arboreal	-23.0346	0.5439
Fossorial	3.1506	2.3177
Terrestrial	-11.2799	1.3568
Aquatic	-20.9494	0.9386
Torrent	-29.5412	0.1352

1.3.2. Relationship among morphological variables, microhabitat use and locomotor performance

Descriptive statistics of morphological variables from each species are presented in Table 1-6 as the mean $\pm$ standard deviation. The phylogenetic Principal Component Analysis performed on morphometric traits resulted in two components. Component 1 of the morphological data set for 19 species (PC1 19) explained 47.77% of the total variance and was related to a positive association between measurements taken from hindlimb (femur, tibiofibular, tarsus and foot) and hand. The second component (PC2 16) explained 23.87% of total variance and was mostly related to a positive association between forelimb elements (humerus and radioulnar) (Table 1-7).

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Table 1-6. Morphological traits (means $\pm$ standard deviations, all in mm) of 15 Brazilian species and 4 Australian species of anurans that differ in habits.

Species	Country	Microhabitat	(n)	SVL	HUM	RAD	HAND	FEM	TBFB	TAR	FOOT
<i>Rhinella schneideri</i>	BR	TERR	3	115.09 $\pm$ 17.95	15.03 $\pm$ 1.37	24.79 $\pm$ 2.79	28.22 $\pm$ 3.46	45.15 $\pm$ 6.31	43.17 $\pm$ 4.54	29.19 $\pm$ 5.25	44.42 $\pm$ 6.00
<i>Cycloramphus boraceiensis</i>	BR	TORR	7	44.29 $\pm$ 2.97	8.26 $\pm$ 1.01	9.63 $\pm$ 0.87	13.01 $\pm$ 1.42	25.05 $\pm$ 2.31	23.47 $\pm$ 2.03	13.42 $\pm$ 1.14	22.89 $\pm$ 2.22
<i>Thoropa taophora</i>	BR	TORR	6	64.64 $\pm$ 5.95	13.77 $\pm$ 2.21	13.82 $\pm$ 1.13	17.95 $\pm$ 2.05	34.14 $\pm$ 4.68	36.53 $\pm$ 3.80	20.24 $\pm$ 2.40	33.35 $\pm$ 3.60
<i>Aplastodiscus leucopygius</i>	BR	ARBO	5	40.34 $\pm$ 2.32	5.65 $\pm$ 0.44	7.32 $\pm$ 0.37	11.87 $\pm$ 0.56	19.38 $\pm$ 0.87	20.14 $\pm$ 0.51	13.53 $\pm$ 0.53	17.13 $\pm$ 0.66
<i>Hypsiboas faber</i>	BR	ARBO	9	93.29 $\pm$ 3.28	14.23 $\pm$ 1.04	17.04 $\pm$ 1.29	31.32 $\pm$ 1.45	49.18 $\pm$ 3.43	51.21 $\pm$ 2.77	31.76 $\pm$ 1.14	41.00 $\pm$ 1.88
<i>Scinax crospedospilus</i>	BR	ARBO	6	29.60 $\pm$ 1.01	4.63 $\pm$ 0.29	5.42 $\pm$ 0.68	8.73 $\pm$ 0.56	13.47 $\pm$ 1.38	15.41 $\pm$ 0.82	9.38 $\pm$ 0.38	13.09 $\pm$ 0.75
<i>Pseudis platensis</i>	BR	AQUA	8	44.43 $\pm$ 4.71	6.41 $\pm$ 1.11	8.24 $\pm$ 0.83	13.92 $\pm$ 1.73	27.18 $\pm$ 2.72	25.24 $\pm$ 2.85	13.90 $\pm$ 1.23	25.95 $\pm$ 3.10
<i>Hylodes asper</i>	BR	TORR	3	42.22 $\pm$ 0.36	8.83 $\pm$ 0.03	8.82 $\pm$ 0.26	13.32 $\pm$ 0.51	23.02 $\pm$ 0.80	24.41 $\pm$ 0.42	12.81 $\pm$ 0.34	21.58 $\pm$ 0.21
<i>Physalaemus cuvieri</i>	BR	TERR	8	27.47 $\pm$ 4.56	5 $\pm$ 0.66	5.52 $\pm$ 0.96	7.38 $\pm$ 0.98	11.64 $\pm$ 1.25	12.95 $\pm$ 1.06	7.54 $\pm$ 1.22	14.52 $\pm$ 1.78
<i>Physalaemus olfersii</i>	BR	FOSS	4	34.94 $\pm$ 1.49	5.71 $\pm$ 0.51	7.31 $\pm$ 0.89	9.75 $\pm$ 0.82	15.02 $\pm$ 0.34	17.58 $\pm$ 0.58	9.42 $\pm$ 0.55	18.31 $\pm$ 0.57
<i>Leptodactylus fuscus</i>	BR	TERR	5	46.01 $\pm$ 3.04	5.45 $\pm$ 0.91	8.00 $\pm$ 0.53	9.86 $\pm$ 0.37	21.04 $\pm$ 1.84	23.95 $\pm$ 1.66	14.82 $\pm$ 1.08	22.77 $\pm$ 1.03
<i>Leptodactylus labyrinthicus</i>	BR	TERR	5	149.33 $\pm$ 6.72	28.11 $\pm$ 5.22	29.24 $\pm$ 3.18	35.4 $\pm$ 1.82	62.35 $\pm$ 7.77	70.08 $\pm$ 6.91	43.9 $\pm$ 2.34	69.78 $\pm$ 6.00
<i>Leptodactylus podicipinus</i>	BR	TERR	5	33.65 $\pm$ 2.04	4.46 $\pm$ 0.68	5.70 $\pm$ 0.28	8.58 $\pm$ 0.99	14.61 $\pm$ 0.77	14.50 $\pm$ 0.96	8.99 $\pm$ 0.82	17.05 $\pm$ 0.86
<i>Dermatonotus muelleri</i>	BR	FOSS	5	55.03 $\pm$ 2.74	6.35 $\pm$ 1.21	11.76 $\pm$ 1.12	14.46 $\pm$ 1.10	21.91 $\pm$ 3.53	17.21 $\pm$ 1.27	12.12 $\pm$ 1.49	21.22 $\pm$ 4.19
<i>Proceratophrys boiei</i>	BR	FOSS	5	54.28 $\pm$ 7.48	9.31 $\pm$ 2.08	12.20 $\pm$ 1.61	15.13 $\pm$ 1.72	22.75 $\pm$ 2.50	19.66 $\pm$ 2.62	12.56 $\pm$ 1.24	22.65 $\pm$ 2.40
<i>Litoria caerulea</i>	AU	ARBO	4	60.07 $\pm$ 2.69	9.94 $\pm$ 0.38	11.44 $\pm$ 1.19	19.13 $\pm$ 1.20	28.42 $\pm$ 0.63	28.03 $\pm$ 1.13	18.50 $\pm$ 1.66	26.09 $\pm$ 1.86
<i>Litoria ewingi</i>	AU	ARBO	6	33.88 $\pm$ 2.82	5.92 $\pm$ 1.23	6.33 $\pm$ 0.89	10.73 $\pm$ 1.29	15.47 $\pm$ 3.50	16.61 $\pm$ 2.02	10.64 $\pm$ 1.02	15.08 $\pm$ 1.26
<i>Crinia signifera</i>	AU	TERR	8	22.09 $\pm$ 1.67	4.03 $\pm$ 0.75	5.01 $\pm$ 0.42	6.12 $\pm$ 0.61	10.10 $\pm$ 1.10	10.25 $\pm$ 0.62	6.09 $\pm$ 0.59	12.25 $\pm$ 1.51
<i>Limnodynastes tasmaniensis</i>	AU	TERR	9	40.15 $\pm$ 5.78	6.28 $\pm$ 1.26	7.42 $\pm$ 1.09	10.26 $\pm$ 1.14	17.78 $\pm$ 2.05	17.46 $\pm$ 1.99	10.70 $\pm$ 1.15	19.76 $\pm$ 2.32

BR=Brazil, AU= Australia, AQUA= aquatic; ARBO= arboreal; FOSS= fossorial; TORR= torrent and TERR= terrestrial. SVL= snout-vent-length; HUM= humerus length, RAD= radioulnar length; FEM= femur length; TBFB= tibiofibular length; TAR= tarsus length.

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Table 1-7. Component loadings from the phylogenetic Principal Component Analysis (pPCA) performed on morphological variables for 19 anuran species. Values with higher loadings in each component are highlighted in asterisk.

Component	1	2
Eigenvalues (λ)	3.34	1.67
Total variance explained (%)	47.77	23.87
Cumulative variance (%)	47.77	71.64
Humerus length	-0.425	0.768*
Radioulnar length	0.201	0.877*
Hand length	-0.515*	0.312
Femur length	-0.891*	-0.034
Tibiofibular length	-0.948*	-0.103
Tarsus length	-0.808*	-0.403
Foot length	-0.715*	0.201

Results from best-fit models showed that interspecific differences in locomotor performance relates to both morphological components (Table 1-8). Species with higher jumping performance were characterized by lower scores from the morphological component 1 [PC1 19] (longer hindlimbs and hands). Accordingly, arboreal, aquatic and torrent species were characterized by higher jumping performance and longer hindlimbs, when compared to fossorial and terrestrial species (Figure 1-2A). Species with higher jumping performance were also characterized by higher scores from the morphological component 2 [PC2 19] (longer humerus and radioulnar lengths), with a less clear association with differences in habits. However, it is noteworthy that torrent species are characterized by short forelimbs for their high locomotor performance (Figure 1-3B).

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Table 1-8. Results for best-fit models testing if locomotor performance is related to morphological variation and microhabitat use. PC1 19 and PC2 19 =Principal Component 1 and 2 respectively, of the morphological data set for 19 species; HAB=microhabitat use (arboreal, fossorial, terrestrial, torrent, aquatic); AICc = modified Akaike information criterion; dAICc= delta Akaike information criterion; df= number of parameters in the model.

Dependent variation	Models	AICc	dAICc	df	weight
Jumping distance	PC1 19*HAB+PC2 19*HAB	48.8	42.5	8	<0.001
	PC1 19*HAB+PC2 19	32.4	26.1	7	<0.001
	PC1 19+PC2 19+HAB	16.6	10.3	6	0.0058
	PC1 19+PC2 19	6.3	0	5	0.9941

(A)

(B)

Figure 1-3: Scatterplots of morphological components and size-free locomotor performance (A) Describe here better of PC1 19 (Hindlimb: femur, tibiofibular, tarsus and foot) and size free jump performance; B) Describe here better PC2 19 (humerus and radioulnar) and size free jump performance.

1.4. Discussion

Our comparative analysis supports the hypothesis of adaptive morphological diversification within anuran lineage, associated with microhabitat use. Additionally, we show that interspecific morphological diversity is associated with differences in locomotor performance, corroborating functional morphological analyses from previous studies (Emerson, 1985; Nauwelaerts et al., 2007; Gomes et al, 2009). In general, arboreal, aquatic, and torrent species are characterized by longer hindlimbs and higher jumping ability than terrestrial and, mainly, fossorial ones. These results emphasize that the inclusion of locomotor performance measurements is highly informative on comparative studies that seek to understand the evolutionary relationship between ecology (e.g. microhabitat use) and morphology (Losos, 1990; Wainwright, 1991; Herrel et al, 2004). Moreover, anurans from two different geographic regions (Brazil and Australia) were included in the morphological analyses, maximizing transitions between microhabitat uses across the anuran phylogenetic history.

Our analyses suggest that the evolutionary trajectories of morphological traits have changed in a predictable manner associated with changes in microhabitat use. The evolution of morphological components related to relative overall limb length was best explained by OUM. It indicates that the adaptive optima differed between microhabitat types. Biomechanical studies predict distinct adaptive optima associated to the gradient of microhabitat use (Zug, 1972, 1978; Emerson, 1978, 1985; Gomes et al., 2009; Moen et al., 2013). While torrent and arboreal habits are associated with evolutionary trajectories towards longer relative overall limbs, fossorial habits lead to opposed trajectories. The long limbs that characterized riparian and stream breeder anurans

might represent the selective outcome associated with improved ability to swim under high flow rates (Dennis & Mahony, 1994; Lewis & Rohweder, 2005; Hoskin, 2010, Vidal-García et al., 2014). *Heleophryne hewitti* (Heleophrynidae), for example, is an African anuran that inhabits fast-flowing mountain streams and has disproportionately long and muscular legs, which may have evolved in association with improved swimming ability against high water resistance (Laurent, 1964). Longer hindlimbs also increase the time of muscular force application against the substrate and thus the distance jumped. Interestingly, our results highlight an interspecific variation in jumped distance associated with the microhabitat use, and corroborate previous studies showing that hindlimbs evolve in association with higher jumping performance in anurans (Zug, 1972; Emerson, 1985, Gomes et al., 2009). In addition to relative limb length, variation in several physiological and morphological characters has been also associated to anuran jumping ability at both intra and interspecific levels. By comparing different individuals of *Rhinella marina*, Longphre & Gatten (1994) found that those showing higher maximum aerobic speed of locomotion were characterized by higher activity of the aerobic enzyme citrate-synthase in muscles from hind limbs and higher masses of ventricles and the muscle iliofibularis. Moreover, 62% of interindividual variation in maximum jumping distance was associated to hindlimb muscle mass and activity of the glycolytic enzyme pyruvate kinase in plantaris from *Hypsiboas bischoffii* (James et al., 2005). At interspecific level, increased jumping performance in anurans has been associated to differences in muscle contractile properties associated with muscle speed (e.g. time to peak tetanus, maximum shortening speed, peak isotonic power) in addition to hindlimb length (Astley, 2016). Our results confirm those from previous studies, showing that fossorial species possess tibiofibula particularly shortened in comparison

to jumping anurans (Enriquez-Urzelai et al., 2015). In opposition to the longer hindlimbs, shorter tibiofibula function as out-levers (Hildebrand, 1974). In this way shorter hindlimbs increase the force generated during scooping (Emerson, 1976), improving burrowing performance (Duellman and Trueb, 1986; Wells, 2007, Vidal-García et al., 2014). Reduced hindlimb lengths can be found in species that burrow either forwards or backwards (Vidal-García et al., 2014). Moreover, shorter legs are found in species that walk rather than hop as demonstrated in two species of *Arenophryne* (Tyler et al., 1980; Vidal-García et al., 2014).

In contrast to the hindlimbs, the forelimbs are generally considered to be conserved among anurans (Manzano et al., 2008) and their main function is associated to the absorption of impact forces during landing (Emerson, 1983). Moreover, frogs often use their forelimbs to capture and transport prey (Gray et al. 1997). Our results show that fossorial species indeed exhibit higher variation in radioulnar length, which may explain their higher morphological forelimb disparity when compared to the hindlimbs. The best evolutionary model for morphological component related to forelimbs (mainly radioulnar length) in our study was the BM process with different rates of evolution among selective regimes. Vidal-García et al. (2014) observed that Australian forward burrowers (*Arenophryne*, *Myobatrachus*) had relatively longer arms. Otherwise, their sister taxon *Metacrinia* (Read et al., 2001), which does not burrow (Tyler & Doughty, 2009), had relatively shorter arms, suggesting that longer arms are important in burrowing forward (Vidal-Garcia et al., 2014). Within the species included in the present study, information on digging mode is available for the Brazilian *Dermatonotus muelleri*, which is described as a forward burrowing frog by Nomura et al. (2009). It is worth noting that *D. muelleri* is one of the species characterized by

longer forelimbs in our data set. However, precise information of burrowing behavior is lacking for most of the species included in our data set, limiting our ability to detect patterns of association between forelimb lengths and digging mode. Otherwise, we show that species associated with fast flowing streams and waterfalls exhibit lower variation in radioulnar length and short forelimbs for their high locomotor performance. Additional studies on biomechanics of locomotion in riparian anurans are necessary to understand the functional implications of this morphological pattern.

Regarding body size, we found just one adaptive optimum among all microhabitat use (OU1), corroborating previous results from an interspecific comparison of Western Mediterranean anurans varying in locomotory mode (Enriquez-Urzelai et al. 2015). In fact, interspecific variation in body size is larger within than between categories of microhabitat use in our data set, with SVL ranging from 18.01 to 94.33 mm, 17.07 to 149.33 mm, 35.12 to 51.70 mm, 24.70 to 82.87 mm, and 17.48 to 91.49 mm, respectively for fossorial, terrestrial, aquatic, torrent and arboreal anurans (Table 1-2). In this way, our analyses suggest that variation in anuran body size is not particularly associated with diversification in microhabitat use.

Anuran locomotor modes comprise burrowing, swimming, walking, hopping, jumping, climbing and gliding (Jorgensen and Reilly, 2013), and anuran interspecific variation in locomotor mode has been frequently associated with morphological diversity (Rand, 1952; Zug, 1972, 1978; Emerson, 1979, 1988; Gomes et al, 2009; Jorgensen and Reilly, 2013 Moen et al, 2013, Vidal-García et al., 2014). Our analyses suggest that hindlimb morphological traits have changed in association with jumping performance, and diversification in microhabitat use is an important selective pressure molding these evolutionary trajectories. According to our results, torrent and arboreal

anurans are characterized by longer hindlimbs and higher jumping ability. Among the torrent anurans from the Atlantic Forest (Brazil), males from the genus *Hylodes* are territorial and call from rocks along the streams to attract females and to keep conspecific males at distance. These frogs are wary, generally escaping through one long jump to the water when disturbed (Pavan et al., 2001). Calling behavior in this genus is also complemented by leg stretching and foot-flagging (Narvaes, 1997), a behavioral pattern associated with frogs that inhabit noisy streams, facilitating the transmission of information about location of the signaler (Hödl and Amézquita, 2001). However, a possible cost of this type of behavior would be greater exposure to predators, and this disadvantage is possibly circumvented by the rapid escape through diving into the water (Narvaes, 1997). In this way, longer hindlimbs and higher jumping ability might have evolved in the context of elaborated courtship and escape behavior in torrent anurans. We recognize that a limitation of the present study is that only Brazilian torrent species were included in the comparative analyses. The inclusion of torrent anurans from different phylogenetic groups and zoogeographical areas might provide stronger evidences of convergent evolution of post-cranial skeleton in association to torrent microhabitat use.

Our results corroborate previous studies suggesting that hindlimb length evolved in association with a higher jumping performance in anurans (Zug, 1972; Emerson, 1985, Gomes et al., 2009). The origin and diversity of adaptations for jumping performance in anurans have been associated with different selective pressures, among which predation stands (Gans and Parsons, 1966, Zug, 1972, Emerson, 1978, Gomes et al., 2009). Predation is intense during metamorphosis and early juvenile life of amphibians (Arnold and Wassersug, 1978), and the origin of saltatory locomotion in

anurans has been suggested to be an adaptation to escape predation (Gans and Parsons, 1966). However, it is important to consider that although anurans show a clear pattern of coevolution of hindlimb length and saltatory performance, the proportion of the maximum jumping performance elicited in response to predators is affected by several factors, including microhabitat structure (Gomes et al., 2002; Martín et al., 2005). Rapid adaptive increase in anuran hindlimb length has been also observed in alternative ecological contexts, such as the coevolution with increased rates of movement during the invasion of *Rhinella marina* in Australia (Phillips et al., 2006).

In conclusion, our results show that microhabitat use is an important selective pressure associated with jumping performance and post-cranial morphological diversification in anurans. Particularly, arboreal, aquatic and torrent anurans are characterized by overall limb lengths and higher jumping performance when compared to terrestrial and, particularly, fossorial species. In this way, these results corroborate the hypothesis that evolutionary modifications of overall limb morphology have been important in the diversification of locomotor performance across the anuran phylogeny, and that such evolutionary changes converged in different phylogenetic groups adapted to similar microhabitat use in different zoogeographical regions. The discussion presented here emphasizes that different or even conflicting selective pressures, such as jumping ability and burrowing efficiency, might act on the evolution of complex structures such as vertebrate hindlimbs in association to diversification in microhabitat use. Further studies investigating possible associations between pelvic morphology and habit, together with data on different patterns of antipredator behavior would be interesting to a better understanding of the functional implications of morphological divergence in this phylogenetic group.

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**2. CAPÍTULO 2: Modulation of anuran antipredator behavior by
microenvironmental complexity and predatory stimulus type: intra
and interspecific variation**

Modulation of anuran antipredator behavior by microenvironmental complexity and predatory stimulus type: intra and interspecific variation.

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Abstract

The antipredator behavior is strikingly diverse in anurans, with dozens of patterns of alternative passive and active antipredator behaviors. While immobility might often prevent detection when a predator approaches, active escape represents a more effective response when anurans have been detected. Antipredator responses might also be modulated by characteristics of the microenvironment occupied, and this modulation might be differentially expressed in anurans with different microhabitat use. We investigated: (A) at intraspecific level, the influence of predatory stimulus and the complexity of the microenvironment in the manifestation of antipredator behavior; (B) at interspecific level, the role of microhabitat use in the evolution of antipredator behavior and locomotor performance in anuran amphibians. To achieve our goals, we simulated predation events in the laboratory using a snake as the predator, and we ran predation tests at three sets of environmental complexity (open area, leaf litter or bush

vegetation), and with two types of stimulus (approach versus touch of predator) within an arena maintained at 25°C. We examined the responses to simulated predation of anuran species with different microhabitat use (arboreal, aquatic, fossorial, terrestrial or torrent). The types of stimulus and microenvironment complexity influenced the escape responses. Passive behaviors such as immobility were mainly observed with the approach of the predator, while active behaviors were the most frequent response to predator's touch. The percentage of active responses decreased in microenvironment characterized with greater structural complexity. Anurans also modulated distance jumped in response to predator's touch according to environmental complexity, and an interspecific variation associated with microhabitat use was observed: arboreal species jumped lower relative distances in three-dimensionally complex microenvironments.

Keywords: amphibian, frogs, defensive behavior, complexity of environment, jumping performance

2.1. Introduction

Predation is one of the major selective agents influencing the evolution of several morphological and behavioral patterns (Lima & Dill, 1990). An individual whose form and behavior reduces its risk of predation is more likely to survive to breed, therefore showing increased fitness (Lind & Cresswell, 2005). Two different broad classes of behavioral strategies have been selected in the context of predation events: avoiding detection by the potential predators and escaping after detection by predators

(Langerhans, 2006). The expression of antipredator behavior may change depending on the ecological context, given that the success of a defensive strategy depends on several factors such as the availability of shelter, the type of predator and the distance between the predator and the prey (Duvall et al., 1985). Escaping from predators, along with other ecologically relevant behaviors with foraging and searching for reproductive partners, involves locomotion (Dickinson et al., 2000). Given the ecological importance of locomotion, it is expected a direct relation between individual locomotor performance and fitness (Arnold, 1983). Although the original proposal by Arnold (1983) considered locomotor performance as the main target of natural selection, Garland & Losos (1994) posteriorly considered behavior as a potential "filter" between whole-organism performance and the direct effects of natural selection. When faced with any environmental challenge, such as facing a predator, an animal can opt between diverse motor patterns that vary in the intensity of maximum performance (Garland & Losos, 1994). Predator escape behavior is commonly invoked as a selective force acting on the morphology and physiology that directly influence performance and, in the latter instance, an individual's fitness (Arnold, 1983; Garland & Losos, 1994, Barros et al, 2016).

Habitat structural complexity may have a profound effect on ecological interactions, playing an important role in predator-prey interactions (Lima & Dill, 1990, Martín et al., 2005). Variation in habitat physical structure can influence prey detectability, given that more complex habitats usually provide physical and visual refuges for prey, while open habitats increase prey conspicuousness (Lima & Dill, 1990). Gomes et al. (2002), for example, observed that tree frogs (*Scinax hiemalis*) jumped shorter distances when exposed to a snake in their naturally complex

microenvironments in field than those distances jumped in response to disturbance in the open floor of the laboratory. Possibly, when these hylids are in their natural habitat, they jump just enough to reach the safety of another branch or leaf litter habitat, where they are camouflaged due to cryptic coloration (Gomes et al., 2002). Additionally, Vanhooydonck & Van Damme (2003) observed that lacertid lizards fled more when confronted with an aerial predator in open microhabitats than in densely vegetated ones. Moreover, when fleeing, most species sought refuge in the vegetation, supporting the hypothesis that vegetated patches may constitute a safe harbor for lacertids (Vanhooydonck & Van Damme; 2003).

Anurans represent a group of vertebrates whose body plan presents a series of phylogenetically conserved adaptations functionally associated with their high jumping performance (Zug 1972; Emerson 1978; Shubin & Jenkins, 1995). Several studies have suggested that the high anuran jumping performance evolved in response to selective pressures associated with predator avoidance (Gans & Parsons, 1966; Emerson, 1978; Marsh, 1994; but see Gans, 1961; Sandusky & Deban, 2012; for considerations involving prey capture). However, anurans present a series of stationary defensive strategies used alternatively to active escape in order to deal with the risk of predation (Williams et al., 2000; Toledo et al., 2005, 2010). Cryptic coloration, immobility, and antipredator postures serve as primary defenses by helping prevent detection or reducing predator attack success (Licht 1986; Williams et al. 2000). Accordingly, stationary strategies are more commonly observed in response to the approach of a snake, active escape is more frequently observed in response to physical contact with this predator (Gomes et al., 2002; Nishiumi & Mori, 2015)

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Although one hyliid (*Scinax hiemalis*) modulates distance jumped in response to predator stimulus according to microhabitat complexity (Gomes et al., 2002), the level of generalization this modulation for other anurans remains unknown. The level of three-dimensional microenvironmental complexity probably varies among arboreal, terrestrial and semi-aquatic anurans, offering different possibilities to avoid predation without using maximum jumping performance. In this way, it is possible that anuran species differing in microhabitat use can also differ in their plasticity of antipredator behavior. In the last decades, a large number of comparative studies have been conducted on the evolution of jumping performance in anurans, their correlations with microhabitat use and life history, as well as their morphological and physiological bases (Gans & Parsons, 1966, Zug, 1972, Emerson, 1978, Marsh, 1994, Gomes et al., 2009, Moen et al., 2013). However, the relations between the locomotor performance and antipredator behavior within the context of diversification of habitats and microhabitat use remain poorly explored for this phylogenetic group. This gap in knowledge about the evolutionary history of frogs is paradoxical, given the potential of behavior for acting as a filter between selection and performance as emphasized by Garland & Losos (1994). Here, we investigated: (A) at intraspecific level, the influence of the microenvironmental complexity in the manifestation of antipredator behavior; and (B) at interspecific level, the role of microhabitat use in the evolution of antipredator behavior and locomotor performance in Brazilian anuran amphibians. The following hypotheses were tested: (1) Individuals of a given species more likely display passive response to approach, and active responses to touch by a predator snake; (2) Individuals of a given species more likely exhibit passive responses (in opposition to flee) when stimulated by a predator in microenvironments characterized by a greater structural

complexity; (3) Individuals of a given species more likely exhibit shorter jumps (relative to maximal performance) in response to predatory stimulus in microenvironments characterized by a greater structural complexity; (4) Anuran species characterized by different microhabitat use should differ in the proportion of active responses to predatory stimulus in microenvironments with different levels of complexity; and (5) Anuran species characterized by different microhabitat use should differ in the relative distances jumped in response to predatory stimulus in microenvironments with different levels of complexity.

2.2. Materials and methods

2.2.1. Species and collection localities

Males from 17 species of anurans were collected in several localities from Brazil: *Leptodactylus labyrinthicus* (N= 5), *Hypsiboas faber* (N= 9) e *Physalaemus cuvieri* (N= 3) were collected at the countryside in Luiz Antônio, SP (21°33'05"S; 47°39'16"W) in 30 November 2013. *Physalaemus cuvieri* (N= 5) and *Hypsiboas bischoffii* (N=2) were collected at the Estação Biológica de Boracéia, Salesópolis, SP (23°39'13"S; 45°53'22"W) between 20 and 23 January 2014. *Physalaemus olfersii* (N= 5), *Aplastodiscus leucopygius* (N= 8), *Scinax hayii* (N=5) and *Scinax crospedospillus* (N= 8) were collected in Botanical Garden of São Paulo, São Paulo, SP (23°32'51" S; 46°38'10" W) in 15 February 2014. *Thoropa taophora* (N= 6) were collected at the coast in Ubatuba, SP (23°26'02"S; 45°04'16"W) between 26 and 28 March 2014. *Proceratophrys boiei* (N=5) were collected at the Parque Estadual Intervales, Ribeirão Grande, SP (24°16'24"S; 48°25'2"W) between 13 and 17 October 2015. *Leptodactylus*

fuscus (N=5) was collected at the University of São Paulo, São Paulo, SP (23°33'51.6"S; 46°43'48.1"W) between 1 and 3 November 2015. *Leptodactylus podicipinus* (N= 6), *Dermatonotes muelleri* (N= 5), *Pseudis platensis* (N=8), and *Rhinella schneideri* (N=3) were collected at the countryside of Selvíria, MS (20°22'02"S; 51°25'08"W) between 23 and 24 November 2015. *Hylodes asper* (N=5) and *Cycloramphus boraceiensis* (N=6) were collected at the coast in Ubatuba, SP (23°26'02"S; 45°04'16"W) between 15 and 19 December 2015.

2.2.2. Animal maintenance in the laboratory

Anurans were placed in plastic boxes and transported to the laboratory in the University of São Paulo (Brazil), where they were housed individually, in terraria of 13x30x28 cm for the smaller species and in terraria of 27 x45 x 32 cm for the for larger species (more than 80 mm SVL). Each terrarium contained fragments of vegetation, some stones and fresh water permanently available, and animals were fed weekly with small live cockroaches. All experiments were carried out within two weeks of the animals being captured. When individuals did not appear visually healthy, the next measurements were cancelled. The terraria were maintained in a climate-controlled room at 25°C ($\pm$ 1°C) and 12h: 12h dark: light cycle, with the darkness starting at 7:40 pm. The collections were performed under approved permission of Ministério do Meio Ambiente, ICMBio, SISBio (License number: 49250-1) and laboratory procedures were performed under the approval of the Comissão de Ética no Uso de Animais (CEUA) do Instituto de Biociências da Universidade de São Paulo (Protocol number: 192/2013). Field work at Parque Estadual Intervales was conducted under authorization of the

“Coordenadoria de informações técnicas, documentação e pesquisa ambiental” (COTEC, process number 611/2015), Instituto Florestal, Secretaria do Meio Ambiente. For the other localities, no specific authorizations were required.

2.2.3. Microhabitat use classification

Based on Haddad et al. (2008), each of the 17 species was classified into one of the following microhabitat use: aquatic (living predominantly in or near water), arboreal (living above the ground on vegetation), fossorial (living predominantly amid leaf litter or in burrows), terrestrial (living on the ground) and torrent (living closely associated with rocky, fast-flowing streams).

2.2.4. Phylogenetic tree

A composite tree for the 17 anuran species (Figure 2-1) was compiled based mainly on topology and divergence times proposed by Pyron (2014), which is the most comprehensive current phylogenetic hypothesis for anurans. Phylogenetic information was available for 11 species in Pyron (2014). Some adjustments were necessary to include the other 6 species, as follows: to include *Leptodactylus fuscus*, the topological position of *L. longirostris* was assumed (Pyron, 2014), given that *Leptodactylus fuscus* is a sister species of *L. longirostris* according by Pyron & Wiens (2011). *Physalaemus olfersii* was inserted diverging from *P. cuvieri* and considering the divergence time for the genus *Physalaemus* (Pyron, 2014). *Hylodes asper* was inserted diverging from *H. phyllodes* and considering the divergence time for the genus *Hylodes* (Pyron, 2014). To include *Pseudis platensis*, the divergence time for the genus *Pseudis* and the other clade

formed by species of the family Hylidae in Pyron (2014) was assumed. *Scinax hayii* was inserted diverging from *S. crospedospilus* and considering the divergence time for the genus *Scinax* (Pyron, 2014).

Figure 2-1: Composite phylogenetic tree for the 17 anuran species included in the present study, with topology and divergence times based on the literature (Pyron & Wiens, 2011; Pyron, 2014). Each microhabitat use is represented by a different color symbols: blue triangles = aquatic, green lozenge= arboreal, brown square= fossorial, yellow triangle= torrent, red circle= terrestrial.

2.2.5. Antipredator behavior tests in the laboratory

To standardize and quantify the occurrence of different antipredator responses in the lab at different microenvironments, we used as predator the snake *Liophis miliaris* (Dipsadidae), which is widely distributed in the state of São Paulo. This is a medium size, semiaquatic and diurnal-nocturnal snake, usually associated with streams, lakes, and other bodies of water, and its diet is based mainly on anurans and fishes (Marques

et al., 2001). All snakes (N= 8) used in the present study came from donations to the Butantan Institute (state of São Paulo, Brazil) and were borrowed from this Institute. We maintained the individuals in the Ectothermic Animals Facility of the Department of Physiology of the Biosciences Institute, University of São Paulo. Snakes were individually maintained in 27 cm × 45 cm×32 cm transparent plastic boxes provided with fragments of vegetation, some stones, resting place and fresh water permanently available, and animals were fed weekly with small live fishes. The terraria were maintained in a climate-controlled room at 25°C ($\pm$ 1°C) and 12: 12 dark: light cycle, with the darkness starting at 07:40 pm. For the tests, the snakes had the mouth surrounded by sealing tape thread, avoiding predation but allowing the snake to touch the anurans with the tongue. A rubber ring was fitted around the snake, which was then tethered to a fishing rod (100 cm) using 10 cm of string.

Antipredator behavior tests, as well as the locomotor performance tests (described below), were carried out behavioral tests during the night (08:00pm - 01:00am) because the most species are predominantly nocturnal (Haddad et al., 2008). The only exception was the diurnal species *Hylodes asper*, which had their behavioral tests carried out during the day (02:00 - 04:00pm). To conduct the data collection, the observer used a headlamp with red light (500 Lumens), and data collection was performed within two weeks of the animals being captured. Simulated predation tests were conducted in an arena with 200 x 90 x 70 cm in a climate-controlled room at 25°C ($\pm$ 1°C). The tests were repeated for each anuran, in three environments of distinct complexity: (1) experimental arena completely empty; (2) leaf litter was used to cover the floor of the experimental arena; (3) leaf litter and plastic bushes with 50 centimeters

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height and spaced per 10 centimeters were used to cover the experimental arena. The order of tests in different arenas was randomized for each individual, allowed a resting day between experimental rounds to minimize handling effects. Manipulations during the experiment were performed by the same experimenter (JMC) in order to control for experimental conditions, and care was taken to minimize disturbance to the frogs.

Each anuran was inserted in the test arena through a small lateral door, being positioned at a distance of five centimeters from the entrance. After 30 minutes, the anuran was located by visual inspection in the arena, and the antipredator behavior was elicited by carefully placing the snake near a frog, always attempting a first contact between the snake and the rear end of the frog (Gomes et al., 2002). Frogs reacted to the mere approach of the snake, exhibit passive response by immobility or/and antipredator postures, or an active escape response by jumping away, even before any physical contact had occurred. Accordingly, behaviors were recorded as soon as the snake was released. The snake consistently moved its head toward frogs and tongue-flicked. Three consecutive contacts were allowed between the anurans and the snake before categorizing the anurans' behavior as either active or passive. Passive behavioral responses were considered when anurans remained immobile and active responses were considered when frogs jumped within 3 sec of one of three contacts. When anurans jumped away, the number of jumps was counted and fleeing distance was estimated (Gomes et al., 2002). Anurans were stimulated to jump on the open floor of the laboratory (maximal jumping performance), and in response to the predator in arenas with different micro environmental complexity. We calculated the proportion of

distance jumped in response to the predator in each arena relative to the maximal jumping performance.

2.2.6. Locomotor performance tests in the laboratory

Individuals were maintained in plastic containers provided with freely available water and some type of shelter in a climate-controlled room at 25°C. Two days after the end of antipredator tests, locomotor tests were performed on the open floor (120 cm width and 285 cm length). The animals were stimulated to jump on the floor covered by Ethylene Vinyl Acetate sheets (EVA) by tapping gently in the posterior region of the anuran for six consecutive times. Starting and landing points were marked on the floor, and later, the distance between marks was measured. The longest jump of the series was used as the best estimate of maximum jumping performance. Snout-to-vent length (SVL) was then measured from proximal edge of inner metatarsal tubercle to tip of outstretched fourth toe tip of snout to posterior end of the ischium for all individuals, using digital calipers to the nearest 0.01 mm.

2.2.7. Statistical analysis

2.2.7.1. Intraspecific analyses

We coded the antipredator response types as 0 or 1, where 0= passive antipredator response and 1= active antipredator response. To test the hypothesis 1 (about individual variation in antipredator responses to approach and touch by predator), we compared the proportions of 0 and 1 between the stimuli in each

microenvironment by using the nonparametric Cochran Q-test. The Cochran Q-test is derived from randomized blocks experimental designs, being specific for dichotomous nominal data that can be coded as 0 or 1 (Zar, 1984). To test the hypothesis 2 (about individual variation in antipredator responses among the microenvironments to the same stimulus), we compared the proportions of 0 and 1 among the three microenvironments for the first (approach) and second (touch) stimuli independently. Here we also used Cochran test, followed by the multiple comparison procedure of Marascuilo and McSweeney (1967). To test the hypothesis 3 (about the individual variation in jumped distance among the microenvironments to the same stimulus), we compared the jumped distances by individuals in the three microenvironments by using an ANCOVA, in which body size were entered as covariate.

2.2.7.2. Interspecific analyses

Anurans were exposed to predatory simulation tests in response to the predator. In order to test if anuran species characterized by different microhabitat use differ in proportional changes in active responses among microenvironment with different levels of complexity, we calculated the proportion, for each species, of active responses to the predator in each arena (AR), and for both stimuli (approach and touch). We implemented multiple linear models with AR as the dependent variable, SVL as an independent variable, and microhabitat use as a factor. An interaction term between SVL and microhabitat use was additionally included in the model. These multiple linear models were implemented in the following contexts: (1) Proportion of **Active**

Responses by frogs exposed to **Approach** by a predator snake in **Empty** arena (ARAE); (2) Proportion of **Active Responses** by frogs exposed to **Approach** by a predator snake in arena with **Leaf** litter (ARAL); (3) Proportion of **Active Responses** by frogs exposed to **Approach** by a predator snake in arena with **Bushes** (ARAB); (4) Proportion of **Active Responses** by frogs exposed to **Touch** by a predator snake in **Empty** arena (ARTE); (5) Proportion of **Active Responses** by frogs exposed to **Touch** by a predator snake in arena with **Leaf** litter (ARTL), and (6) Proportion of **Active Responses** by frogs exposed to **Touch** by a predator snake in arena with **Bushes** (ARTB).

In order to test if anuran species characterized by different microhabitat use differ in proportional changes in relative jumping distances among microenvironment with different levels of complexity, we calculated the proportion, for each species, of jumped distance in each arena relative to the maximal jumping performance (JD), and for both stimuli (approach and touch). We implemented multiple linear models with JD as the dependent variable, SVL as an independent variable, and microhabitat use as a factor. An interaction term between SVL and microhabitat use was additionally included in the model. These multiple linear models were implemented in the following contexts: (1) Proportion of relative **Jumping Distances** by frogs exposed to **Approach** by a predator snake in **Empty** arena (JDAE); (2) Proportion of relative **Jumping Distances** by frogs exposed to **Approach** by a predator snake in arena with **Leaf** litter (JDAL); (3) Proportion of relative **Jumping Distances** by frogs exposed to **Approach** by a predator snake in arena with **Bushes** (JDAB); (4) Proportion of relative **Jumping Distances** by frogs exposed to **Touch** by a predator snake in **Empty** arena (JDTE); (5) Proportion of

relative **Jumping Distances** by frogs exposed to **Touch** by a predator snake in arena with **Leaf** litter (JDTL), and (6) Proportion of relative **Jumping Distances** by frogs exposed to **Touch** by a predator snake in arena with **Bushes** (JDTB).

The composite tree for the species tested (Figure 2-1) was incorporated into these regressions by using generalized least squares (GLS) regression techniques. Analyses were performed using R statistical computing environment (R Development Core Team 2010) and SPSS version 17 (SPSS Inc., Chicago, IL, USA).

2.3. Results

2.3.1. Intraspecific results

Anurans differ in the proportion of passive and active antipredator responses between the stimuli (approach and touch) in each microenvironment, as observed in Table 2-1 (Cochran test; p-value). Active antipredator responses (jumping away) were more frequent to the touch than predator's approach for most of the species in three microenvironments tested. The contrast between stimuli is amplified in microenvironments characterized by a greater structural complexity, as significant differences in antipredator responses were observed for a higher number of species in leaf litter and bushes arenas (9 and 8 species respectively), when compared with empty arena (5 species) in Table 2-1. Additionally, individuals of some species were consistently more likely to exhibit either an active or passive antipredator response, regardless of the type of stimulus or microenvironment. For example, individuals of *L. labyrinthicus* showed just passive responses in empty or leaf-litter microenvironment for both stimuli (Table 2-1). Otherwise, only active responses were observed for

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individuals of *L. fuscus* in empty arena; for individuals of *P. boiei* in leaf litter, and for *P. platensis* in bushes arena (Table 2-1).

The microhabitat complexity affected the frequency of active responses to the predator approach in only three of the 17 species tested: *P. platensis* ($p= 0.050$), *L. fuscus* ($p= 0.041$) and *P. boiei* ($p= 0.050$) (Cochran test; p-value - Table 2-2). The Marascuilo test showed that, for *P. platensis* and *L. fuscus*, the frequency of active responses was higher in empty arena when compared to the arenas with leaf litter and bushes. For *P. boiei*, the Marascuilo test showed that the frequency of active responses was higher in the leaf litter arena than in empty arena and arena with bushes. In response to the snake's touch, most of the anurans fled, regardless of the microenvironments tested (Table 2-2).

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Table 2-1. Percent of passive and active antipredator responses in anurans species exposed to approach and touch by a predator snake in each microenvironment (empty/ leaf litter /bushes).

Species	Responses	Microenvironments								
		Empty			Leaf litter			Bushes		
		Stimulus		p-value	Stimulus		p-value	Stimulus		p-value
		approach	touch		approach	touch		approach	touch	
<i>Rhinella schneideri</i>	P	67%	33%	0.317	67%	67%	1.000	100%	33%	0.157
	A	33%	67%		33%	33%		0%	67%	
<i>Cycloramphus boraceiensis</i>	P	50%	17%	0.317	100%	33%	0.045	83%	0%	0.025
	A	50%	83%		0%	67%		17%	100%	
<i>Thoropa taophora</i>	P	33%	0%	0.157	17%	0%	0.317	67%	0%	0.045
	A	67%	100%		83%	100%		33%	100%	
<i>Aplastodiscus leucopygius</i>	P	38%	0%	0.083	75%	0%	0.014	75%	0%	0.014
	A	63%	100%		25%	100%		25%	100%	
<i>Hypsiboas bischoffii</i>	P	33%	0%	0.317	67%	0%	0.157	67%	0%	0.157
	A	67%	100%		33%	100%		33%	100%	
<i>Hypsiboas faber</i>	P	89%	11%	0.008	67%	22%	0.045	78%	33%	0.102
	A	11%	89%		33%	78%		22%	67%	
<i>Scinax crospedospilus</i>	P	63%	13%	0.045	63%	13%	0.045	88%	0%	0.008
	A	38%	88%		38%	88%		13%	100%	
<i>Scinax hayii</i>	P	50%	0%	0.083	33%	0%	0.045	50%	0%	0.083
	A	50%	100%		67%	100%		50%	100%	
<i>Pseudis platensis</i>	P	63%	13%	0.045	0%	25%	0.014	0%	0%	0.004
	A	38%	88%		100%	75%		100%	100%	

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<i>Hylodes asper</i>	P	83%	0%	0.025	33%	0%	0.045	67%	33%	0.157
	A	17%	100%		67%	100%		33%	67%	
<i>Physalemus cuvieri</i>	P	25%	0%	0.157	50%	0%	0.045	50%	0%	0.045
	A	75%	100%		50%	100%		50%	100%	
<i>Physalemus olfersii</i>	P	20%	0%	0.317	40%	0%	0.157	60%	0%	0.083
	A	80%	100%		60%	100%		40%	100%	
<i>Leptodactylus labyrinthicus</i>	P	100%	100%	1.000	100%	100%	1.000	100%	80%	0.317
	A	0	0		0	0		0	20%	
<i>Leptodactylus fuscus</i>	P	0%	0%	1.000	80%	0%	0.045	80%	0%	0.045
	A	100%	100%		20%	100%		20%	100%	
<i>Leptodactylus podicipinus</i>	P	33%	0%	0.157	50%	0%	0.083	100%	0%	0.014
	A	67%	100%		50%	100%		0%	100%	
<i>Dermatonotus muelleri</i>	P	100%	60%	0.157	100%	60%	0.157	100%	80%	0.317
	A	0%	40%		0%	40%		0%	20%	
<i>Proceratophrys boiei</i>	P	60%	0%	0.083	0%	0%	1.000	60%	0%	0.083
	A	40%	100%		100%	100%		40%	100%	

Cochran test, $p < 0.05$

Values with p-value > 0.05 are highlighted in boldface

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Table 2-2. Percent of passive and active antipredator responses in anurans at three different microenvironments (empty/ leaf litter/bushes) for the two stimuli: approach and touch independently.

Family	Species	n	Responses	Approach			p-value	Touch			p-value
				Microenvironments				Microenvironments			
				Empty	Leaf-litter	Bushes		Empty	Leaf-litter	Bushes	
Bufonidae	<i>Rhinella schneideri</i>	3	P	67%	67%	100%	0.368	33%	67%	33%	0.607
			A	33%	33%	0%		67%	33%	67%	
Cycloramphidae	<i>Cycloramphus boraceiensis</i>	6	P	50%	100%	83%	0.097	17%	33%	0%	0.223
			A	50%	0%	17%		83%	67%	100%	
	<i>Thoropa taophora</i>	6	P	33%	17%	67%	0.097	0%	0%	0%	1,000
			A	67%	83%	33%		100%	100%	100%	
Hylidae	<i>Aplastodiscus leucopygius</i>	8	P	38%	75%	75%	0.223	0%	0%	0%	1,000
			A	63%	25%	25%		100%	100%	100%	
	<i>Hypsiboas bischoffii</i>	3	P	33%	67%	67%	0.717	0%	0%	0%	1,000
			A	67%	33%	33%		100%	100%	100%	
	<i>Hypsiboas faber</i>	9	P	89%	67%	78%	0.549	11%	22%	33%	0.472
			A	11%	33%	22%		89%	78%	67%	
	<i>Scinax crospedospilus</i>	8	P	63%	63%	88%	0.449	13%	13%	0%	0.607
			A	38%	38%	13%		88%	88%	100%	
	<i>Scinax hayii</i>	6	P	50%	33%	50%	0.607	0%	0%	0%	1,000
			A	50%	67%	50%		100%	100%	100%	
	<i>Pseudis platensis</i>	8	P	63%	0%	0%	0.050	13%	25%	0%	0.223
			A	38%	100%	100%		88%	75%	100%	

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Hylodidae	<i>Hylodes asper</i>	6	P	83%	33%	67%	0.717	0%	0%	33%	0.135
			A	17%	67%	33%		100%	100%	67%	
Leptodactylidae	<i>Physalemus cuvieri</i>	8	P	25%	50%	50%	0.449	0%	0%	0%	1,000
			A	75%	50%	50%		100%	100%	100%	
	<i>Physalemus olfersii</i>	5	P	20%	40%	60%	0.223	0%	0%	0%	1,000
			A	80%	60%	40%		100%	100%	100%	
	<i>Leptodactylus labyrinthicus</i>	5	P	100%	100%	100%	1,000	100%	100%	80%	0.368
			A	0	0	0		0	0	20%	
	<i>Leptodactylus fuscus</i>	5	P	0%	80%	80%	0.041	0%	0%	0%	1,000
			A	100%	20%	20%		100%	100%	100%	
Microhylidae	<i>Dermatonotus muelleri</i>	5	P	100%	100%	100%	1,000	60%	60%	80%	0.717
			A	0%	0%	0%		40%	40%	20%	
Odontophrynidae	<i>Proceratophrys boiei</i>	5	P	60%	0%	60%	0.050	0%	0%	0%	1,000
			A	40%	100%	40%		100%	100%	100%	

Cochran test, multiple pairwise comparisons comparison: Marascuilo test, $p < 0.05$

Values with p-value > 0.05 are highlighted in boldface

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Anurans modulated distance jumped in response to predator according to environmental complexity. The data in Table 2-3 reflect average values for jumped distance in anurans species exposed to approach and touch by a predator snake in each microenvironment. For the majority of the species, the fleeing distance decreased with complexity of environmental. Furthermore, fleeing distance was higher in response to snake's touch when compared with snake's approach in the three microenvironments tested, for most of the species (Table 2-3). Only four species jumped in response to the snakes' approach (Table 2-3). Individuals of *P. boiei* performed longer relative jumps in the empty arena when compared to the arenas with leaf litter and bushes (ANCOVA $F_{2,3} = 45.771$; $p=0.021$), while individuals of *P. cuvieri* (ANCOVA $F_{2,3} = 2.589$; $p=0.291$), *S. hayii* ($F_{2,3} = 0.097$; $p=0.955$), and *T. taophora* ($F_{2,3} = 0.913$; $p=0.561$) did not differ in relative jumping distances among the microenvironments tested. In response to snakes' touch, individuals of *H. faber* (ANCOVA $F_{2,12} = 6.537$; $p=0.008$) and *P. cuvieri* ($F_{2,21} = 22.830$; $p=0.0001$), performed longer relative jumps in the empty arena when compared to the arenas with leaf litter and bushes. Individuals of *L. fuscus* ($F_{2,12} = 7.164$; $p=0.006$) performed longer relative jumps in the arena with bushes, while for individuals of *Cycloramphus boraceiensis* ($F_{2,9} = 2.891$; $p= 0.102$), *Aplastodiscus leucopygius* ($F_{2,12} = 0.677$; $p=0.584$), *Hypsiboas bischoffii* ($F_{2,3} = 1.719$; $p=0.388$), *Scinax hayii* ($F_{2,12} = 2.352$; $p=0.128$), *Scinax crospedospilus* ($F_{2,9} = 0.836$; $p=0.511$), *Thoropa taophora* ($F_{2,12} = 1.274$; $p=0.331$) and *Leptodactylus podicipinus* ($F_{2,12} = 2.486$; $p=0.115$), *P. boiei* ($F_{2,12} = 3.049$; $p=0.074$), *P. olfersii* ($F_{2,9} = 3.418$; $p=0.073$) and *P. platensis* ($F_{2,15} = 3.310$; $p=0.051$) there was no significant differences in the relative jumping distances among the micro-environments.

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Table 2-3. Jumping distance (mean $\pm$ standard deviation) of anuran species exposed to approach or touch by a predator snake in the three microenvironments.

Species	n	Empty	Microenvironments			
			n	Leaf-litter	n	Bushes
Approach						
<i>Rhinella schneideri</i>	1	1.96	1	1.23	0	
<i>Cycloramphus boraceiensis</i>	3	9.00 ± 3.84	0		1	6.43
<i>Thoropa taophora</i>	4	5.23± 2.17	5	9.04 ± 2.31	2	4.10 ± 1.43
<i>Aplastodiscus leucopygius</i>	3	8.57 ± 0.50	3	6.27 ± 0.63	1	12.95
<i>Hypsiboas bischoffii</i>	1	9.37	1	10.31	0	
<i>Hypsiboas faber</i>	1	12.98	3	6.67 ± 1.67	2	6.62 ± 2.63
<i>Scinax crospedospilus</i>	3	15.1 ± 2.38	1	14.47	1	13.91
<i>Scinax hayii</i>	3	19.79 ± 8.08	2	20.84 ± 13.88	2	15.20 ± 11.40
<i>Pseudis platensis</i>	3	8.91 ± 1.17	0		0	
<i>Hylodes asper</i>	1	9.03	1	8.21	1	4.27
<i>Physalaemus cuvieri</i>	6	8.44 ± 1.17	4	6.94 ± 1.16	4	4.89 ± 0.76
<i>Physalaemus olfersii</i>	3	8.85 ± 5.43	2	7.86 ± 2.28	1	4.76
<i>Leptodactylus fuscus</i>	1	5.49	1	4.21	5	12.06 ± 4.61
<i>Leptodactylus podicipinus</i>	3	4.79 ± 1.40	3	9.66 ± 2.92	0	
<i>Proceratophrys boiei</i>	2	3.96 ± 0.45	5	3.14 ± 0.52	2	2.58 ± 0.07
Touch						
<i>Rhinella schneideri</i>	1	2.27	1	1.85	1	1.54
<i>Cycloramphus boraceiensis</i>	5	8.65 ± 2.75	4	8.12 ± 2.06	6	10.03 ± 2.15
<i>Thoropa taophora</i>	5	9.28 ± 2.56	5	9.9 ± 2.33	5	9.76 ± 3.17
<i>Aplastodiscus leucopygius</i>	5	9.16 ± 2.91	5	8.14 ± 3.63	5	9.94 ± 2.67
<i>Hypsiboas bischoffii</i>	2	11.8 ± 0.89	2	6.72 ± 1.44	2	7.25 ± 3.57
<i>Hypsiboas faber</i>	8	12.36 ± 3.14	7	6.12 ± 1.44	6	6.28 ± 1.82
<i>Scinax crospedospilus</i>	5	13.13 ± 4.07	5	15.97 ± 6.22	6	11.94± 1.86
<i>Scinax hayii</i>	5	15.56 ± 3.73	5	19.81 ± 7.26	5	9.91 ± 2.72
<i>Pseudis platensis</i>	7	20.68± 2.94	6	13.64 ± 5.96	8	14.63 ± 2.96
<i>Hylodes asper</i>	3	9.22± 4.77	3	9.87 ± 2.61	1	7.84
<i>Physalaemus cuvieri</i>	8	9.97 ± 2.12	8	6.73 ± 1.49	8	5.92 ± 1.46
<i>Physalaemus olfersii</i>	4	7.7 ± 4.25	4	6.96 ± 1.18	4	10.5 ± 6.26
<i>Leptodactylus fuscus</i>	5	9.59 ± 4.15	5	15.35 ± 3.10	5	15.77 ± 2.62
<i>Leptodactylus podicipinus</i>	5	10.26 ± 2.12	5	9.95 ± 2.09	5	7.43 ± 2.28
<i>Dermatonotus muelleri</i>	2	2.26 ± 0.30	2	2.17 ± 0.06	1	2.70
<i>Proceratophrys boiei</i>	5	4.72 ± 0.66	5	3.57 ± 0.94	5	3.50 ± 0.63
Distance jumped (body lengths)						

Distance jumped (body lengths)

2.3.2. Interspecific results

Body size influences the proportion of active responses by anurans exposed to the snake's approach in the empty arena ($p < 0.045$) and touch in the arena with bushes ($p < 0.032$; Table 2-4). Larger species in our study ($SVL > 93.29$ mm), such as

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Leptodactylus labyrinthicus, *Hypsiboas faber*, *Rhinella schneideri*, were more likely to exhibit passive responses in these situations. Species differences in microhabitat use in nature do not influence the proportion of active responses to snake's approach or touch ($p > 0.194$). In addition, *Dermanotonus muelleri* (SVL ranging from 52.56 to 57.77 mm, see Table 1-6 in Chapter 1) did not flee from the snake in the arena with bushes.

Table 2-4. Results for phylogenetic regression to the proportions of active responses by anuran species exposed to approach or touch by a predator snake at three microenvironments (empty/ leaf litter /bushes).

Dependent variable	<i>P</i> (SVL)	<i>P</i> (MH)	<i>P</i> (SVL : MH)
ARAE	0.045	0.218	0.193
ARAL	0.976	0.910	0.893
ARAB	0.732	0.885	0.859
ARTE	0.877	0.194	0.200
ARTL	0.295	0.465	0.454
ARTB	0.032	0.370	0.398

Values in each cell represent the P-value of pGLS (phylogenetic generalized least squares regression)

Values with p-value > 0.05 are highlighted in boldface

ARAE- Active Responses Approach Empty; ARAL- Active Responses Approach Leaf litter;

ARAB- Active Responses Approach Bushes; ARTE- Active Responses Touch Empty;

ARTL- Active Responses Touch Leaf litter; ARTB- Active Responses Touch Bushes;

SVL- snout-vent-length; MH- ecological classification of microhabitat use

Body size also influences the relative jumping distances performed by anurans in response to the snake's approach in the arena with bushes ($p < 0.0472$; Table 2-5). Relative jumping distances performed by anurans in response to snake's touch in the

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arena with leaf litter and bushes is influenced by interspecific differences in body size and microhabitat use ($p < 0.024$ and $p < 0.0325$ for the interaction term in the arenas with litter and bushes, respectively; Table 2-5). Larger species of arboreal anurans performed shorter relative jumping distances in arenas with bushes and litter (Figure 2-2).

Table 2-5. Results for phylogenetic regression to the proportions of relative jumping distances by frogs exposed to approach or touch by a predator snake at three microenvironments (empty/ leaf litter/bushes).

N	Dependent variable	<i>P</i> (SVL)	<i>P</i> (MH)	<i>P</i> (SVL : MH)
15	JDAE	0.453	0.893	0.821
13	JDAL	0.163	0.308	0.290
13	JDAB	0.047	0.146	0.179
16	JDTE	0.383	0.716	0.715
16	JDTL	0.036	0.026	0.024
17	JDTB	0.016	0.040	0.032

Values in each cell represent the P-value of pGLS (phylogenetic generalized least squares regression)

Values with p-value > 0.05 are highlighted in boldface

JDAE- Jumped DistanceApproach Empty ; JDAL- Jumped DistanceApproach Leaf litter ;

JDAB- Jumped DistanceApproach Bushes; JDTE- Jumped DistanceTouch Empty ;

JDTL- Jumped DistanceTouch Leaf litter; JDTB- Jumped DistanceTouch Bushes;

N- number of species; SVL- snout-vent-length; MH- ecological classification of microhabitat use

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(a) Leaf litter

(b) Bush

Figure 2-2: Relationship between the relative jumping distances and body size (SVL) by different anuran species that differ in microhabitats use exposed to touch by a predator snake in the environment with: (a) leaf litter and (b) bush.

2.4. Discussion

Our comparative study about antipredator behavior in anurans revealed that, when disturbed, individuals of different species display a variety of antipredator behaviors from flight and immobility, to defensive postures. Our approach, encompassing analyses across individuals and across species, allow us to provide insights into how the microenvironmental complexity influences the behavioral responses of anurans adapted to different microhabitat use. Intraspecific e interspecific analyses showed that the relative frequency of active/passive responses is heavily dependent on the type of predatory stimulus applied to the anurans (approach/touch), and varies according to structural complexity of the environment. Additionally, anurans modulated distance jumped in response to predator's touch according to environmental complexity, and an interspecific variation associated with microhabitat use was observed.

Our analyses suggest that anuran species are more likely to exhibit passive antipredator responses following the approach of a predator, such as a snake, whereas they are more likely to show an active antipredator response after physical contact with a predator. These results are consistent with experiments on salamanders (Ducey & Brodie, 1983), and corroborate the study with treefrogs (Gomes et al., 2002) which show that individuals respond in different ways to different stimuli and active antipredator responses (jumping away) were more frequent to the touch than predator's approach. In our investigation, immobility was exhibited more frequently by anurans exposed to approach by a predator. For many anuran species, immobility is crucial to maintain crypsis and it is a major component of behavioural defence against predation

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(Cooper et al., 2008b). In this way, immobility was expected to be more frequent in response to approach than to touch by a predator. Previous studies have shown the habitat structural complexity can also play an important role in predator-prey interactions. In general, preys foraging in microhabitats with greater vegetation cover tend to allow greater proximity of predators than those foraging in open areas (Lima & Dill 1990; Martin & López, 2000). In our study, immobility was also exhibited more frequently when anurans were exposed to predatory stimulus in structurally complex environments, whereas fleeing decreased with microenvironmental complexity. These results suggest that microhabitat covering is associated with greater protection against detection of anurans by predators. Thus, immobility, associated with remaining cryptic, is more important and probably effective, to the approach than predator's touch, and also in environments more structurally complex.

Our results suggest that, in general, anurans from different species jump short distances in response to predators 'approach, whereas relative jumping distance increases after physical contact with a predator. Gomes et al, (2002) observed that individuals of treefrogs (*Scinax hiemalis*) that are able to jump quickly are more likely to exhibit active responses when threatened by snakes (Gomes et al., 2002). We also observed that relative jumping distances was also influenced by microenvironmental complexity; given that shorter jumping distances were more frequently observed in microenvironments with leaf litter or bush vegetation when compared to open arena. These results are consistent with those observed by Martin et al. (2005) for *Rana perezii*, in which individuals jumped relatively greater distances in microenvironments without aquatic vegetation than when aquatic vegetation was present (Martin et al., 2005). In

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this way, anurans seem to modulate both the type of behavioral response and the distance jumped in relation to the characteristics of the microenvironment.

Antipredator behavior was also affected by body length. Our interspecific comparative analysis showed that larger species remained motionless more frequently than smaller ones when approached by a potential predator. Several studies have emphasized that passive antipredator behavior is commonly associated with the presence of the production of granular secretions and these secretions can be viewed as the ultimate in secretory defense (Toledo et al., 2005; Maciel, 2008). Accordingly, two of the larger anuran species tested in our study (*Leptodactylus labyrinthicus* and *Rhinella schneideri*), are characterized by the production of secretory defenses and frequently remained motionless when stimulated with the snake. However, it is also possible that these results are due to the fact that all the species, independently of their mean sizes, were tested in the same arena, and using the same snakes as predators. It is possible that arena size, mainly when arranged for complex microenvironments, limited the antipredator behavior in some way. It is equally plausible that the predatory stimulus given by the snakes used were not equivalent for all the anuran species along the size range tested, since predator-prey interactions are inherently size-dependent (Nakazawa et al., 2013). Further research is required to test the importance of scaling of the arenas and predators used on antipredator behavior of anurans of different body sizes.

Interestingly, anuran species adapted to different microhabitat use vary in distances jumped relative to maximum jumping performance in microenvironments characterized by different structural complexities, when threatened by a predatory snake. Particularly, the arboreal species tested tended to flee from the snakes by jumping shorter relative distances when subjected to arenas with bushes. A similar

situation has been found, at intraspecific level, in treefrog (*Scinax hiemalis*), in which individuals exposed to a snake in the field jumped distances that were about 40% shorter than those observed in frogs exposed to predatory stimulus at open floor in the laboratory (Gomes et al., 2002). In this way, these treefrogs jumped shorter relative distances, targeted to specific sites of landing, when exposed to three-dimensionally complex environments, (Gomes et al., 2002). The results from the present interspecific analysis allow the generalization of this modulation of relative jumping distances by microenvironmental complexity, previously observed for *S. hiemalis*, for different phylogenetic groups of hylids. This difference in relative distances jumped in arboreal hylids when threatened by predators in different microenvironments may be related to the efficiency of camouflage under different conditions. Many green arboreal anurans, for example, are frequently found at the upper surfaces of leaves, where its green coloration makes it inconspicuous (Glime & Boelema, 2015).

According to Duellman & Trueb (1986), species with cryptic coloration tend to jump less, fleeing predators with only one jump and remaining motionless. Martín et al. (2005) found that individuals of the Iberian green frogs (*Rana perezi*) in the field also modulate relative distances jumped when threatened according to the presence of vegetation cover within and at the edge of the water. In the absence of aquatic vegetation, the individuals jumped relatively greater jumped distances (Martin et al., 2005). In the present study, only arboreal species modulated their relative jumped distances according to the microenvironmental complexity. The absence of association between relative jumped distances and microenvironmental complexity observed in the present study for anuran species adapted to other microhabitat use may be to lower representation of species in certain categories (aquatic species, for example). In

addition, we did not use a large array of microenvironmental types in the present study. The inclusion of a water body in the arena, for example, might amplify the chances to observe modulatory responses of relative jumped distances under predatory stimulation in anurans adapted to other microhabitat uses.

In summary, our study used an integrative approach to understand the interaction of factors influencing the antipredator responses of anurans. We showed that the antipredator behavior in anurans is strongly influenced by ecological factors, such as environmental complexity and stimulus type. Antipredator behaviors classified as passive, such as, immobility were mainly observed with the approach of the predator, while active behaviors were the most frequent response to predator's touch. Additionally, the percentage of active responses decreased in microenvironments characterized with greater structural complexity. The distance jumped was also modulated in response to the type of predatory stimulus, given that relative jumping distances were higher in response to the predator's touch than approach. Moreover, anurans jumped lower relative distances in response to predator's touch in microenvironments characterized by greater structural complexity. At interspecific level, arboreal species jumped lower relative distances in three-dimensionally complex microenvironments when compared to species adapted to other microhabitat use. Thus, our results also support an evolutionary association between antipredator responses in anurans and microhabitat diversification.

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**3. CAPÍTULO 3: Escape strategies and refuge use by Australian
frogs under simulated predation risk**

Escape strategies and refuge use by Australian frogs under simulated predation risk

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Abstract

We investigated whether Australian frogs modify their antipredator strategies under simulated predation risk in areas with different microhabitat structure. We examined, in the laboratory, the responses to simulated predation events of four species that differ in microhabitat use. When confronted with an open area, anurans preferred to jump to a refuge. However, when given the option to jump towards different types of refuges, individuals of *Litoria ewingii* and *Limnodynastes tasmaniensis* most often selected bush vegetation; *Crinia signifera* escaped preferentially to leaf litter and *Neobatrachus pictus* adopted immobility when stimulated instead of fleeing. Our data show that Australian frogs use bush vegetation, leaf litter and water as refuge areas when subjected to simulated predator. Moreover, there is interspecific variation in the choice of refuges potentially associated to diversification of microhabitat use.

Short communication

A number of factors can affect the risk of detection of prey by a predator. Several studies show that prey usually do not flee immediately upon detecting an approaching predator, but instead adjust their escape response to minimize costs of flight (Ydenberg & Dill, 1986; Martín, Luque-Larena, & López, 2005). Several factors have been shown to affect the escape decisions of prey such as microhabitat structure and the relative safety of potential refuges. Microhabitat structure can affect the probability of being detected by predators and may hinder their movements, decreasing predation risk (Lima, 1990; Martín & López, 1995a, 2000; Cooper, 1998). Additionally, several studies have shown that prey often respond to predator presence by increasing their use of refuges (Werner, Gilliam, Hall, & Mittelbach, 1983; Martín, 2001).

Anuran amphibians (frogs) present several phylogenetically conserved specializations of the body plan for saltatory locomotion (Shubin & Jenkins, 1995), and several studies have linked these with selective pressures related to escape from predators (Gans & Parsons, 1966; Zug, 1972, Emerson, 1978; Gomes, Rezende, Grizante, & Navas, 2009). Despite the constituent basic aspects of body shape in anurans being highly conserved, this group displays a wide variety of microhabitat occupation, including leaf litter, underground chambers, edges of ponds, reservoirs, streams and rivers, inside phytotelmata such as bromeliads, and on bush vegetation and tree branches (Haddad, Toledo, & Prado, 2008). This diversity in microhabitat preference is associated with the evolution of locomotor performance and hindlimb morphology. Terrestrial and fossorial species are generally characterized by lower jumping performance and shorter hindlimbs when compared with aquatic and arboreal

species (Zug, 1972, 1978; Emerson, 1979; Gomes et al., 2009; Moen, Irschick, & Wiens, 2013).

Antipredator behavior is also strikingly diverse in frogs, with alternative antipredator behaviors used in diverse behavioral contexts including active predator avoidance, escape behaviors and use of refuge sites (Lima & Dill 1990; Williams, Brodie, Tyler, & Walker, 2000, Toledo, 2005; Toledo, Sazima, & Haddad, 2010, 2011). Cryptic coloration, immobility, and antipredator postures serve as primary defenses by helping prevent detection or reducing predator attack success, but active escape behaviour is often needed (Licht 1986; Williams et al., 2000). Gomes, Bevier, and Navas (2002) observed that tree frogs (*Scinax hiemalis*) jumped shorter distances when exposed to a snake in the field than when disturbed in the open floor of the laboratory. Possibly, when these hylids are in their natural habitat, they jump just enough to reach the safety of another branch or leaf litter habitat, where they are camouflaged due to cryptic coloration (Gomes et al., 2002). Martín et al. (2005) also showed that escape decisions of Iberian green frogs (*Rana perezi*) in the field are influenced by microhabitat variables. Both the approach distance allowed to the predator and the distance jumped by the frogs in response to the predators' approach were positively correlated with the initial distance of the frog from the water's edge. Additionally, responses to predators were also dependent on vegetation cover within and at the edge of the water (Martín et al., 2005). Martín et al. (2005) concluded that such flexibility in the escape response might allow frogs to reduce predation risk without incurring excessive costs.

The importance of refuge sites in predator-prey interactions is widely recognized (Werner et al., 1983; Martín, 2001). However, the investigation of refuge site choice

remains largely unexplored in studies of antipredator behavior in frogs. We suggest that the choice between refuges should be modified by environmental factors that affect the level of predation risk, and that this choice varies between species with different microhabitat use (i.e. arboreal, terrestrial or fossorial). This study aimed to determine whether adult Australian frogs modify their antipredator strategies (escape strategies and refuge use) under simulated predation risk in areas with different microhabitat structure. Specifically, we tested if (1) frogs prefer to jump to a refuge (bush vegetation, leaf litter or water) instead of to an open area when stimulated, and if so, (2) individuals from species with different habits (arboreal, terrestrial or fossorial) differ in their preferences among the three types of refuges.

We collected data for four Australian frog species from November 2014 to January 2015. All individuals were adult males: *Litoria ewingii* (n=6) (Hylidae); *Limnodynastes tasmaniensis* (n=9) and *Neobatrachus pictus* (n=2) (Limnodynastidae); *Crinia signifera* (n=8) (Myobatrachidae). Specimens were captured by hand in South Australia (Adelaide metropolitan area and the southern Flinders Ranges, approximately 450km to the north). Frogs were placed in cloth bags and transported to the laboratory to the University of South Australia (Australia), where they were housed individually, in terraria of 130 x 130 x 160 mm for the small species (snout-vent length - SVL, in mm): *L. ewingii* (SVL= 33.88 ± 2.82), *L. tasmaniensis* (SVL= 40.15 ± 5.78) and *C. signifera* (SVL=22.09 ± 1.67) and in terraria of 280 x 190 x 360 cm for the larger species: *Neobatrachus pictus* (SVL=45.54 ± 1.85). Each terrarium contained a sandy substrate, some stones and fragments of vegetation, fresh water permanently available, and animals were fed weekly with small live crickets. All experiments were carried out within two weeks of the animals being captured. The terraria were maintained in a

climate-controlled room at 25°C ($\pm$ 1°C) and 12: 12 dark: light cycle. Frogs were acclimatized to these conditions for over 1 week prior to the experiments. All data were collected during darkness, when frogs were fully active (08:00 am and 12:00 am). For the behavioral observations, the observer used a headlamp with red light (500 Lumens). Animals were collected, housed and studied under a permit from the University of South Australia Animal Ethics Committee (protocol n° 33636). Previous authors (Tyler & Walker, 2011), have classified *L. ewingii* as arboreal (living above the ground on vegetation), *L. tasmaniensis* and *C. signifera* as terrestrial (living on the ground), and *N. pictus* as fossorial (living predominantly in burrows).

In order to test if anurans prefer to jump to a refuge (bush vegetation, leaf litter or water) instead of to an open area when stimulated, behavioural choice experiments were conducted in a 72 x 58 x 61cm arena with two options of escape route: the empty area (E) in one side of the arena and one of three micro-environments [bush vegetation (B), body of water (W) and leaf litter (L)] at the other side. As bush vegetation, were used artificial plants, because natural plants may exhibit changes in coloration and in leaves shape and we opted to standardize the methodology; as leaf litter were used dried leaves. The positions of the empty area and the microenvironment were random, resulting in the following possible combinations: E-B; E-W; E-L; B-E; W-E; L-E. All individuals were exposed to the six refuge combinations, and each individual was tested once a day. The order of exposure to each combination was random.

With the intention of testing if individuals from species with different microhabitats use (arboreal, terrestrial, fossorial) differ in their preferences among the three types of refuges, choice experiments were conducted one week later, in another arena measuring 72 x 80 x 61 cm with three microenvironment landing options: bush

vegetation (B), body of water (W) and leaf litter (L). Each combination of microenvironments created a group; all possible combinations resulted in six refuge groups for testing (B-W-L, B-L-W, W-B-L, W-L-B, L-B-W, L-W-B). All individuals were exposed to the six groups, and each individual was tested once a day. The order of exposure to each group was random.

Individuals were inserted in both arenas through a small door on the opposite side of microenvironment choice area. They were centrally positioned in the floor at 10 cm of distance and heads oriented to the microenvironment choices area. In order to simulate a predatory attack, individuals were encouraged to jump towards the microenvironments choice area by gently stimulating the posterior region of the anuran with a large pair of forceps (Brodie, 1977; Brodie, Williams and Tyler, 1998). In order to control for experimental conditions, the stimuli were always performed by the same experimenter (JMC), and care was taken to minimize disturbance of the frogs.

The results from both behavioral experiments were analyzed by using the nonparametric Cochran Q-test (Zar, 1984), where the response variable is a binary variable (i.e., there are only two possible outcomes, which are coded as 0 and 1). In this study: (1) when given refuge type was selected and (0) when the refuge was not selected. If the Cochran test rejects the null hypothesis of equally selected refuges, pairwise multiple comparisons can be made by applying the Marascuilo test ($p < 0.05$) which can be used to simultaneously examine comparisons between all refuges. Data from individuals of *N. pictus* were not included in the analysis because they did not choose fleeing in this context perhaps due to its body dimensions and fossorial habit (Tyler & Walker, 2011).

In the first experiment, for each species, Cochran's Q showed significant variation among the six refuge groups: *L. tasmaniensis* ($Q = 10.667$, $P < 0.001$), *L. ewingii* ($Q = 138.16.000$, $P < 0.0001$), *C. signifera* ($Q = 10.083$, $P < 0.001$). By inspection (Figure 3-1) all three species preferred to jump to a refuge (bushes, leaf litter or water) instead of to an open area when stimulated.

Figure 3-1: Number of choice by species with two possible routes of a potential predator escapes (refuge area x empty area). Refuges consisted of bush vegetation, leaf litter or water. *Limnodynastes tasmaniensis* (n=9), *Litoria ewingii* (n=6) and *Crinia signifera* (n=8).

In the second experiment, for each species: *L. tasmaniensis* ($Q = 14.111$, $P < 0.001$), *L. ewingii* ($Q = 25.500$, $P < 0.0001$) and *C. signifera* ($Q = 10.500$, $P < 0.005$), Cochran's Q showed significant variation in selection among the three types of refuges (bush vegetation, leaf litter or water). The Marascuilo test showed that, for *L. tasmanienis* and *L. ewingii*, the frequency of choice was higher in bush refuge when compared to the refuges with water and leaf litter. For *C. signifera* the Marascuilo test

showed that the frequency of choice was in the leaf litter refuge than in water and bush refuge. Figure 3-2: (a), (b) and (c).

Figure 3-2: Number of choice by species with three possible routes of a potential predator escapes (bush, leaf litter or water). Horizontal bars with an asterisk indicates significant difference between groups (Cochran test, multiple pairwise comparisons comparison: Marascuilo test, $p < 0.05$).

Our results showed that individuals from different species of Australian frogs prefer to seek refuge in more complex microhabitat as opposed to open area when exposed to a simulated predation. Bush vegetation, leaf litter and water were selected as refuges 75% of the time when opposed to open areas, suggesting that refuge selection was primarily determined by the need to find shelter from the simulated predator. Microhabitat characteristics, especially microhabitat cover, have been identified as an important factor determining risk assessment by prey (Martín et al., 2005). Generally, cryptic behavior offers greater protection against detection by predators when associated with microhabitat, while in open habitats the animals are more conspicuous (Lima & Dill, 1990). Accordingly, prey that forage in covered microhabitats permit shorter approach distances to the predator when compared to individuals foraging in open areas (Lima & Dill, 1990; Martín & López, 2000).

Although bushes, leaf litter and water can be considered refuges by individuals from different anuran species, our results emphasize the existence of interspecific variation in microenvironment choice as a refuge, when options are given. Individuals of *L. tasmaniensis*, a species classified as terrestrial by Tyler and Walker (2011), jumped more frequently to bush areas when stimulated. This species can be found in a number of habitats, particularly in grassy areas near waterholes (Tyler & Walker, 2011), and individuals from the genus *Limnodynastes* can change the colour of both the dark and the light body regions to match the background shades (Porter, 1972; Osorio & Srinivasan, 1991). Otherwise, individuals of the arboreal *L. ewingii* more often selected bush vegetation and frequently jumped to the top of the artificial plants when stimulated in the arena, where microenvironment was structurally more complex. Choosing

microenvironmental patches characterized by higher three-dimensional complexity possibly offer higher quality refuge from predation (Ydenberg & Dill, 1986, Cooper, 1998). Individuals of *C. signifera* more often sought microenvironment with leaf litter when stimulated, which might be related to the fact that they are small ground dwelling frogs with cryptic coloration which remains camouflaged among leaves that implies avoidance of detection by possible predators (Tyler & Knight, 2011).

Previous studies have emphasized that fossorial species are characterized by shorter hind limbs and shorter jumping distances when compared to arboreal and terrestrial species, suggesting the existence of a tradeoff between digging efficiency and jumping performance (Zug, 1972, 1978; Emerson, 1976; Gomes et al., 2009; Enriquez-Urzelai et al., 2015). Accordingly, the fossorial *N. pictus* adopted immobility in both simulated predation tests instead of fleeing, and displayed stationary anti-predator postures and body inflation, anti-predator behavioral patterns previously reported for this species (Williams et al., 2000).

Although fleeing by jumps was the most common reaction to the predation simulation for the other species studied, individuals of *L. tasmaniensis* exhibited a variety of behavioural responses that ranged from body inflated and elevated, or flattening the body with the touch of stimulus, following a single jump or series of jumps. Defensive calls were emitted by individuals of *L. ewingii* and individuals of *C. signifera* flattened the body against the substrate. These observations corroborate previous studies showing that Australian frogs display a great variety of defensive tactics against predation, from immobility to jumping (Williams et al., 2000).

In conclusion, our data show that Australian frogs use bush vegetation, leaf litter and water as refuge areas when subjected to simulated predator in the laboratory.

Moreover, our results show that there is interspecific variation in the choice of potential refuge when alternatives are given, and we observed a tendency associated with microhabitat, with arboreal species tending to prefer bushes while terrestrial species prefer leaf litter. However, more studies with more number of species and individuals are necessary to confirm these data. Although it is known that anurans may behave differently in a natural environment, however our experimental protocol provides information that antipredator behavior, including microhabitat choice when fleeing, can be studied effectively using the laboratory environment. Furthermore, here are some examples of antipredator behaviors that have been seen in nature. Both laboratory experiments and field observation play an important role and are complementary in behavior research.

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DISCUSSÃO E CONCLUSÕES GERAIS.

4. DISCUSSÃO E CONCLUSÕES GERAIS

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O presente estudo comparativo buscou investigar a associação entre caracteres morfológicos, desempenho locomotor e comportamento antipredatório com o uso do micro-habitat em anfíbios anuros. Nossos resultados mostram que o uso do micro-habitat representa uma importante pressão seletiva associada ao desempenho locomotor e a diversificação morfológica dos membros nos anuros, destacando a presença de múltiplos ótimos adaptativos para comprimento dos membros associados aos diferentes micro-habitats. Particularmente, espécies de anuros aquáticas, arborícolas e reofílicas (que vivem sobre pedras em pequenos riachos de corredeira), tendem a apresentar patas traseiras mais longas e saltar maiores distâncias, enquanto espécies fossoriais e terrestres apresentam membros posteriores mais curtos e menor desempenho de salto.

O atual estudo investigou também a influência do tipo de estímulo e a complexidade do microambiente na manifestação do comportamento antipredatório, assim como, o papel do uso de micro-habitat na evolução do comportamento antipredatório em anfíbios anuros. Os nossos resultados mostram que o comportamento antipredatório foi influenciado tanto pela complexidade ambiental quanto pelo tipo de estímulo apresentado. Comportamentos passivos, como a imobilidade, foram mais frequentes com a aproximação de um predador, enquanto comportamentos ativos, como a fuga, foram mais frequentes frente ao toque deste. Adicionalmente, a porcentagem de respostas ativas diminuiu em microambientes mais complexos. Anuros de diferentes espécies também saltaram maiores distâncias em resposta ao toque do predador, quando comparadas às distâncias saltadas em resposta à aproximação deste. Além disso, os anuros saltaram menores distâncias relativas em resposta ao toque do predador em microambientes caracterizados por maior complexidade estrutural. Nossas análises interespecíficas revelam também que espécies arborícolas saltaram distâncias relativas

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menores em microambientes tridimensionalmente complexos, quando comparadas às espécies adaptadas para outro uso de microhabitat.

Adicionalmente, habitats com diversidade espacial possuem uma maior disponibilidade de micro-habitats que podem ser utilizados como refúgios pelos anuros e, portanto, podem diminuir o risco de predação e afetar as decisões de escape da presa. Nesta etapa do estudo foi investigado se os anuros modificam suas estratégias antipredatórias (estratégias de escape e uso de refúgio) em áreas com diferentes estruturas de micro-habitat. Nossos resultados mostram que diferentes espécies de anuros selecionam microambientes com vegetação arbustiva, folhiço e água como áreas de refúgio, ao invés da área aberta quando submetidos a uma simulação predatória em laboratório. Além disso, há uma variação interespecífica na seleção dos refúgios, potencialmente associada à diversificação do uso de micro-habitat. Para as espécies terrícolas, a frequência de seleção foi maior no ambiente com arbustos ou folhiço quando comparadas com o ambiente com água. Já para a espécie arborícola, a frequência de seleção foi maior no ambiente com arbustos ao invés dos ambientes com folhiço ou água.

Desta forma, nossos resultados corroboram a hipótese de que modificações evolutivas da morfologia têm sido importantes na diversificação do desempenho locomotor ao longo da filogenia dos anuros, e que tais mudanças evolutivas estão associadas com a diversidade de micro-habitats. Adicionalmente, nossos resultados também suportam uma associação evolutiva entre respostas antipredatórias e a diversificação do micro-habitat nos anuros. Dado que anfíbios anuros exibem extensa variação fenotípica, ecológica e comportamental, estudos futuros investigando possíveis associações entre morfologia pélvica e uso micro-habitat, juntamente com dados de

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frequência de expressão de diferentes padrões de comportamento antipredatório, poderiam trazer novas informações sobre a evolução da morfologia associada ao uso do micro-habitat dos anuros e poderiam fornecer importantes considerações sobre os padrões de diversificação adaptativa.

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