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Jéssica Stephanie Kloh

TADPOLE DANCE:

Estratégias alimentares em girinos

Belo Horizonte

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"Tadpole dance: estratégias alimentares em girinos"

JÉSSICA STEPHANIE KLOH

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“...de baixo d´água tudo era mais bonito
mais azul, mais colorido …”

Arnaldo Antunes

Resumo geral

Os girinos são considerados importantes componentes das teias alimentares em ecossistemas aquáticos, no entanto, seus papéis tróficos permanecem pouco estudados em comparação com outros grupos de vertebrados aquáticos. A morfologia das larvas indica aspectos gerais das dietas, porém poucos estudos até então exploraram de fato as composições alimentares dos girinos, principalmente na região Neotropical. Ao longo do desenvolvimento dessa tese, buscamos explorar essas lacunas, desenvolvendo uma serie de experimentos com foco nas diferentes estratégias alimentares utilizadas por girinos, principalmente em relação ao consumo de grãos de pólen. Elaboramos 4 capítulos, intitulados, Capítulo 1: Tadpole dance: the gymnastics of *Scinax machadoi* larvae to feed on pollen, onde buscamos avaliar e descrever alterações no comportamento de forrageio dos girinos de *Scinax machadoi* em favor de grãos de pólen na superfície d'água. Capítulo 2: No choice is not a choice: food selectivity occurs in different ontogenetic stages of *Phasmahyla jandaia* (Anura, Phyllomedusidae), capítulo no qual estudamos a dieta de três estágios de desenvolvimento das larvas de *Phasmahyla jandaia* e avaliamos os principais itens consumidos por cada estágio, comparando, assim, os itens disponíveis no ambiente com os itens ingeridos por cada fase. Capítulo 3: Pollen as food: Effects of consumption on tadpole growth, development, and mobility, no qual testamos a influência do consumo de grãos de pólen sobre o crescimento, desenvolvimento e mobilidade de larvas de *Scinax machadoi*. Capítulo 4: Taste for pollen comes in different shapes: consumption by tadpoles from three divergent ecomorphotypes, no qual avaliamos por fim, a capacidade de ingestão e quebra de grãos de pólen por três tipos ecomorfológicos de larvas de anuros.

Palavras-chave. Anfíbios, Anuros, dieta de girinos, comportamento alimentar, alimentos flutuantes, forrageamento ótimo.

Abstract

Tadpoles are considered important components of food webs in aquatic ecosystems, however, their trophic roles remain poorly studied compared to other groups of aquatic vertebrates. The morphology of the larvae indicates general aspects of the diets, but few studies so far have actually explored the food composition of tadpoles, especially in the Neotropics. Throughout the development of this thesis, we sought to explore these gaps, developing a series of experiments focusing on the different feeding strategies used by tadpoles, mainly in relation to the consumption of pollen grains. We developed 4 chapters, entitled, Chapter 1: Tadpole dance: the gymnastics of *Scinax machadoi* larvae to feed on pollen, where we seek to evaluate and describe changes in the foraging behavior of *Scinax machadoi* tadpoles in favor of pollen grains on the water surface. Chapter 2: No choice is not a choice: food selectivity occurs in different ontogenetic stages of *Phasmahyla jandaia* (Anura, Phyllomedusidae), in which we studied the diet of three developmental stages of *Phasmahyla jandaia* larvae and evaluated the main items consumed by each stage, thus comparing the items available in the environment with the items ingested by each stage. Chapter 3: Pollen as food: Effects of consumption on tadpole growth, development, and mobility, in which we tested the influence of pollen grain consumption on the growth, development and mobility of *Scinax machadoi* larvae. Chapter 4: Taste for pollen comes in different shapes: consumption by tadpoles from three divergent ecomorphotypes, in which we finally evaluated the ability to ingest and break pollen grains by three ecomorphological types of anuran larvae.

Keywords. Amphibia, Anura, tadpole, feeding behaviour, buoyant food, optimum foraging, pollen.

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INTRODUÇÃO GERAL

Aspectos gerais sobre os estudos de dieta de girinos

Os anfíbios são atualmente um dos grupos de vertebrados mais ameaçados de extinção no mundo (PRYOR, 2014). A perda de habitats, poluição dos corpos d´água, mudanças climáticas, introdução de espécies exóticas e disseminação de doenças têm sido apontadas como as principais causas para a diminuição das populações mundiais de anfíbios (SCHIESARI, 2003). Das mais de 6000 espécies de anfíbios conhecidas a maior parte pertence à ordem Anura (FROST, 2015), e cerca de 75% dessas espécies apresentam um estágio larval aquático (ALTIG & MCDIARMID, 1999). O Brasil é líder em riqueza de espécies de anuros (SEGALLA et al., 2021), registrando 1144 espécies (SBH, 2021), e a maioria delas também apresenta um estágio larval aquático.

Estudos que envolvem larvas de anuros são relativamente escassos, sendo um dos primeiros trabalhos realizados com esses organismos registrado no velho continente no século VXIII, correspondendo principalmente aos estudos de desenvolvimento larval feitos por Rosel Von Rosenhof. Os principais pioneiros nos estudos com larvas de anuros no Brasil são Alípio de Miranda-Ribeiro (1920), Antenor Leitão de Carvalho (1937), Adolpho Lutz (1926) e Bertha Lutz (1949), os quais contribuíram inicialmente para o conhecimento da história natural das larvas das espécies brasileiras. Entretanto, os primeiros estudos focados na dieta de girinos são relacionados aos trabalhos de Carvalho (1939), Cardoso & Sazima (1977), Cardoso (1981), Wassersug & Heyer (1983) e Rossa-Feres (1997), que envolveram análise de conteúdos intestinais associados às morfologias orais e ao uso dos ambientes.

Atualmente, a maior parte dos trabalhos publicados concentram dados de análises taxonômicas e de padrões de distribuição (ANDRADE et al., 2007) das larvas. Muito desse cenário pode ser explicado pelo baixo número de pesquisadores dedicados aos estudos com girinos (ANDRADE et al., 2007). Nos últimos 21 anos foram produzidos poucos trabalhos visando investigar os aspectos relacionados à dieta de girinos (Figura 1), que, apesar de estarem em um grupo ameaçado, têm sua ecologia alimentar historicamente incompreendida (PRYOR, 2014).

A análise de conteúdo intestinal é o método mais utilizado para descrever a composição alimentar das larvas de anuros (MONTAÑA et al., 2019). É considerada uma técnica eficiente, uma vez que fornece informações importantes sobre aspectos da ecologia funcional e comportamentos de forrageio das larvas (ALTIG et al., 2007; MONTAÑA et al., 2019). Atualmente outras técnicas foram desenvolvidas e estão sendo utilizadas para esse tipo de estudo (Figura 2). A análise de isótopos estáveis é uma técnica que leva em consideração as assinaturas isotópicas de carbono e nitrogênio presentes nos tecidos de consumo, de modo que quanto maior a presença desses elementos, maior é a posição que aquela espécie ocupa na cadeia trófica (SCHALK et al., 2017). Outra possibilidade é a análise de ácidos graxos, que considera marcadores bioquímicos presentes nos alimentos, auxiliando no entendimento das dinâmicas de plasticidade alimentar nas larvas (WHILES et al., 2010). Outro exemplo é o uso das técnicas da metagenômica para identificação de grupos pertencentes à microbiota intestinal, a qual influência nos processos digestivos das larvas entre outras funções (KOHL et al., 2013). Outros procedimentos, como análise de conteúdo fecal é estequiometria ecológica, também podem ser aplicados para avaliação de dietas. Porém, até então, essas são linhas de pesquisa pouco desenvolvidas para o estudo de dietas de girinos.

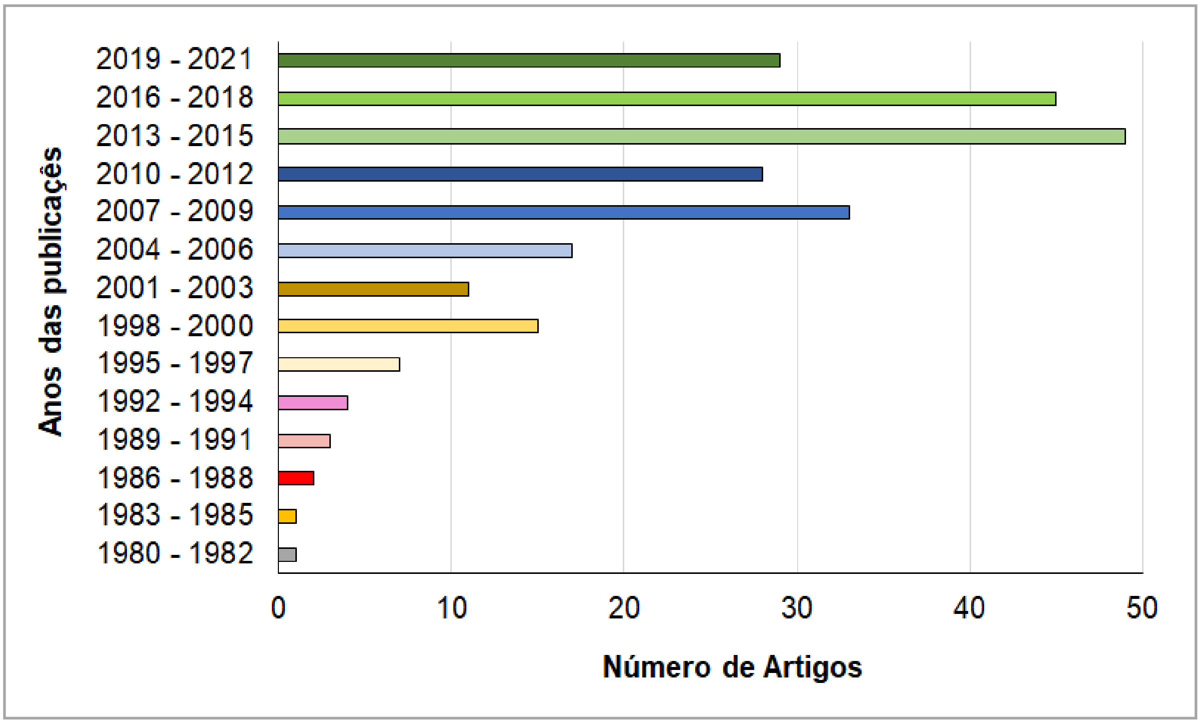


Figura 1. Artigos publicados com o tema “Dieta de girinos” entre os anos de 1980 a 2021. Fonte de dados: Web of Science.

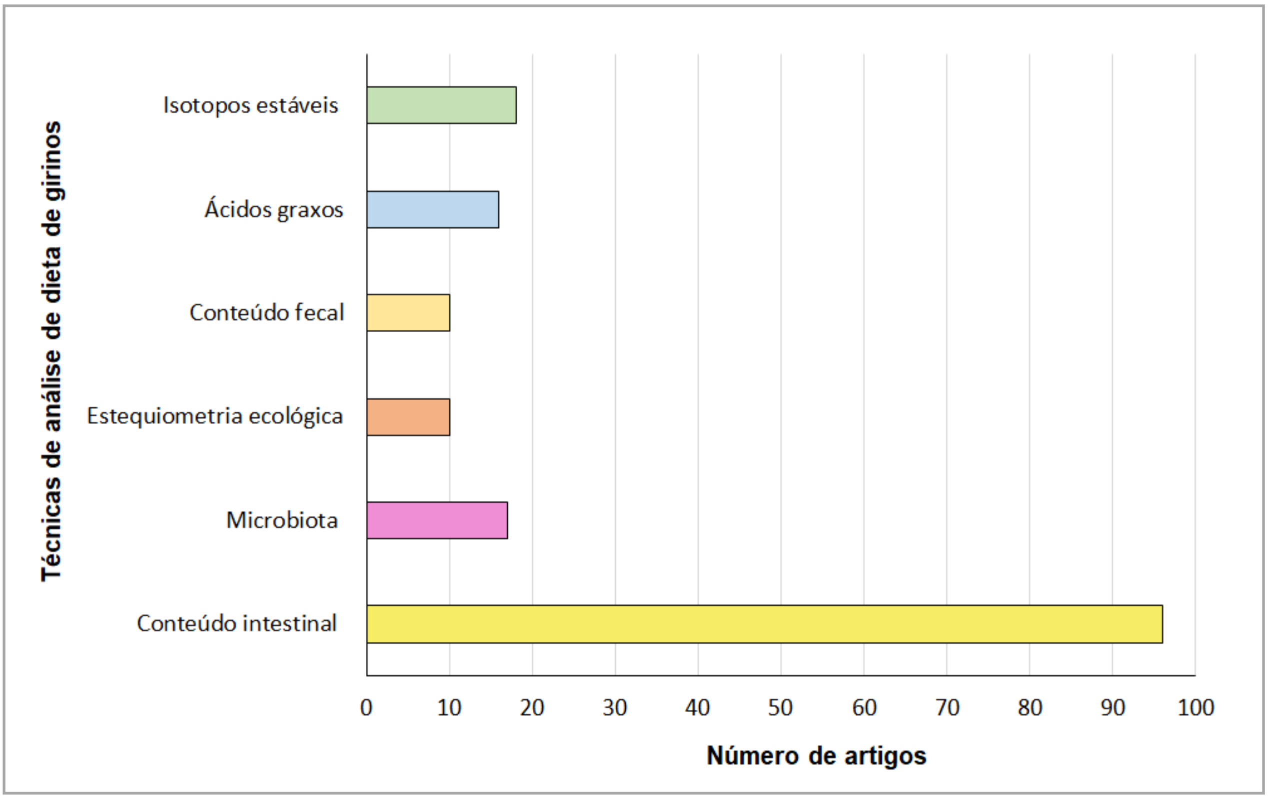
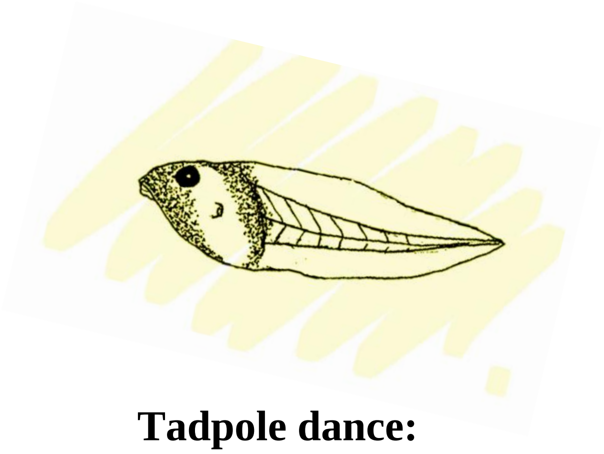


Figura 2. Principais técnicas de análise de dieta de girinos utilizadas em estudos publicados entre os anos de 1980 a 2021. Fonte de dados: Web of Science.

Essa tese foi estruturada em 4 capítulos, que correspondem as investigações por diferentes estratégias alimentares e a influência do consumo de grãos de pólen e outros itens no comportamento, crescimento, desenvolvimento e mobilidade das larvas de espécies de anuros encontradas nos Campos Rupestres no sudeste de Minas Gerais. Busca-se também contribuir no preenchimento de lacunas no conhecimento sobre ecologia de girinos, auxiliando assim, na conservação dos anfíbios em geral, uma vez que atualmente são um grupo extremamente ameaçado.

Capítulo 1



Tadpole dance:

The gymnastics of *Scinax machadoi* larvae to feed on pollen

ABSTRACT

A species diet offers information on its trophic level and its roles in the community. Tadpoles are known to be important components of aquatic food webs, however little is known about their feeding habits and food preferences. Tadpole ecomorphotypes have been shown to relate to their diet, but some consumed food resources are not available at preferred feeding microhabitats, such as pollen for benthic tadpoles. Pollen has high nutritive value, thus tadpoles may change their feeding behavior in order to explore it. We tested this hypothesis by offering fed or hungry tadpoles pollen on the water surface at three different depths, with or without an alternative food source. We noticed that pollen consumption decreased with increased depth, what is in accordance with optimal foraging theory, as the tadpoles have to make longer manouvers to achieve the pollen. That is likely also the reason why tadpoles decreased pollen consumption when provided with alternative food on the bottom, where they usually forage. However, increased difficulty and alternative food sources did not prevent tadpoles to feed on pollen, showing that they can be flexible enough to change their feeding habits in order to oportunistically explore new nutritive food sources.

Keywords. Amphibia, Anura, Hylidae, *Ololygon machadoi*, tadpole, feeding behaviour, buoyant food, optimum foraging, pollen.

RESUMO

A dieta de uma espécie oferece informações sobre seu nível trófico e seus papéis na comunidade. Os girinos são conhecidos por serem importantes componentes das teias alimentares aquáticas, porém pouco se sabe sobre seus hábitos e preferências alimentares. Foi demonstrado que os ecomorfotipos de girinos estão relacionados à sua dieta, mas alguns recursos alimentares consumidos não estão disponíveis em microhabitats preferidos, como pólen para girinos bentônicos. O pólen tem um alto valor nutritivo, portanto os girinos podem mudar seu comportamento alimentar para explorá-lo. Testamos essa hipótese oferecendo pólen a girinos alimentados e famintos na superfície da água em três profundidades diferentes, com ou sem uma fonte alternativa de alimento. Observamos que o consumo de pólen diminuiu com o aumento da profundidade, o que está de acordo com a teoria de forrageamento ideal, pois os girinos precisam fazer manobras mais longas para atingir o pólen. Essa também é provavelmente a razão pela qual os girinos diminuíram o consumo de pólen quando fornecidos com alimentos alternativos no fundo, onde geralmente se alimentam. No entanto, o aumento da dificuldade e fontes alternativas de alimentos não impediram os girinos de se alimentarem de pólen, mostrando que eles podem ser flexíveis o suficiente para mudar seus hábitos alimentares a fim de explorar oportunisticamente novas fontes de alimentos nutritivos.

Palavras-chave. Anfíbios, Anuros, Hylidae, *Scinax machadoi*, dieta de girinos, comportamento alimentar, alimentos flutuantes, forrageamento ótimo

1. INTRODUCTION

A species diet indicates its role in the trophic web and reveals important connections among species in communities (DUTRA & CALLISTO 2005; SCHIESARI et al., 2009). Amphibian larvae have important roles in aquatic food chains and connect aquatic and terrestrial energy cycles (SEALE, 1980; OSBORNE & MCLACHLAN, 1985; ALTIG et al., 2007). However, knowledge on tadpole trophic ecology is relatively limited (ALTIG et al., 2007; MONTAÑA et al., 2019).

Contrary to old assumptions that considered tadpoles as non-selective feeders with little niche differentiation, tadpole diets are diverse (SCHIESARI et al., 2009) and can include algae, fungi, amoebae, eggs of aquatic invertebrates, vegetal debris, pollen, amphibian eggs and larvae (ALTIG et al., 2007; WELLS, 2007; KLOH et al., 2019). Pollen from Gymnosperma and Angiosperma are rich in amino acids, vitamin, lipids, and thus a nutritive food resource (PALANIVELU & TSUKAMOTO, 2011; FRIAS et al., 2016). Pollen enters aquatic food webs through pollen rains that deposit on water surface. It is an important allochtonous carbon and phosphorus source that boosts microbial growth and is ultimately transferred to zooplankton consumers (MASCLAUX et al., 2013). Although it has received little attention as a component of tadpole diets, is commonly present in the diet of some species (WAGNER, 1986; KLOH et al., 2018; 2019), such as *Scinax machadoi* (BOKERMANN & SAZIMA, 1973), which search for and intentionally eat pollen.

The tadpoles of *S. machadoi* are benthic (BOKERMANN & SAZIMA, 1973) and would thus be expected to feed on the bottom (ALTIG & JOHNSTON, 1989). They occur in rocky streams in montane meadows (“Campos Rupestres”) in southeastern Brazil, which are considered oligotrophic habitats (MENDES, 2003). In that habitat, pollen could be an important nutritive resource for the tadpoles. However, because pollen may have bladders that make them buoyant (MASCLAUX et al., 2013), they may take time to sink and lose nutritious value with time (KELLER et al., 2005). To maximize the value of feeding on pollen, tadpoles may benefit from ingesting pollen while it is fresh on the water’s surface.

In this study, we explore the circumstances when *S. machadoi* tadpoles alter their benthic behavior in order to capture and profit from pollen on the water’s surface. We tested tadpoles in two developmental stages, and under satiety and hunger conditions. Tadpoles were subject to one of three water levels and had an alternative food source or not during the trials. We expected larger tadpoles to eat more pollen than smaller tadpoles due to likely greater energy needs. We expected hungry tadpoles to consume more pollen, especially at low water level, where they needed to spend less energy to reach the water surface. We also expected tadpoles to eat less pollen when an alternative food source was available on the bottom, with easier access.

2. MATERIALS AND METHODS

2.1 Study system and sampling

Tadpoles of *Scinax machadoi* were obtained for this study at a third order stream (*sensu* STRAHLER, 1957) (19°16’3.35” S, 43°30’56.23” W, 1,000 m a.s.l.) from the Doce water basin located in the Parque Nacional (National Park) Serra do Cipó (PNSC), at the southern portion of the Espinhaço mountain range, in southeastern Brazil. The stream has low nutrient concentrations and can be considered as oligotrophic (MENDES, 2003). The climate at the PNSC presents a dry, cold season from April to September and a wet, warm season from October to March. Mean monthly temperatures vary between 13 and 29°C and mean annual rainfall, between 1460 and 2490 mm (VIVEROS, 2010).

Scinax machadoi is endemic from the Espinhaço mountain range, a priority area for conservation in Brazil due to its great species diversity and levels of endemism for varied taxa (LEITE et al., 2008). It breeds year-round in permanent streams (ETEROVICK & SAZIMA, 2004). The tadpoles have slow development, taking about five months to reach metamorphosis (ETEROVICK & SAZIMA, 2004). They are easily recognizable due to their dark body crossed by bright yellow bars (ETEROVICK et al., 2018; Fig. 1A). They have an ovoid body, ventral mouth and dorsal eyes (Fig. 2A), being characterized as benthic (PIMENTA et al., 2014). They eat algae (Zygnematophyceae, Euglenophyceae, Bacillariophyceae, and Cyanophyceae), testate amoeba, vegetal debris, and pollen at the study site (KLOH et al., 2019), being normally seen at the bottom of streams (ETEROVICK & SAZIMA, 2004). Their oral disc is not laterally emarginated, with biseriate marginal papillae around the whole perimeter, a keratinized beak, and labial tooth row formula (LTRF) 2(2)/3 (PEZZUTI, 2019; Fig. 2B), characterizing a benthic scraper. It is likely adapted to microhabitats with little water flow due to the absence of attaching structures in the mouth (ALTIG et al., 2007). The oral disc is more prominent compared to other species with similar body features and biology, what could favor pollen capture on the water surface, when tadpoles can use their oral discs as a basal funnel. The marginal papillae could also aid to pollen sorting and capture.

Tadpoles of *S. machadoi* can be observed year-round, using varied types of microhabitats. We observed these tadpoles to swim to the surface and turn their ventral region upwards to capture floating pollen grains at sites with slow or no water flow close to flowering plants at the margins.

We collected *S. machadoi* tadpoles and pollen from *Tibouchina martiusiana* (DC.) Cogn. (Melastomataceae) for our experiments at the aforementioned stream in January 2019. *Tibouchina martiusiana* grows up to about 1.50 m high and flowers year-round. This shrub is adapted to high sun incidence at the “Campos rupestres” and is common alongside streams at the PNSC (Martens 2008). We collected 30 flowers for pollen from plants located up to 1 m from stream margins. We stored the flowers in plastic bags within polystyrene foam boxes with ice for transport to the laboratory (a 2-hour-drive). At the laboratory, we extracted pollen from the flowers with tweezers under a stereomicroscope and stored it at -4°C to avoid nutrient loss / degradation.

We captured tadpoles with dipnets while progressing upstream to avoid disturbing them with our movement in the water. We collected 120 tadpoles along a 150 m extension of the stream, including 60 tadpoles in developmental stage 25 and 60 in developmental stage 30 (*sensu* GOSNER, 1960). We transported the tadpoles in plastic bags with aged mineral water, which were accommodated amidst ice packs within polystyrene foam boxes.

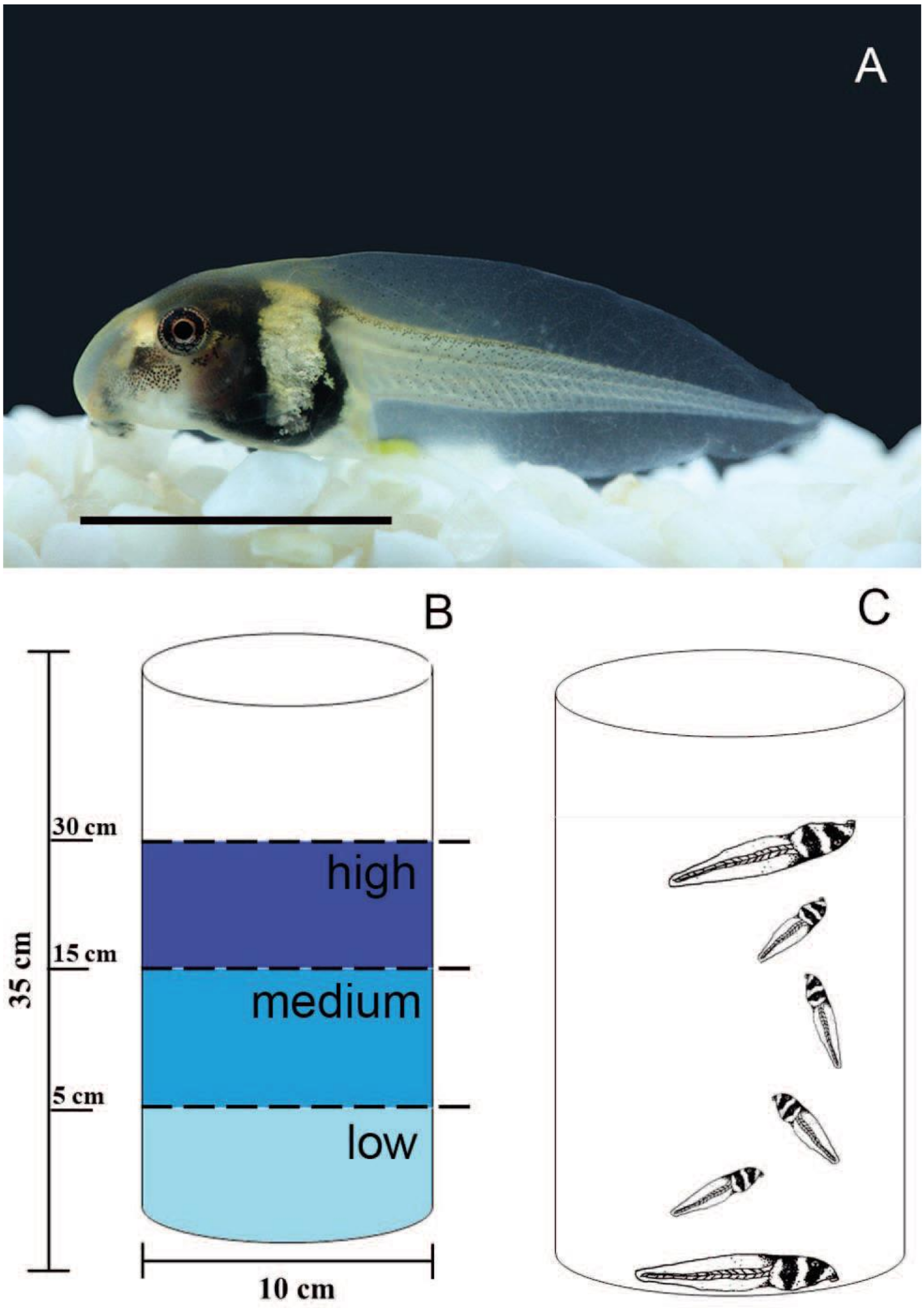


Figure 1. Tadpole of *Scinax machadoi* in developmental stage 25 (A, photography by Tiago Leite Pezzuti), schematic representation of water levels in experimental containers (B) and tadpole maneuver to reach pollen on the water surface (C). The scale bar in A represents 10 mm. The tadpole in C is represented out of scale for greater detail

2.2 Experimental design

At the laboratory, tadpoles were individually allocated in polyethylene transparent containers (35 cm high, 10 cm diameter) with aged mineral water and randomly assigned to two experimental groups: one group received commercial fish food (Probetta Show®, 0.0215 g per individual every 3 hours) and the other received no food for 12 hours, before we started the experiments. Each group included 30 tadpoles in each of the two developmental stages. We have previously used this fish food to feed tadpoles in the laboratory and they showed normal growth during limited periods (up to a month). Colleagues have successfully used this and similar fish commercial foods for longer maintenance of tadpoles of other species (C. Vinicius, C. Lisboa, I. F. Machado, T. Kohlsdorf pers. comm.). We considered it to be a nutritive food, as it contains components from fungi, animal and vegetal origins (fish, shrimp, corn, soy protein, yeast, algae, spirulin, vitamins, and antioxidants according to the manufacturer).

For the experiments we used 120 of the same polyethylene containers with three different amounts of water to produce: 1) low water level (400 ml of water to fill the container up to 5 cm); 2) intermediate water level (1 liter of water, to 15 cm) and 3) high water level (2 liters of water, to 30 cm; Fig. 1B). We manipulated water depths based on depths of microhabitats where *S. machadoi* tapdoles normally occur within their natal stream (PCE, unpublished data). We assigned 20 tadpoles (10 of each developmental stage) from the two dietary groups (tadpoles with or without food for 12 hours) to each water level treatment. The 10 tadpoles within the same developmental stage, previously determined hunger level, and water level were divided between two food treatments, that were introduced two minutes after tadpole placement in the recipient: (1) they either received five pollen grains (± 0.198 g) and five fish food pellets (± 0.0215 g) or (2) just five pollen grains (± 0.0198 g). The pellets sink to the bottom, whereas the pollen remains on the water surface.

We then observed the tadpoles at a distance (2 m) during one hour and at the end, we counted the remaining pollen grains and fish food pellets at each recipient. No pollen grains sank during the experiment. At the end of the experiment, we euthanized the tadpoles with xylocain 10% and deposited them in the Amphibian Collection of the Museu de Ciências Naturais of the Pontifícia Universidade Católica de Minas Gerais (MCN-AM) as testimony material (MCN 21149). We did not return the tadpoles to their original habitat after taking them to the laboratory to avoid any potential contamination with diseases, because we have no information about such risk.

We tested the 120 tadpoles (5 tadpoles × 2 developmental stages × 2 hunger levels × 3 water levels × 2 food treatments) simultaneously under natural day light at ambient temperature (22°C).

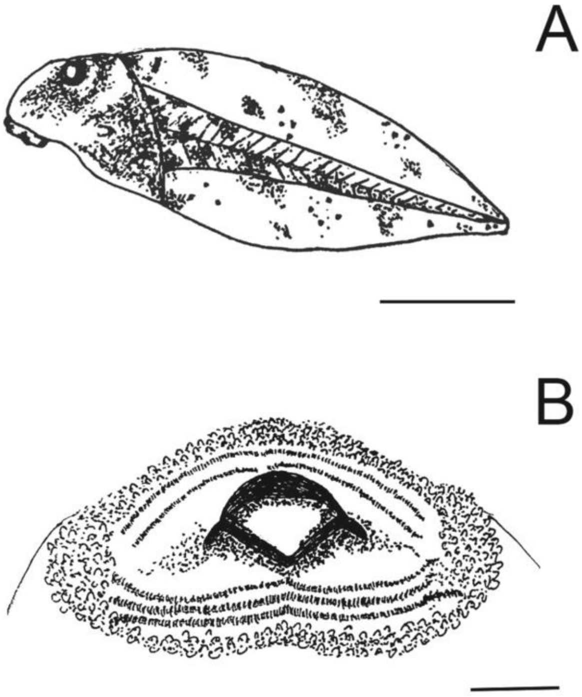


Figure 2. Lateral view (A) and details of the oral disc (B) of the tadpole of *Scinax machadoi*.

3. STATISTICAL ANALYSES

We compared pollen consumption (i.e., number of pollen grains ingested as a dependent variable) among tadpoles from two developmental stages (25 and 30) subject to two previously determined hunger levels (hungry or fed), three water levels (low, medium, and high) and in the presence or absence of an alternative food source (i.e., fish food) as explanatory variables.

We built Generalized Linear Models (GLMs) with the packages car (FOX & WEISBERG, 2011) and MASS (VENABLES & RIPLEY, 2002) in R (R CORE TEAM, 2020). We then used the package MuMIn (BARTÓN, 2019) for R (R Core Team 2020) to select the best models controlling the overall type I error rate (HOTHORN et al., 2008). Lastly, we conducted Tukey post hoc tests with the package emmeans in R (RUSSELL, 2020).

4. RESULTS

Scinax machadoi tadpoles fed on pollen under all experimental conditions, but in different amounts. When reaching for pollen (Fig. 1C), tadpoles initiate their movement turning their heads to the right (70% of individuals, Fig. 3) or to the left (30% of individuals). Afterwards they swim bending their bodies in pitch up (*sensu* Fish & Lauder 2017) to the left or to the right respectively (depending on the side they initiated the movement) until they reach ⅓ of the distance to the surface. Then they turn to the right or to the left respectively, and continue in pitch up until they approach the surface, where they perform a pitch back movement turning their ventral region towards the surface at an angle of 30 degrees, their mouths touching the water surface. The tadpoles then perform continuous yaw movements, when their tails wag laterally, allowing them to move at the surface while they feed on pollen. When they are done feeding, they make a fast roll twisting the tail and turning the ventral region towards the bottom. During this movement, 80% of the tadpoles turn to the left, including all the ones that started the movement turning their heads to the right and some the ones that started turning their heads to the left.

The best model to explain pollen consumption included all variables, but no interactions among them (Table 1). Tadpoles in stage 25 consumed more pollen (n = 81 grains) than tadpoles in stage 30 (n = 28 grains; deviance = 26.896, df = 1, $p < 0.001$).

Tadpoles that were kept without food for 12 hours before the experimental trials ate more pollen (n = 84 grains) than tadpoles that were fed during this period (n = 25 grains; deviance = 33.713, df = 1, $p < 0.001$). Tadpoles that were offered fish food as an alternative food item during the experiments ate less pollen (n = 44 grains) than those that received just pollen (n = 65 grains; deviance = 4.071, df = 1, $p = 0.044$). Pollen consumption increased as water level decreased (deviance = 28.682, df = 2, $p < 0.001$). Pollen consumption at low water level (n = 63 grains) was greater than both at medium (n = 27 grains; Tukey estimate = 0.847, z = 3.684, $p < 0.001$) and high water levels (n = 19 grains; Tukey estimate = -1.199, z = -4.580, $p < 0.001$). Consumption at medium and high water levels did not differ (Tukey estimate = -0.351, z = -1.173, $p = 0.469$; Fig. 2).

5. DISCUSSION

Scinax machadoi ate pollen under all experimental conditions, even when they suffered no food deprivation and had an easily accessible alternative food source. Pollen can be very important in some freshwater food chains (MASCLAUX et al., 2013) and is considered an important nutrient source for several animals (KELLER et al., 2005; FRIAS et al., 2016). Pollen is estimated to provide 240–305 kcal per 100g dry weight, which makes it an exceptional high caloric food compared to phytoplankton with 30–60 kcal for the same dry weight (PINTO-COELHO, 2009). In bees, pollen consumption can increase life span, boost hypopharyngeal gland development and the immune system, making the insects more resistant to parasites and viruses (FRIAS et al., 2016). The effects of pollen on tadpoles is still unknown, but it may be an important and diverse food source, especially in the tropical region rich in pollen producing plant species (PALANIVELU & TSUKAMOTO, 2011).

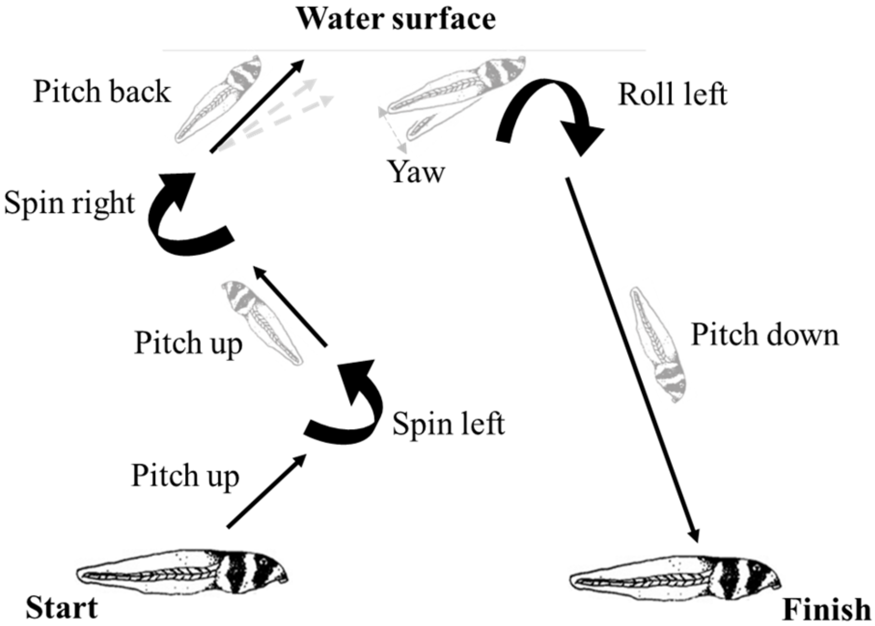


Figure 3. Schematic representation of *Scinax machadoi* tadpole maneuver to reach pollen at the water surface.

The tadpoles of *S. machadoi* exhibited lateralization in their maneuvers to reach for pollen. Lateralization has already been described to occur in fish, amphibians, and reptiles and related to feeding behavior (SOVRANO, 2007; STANCHER et al., 2018). Most individuals of *S. machadoi* turned to the left during spin and roll movements. This could be related to the presence of the spiracle at the left side (GOREE & WASSERSUG, 2001), what could optimize oxygen flow during the movements (GOREE & WASSERSUG, 2001; STANCHER et al., 2018). However, some studies suggest that side preferences would be more strongly related to phylogeny than to external morphology (WASSERSUG et al., 1999). Lateralization studies are still incipient in tadpoles, with most studies on amphibians focusing on post-metamorphic individuals (WASSERSUG et al., 1999).

Contrary to our expectations, tadpoles in developmental stage 25 ate more pollen than larger tadpoles in developmental stage 30. Tadpoles of *S. machadoi* hatch at stage 25 and are susceptible to predators due to their small size. Tadpoles have a great nutrient need in order to be active and grow quickly (WASSERSUG, 1975). Tadpoles in developmental stage 30 are heavier and likely must expend more energy to turn and move their bodies up to the surface, increasing the energetic costs of pollen acquisition. This is in accordance with the optimal foraging theory (MACARTHUR & PIANKA, 1966), which predicts that foragers should invest in food items that provide the greatest nutritional advantage (energy gain) with the lowest costs (energy expenditure for food acquisition). This theory is also compatible with the decrease of pollen consumption as water level increased, representing a longer distance upwards to be covered by tadpoles trying to feed on pollen at the surface. Greater pollen proximity provided by lower water levels may also have intensified the cues used by tadpoles to detect it, encouraging its pursue.

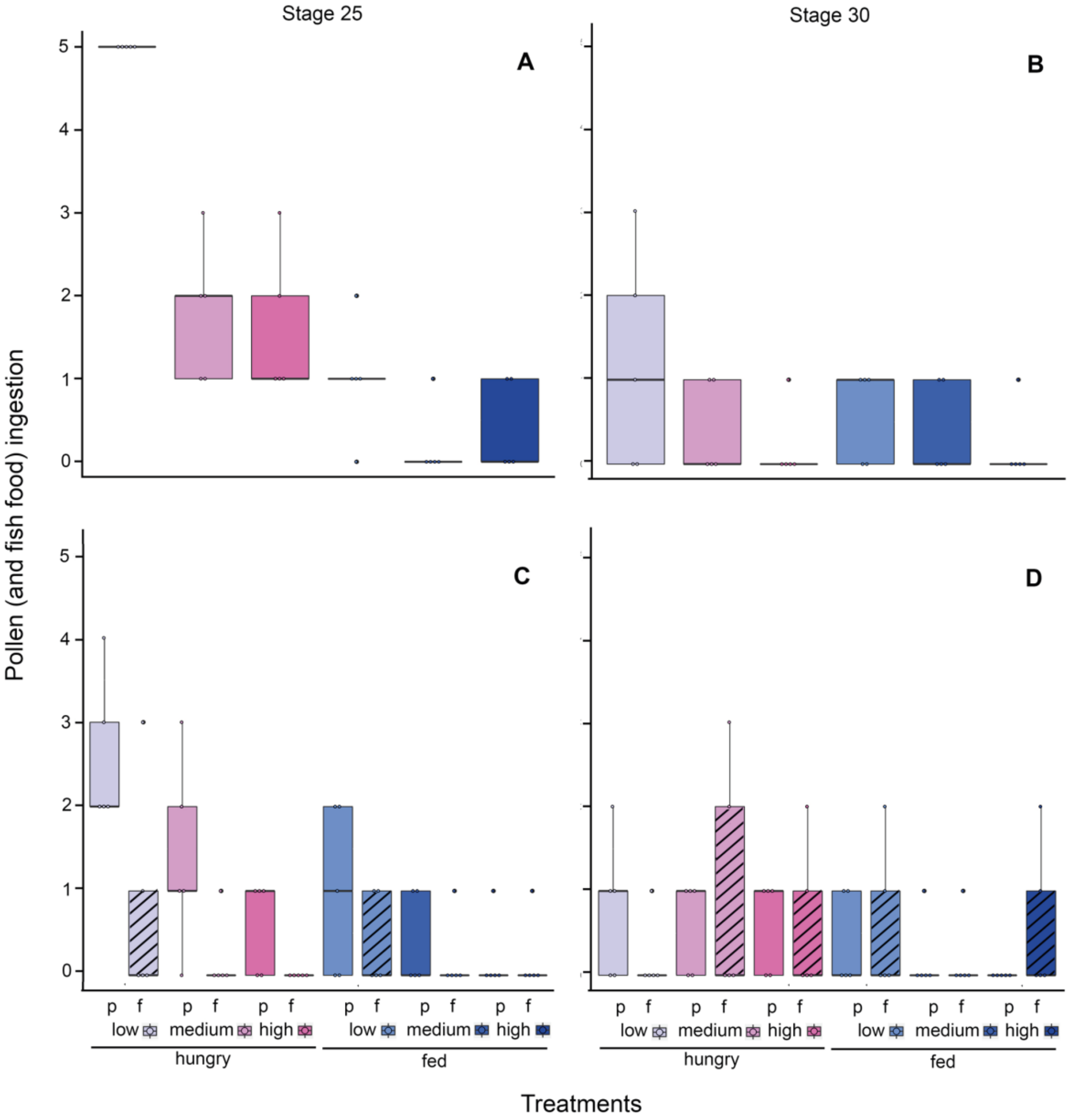


Figure 4. Box plots representing ingestion of pollen (p, plain bars) and fish food pellets (f, dashed bars) by *Scinax machadoi* tadpoles in developmental stages 25 (A, C) and 30 (B, D) offered just pollen (A, B) or pollen and fish food (C, D). Tadpoles were submitted to two previous feeding regimes that consisted in receiving no food (hungry) or fish food (fed) for 12 hours before the experimental trials and three water levels (low, medium, and high) in the experimental containers.

In *S. machadoi* natural habitats, food abundance varies spatially (KLOH et al., 2019) and likely also temporally. The periphyton algal community, for instance, can be influenced by water flow, that depends on rainfall and discharge (NECCHI-JR. et al. 1991). Pollen availability on the water surface may be influenced by both water flow and pollen production as a result of plant reproductive cycles. *Tibouchina martiusiana* flowers year-round, but an increase in the number of flowers can be noticed during the rainy season, also coinciding with the presence of the highest numbers of newly hatched *S. machadoi* tadpoles (J.S. KLOH, pers. obs.). Thus, pollen is likely to represent an important food source for initial tadpole growth, corroborating our results of higher likeliness of smaller tadpoles to feed on pollen.

Different feeding strategies are linked to different phenotypes (BEGON et al. 2007), and this also applies to tadpoles (CANDIOTI, 2007). Being benthic, tadpoles of *O. machadoi* are adapted to feed on the bottom, but their behavioural plasticity demonstrated here corroborates the importance of flexible feeding strategies for better food harvesting. Waiting for pollen grains to sink would not be so profitable, as pollen loses its nutritional value over time and it can be carried away by the water flow before it sinks (KELLER et al., 2005). *Pseudacris regilla* (BAIRD & GIRARD, 1852) tadpoles are also able to somehow detect pollen on the water surface and evaluate its quality. These tadpoles adjust their feeding behavior during gymnosperm pollen dispersion, when they elect pollen as a preferential food resource (WAGNER, 1986).

This study demonstrates that tadpoles can actively select their food to the point of altering their feeding behavior in order to opportunistically acquire nutritive food items with more difficult access. The accessibility of food for tadpoles is intimately dependent on their morphological attributes (ANNIBALE et al., 2019), and in our study we have shown that the tadpoles of *S. machadoi* can overcome morphological limitations (i.e., the ventral position of the oral disc as an unfavorable feature for surface feeding) with behavioural maneuvers in order to reach a profitable resource. Our study represents an example of optimal foraging theory, in which the energetic costs of food acquisition may be compensated by its nutritional value. Tadpoles are under strong pressure to acquire nutrients for growth and development, and here we have shown that they can be flexible and adapt to explore occasional profitable food sources via behavioural plasticity.

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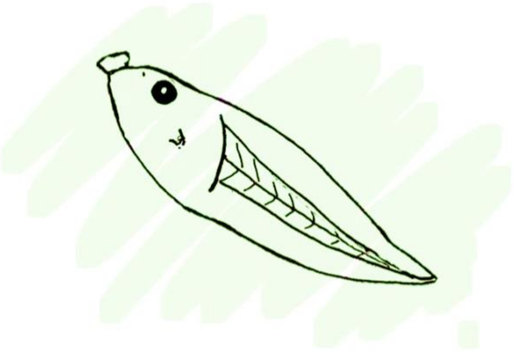
Capítulo 2



No choice is not a choice:
food selectivity occurs in different ontogenetic stages of *Phasmahyla jandaia* (Anura: Phyllomedusidae)

ABSTRACT

Amphibians play important roles in aquatic habitats and also constitute important elements connecting aquatic and terrestrial food webs. The majority of tadpoles consume a large amount of aquatic resources before they metamorphose and move to the terrestrial habitat. However, the diets and specially the foraging behavior of tadpoles are largely unknown. Some studies have been showing that they can respond to factors as varied as tadpole ecomorphotype, seasonality, habitat structure, and tadpole developmental stage. Here we studied the diet of the morphologically specialized neustonic tadpoles of *Phasmahyla jandaia* in three different developmental stages to test for ontogenetic differences in their diet and food preferences. We found tadpoles in all stages to feed on food items available at the water surface as expected based on their morphological features, leading to great niche overlap among them. However, tadpoles in different stages still showed different electivity values related to consumed food items, showing active food choice, even within ecomophotype restrictions. Our results corroborate the complexity of tadpole foraging behavior and its influencing factors, advocating for more attention to these aquatic consumers.

Key words. Amphibia, *Phasmahyla jandaia*, tadpole diet, tadpole ecomorphotype, optimum foraging.

RESUMO

Os anfíbios desempenham papéis importantes nos habitats aquáticos e também constituem elementos importantes de ligação das redes alimentares aquáticas e terrestres. Os girinos consomem uma grande quantidade de recursos aquáticos antes de se metamorfosear e se moverem para o habitat terrestre. No entanto, as dietas e principalmente o comportamento de forrageamento dos girinos são amplamente desconhecidos. Alguns estudos têm mostrado que eles podem responder a fatores tão variados como o ecomorfotipo dos girinos, sazonalidade, estrutura do habitat e estágio de desenvolvimento dos girinos. Em seguida, estudamos a dieta dos girinos neustônicos morfologicamente especializados de *Phasmahyla jandaia* em três diferentes estágios de desenvolvimento para testar as diferenças ontogenéticas em sua dieta e preferências alimentares. Encontramos girinos em todos os estágios para se alimentar de alimentos disponíveis na superfície da água, conforme esperado com base em suas características morfológicas, levando a uma grande sobreposição de nicho entre eles. No entanto, girinos em diferentes estágios ainda apresentaram diferentes valores de eletividade em relação aos itens alimentares consumidos, apresentando escolha alimentar ativa, mesmo dentro das limitações do ecomofotipo. Nossos resultados corroboram a complexidade do comportamento de forrageamento dos girinos e seus fatores de influência, defendendo mais atenção a esses consumidores aquáticos.

Palavras-chave. *Phasmahyla jandaia*, dieta de girinos, ecomorfotipo, forrageamento ótimo.

1. INTRODUCTION

Anurans are important invertebrate predators and are responsible for a relevant amount of energy transfer between aquatic and terrestrial habitats due to the biphasic life cycle of most species (ALFORD, 1999; DUTRA & CALLISTO 2005). In this context, the larval phase of most anurans is extremely important, as they consume large amounts of aquatic organisms (ALTIG et al., 2007; SCHIESARI et al., 2009) and experience extensive growth and development in bodies of water until they become terrestrial adults. Besides, tadpoles experience very high predation rates, an additional contribution to aquatic food webs (WELLS, 2007). However, the diets of tadpoles are understudied, and even their trophic roles are often difficult to establish (SCHIESARI et al., 2009). Diet is an important aspect of a species natural history, as consumed items provide information on species interactions within the food web, whose comprehension is fundamental for biodiversity conservation (ALTIG et al., 2007; MONTAÑA et al., 2019), as well as for individual species conservation itself (GELWICK & MATTHEWS, 2006; CHIPPS & GARVEY, 2007). Tadpoles have been shown to have diversified diets and choose their food items under the influence of used microhabitats, seasonality, and developmental stage (KLOH et al. 2018, 2019). Contrary to previous preconceptions, they can also actively feed on preferential, more nutritive items, even when it requires foraging at microhabitats not normally occupied by them (KLOH et al., 2021).

Tadpole ecomorphotypes have been defined based on morphological features (e.g., oral disc position and direction, body shape) that favor feeding on specific microhabitats (e.g., bottom vs. water column; ALTIG & JOHNSTON, 1989). Tadpoles with different morphologies are thus expected to have different diets (CANDIOTI, 2007), that they acquire in different microhabitats (KLOH et al., 2019). However, we recently demonstrated that this rule has exceptions, as the benthic tadpoles of *Scinax machadoi* (BOKERMANN & SAZIMA, 1973) actively swim to the water surface to consume large amounts of pollen (KLOH et al., 2021). Pollen has been shown to be an important source of proteins and lipids for pollinators (HUANG, 2012; RUEDENAUER et al., 2019) and could therefore be a nutritive food for other animals as well (ROULSTON & CANE, 2000), what would justify the behavior of *S. machadoi* tadpoles according to the optimal foraging theory (MACARTHUR & PIANKA, 1966; KLOH et al. 2021). This shows that the behavior of tadpoles can be complex and ultimately influence energy transfer outcomes both within aquatic food webs and between aquatic and terrestrial species. Changes in diet composition could occur depending on the ontogenetic stage (KLOH et al., 2019) and the acquired nutrients are likely to influence growth and metamorphosis (BOWEN et al., 1995; KUPFERBERG, 1997).

Considering the complexity of factors influencing tadpole diets, much of which remains to be studied, we chose to investigate the composition of the diet of a species with very specialized morphology and its variation among different developmental stages throughout the tadpole phase. We expected that tadpole morphology would influence diet composition and that diet composition would change with developmental stage, reflecting possible varying nutrient needs along tadpole growth/development. We have previously shown that tadpoles with more generalized ecomorphotypes can diversify their diets through behavioral plasticity (KLOH et al., 2021). Here we also intended to test whether this rule would apply to species with more specialized ecomorphotypes such as the neustonic *Phasmahyla jandaia* tadpole.

2. MATERIALS AND METHODS

2.1 Study system and sampling

We collected tadpoles of *Phasmahyla jandaia* in a permanent stream (19°15’24.25” S, 43°32’49.64” W – 1374 m a.s.l.) bordered by riparian forest and close to a two-lane paved road (MG-010) with constant flow of trucks, buses, and cars. The region has a marked rainy season from October to March and a dry season from April to September. Mean monthly rainfall varies between 1460 and 2490 mm, mean temperatures range from 13 to 29°C (VIVEROS, 2010). The genus *Phasmahyla* contains only eight species, *P. cochranae* (BOKERMANN, 1966), *P. cruzi* (CARVALHO-E-SILVA, SILVA & CARVALHO-E-SILVA, 2009), *P. exilis* (CRUZ, 1980), *P. guttata* (A. LUTZ, 1924), *P. jandaia* (BOKERMANN & SAZIMA, 1978), *P. lisbella* (PEREIRA, ROCHA, FOLLY, SILVA & SANTANA, 2018), *P. spectabilis* (CRUZ, FEIO & NASCIMENTO, 2008), and *P. timbo* (CRUZ, NAPOLI & FONSECA, 2008), all of them typical from Atlantic Forest formations or remnants (LEITE et al., 2008; ETEROVICK et al., 2020). The tadpoles of all species have a neustonic ecomorphotype (*sensu* ALTIG & JOHNSTON, 1989). Their umbelliform oral disc is projected towards the water surface, from where they seem to filter food particles (LEITE et al., 2008; COSTA & CARVALHO-E-SILVA, 2008). At the

Serra do Cipó, adult males of *P. jandaia* call at the onset of the rainy season (October–December) from the vegetation (ETEROVICK et al., 2020). The eggs are laid on leaves hanging over the water, so that tadpoles fall directly into the stream when they hatch (ETEROVICK et al., 2020; COSTA & CARVALHO-E-SILVA, 2008). The tadpoles show tones of yellow and grey and usually aggregate in groups of 10 to 15 individuals (LEITE et al. 2008; COSTA & CARVALHO-E-SILVA, 2008) (Fig. 1). These groups are mixed, including individuals from different clutches, what is evidenced by their diversified developmental stages (J. S. KLOH, pers. obs.). We collected a total of 60 tadpoles of *P. jandaia* from mixed groups, 20 at each of three developmental stages (stages 25, 30 and 40 *sensu* GOSNER, 1960). Tadpoles were identified based on the original description (BOKERMANN & SAZIMA, 1978) and this is the only species of *Phasmahyla* at the study site, preventing misidentifications. All samplings were conducted by two persons in three occasions: 14 October and 11 December 2018, 24 March 2019.

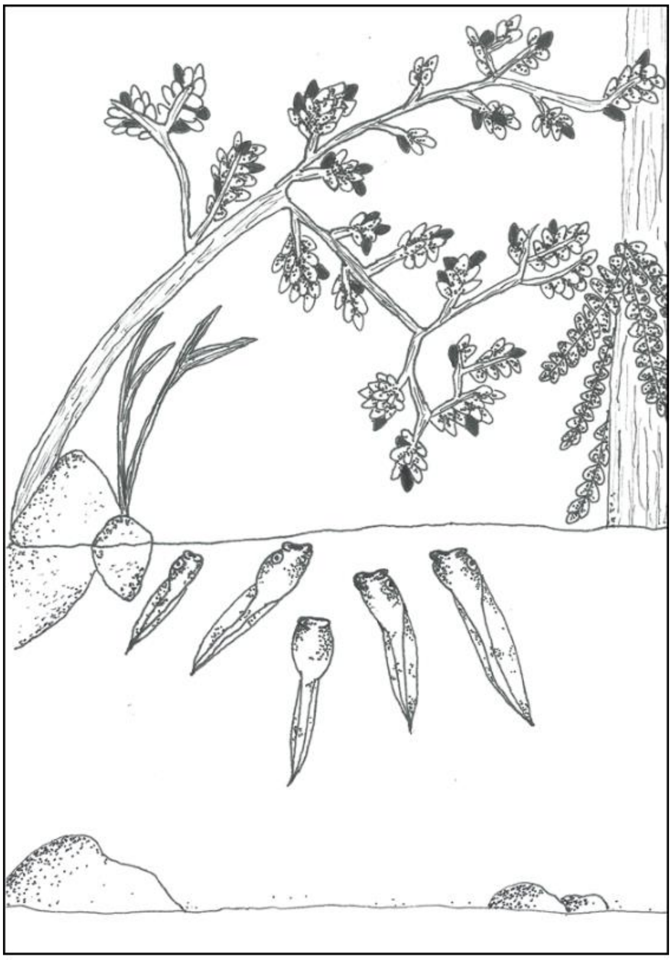


Figure 1. Group of *Phasmahyla jandaia* tadpoles in many stages.

We captured tadpoles with dipnets throughout an extension of 150 m along the stream, with tadpoles at different stages being collected from the same groups in order to randomize the effect of specific groups and locations throughout these 150 m stream section. We also collected tadpoles from the three different stages at each sampling occasion, to standardize any possible temporal effect in diet composition (Table 1). Tadpoles were immediately euthanized with 10% xylocaine and preserved in 10% formalin to retain all gut contents (see KLOH et al., 2019). All tadpoles were meas ured and had their developmental stages confirmed under a stereomicroscope in laboratory (Table 1). After that we removed tadpole guts and separated the first ⅓ of their length that corresponds to the manicoto and initial gut section (*sensu* PRYOR & BJORNDAL, 2005), as food items in this portion of the gut are still little digested and can be better identified (KLOH et al. 2018). We diluted the contents of this gut portion in 1 ml distilled water and quantified included food items in a Sedgewick rafter counting chamber under an optic microscope (Olympus cx40) under 400 × magnification (as in KLOH et al., 2019).

In order to estimate food availability for tadpoles, we collected three water samples equally distributed (every 50 m) along the 150 m sampled stream section at each of the three sampling occasions. We moved a 300 ml flask with a 4.8 cm opening perpendicularly from the stream surface to the bottom and back, touching the sediment (see Fig. 2), in order to include food items available throughout the water column, as well as on the stream bottom. We moved the water to another container and added 50 ml of 10% formalin to preserve food items for posterior identification under a microscope. In the laboratory, we stirred the container to homogenize the solution and used 1 ml of it in a Sedgewick rafter counting chamber for quantification of available potential food items as we did for gut contents.

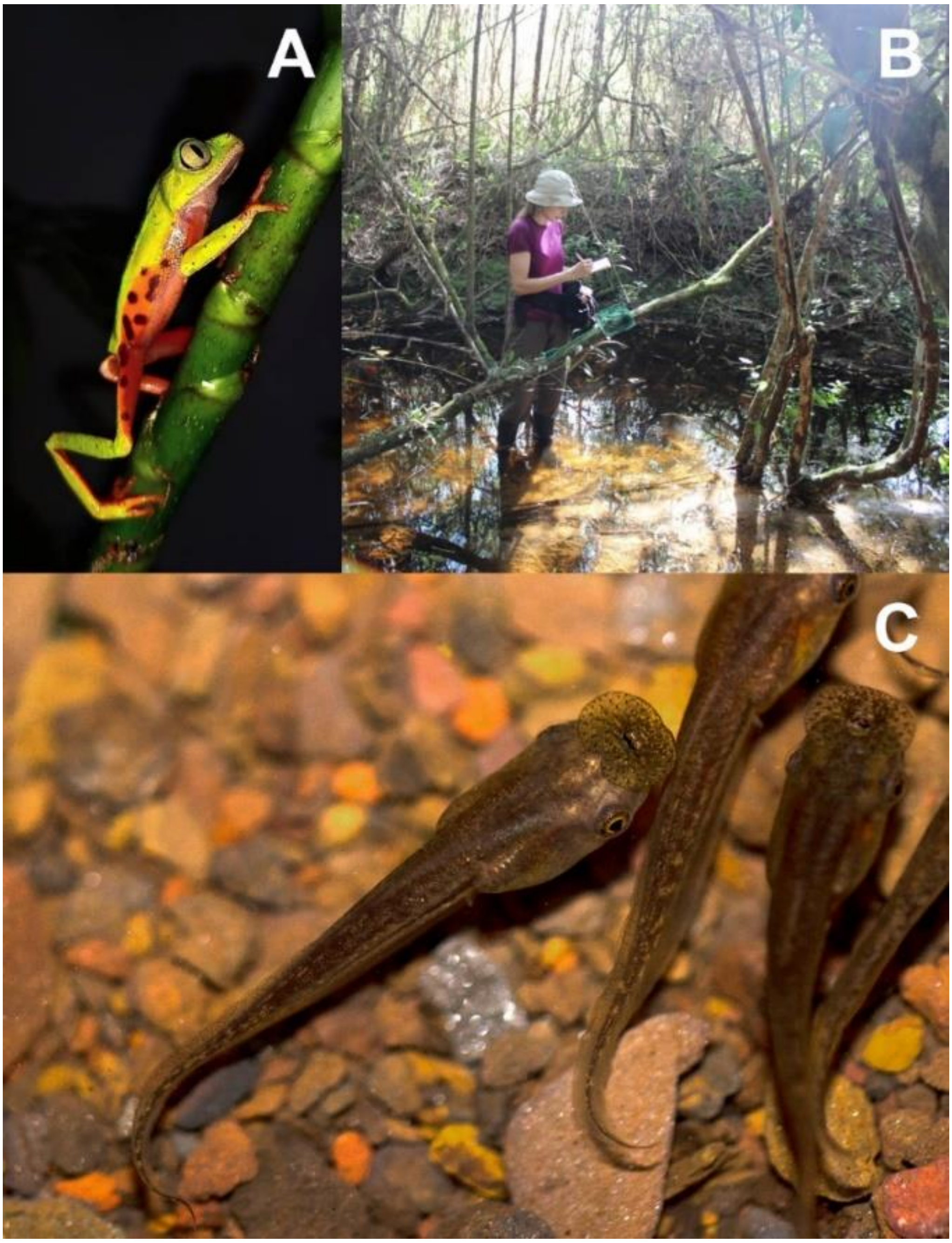


Figure 2. Adult male (A), natural habitat (B) and tadpoles (C) of *Phasmahyla jandaia* at the Serra do Cipó, Southeastern Brazil. Habitat and tadpole pictures (B and C) by Ítalo M. Martins and Bárbara Zaidan, respectively.

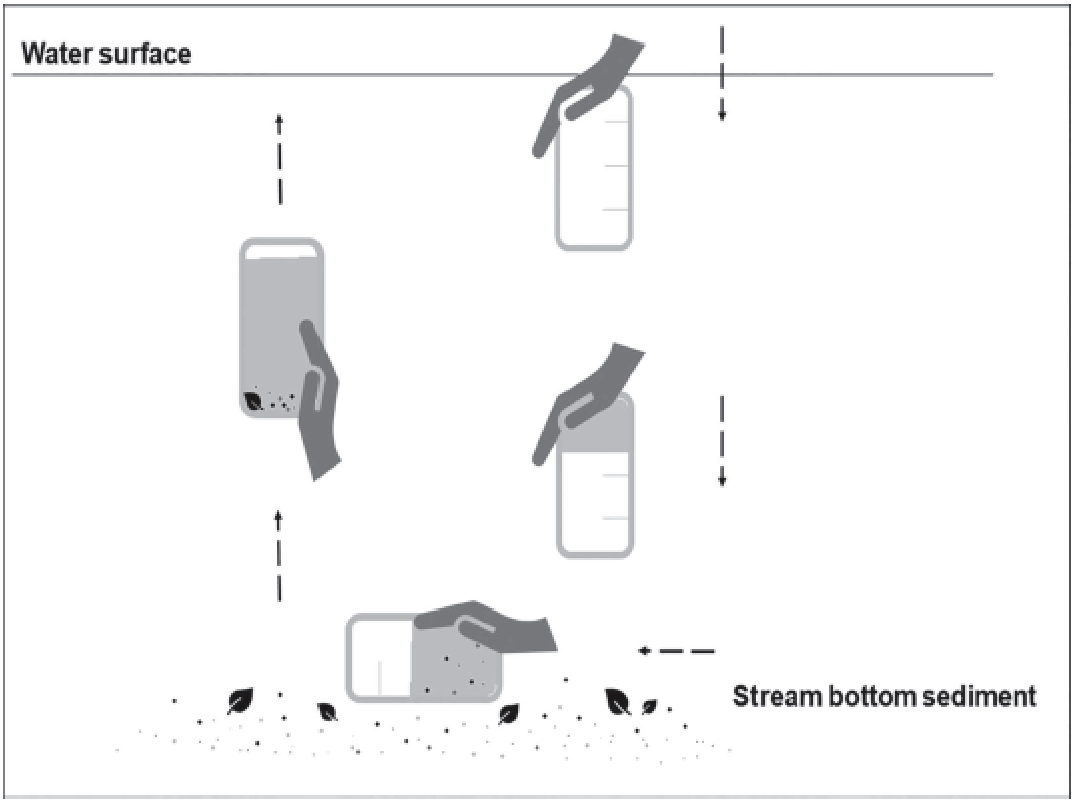


Figure 3. Water sampling procedure for estimation of food availability for tadpoles. The flask had 300 ml capacity and an opening width of 4.8 cm.

3. STATISTICAL ANALYSES

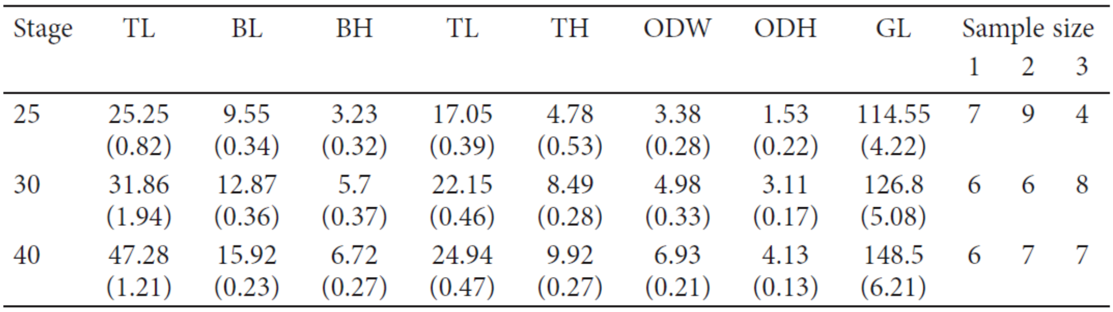
We tested whether developmental stages 25, 30, and 40 had different diets using a discriminant analyses with the packages MASS (VENABLES & RIPLEY, 2002), tidyverse (WICKHAM et al., 2019), and caret (KUHN, 2020) for R (R Core Team 2016). We also calculated niche overlap between pairs of developmental stages and compared mean niche overlap to randomly expected values based on availability of the food items in the environment using EcoSim 7.0 (GOTELLI & ENTSMINGER, 2001). We conducted 1000 simulations of random niche overlap values with the algorithm RA3 (zero values reshuffled and niche breadth retained, PIANKA, 1973). We calculated, for each developmental stage, electivity indexes for each consumed food item based on JACOBS (1974).

4. RESULTS

Tadpoles of *Phasmahyla jandaia* grew along the developmental stages considered, what was followed by a concomitant increase in gut length (Table 1). They hatch in stage 25, in stage 30 their posterior members are being formed and in stage 40 they are completely formed and the anterior members are about to come out.

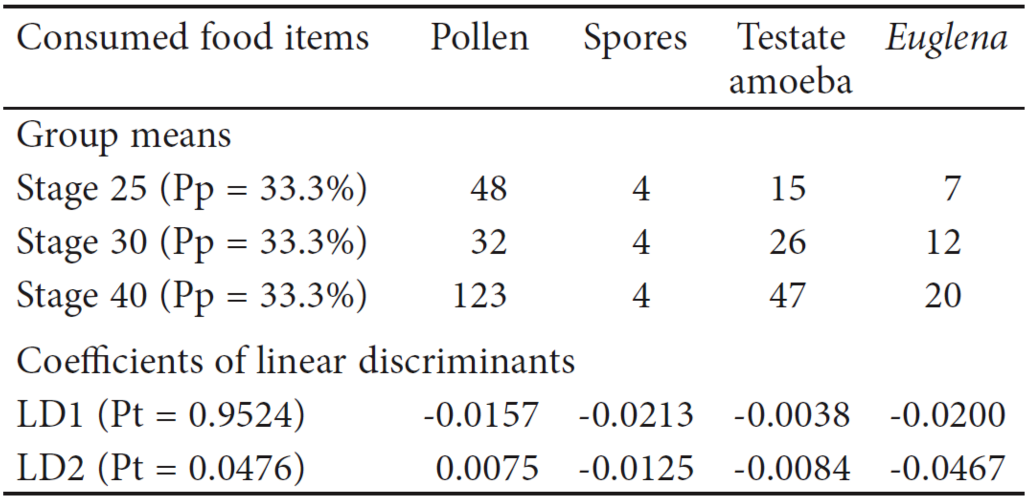
The diets of tadpoles in all developmental stages considered were in accordance with the neustonic ecomorphotype, as tadpoles did not include periphytic algae in their diets, although these autotrophs were largely available in the environment (Fig. 3). In water samples we identified cyanobacteria, three classes of algae (belonging to three Phyla), a heterotrophic protist (testate amoeba), pteridophyte spores and pollen. We classified these items as filamentous and unicellular Bacillariophyceae, filamentous and unicellular Zygnematophyceae, Euglenophyceae, Cyanophyceae, testate amoeba, spores from Pteridophyta, and pollen. Items ingested by tadpoles included only Euglenophyceae, testate amoeba, spores from Pteridophyta, and pollen.

The different developmental stages of *Phasmahyla jandaia* tadpoles showed subtle variations in their diets, which were not enough to classify tadpoles correctly to developmental stage based on what they ate (chances of correct classification were only 33% for any stage; Table 2, Fig. 4). Indeed, niche overlaps between developmental stages were higher than expected by chance ($p = 0.008$; Table 3). However, tadpoles still varied in their preferences for different food items (Fig. 5), with stage 30 showing higher preference for Euglenophyceae and testate amoeba compared to the other stages, as well as a less pronounced preference for pollen. Pteridophyte spores were rejected by tadpoles in all stages (mainly stage 40). Pollen was always the preferred item, although for stage 30 its preference was comparable to Euglenophyceae and testate amoeba.



Stage	TL	BL	BH	TL	TH	ODW	ODH	GL	Sample size		
									1	2	3
25	25.25 (0.82)	9.55 (0.34)	3.23 (0.32)	17.05 (0.39)	4.78 (0.53)	3.38 (0.28)	1.53 (0.22)	114.55 (4.22)	7	9	4
30	31.86 (1.94)	12.87 (0.36)	5.7 (0.37)	22.15 (0.46)	8.49 (0.28)	4.98 (0.33)	3.11 (0.17)	126.8 (5.08)	6	6	8
40	47.28 (1.21)	15.92 (0.23)	6.72 (0.27)	24.94 (0.47)	9.92 (0.27)	6.93 (0.21)	4.13 (0.13)	148.5 (6.21)	6	7	7

Table 1. Mean (and standard deviation) of *Phasmahyla jandaia* tadpole measurements according to studied developmental stages. TL = total length; BL = body length; BH = body height; TL = tail length; TH = tail height; ODW = oral disc width; ODH = oral disc height; GL = gut length. Measurements follow DUELLMAN (1970). Sampling dates (1, 2, 3) correspond to 14 October and 11 December 2018, 24 March 2019, respectively.



Consumed food items	Pollen	Spores	Testate amoeba	*Euglena*
Group means				
Stage 25 (Pp = 33.3%)	48	4	15	7
Stage 30 (Pp = 33.3%)	32	4	26	12
Stage 40 (Pp = 33.3%)	123	4	47	20
Coefficients of linear discriminants				
LD1 (Pt = 0.9524)	-0.0157	-0.0213	-0.0038	-0.0200
LD2 (Pt = 0.0476)	0.0075	-0.0125	-0.0084	-0.0467

Table 2. Discriminant analyses results for the composition of the diet of *Phasmahyla jandaia* tadpoles in stages 25, 30, and 40 (*sensu* GOSNER, 1960). Pt = Proportion of trace; Pp = Prior probabilities.

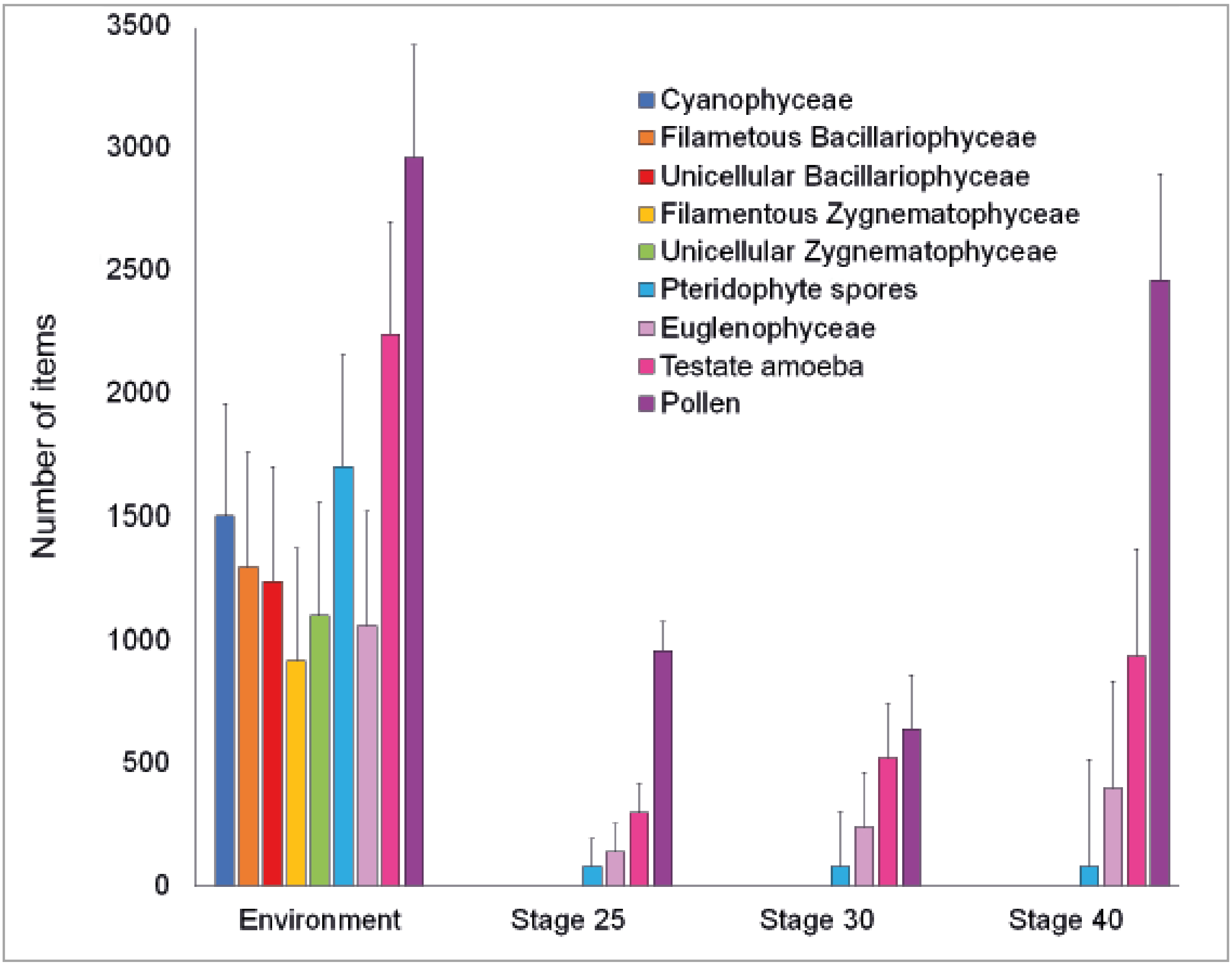


Figure 4. Food item availability and consumption by *Phasmahyla jandaia* tadpoles in developmental stages 25, 30 and 40 (*sensu* GOSNER, 1960), in a stream at the Serra do Cipó, Southeastern Brazil.

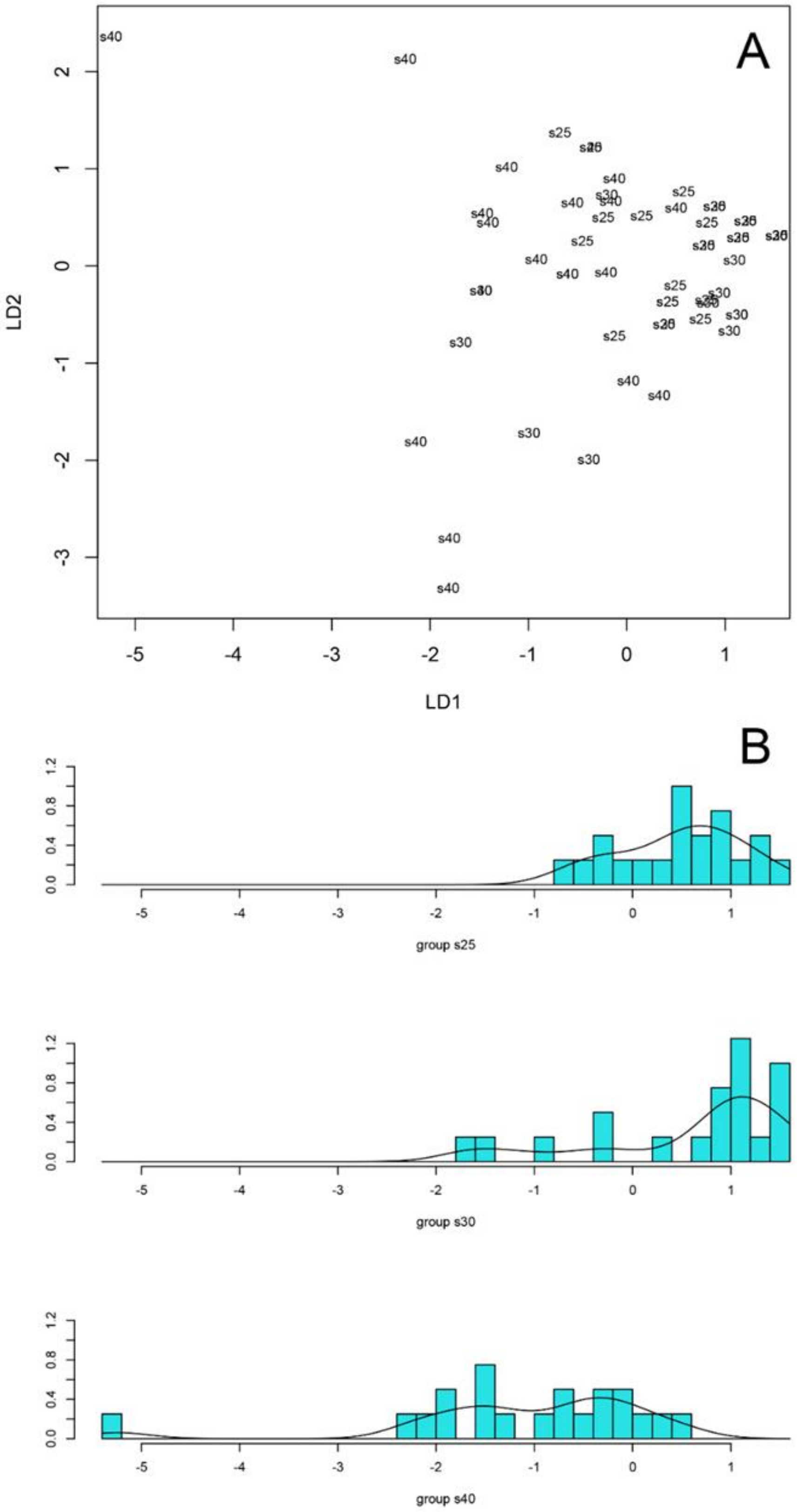


Figure 5. Representation of (A) the two first axes of the discriminant analyses to compare diet composition of three developmental stages of *Phasmahyla jandaia* tadpoles (25, 30 and 40; *sensu* GOSNER, 1960) and (B) the linear distribution of groups in the first axis, that explained 95.24% of the variation.

Niche overlap	Stage 25	Stage 30	Stage 40
Stage 25	1	0.899	0.993
Stage 30		1	0.928
Stage 40			1

Table 3. Niche overlap results for the composition of the diet of *Phasmahyla jandaia* tadpoles in stages 25, 30, and 40 (*sensu* GOSNER, 1960). Expected value based on 1000 simulations (RA3 algorithm): 0.754.

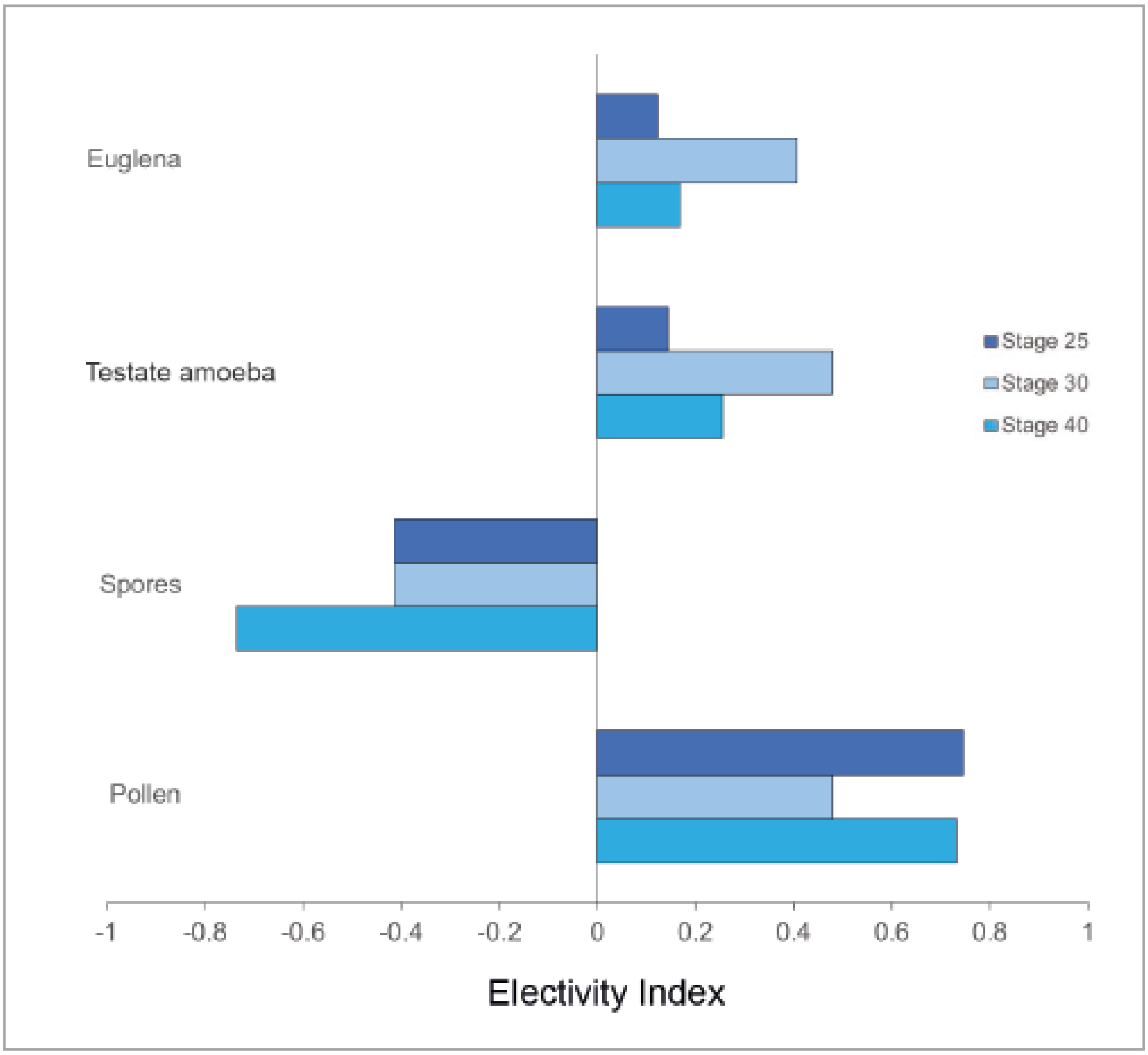


Figure 6. Electivity indexes of the food items consumed by *Phasmahyla jandaia* tadpoles in developmental stages 25, 30 and 40 (*sensu* GOSNER, 1960), in a stream at the Serra do Cipó, Southeastern Brazil.

5. DISCUSSION

The majority of the diet of *Phasmahyla jandaia* tadpoles was composed of pollen. Although the anatomy of the oral disc of these tadpoles favors pollen consumption at the water surface and its availability in the sampled stream was great, tadpoles in all developmental stages still showed a positive, high electivity for this food item, meaning a remarkable preference for it. Several groups of vertebrates have pollen as an important component of their diets as it provides lipids, proteins, vitamins, and mineral salts, among other essential elements for their development (ROULSTON & CANE, 2000; FRIAS et al., 2016).

The highest absolute food intake in Gosner stage 40 may be related to a higher energy demand for tadpoles in that stage. It is probably important to maximize nutrient intake in stage 40, as this is the last stage in which tadpoles can be considered as maintaining a normal tadpole diet, followed by atrophy of mouthparts from stage 41 on (ALTIG & JOHNSTON, 1989). Besides, an increased oral disc (see Table 1) and gained experience may turn stage 40 tadpoles more efficient in pollen acquisition than smaller stage 30 and 25 tadpoles. Tadpoles in stage 25 can probably supply their nutritional needs with smaller pollen quantities due to their smaller size. On the other hand, stage 30 tadpoles may need a greater amount of pollen than stage 25 tadpoles, but they may not be efficient enough to acquire it before pollen availability decreases. As pollen becomes less profitable to harvest, stage 30 tadpoles may switch to other food items and complement their diets with proportionally larger amounts of Euglenophyta and testate amoeba, which could be easier to acquire as the amount of pollen available for consumption is gradually reduced at the vicinities of the tadpole group.

It is interesting to notice that the gut of *P. jandaia* tadpoles (107–163 cm total length through stages 25–40) is shorter than those of benthic hylid tadpoles of similar size [Bokermannohyla saxicola (BOKERMANN, 1964): 230– 290 cm in stages 25–30, Scinax machadoi: 180–250 cm in stages 25–30; J. S. KLOH, pers. obs.]. This could be related to a great consumption of pollen, whose high nutritional value (ROULSTON & CANE, 2000) may supply tadpole needs with lower consumed amounts. Although pollen is considered to be difficult to digest (ROULSTON & CANE, 2000), a shorter gut could reduce tadpole weight and minimize the energy needed to keep their bodies close to the surface, where they can easily eat pollen, in accordance with the optimal foraging theory (MACARTHUR & PIANKA, 1966).

Euglenophyceae also constituted an important component of the diet of *P. jandaia* tadpoles. As flagellates, they remain for more time close to the water surface (CHINDIA & FIGUEREDO, 2018), where they can be easily captured by the tadpoles of *P. jandaia*. These tadpoles move the water with their tail tips (LEITE et al., 2008) and the water flow promoted by groups of tadpoles may facilitate acquisition of suspended food items. Testate amoeba are amoeboid protists with a test that partially encloses the cell. They usually occupy the water surface and are indicators of low habitat quality (SOUSA, 2008). Their presence in the studied stream may be related to the occasional presence of cattle in its vicinities, as well as the increasing traffic at the road, sewage irregular disposal, and other human activities that lead to water pollution and eutrophication at the Serra do Cipó, although still with low intensity. The presence of testate amoeba as a preferential item in the diet of *P. jandaia* tadpoles in all developmental stages may relate to the high iron and manganese contents in their tests (SOUSA, 2008), as these elements play important roles as nutrients for vertebrate immunity and metabolism (PINTO-COELHO, 2009). Pteridophyte spores, on the other hand, were not preferential items, although they may be easily available at the surface. Their nutritional value for tadpoles remains unknown.

We reported here that the diet of neustonic *P. jandaia* tadpoles is largely influenced by their ecomorphotype. They occupy the water surface and have a dorsal mouth with no denticles (COSTA & CARVALHO-E-SILVA, 2008), resulting in the absence of periphyton in their diets. However, it does not mean that they do not select their food items, as even with a relatively specialized morphology, they showed differential selectivity for the potential food items available to them.

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Capítulo 3

Pollen as food: Effects of consumption on tadpole growth, development, and mobility

ABSTRACT

The structure of many aquatic food webs is influenced by tadpole survivorship and seasonal permanence, which depend on tadpole diet. Pollen has been occasionally reported to occur in tadpole diets, and tadpoles seem to show a strong preference for this supposedly nutritive resource. Thus, we aimed to test whether a diet composed of pollen from varied plant species would booster tadpole growth, development, and agility/mobility, compared to a regular artificial balanced and nutritive diet. Tadpoles in initial developmental stages 25 and 30 grew more when fed with pollen, compared to artificial food and a mixture of both diets. Those in stage 30 advanced more developmental stages during one month when fed exclusively with pollen. Tadpoles moved longer distances in response to an aversive stimulus simulating a predatory attack when fed with pollen. Our results corroborate the hypotheses of pollen being a very important food source to aquatic consumers such as tadpoles, being likely to influence the structure of aquatic food webs through tadpole nutrition and growth/development speed, as well as ability to escape predators. These results highlight the importance of the riparian vegetation and its conservation as a resource supplier to aquatic food webs.

Keywords: tadpole diet; growth; development; pollen; mobility

RESUMO

Os girinos são componentes importantes de muitas teias alimentares aquáticas. A estrutura dessas teias alimentares é influenciada por sua sobrevivência e permanência sazonal em corpos d'água, que, por sua vez, dependem da dieta dos girinos. O pólen tem sido ocasionalmente relatado em dietas de girinos, e os girinos parecem mostrar uma forte preferência por este recurso supostamente nutritivo. Assim, nosso objetivo foi testar se uma dieta composta de pólen de espécies vegetais variadas impulsionaria o crescimento, desenvolvimento e agilidade/mobilidade dos girinos, em comparação com uma dieta artificial balanceada e nutritiva regular. Os girinos nos estágios iniciais de desenvolvimento 25 e 30 cresceram mais quando alimentados com pólen, em comparação com alimentos artificiais. Aqueles no estágio 30 avançaram mais estágios de desenvolvimento durante um mês quando alimentados com pólen. Os girinos moveram distâncias maiores em resposta a um estímulo aversivo simulando um ataque predatório quando alimentados com pólen. Nossos resultados corroboram as hipóteses do pólen ser uma fonte de alimento muito importante para consumidores aquáticos como girinos, podendo influenciar a estrutura das teias alimentares aquáticas através da nutrição e velocidade de crescimento/desenvolvimento dos girinos, além da capacidade de escapar de predadores. Esses resultados destacam a importância da mata ciliar e sua conservação como fornecedora de recursos para as teias alimentares aquáticas.

Palavras-chave: dieta de girinos, crescimento, desenvolvimento, pólen, mobilidade

1. INTRODUCTION

Amphibian larvae are key to many aquatic food webs (e.g., O'NEIL & THORP, 2014; SCHMIDT et al., 2017) and their patterns of food consumption, growth, and development influence other trophic levels (COSTA & VONESH, 2013; HITE et al., 2018). Predation on anurans in all life stages can trigger a trophic cascade effect leading to increase in populations of their prey (HITE et al., 2018), whereas their seasonal role as aquatic consumers can increase complexity of food webs (SCHMIDT et al., 2017).

The larval phase is key for growth and development of most amphibians (HEYER, 1979; WELLS, 2007) and its duration is affected by many factors such as nutrition, temperature, species interactions, and environmental features (MONTAÑA et al., 2019). Diet composition can be considered a key factor, as rates of food consumption directly influence size and time to metamorphosis (KUPFERBERG, 1997). Anuran larvae usually adjust their growth to environmental conditions such as resource levels. They can grow until they reach a minimum size to metamorphose and leave an unfavorable aquatic habitat or grow to a maximum size if the aquatic habitat is favorable (WILBUR & COLLINS, 1973).

Although tadpoles are abundant and diverse in many aquatic ecosystems (ALTIG & MCDIARMID, 1999), their diets, trophic ecology, and trophic level are poorly known (MONTAÑA et al., 2019) compared to other freshwater consumers such as fish (UDOH & EKPO, 2017). A variety of items are reported to occur in tadpole diets, including algae, fungi, protozoan (ALTIG et al., 2007). There are not many records of pollen as a food item for vertebrates in general (PRYOR, 2014), but pollen has been recorded in the diets of a few tadpole species (e.g., WAGNER, 1986), including several tropical species at the montane meadows of southeastern Brazil (KLOH et al., 2018; 2019; 2021a). Additionally, *Scinax machadoi* tadpoles were shown to vertically swim through the water column to actively feed on pollen even when presented with other more easily accessible food items (KLOH et al., 2021b).

In this study we investigate the effects of pollen in growth, development, and agility/mobility of tadpoles, compared to a nutritive and balanced artificial diet likely to contain the nutrients obtained by tadpoles in natural habitats. We expected pollen fed tadpoles to present faster growth, development, and performance, corroborating their preference and promptness to consume this food item when available (KLOH et al., 2019; 2021b).

2. METHODS

2.1 Study site

We collected tadpoles from a third order stream (*sensu* STRAHLER, 1957) called Água Escura (19° 16’ 3.35” S, 43° 30’ 56.23” W; 1236 m alt.) within the Doce River basin at the Parque Nacional (National Park) Serra do Cipó (PNSC). The PNSC is inserted in the southern portion of the Espinhaço Mountain Range, an important area for species conservation in Minas Gerais state (ETEROVICK et al., 2020) due to high levels of endemism, including amphibians (LEITE et al., 2008). The climate has marked rainy (October – March) and dry (April – September) seasons, with mean monthly temperatures varying between 13 and 29°C and mean annual rainfall, between 1460 and 2490 mm (VIVEROS, 2010). Montane meadows (Campos Rupestres) are the predominant vegetation, with Asteraceae, Fabaceae, Bromeliaceae, Orchidaceae, Eriocaulaceae, Xyridaceae, Melastomataceae, Lentibulariaceae, and Velloziaceae as the most representative families (GIULIETTI et al., 1997; MACHADO et al., 2007).

2.2 Model species

Scinax machadoi (BOKERMANN & SAZIMA, 1973) is a common species that breeds year-round in permanent streams of the PNSC. Tadpoles take about five months to metamorphose (ETEROVICK et al., 2020). They have ovoid bodies, ventral mouth, and dorsal eyes (PIMENTA et al., 2014), benthic habits, and are active both during the day and at night (ETEROVICK et al., 2020). They have a dark brown body with two bright yellow dorsal bars (ETEROVICK et al., 2018). Their diet includes unicellular and filamentous Zygnematophyceae and Bacillariophyceae, Tecamoeba, Cyanophyceae, and pollen from varied plant families (KLOH et al., 2019; pers. obs.).

Figure 1. *Scinax machadoi* tadpole.

2.3 Sampling procedures and experimental trials

We used 30 tadpoles in each of the stages 25 and 30 (*sensu* GOSNER, 1960), that were collected by a single person with dipnets at a stream backwater on 14th January 2020. After collection, tadpoles were placed in containers with tap water rested for 24h to eliminate chlorine. They remained in these containers for 12h to eliminate previously ingested gut contents. Afterwards tadpoles of each stage (25 or 30) were distributed in three treatments (10 tadpoles each), represented by diets composed of (1) pollen, (2) commercial fish food (intended to simulate the variety of nutrients contained in a diversified diet naturally consumed by tadpoles), and (3) a mixture of both pollen and fish food. Because not many plants were flowering at the study site at the time of the experiment, we used commercial bee pollen from *Apis mellifera* (Santa Bárbara®) produced at the region according to commercial regulations. This pollen consists of a mixture containing essentially pollen from flowers from different plant families collected by bees, with a very small quantity of nectar and bee salivary secretions. It contains a variety of proteins, lipids, sugars, fibers, mineral salts, aminoacids, and vitamins (BRASIL, 2000). We characterized pollen composition by dehydrating the whole content of the container (500 g) at 45°C for two hours. Posteriorly we macerated and homogenized the grains and selected subsamples using the method proposed by ZENEBON et al. (2008) for solid food samples. The whole content was distributed on a squared flat surface and the content of two diagonal quadrats was separated. The process was then repeated three times to obtain a smaller sample, which was treated with acids to remove the cytoplasmatic content of pollen grains as described by ERDTMAN (1960). This treatment makes pollen morphology more visible. We then examined the material on slides under an optic microscope (Olympus BX50) with 400x to 1000x amplification. The relative frequency of pollen grains from different plants families was determined based on counting of at least 1500 grains, as described by LOUVEAUX et al. (1978). We identified 18 different pollen types belonging to 15 plant families by comparison with reference slides from the collection of Fundação Ezequiel Dias, Belo Horizonte, Brazil, and by consulting specific literature, plus eight non identified types (Table 1; Fig. 1).

For the artificial food treatment, we used commercial fish food (Probetta Show®), that has a rich nutrient content, aiming to simulate a combination of nutrients likely to be supplied by the mixture of natural items consumed by tadpoles (KLOH et al., 2019). Probetta Show® contains mollusk, crustacean compounds, meat and fish derivates, vegetal subproducts, yeast, oils, minerals, vitamins, and antioxidants, according to manufacturer specifications. Although the real nutritional composition of *Scinax machadoi* tadpoles' diet is not known, we conservatively aimed for a nutritive commercial food, as colleagues have successfully used this and similar fish commercial foods for maintenance of tadpoles of varied species until metamorphosis (C. Vinicius, C. Lisboa, I. F. Machado, T. Kohlsdorf, pers. comm.). Even if this food has a higher nutritional value than the mixture of items in the natural diet of tadpoles, it would underestimate the difference among treatments, reinforcing possible significant results.

After tadpoles eliminated previously ingested gut contents, they were placed individually in 8 x 8 x 9.5 cm containers with 300 ml of rested tap water. They received standardized 36 g portions (weighed to the nearest 0.0001 g) of macerated food (pollen, fish food, or mixture of both at equal proportions) every three days. The water of the containers was replaced immediately before food delivery to keep them clean. The whole experiment lasted 30 days. Tadpoles had their total length (TL), body length (BL), body width (BW), tail length (TaL), and tail height (TH) measured as described by ALTIG and MCDIARMID (1999) at the beginning and at the end of the experiment to obtain their increase in each measurement. We also recorded developmental stages of all tadpoles at the end of the experiment, to access through how many stages they developed towards metamorphosis starting from their initial stages in the experiment (25 or 30). During the experiment, the containers were kept in a chamber under a 14 h light / 10 h dark regime at 21°C. Water temperature in the containers varied between 16 and 18°C. We chose values within the mean temperature range at the study site to avoid high temperatures that could increase activity and energy expenditure, as well as very low temperatures, that could make tadpoles lethargic and less prone to eat (MACIEL & JUNCÁ, 2009).

By the end of the experiment, we randomly picked five tadpoles of each stage / diet treatment to perform agility/mobility tests. These tests were based on tadpole response to an artificial disturbance simulating a predator attack, as in ETEROVICK et al. (2018). Tadpoles randomly assigned to agility/mobility tests were placed individually at 20 x 25 x 7 cm containers with a grid of 20 5-cm-cells marked and identified with letters at the bottom. Each container was filled with 500 ml rested tap water. Tadpoles were left in the container for two minutes and then subject to an aversive stimulus represented by an approaching stick, simulating a predator attack (ETEROVICK et al., 2018; Fig. 2). The stick was inserted by the same person slowly and at a constant speed towards the head of the tadpole until it moved or until the stick touched its head, and the distance covered by the fleeing tadpole was measured. The side to where the tadpole escaped was also recorded to detect any laterality and its potential influence on fleeing distance.

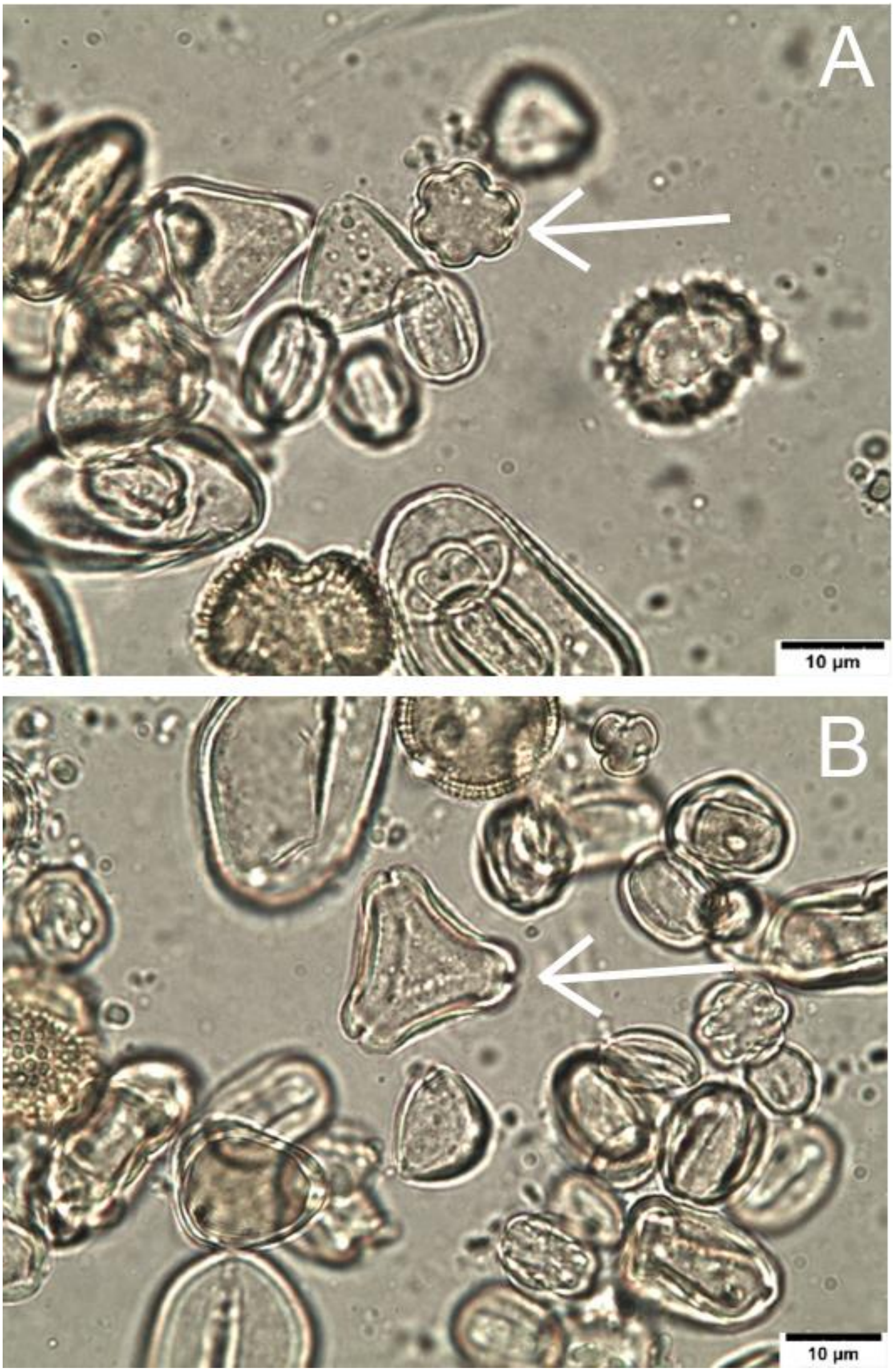

Figure 2. Pollen grains showing examples belonging to plants from the families (a) Melastomataceae and (b) Myrtaceae (arrows). Pictures made under microscope at 400 x magnification, during pollen quantification procedures.

After tadpoles eliminated previously ingested gut contents, they were placed individually in 8 x 8 x 9.5 cm containers with 300 ml of rested tap water. They received standardized 36 g portions (weighed to the nearest 0.0001 g) of macerated food (pollen, fish food, or mixture of both at equal proportions) every three days. The water of the containers was replaced immediately before food delivery to keep them clean. The whole experiment lasted 30 days. Tadpoles had their total length (TL), body length (BL), body width (BW), tail length (TaL), and tail height (TH) measured as described by ALTIG and MCDIARMID (1999) at the beginning and at the end of the experiment to obtain their increase in each measurement. We also recorded developmental stages of all tadpoles at the end of the experiment, to access how much they developed towards metamorphosis. During the experiment, the containers were kept in a chamber under a 14 h light / 10 h dark regime at 21°C. Water temperature in the containers varied between 16 and 18°C. We chose values within the mean temperature range at the study site in order to avoid high temperatures that could increase activity and energy expenditure, as well as very low temperatures, that could make tadpoles lethargic and less prone to eat (MACIEL & JUNCÁ, 2009).

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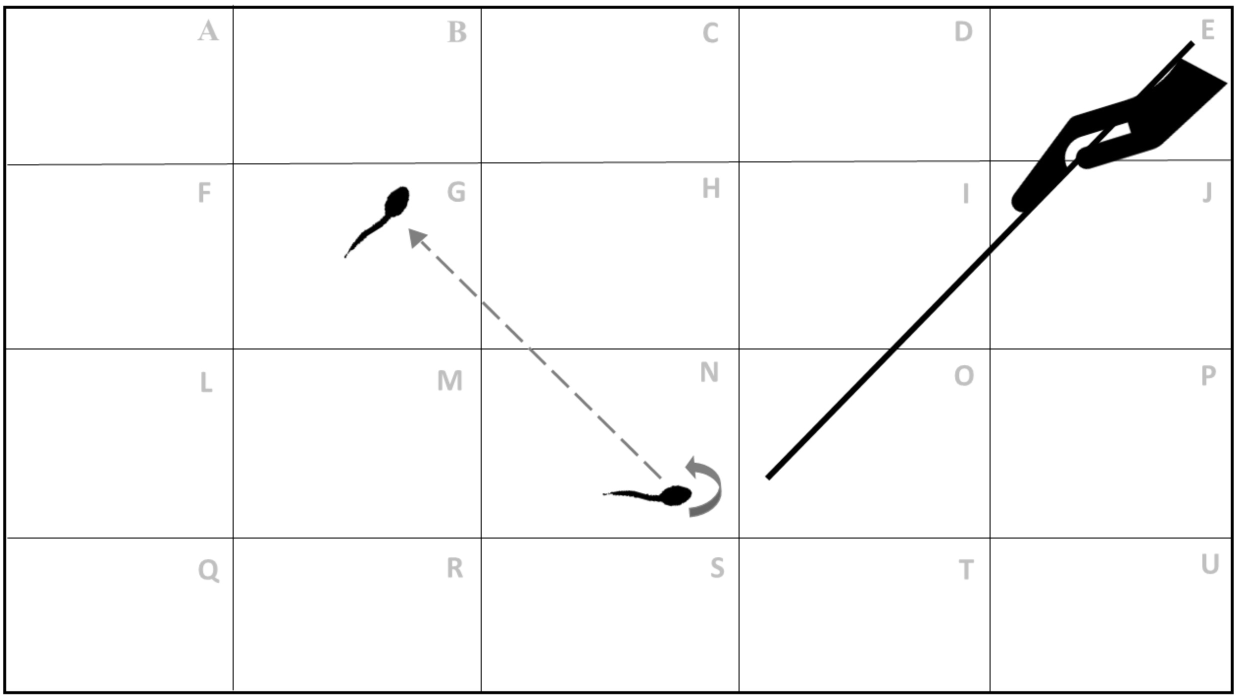


Figure 3. Schematic representation of agility experiments conducted with tadpoles of *Scinax machadoi* in stages 25 and 30 subject to three different diets (pollen, fish food, mixture of pollen and fish food).

3. STATISTICAL ANALYSES

To compare measurement gains of tadpoles in stages 25 and 30 subject to three different diets (1) pollen, (2) fish food, and (3) a mixture of both, we used Non-Metric Multidimensional Scaling (NMDS). NMDS allowed us to combine measurement gains to show not only growth but also potential shape variations under different diets. We used metaMDS function in the package MASS (VENABLES & RIPLEY, 2002) for R (R Core Team, 2020). We used the same data to conduct a PERMANOVA in the package Vegan (OKSANEN et al.,2016) using diet as explanatory variable for growth/shape changes. We performed separated analyses for tadpoles that started the experiment in stage 25 and in stage 30.

We tested whether initial developmental stage (25 or 30), diet, and their interactions explained number of developmental stages advanced by tadpoles during the experiment (starting from either stage 25 or 30) and whether initial developmental stage, diet, or laterality and their interactions explained distance covered by fleeing tadpoles using Generalized Linear Models (GLMs). We used the package MuMIn (BARTÓN, 2022) to select the best model based on AICc values and the package emmeans (LENTH, 2022) for post hoc tests.

4. RESULTS

Tadpoles fed exclusively with pollen were the ones that grew more, followed by tadpoles with the other diets (mixed diets and fish food), regardless of their initial stage (Fig. 3, Table 2). The contributions of all measurements were important to distribute experimental groups in the multi-dimensional space, as represented by the similar length of their corresponding vectors (Fig. 3, Table 3). The spatial distribution of the groups did not indicate any important shape alteration among treatments, except for tadpoles in stage 30 fed with fish food gaining proportionally a little less body width (Fig. 3B).

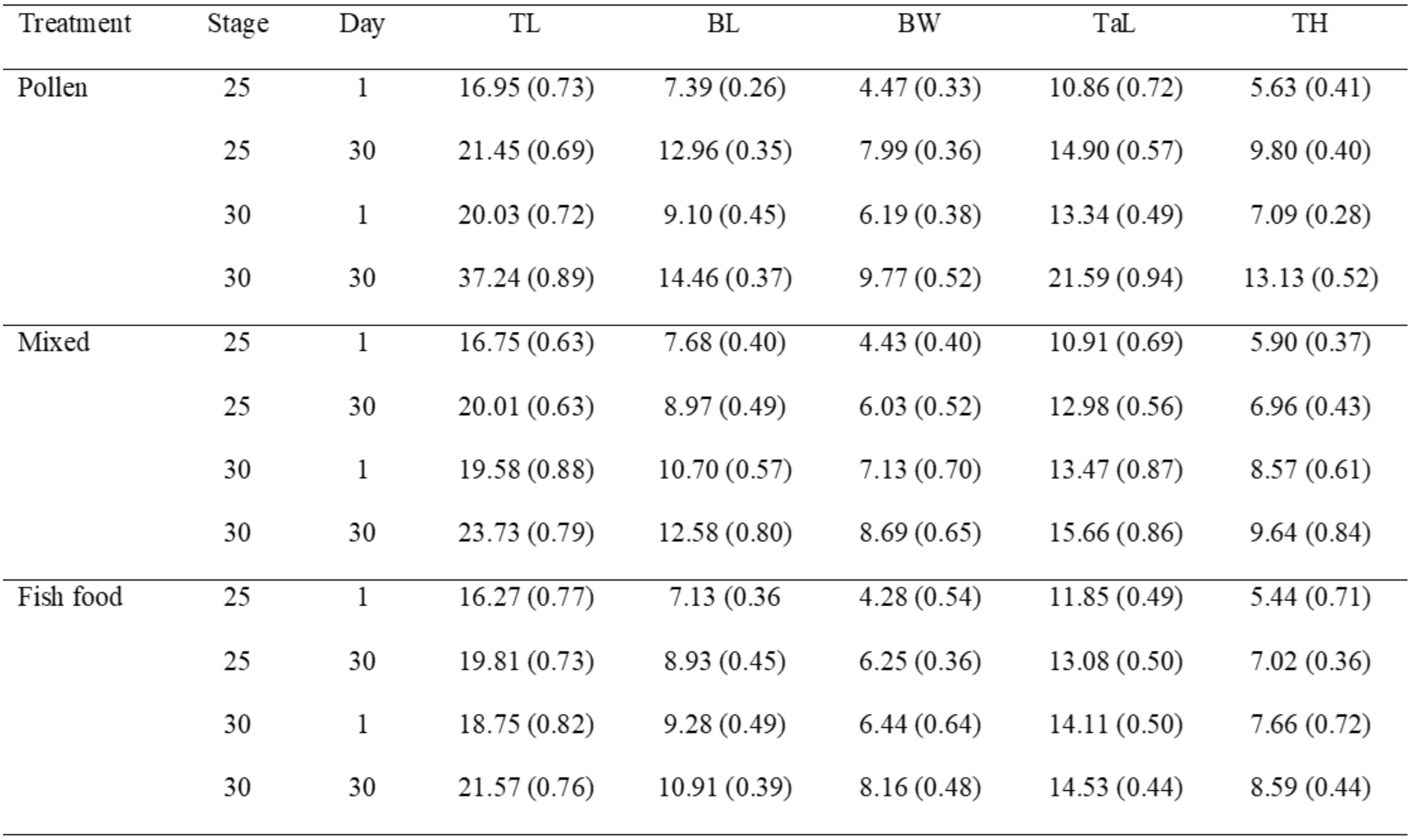
We chose the complete model to explain number of developmental stages advanced by tadpoles during the experiment because both variables, diet (Deviance = 129.0; df = 2, 56; $p < 0.001$) and initial developmental stage: Deviance = 123.3; df = 1, 58; $p < 0.001$), and their interaction (Deviance = 41.0; df = 2, 54; $p = 0.006$) were significant. The development of tadpoles that started in stage 25 did not change with diet, but for tadpoles that started in stage 30, development was greater with the pollen diet than with the other treatments (Fig. 4, Table 4).

The best model to explain fleeing distance included developmental stage at the beginning of the trials (Deviance = 101.2; df = 1, 28; $p = 0.001$) and diet (Deviance = 137.6; df = 2, 26; $p < 0.001$), without interactions (AICc = 161.15). The next better model also included the interaction between developmental stage and diet (AICc = 165.14). Tadpoles that started the trials in stage 25 always covered longer distances than tadpoles that started the trials in stage 30 within the same diet treatment. Tadpoles that started the trials in either stage 25 or 30 covered the longest distances when fed with pollen, differing significantly from tadpoles fed only with fish food. Tadpoles fed with mixed diets had intermediate values (Fig. 5, Table 4). Most tadpoles moved to the left, but laterality did not influence in tadpole performance regarding fleeing distance.

Pollen type and plant family	Number of quantified grains	Relative proportion
Melastomataceae	867	46.7
Eucalyptus type (Myrtaceae)	413	22.3
Areaceae	260	14.0
Eugenia type (Myrtaceae)	88	4.7
Cecropia type (Urticaceae)	29	1.6
Baccharis type (Asteraceae)	14	0.8
Mimosa type (Fabaceae)	14	0.8
Ilex type (Aquifoliaceae)	13	0.7
Anacardiaceae	11	0.6
Convolvulaceae	8	0.4
Vernonia type (Asteraceae)	7	0.4
Poaceae	6	0.3
Borreria type (Rubiaceae)	4	0.2
Bauhinia type (Fabaceae)	1	0.1
Croton type (Euphorbiaceae)	1	0.1
Gomphrena type (Amaranthaceae)	2	0.1
Malpighiaceae	2	0.1
Verbenaceae	1	0.1
Not identified*	114	6.1
Total	1855	100

*Eight pollen types not identified

Table 1. Pollen types and botanical families in decreasing order of relative proportions identified in the commercial pollen from *Apis mellifera* sample.



Treatment	Stage	Day	TL	BL	BW	TaL	TH
Pollen	25	1	16.95 (0.73)	7.39 (0.26)	4.47 (0.33)	10.86 (0.72)	5.63 (0.41)
	25	30	21.45 (0.69)	12.96 (0.35)	7.99 (0.36)	14.90 (0.57)	9.80 (0.40)
	30	1	20.03 (0.72)	9.10 (0.45)	6.19 (0.38)	13.34 (0.49)	7.09 (0.28)
	30	30	37.24 (0.89)	14.46 (0.37)	9.77 (0.52)	21.59 (0.94)	13.13 (0.52)
Mixed	25	1	16.75 (0.63)	7.68 (0.40)	4.43 (0.40)	10.91 (0.69)	5.90 (0.37)
	25	30	20.01 (0.63)	8.97 (0.49)	6.03 (0.52)	12.98 (0.56)	6.96 (0.43)
	30	1	19.58 (0.88)	10.70 (0.57)	7.13 (0.70)	13.47 (0.87)	8.57 (0.61)
	30	30	23.73 (0.79)	12.58 (0.80)	8.69 (0.65)	15.66 (0.86)	9.64 (0.84)
Fish food	25	1	16.27 (0.77)	7.13 (0.36	4.28 (0.54)	11.85 (0.49)	5.44 (0.71)
	25	30	19.81 (0.73)	8.93 (0.45)	6.25 (0.36)	13.08 (0.50)	7.02 (0.36)
	30	1	18.75 (0.82)	9.28 (0.49)	6.44 (0.64)	14.11 (0.50)	7.66 (0.72)
	30	30	21.57 (0.76)	10.91 (0.39)	8.16 (0.48)	14.53 (0.44)	8.59 (0.44)

Table 2. Mean (SD) of tadpole measurements at the beginning (day 1) and at the end (day 30) of the experiment. Tadpoles in stages 25 and 30 were subject to three different diets composed by pollen, fish food, and a mixture of both. TL = total length, BL = body length, BW = body width, TaL = tail length, TH = tail height.

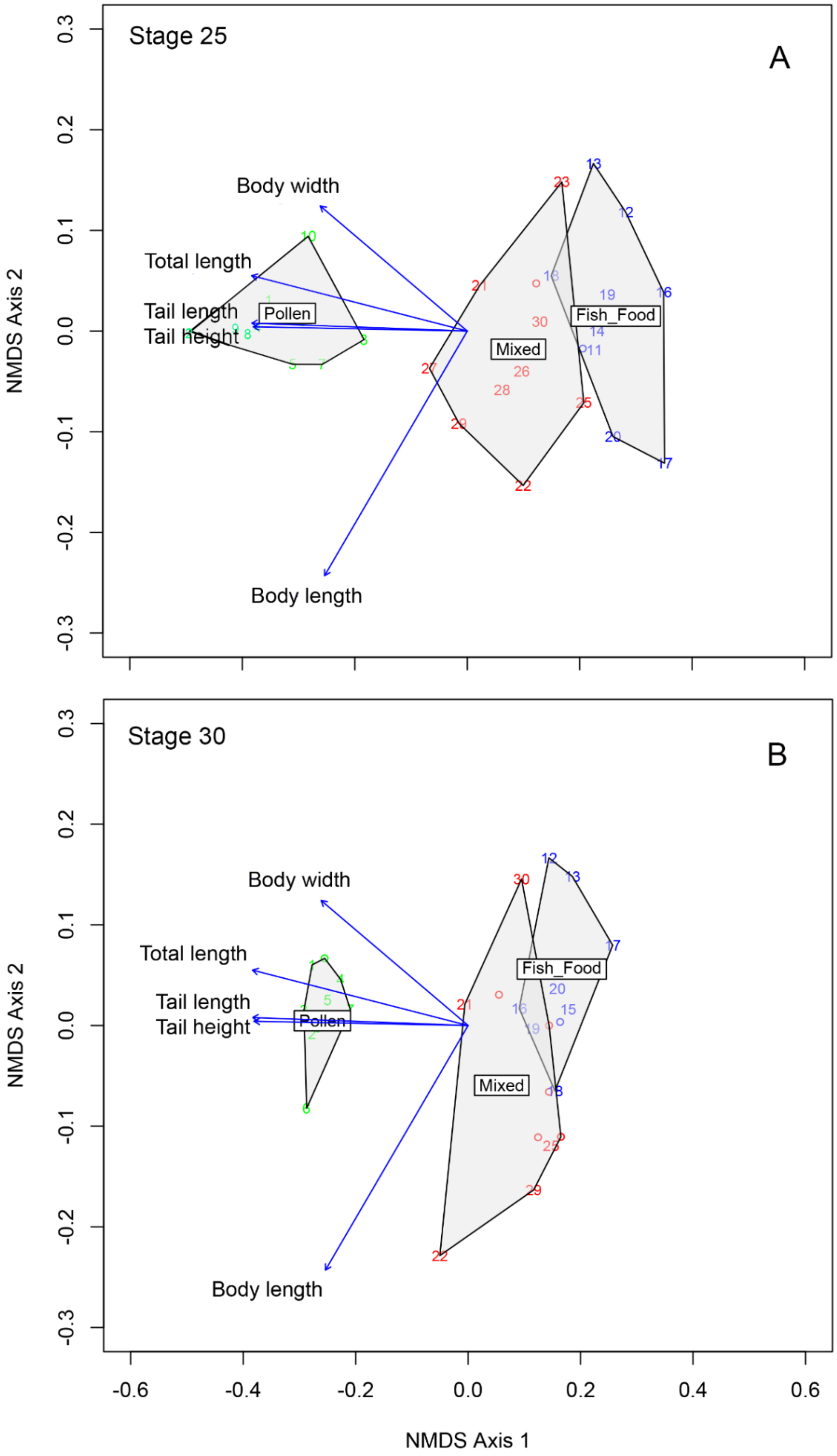


Figure 4. Distribution of tadpoles with initial stages 25 (A) and 30 (B) from different experimental groups (tadpoles fed with pollen, fish food or both) in a multidimensional space representing gain in body measurements (vectors). The contribution of each vector to the two axes can be seem at Table 3.

Measurement	NMDS 1	NMDS 2	r^2	*P*
Total length	-0.990	0.142	0.930	0.001
Body length	-0.722	-0.692	0.765	0.001
Body width	-0.903	0.429	0.519	0.001
Tail length	-0.999	0.021	0.911	0.001
Tail height	-0.999	0.011	0.898	0.001

Table 3. Contribution of tadpole measurement gains to the represented axes of the NMDS (see Fig. 4).

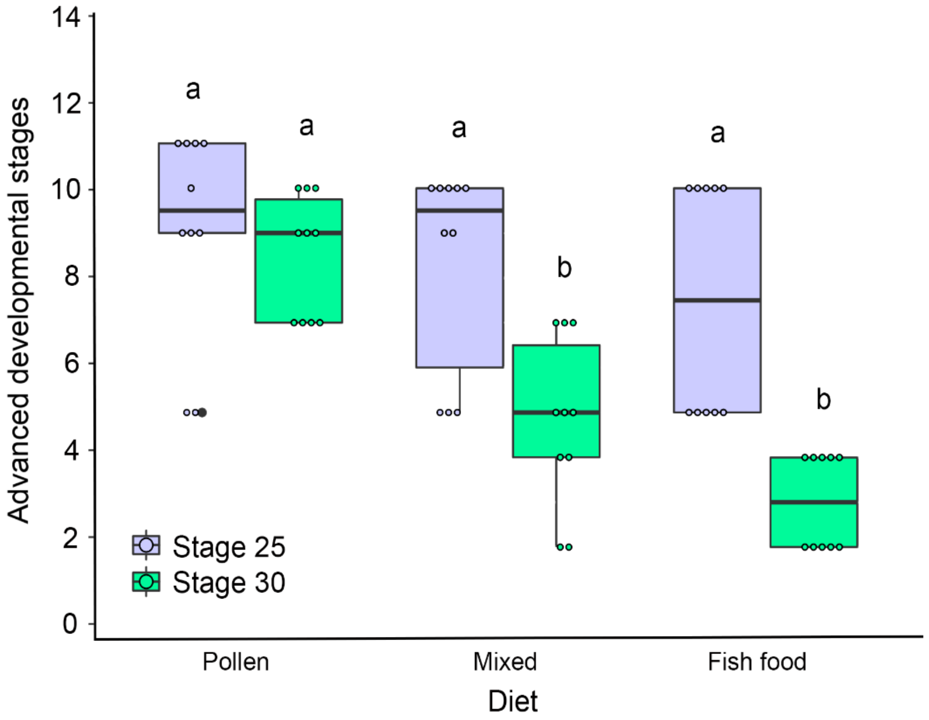


Figure 5. Number of developmental stages advanced by tadpoles with initial stages 25 and 30 after three different diet treatments (pollen, fish food, mixture of pollen and fish food). Different letters represent treatments/initial stages that differed significantly ($P < 0.05$).

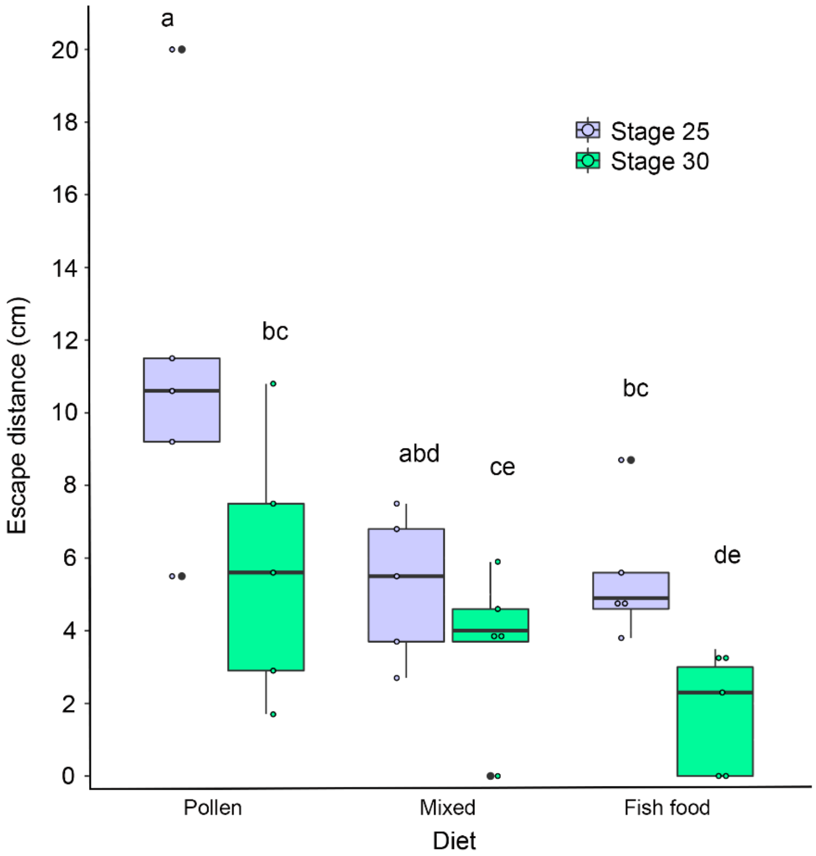


Figure 6. Fleeing distances covered by tadpoles with initial stages 25 and 30 after three different diet treatments (pollen, fish food, mixture of pollen and fish food). Different letters represent treatments/initial stages that differed significantly (*P* < 0.05).

Treatment	Initial stage	Final stage (n = 10)	Fleeing distance mean (SD) (n = 5)	Laterality % left (n = 5)
Pollen	25	30-36	11.36 (5.34)	80
	30	37-40	5.70 (3.64)	60
Mixed	25	30-35	5.24 (2.03)	80
	30	32-34	3.64 (2.20)	50
Fish food	25	30-35	5.52 (1.89)	40
	30	34-37	1.76 (1.66)	100

Table 4. Development, fleeing distance, and laterality of tadpoles of stages 25 and 30 subjected to three food treatments (pollen, fish food, and both mixed).

5. DISCUSSION

Our experiments corroborated the high nutritional value of pollen, considered to be rich in vitamins, mineral salts, lipids, and proteins (ROULSTON & CANE, 2000), for tadpole growth and development. Tadpoles grew more when fed with only pollen compared to a nutritive and balanced commercial food, and showed an intermediate response to a mixture of both diets (pollen and commercial food), regardless of their initial developmental stage (25 or 30). Considering development, tadpoles that started the experiments in stage 25 did not differ significantly in number of further developmental stages reached by the end of the trials. However, tadpoles that started in stage 30 reached more advanced developmental stages when fed with pollen compared to the other diets, reinforcing the value of pollen not only for growth, but also for development in the later stages before metamorphosis. Diet composition is extremely important for tadpole metamorphosis (MONTAÑA et al., 2019), which is influenced by diversity of consumed food items and their protein, carbohydrate, and lipidic proportions (KUPFERBERG, 1997; MONTAÑA et al., 2019). A proper nutrition is key to trigger hormonal changes that favor larval development (KUPFERBERG, 1997), like the regulation of thyroid hormones, responsible for a broad body structure remodeling (BROWN & CAI, 2007).

Tadpoles that started the experiment in stage 25 always covered longer distances while fleeing compared to tadpoles in stage 30 under the same diet treatment. A greater susceptibility to predation could explain a more pronounced fleeing response in vulnerable tadpoles (BATEMAN & FLEMING, 2015), and it is likely that stage 25 *Scinax machadoi* tadpoles may be at higher risk than stage 30 tadpoles at the study site, where the main predators are not so large aquatic insects (ESPANHA et al., 2015). However, when tadpoles were compared among diet treatments, they covered longer distances when fed with pollen compared to the commercial food, likely also indicating higher energy available due to better quality food. The behavior of *Scinax machadoi* tadpoles corroborates the high energetic content of pollen, as they change their feeding behavior, swimming more actively to reach pollen grains at the water surface, despite their usual benthic habits (KLOH et al., 2021b). According to the optimal foraging theory (MACARTHUR & PIANKA, 1966), only a high benefit provided by pollen would justify this higher energy investment for its acquisition. Even with no influence in fleeing distance, the laterality observed in this study, with most of the tadpoles turning to the left to escape, is an interesting record. Laterality consists in a tendency to use preferentially one side of the body due to structural asymmetries of the nervous system (Stancher et al., 2018) and has been recorded for some vertebrate groups (STANCHER et al., 2018; GOREE & WASSERSUG, 2001), including adult amphibians (WASSERSUG et al., 1999; GOREE & WASSERSUG, 2001). For tadpoles, however, there is still little information available about laterality (WASSERSUG et al., 1999). The preferential use of the left side by most *Scinax machadoi* tadpoles had already been recorded for feeding behavior (KLOH et al., 2021b) and is now reinforced by escape behavior, with no signs of influence in tadpole performance.

Our study corroborated a superior performance of tadpoles fed with pollen regarding growth, development, and agility/mobility. In this scenario, pollen input could be considered an important nutrient source for aquatic food webs, reinforcing the importance of preserving riparian vegetation for amphibian conservation. Such habitats not only offer shelter and favorable conditions for amphibian movement among breeding and resting sites (SEMLITSCH & BODIE, 2003), but also the flowering plants that provide tadpoles and likely other consumers with pollen, showed here to be a valuable food item. An impoverished diet can result in longer tadpole development (KUPFERBERG, 1997) with implications for the food web due to longer tadpole permanence in the aquatic habitat (HITE et al., 2018). Tadpole agility to escape predators can also influence survivorship (VAN BUSKIRK & MCCOLLUM, 2000) and reflect in the food web (HITE et al., 2018), as predation is an important mortality source controlling tadpole abundance (WELLS, 2007). Our results indicate that pollen should not be neglected as an important resource in the study of aquatic food webs involving tadpoles, as well as the importance of the riparian vegetation for nutrition of aquatic consumers.

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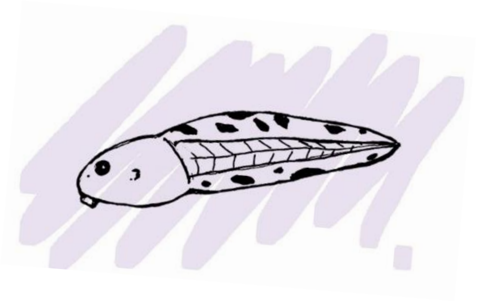
Capítulo 4



Taste for pollen comes in different shapes: consumption by tadpoles from three divergent ecomorphotypes

ABSTRACT

Pollen is a nutritive resource but its role in aquatic food webs is little studied. Successful pollen acquisition may require behavioral adaptations for tadpoles with certain ecomorphotypes, besides, pollen is hard to digest. We investigated the consumption of pollen by tadpoles of three anuran species representing three ecomorphotypes (nektonic, neustonic, and benthic tadpoles). We analyzed pollen content and digestion level in three sections of tadpole guts and in their feces, including in our sample tadpoles in three developmental stage ranges collected at wet and dry seasons at a site in southeastern Brazil. We tested the hypothesis that pollen is more consumed, and consumption is more diversified (including pollen from more plant families) by tadpoles that can reach it more easily from their usual microhabitats, determined by their ecomorphotypes, considering also potential effects of developmental stage range and seasonal differences. We also compared total number of pollen grains and proportion of digested (broken) grains at different gut sections and feces to identify digestion throughout the gut. We found neustonic tadpoles to consume a greater amount of pollen grains and digest them faster, compared to the other ecomorphotypes. However, diversity of plant families in the pollen consumed by tadpoles did not differ. Developmental stage ranges or season did not influence pollen consumption or digestion. We found pollen to be an important component of tadpole diets, being consumed and digested year-round by tadpoles in all developmental stages. The high nutritive value of pollen is likely to booster tadpole growth and development and have an important effect in aquatic food webs, reinforcing the importance of conserving riparian habitats and their vegetation.

Keywords: Pollen, diet, tadpole, ecomorphotype, digestion

RESUMO

O pólen é um recurso nutritivo, mas seu papel nas teias alimentares aquáticas é pouco estudado, embora já tenha sido relatado que girinos de várias espécies de anuros o consomem. No entanto, o pólen é difícil de digerir e pode exigir adaptações comportamentais para aquisição bem-sucedida por girinos com certos ecomorfotipos. Investigamos o consumo de pólen por girinos de três espécies de anuros representando três ecomorfotipos (girinos nectônicos, neustônicos e bentônicos). Analisamos o conteúdo de pólen e o nível de digestão em três seções do intestino de girinos e em suas fezes, incluindo em nossa amostra girinos em três faixas de estágio de desenvolvimento coletados nas estações úmida e seca em um local no sudeste do Brasil. Testamos a hipótese de que o pólen é mais consumido , e o consumo é mais diversificado (incluindo pólen de mais famílias de plantas) por girinos que podem alcançá-lo mais facilmente a partir de seus microhabitats habituais, determinados por seus ecomorfotipos, considerando também os efeitos potenciais da variação do estágio de desenvolvimento e diferenças sazonais. Também comparamos o número total de grãos de pólen e a proporção de grãos digeridos (quebrados) em diferentes seções intestinais e fezes para identificar a digestão em todo o intestino. ecomorfotipos. No entanto, a diversidade de famílias de plantas no pólen consumido pelos girinos não diferiu. Faixas de estágio de desenvolvimento ou estação do ano não influenciaram o consumo de pólen ou a digestão. Descobrimos que o pólen é um componente importante das dietas dos girinos, sendo consumido e digerido durante todo o ano por girinos em todos os estágios de desenvolvimento. É provável que o alto valor nutritivo do pólen impulsione o crescimento e desenvolvimento dos girinos e tenha um efeito importante nas teias alimentares aquáticas, reforçando a importância da conservação dos habitats ribeirinhos e sua vegetação.

Palavras-chave: Pólen, dieta, girinos, ecomorfologia, digestão.

1. INTRODUCTION

Pollen represents an important adaptation for breeding success of Gymnosperma and Angiosperma (VAN DER KOOI & OLLERTON, 2020). As pollen evolved, many organisms also adapted to use this caloric and protein rich structure (ROULSTON & CANE, 2000) as a resource (BOSCH et al., 2009; VAN DER KOOI & OLLERTON, 2020), leading to coevolution and speciation processes that brought mutual benefits to animals and plants involved (VAN DER KOOI et al., 2021). Mutualistic interactions between plants and pollinators are well studied and include varied groups such as insects, hummingbirds (Aves, Trochilidae), and bats (Mammalia, Chiroptera) (STRITONGCHUAY et al., 2019; VAN DER KOOI et al., 2021), which are attracted by specific plant adaptations (FENSTER et al., 2004). The role of pollen in aquatic food webs receive considerably less attention, however the consumption of pollen by amphibian larvae have recently been reported on several occasions (PRYOR, 2014; KLOH et al., 2018, 2019, 2021a, 2021b), indicating that it should not be neglected.

Tadpoles are common components of many aquatic food webs (e.g., O'NEIL & THORP, 2014; SCHMIDT et al., 2017) and their temporary permanence in aquatic habitats brings important consequences to the complexity and structure of such food webs (COSTA & VONESH, 2013; HITE et al., 2018). Their diets include periphytic algae, pollen, arthropod larvae, fungi, protozoan, amphibian eggs and larvae (ALTIG et al., 2007; KLOH et al., 2019). Although pollen is considered a very nutritive food item, it has a very resistant wall mainly composed by cellulose and sporopollenin and it is supposed to be hard to digest, requiring special adaptations (ROULSTON & CANE, 2000). Thus, it remains to be tested whether tadpoles can frequently consume and digest pollen to exclude the possibility of it being an occasional or accidental item in their diets. Additionally, many nutritive substances on pollen grains are hydrophilic, such as starch and the majority of its vitamins (ROULSTON & CANE, 2000), and could be lost as soon pollen falls on the water surface, quickly decreasing its nutritional value before it sinks (WAGNER, 1986). Thus, tadpoles should be able to harvest this resource at the surface, what may be a limiting factor for tadpoles that usually feed at the bottom. Tadpoles are known to feed mostly in specific microhabitats favored by their morphology (KLOH et al., 2019). For instance, tadpoles that feed on the bottom have ventral oral discs, whereas those filtering particles at the water column have frontal oral discs, and those adapted to feed on the surface have dorsal oral discs (ALTIG & JOHNSTON, 1989; ALTIG & MCDIARMID, 1999).

We tested the hypotheses that pollen is more consumed, and consumption is more diversified (including pollen from more plant families) by tadpoles that can reach it more easily from their usual microhabitats, determined by their ecomorphotypes. Thus, we expected pollen to be more abundant and diversified in the diets of neustonic, followed by nektonic tadpoles, and less abundant/diversified in the diets of benthic ones. We also considered developmental stages and season as potential factors influencing pollen consumption, as nutritional needs may change during tadpole development (KUPFERBERG, 1997) and pollen availability may change throughout the year (e.g., MODRO et al., 2007; SILVA et al., 2020a). Because tadpoles consume pollen and seem to do it actively (KLOH et al., 2021a), we expected pollen to be efficiently digested, especially in species that consume more pollen (if it is the case), justifying tadpole investment according to the optimal foraging theory (MACARTHUR & PIANKA, 1966). Thus, we expected the amount of pollen grains to decrease towards gut distal sections (and finally feces) and the proportion of broken grains to increase in the same direction.

2. METHODS

2.1 Study site

The sampling sites included a temporary stream (Fig. 1A), a permanent stream (19° 15’24.25” S, 43°32’39.8” W; Fig. 1E), and a puddle (Fig. 1I) 19°17’28.7” S, 43°35’39.8” W and 19°17’28.7” S, 43°34’39.8” W), all located at 1374 m above sea level at the Parque Nacional (National Park) Serra do Cipó (PNSC). The PNSC is an important conservation area at the southern portion of the Espinhaço Mountain Range (MADEIRA et al., 2008). The climate is seasonal, with rains concentrated between October and March and a dry season extending from April to September. Monthly temperatures vary between 13 and 29°C and mean annual rainfall, between 1460 and 2490 mm (VIVEROS, 2010). The predominating vegetation formation consists of montane meadows (“campos rupestres”), adapted to the rocky soils and limestone outcrops of the region (RIBEIRO et al., 2009). Some important plant families include Melastomataceae, Archariacea, Myrtaceae, Rubiaceae, and Lauraceae, among others (SANTOS et al., 2011).

2.2 Studied species

In order to represent contrasting tadpole ecomorphotypes, we selected three species with tadpoles described as nektonic (*Scinax curicica*; Fig. 1B, D), neustonic (*Phasmahyla jandaia*; Fig. 1F, H), and benthic (*Odontophrynus juquinha*; Fig. 1 J, L; *sensu* ALTIG AND JOHNSTON, 1989; ALTIG & MCDIARMID, 1999). We had already observed all three species to consume pollen in previous studies (KLOH et al., 2018; 2019; Fig. 1C, G, K).

Scinax curicica (PUGLIESE, POMBAL, & SAZIMA, 2004) is a treefrog endemic from the mountains of the southern portion of the Espinhaço Mountain Range, in Minas Gerais state (LEITE et al., 2006). It breeds in puddles in the rainy season and its tadpoles are nektonic (Fig. 1B, D), diurnal. They are found mainly in the rainy season but can occasionally occur at other periods if suitable habitats are available (ETEROVICK et al., 2020). *Phasmahyla jandaia* (BOKERMANN & SAZIMA, 1978) is endemic to Atlantic Forest remnants at the southern portion of the Espinhaço Mountain Range (SILVEIRA et al., 2019).

It breeds in permanent streams from the end of the dry season until the end of the rainy season (LEITE et al., 2008). Its tadpoles are neustonic, nocturnal, and present an expanded oral disc shaped like a funnel that filters food particles from the water surface (ETEROVICK et al., 2020; Fig. 1F, H). They can be found from September to April (LEITE et al., 2008; ETEROVICK et al., 2020). *Odontophrynus juquinha* (ROCHA, SENA, PEZZUTI, LEITE, SVARTMAN, ROSSET, BALDO & GARCIA, 2017) occurs in the states of Minas Gerais and Mato Grosso do Sul (SILVEIRA et al., 2019). It breeds in streams, swamps or temporary puddles during the rainy season and its tadpoles are benthic (Fig. 1J, L) and diurnal, occurring from November to March at the PNSC (ETEROVICK et al., 2020).

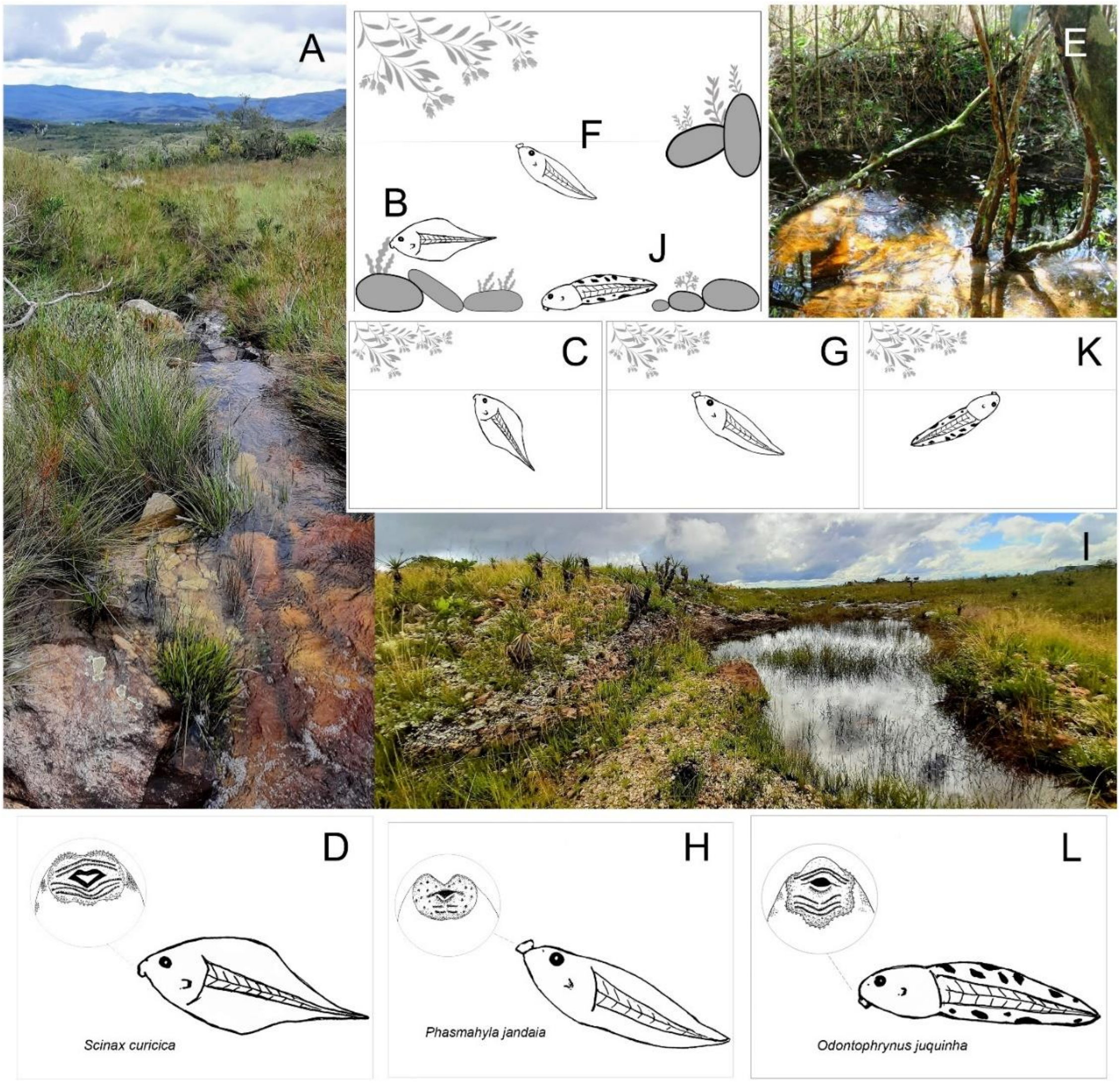


Figure. 1. Sampling sites including a temporary stream (A), a permanent stream (E), and a puddle (I) at the PNSC (Parque Nacional da Serra do Cipó), where tadpoles of *Scinax curicica* (B, C, D), *Phasmahyla jandaia* (F, G, H), and *Odontophrynus juquinha* (J, K, L) were collected, respectively. The tadpoles belong to nektonic (B, D), neustonic (F, H), and benthic (J, L) ecomorphotypes respectively, and all move to the water surface to feed on pollen (C, G, K).

2.3 Sampling procedures

We collected tadpoles in two days, one in January 2020, representing the rainy season, and the other in July 2020, representing the dry season. At each period we collected, for each of the three studied species, 10 tadpoles of three different developmental stage ranges: stages 25–28, stages 29–33, and stages 34–39 (*sensu* GOSNER, 1960). Our sample encompassed a total of 180 tadpoles (3 species x 10 tadpoles x 3 developmental stage ranges x 2 seasons). Tadpoles were immediately euthanized in xylocaine 10% and preserved in 10% formalin to preserve gut contents. Developmental stages were confirmed in the laboratory under a stereomicroscope.

For diet analyses, we removed the guts and divided them in three sections of equivalent length (KLOH et al., 2018; Fig. 2) to compare digestion levels: the proximal section includes the manicotto, the medial section corresponds to a portion of the small intestine, and the distal section includes the colon and the rectum (*sensu* PRYOR & BJORNDAL, 2005). Each section was macerated and diluted in 1 mL distilled water on a Sedgewick-Rafter counting chamber, which we examined under optic microcope at 400x magnification. Besides the 180 collected tadpoles, we captured five additional tadpoles of each species at each developmental stage range considered and at each sampling period and kept them in individual containers with rested tap water for two hours to obtain their feces. This resulted in a total of 90 fecal samples (3 species x 5 tadpoles x 3 developmental stage ranges x 2 seasons). Afterwards these tadpoles were returned to their original habitats. The feces were collected with a syringe with 1 ml water and placed at a Sedgewick-Rafter counting chamber. We counted and identified pollen grains in feces and all gut sections based on CASSINO et al. (2016) and SILVA et al. (2020b).

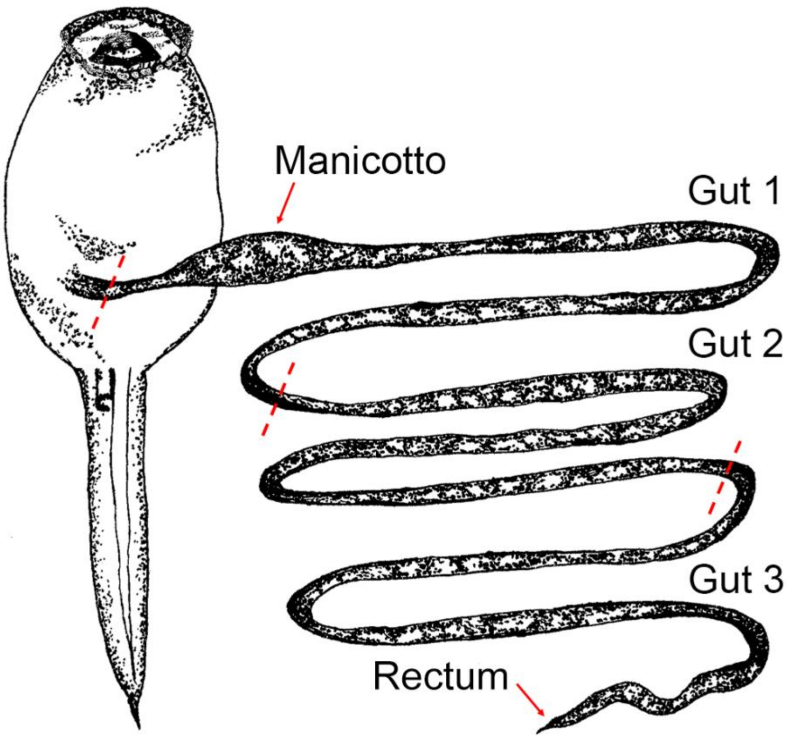


Figure. 2. Schematic representation of the sections considered for the analyses of gut contents in tadpoles of *Phasmahyla jandaia*, *Scinax curicica*, and *Odontophrynus juquinha*. Traced lines represent cutting points between sections.

3. STATISTICAL ANALYSES

We tested for the importance of (1) tadpole ecomorphological type (represented by the three different species), (2) developmental stage, (3) season, and their potential interactions to explain diversity of pollen consumption. We represented pollen diversity (our dependent variable) as the number of consumed pollen grains (log transformed for normality) of each plant family by tadpoles of each species in each developmental stage range. We used generalized linear models (GLMs) in R (R Core Team, 2020). We used the package MuMIn (BARTÓN, 2022) to select the best model based on AICc values.

We tested for differences in total number of pollen grains (including both intact and broken grains, that is, grains with no contents) and proportion of broken grains (as dependent variables) as surrogates for digestion level in each of four digestion phases, represented by the three sections of the gut and feces. We used individual data for all 180 tadpoles plus 90 fecal samples. Besides (1) digestion phase, we also used (2) species, (3) developmental stage range, and (4) season as potential explanatory variables for digestion level. Our data had Tweedie distribution due to the great number of gut sections/feces with no pollen grains in the sample. We thus used statmod (GINER & SMYTH, 2016) and tweedie (DUNN, 2017) packages to build GLMs. We first tested for effects of interactions between species, seasons, and developmental stages. As these showed no interactions, we tested each variable separately. We used the package emmeans (LENTH, 2022) for post hoc tests.

4. RESULTS

We found pollen from seven plant families (Fabaceae, Asteraceae, Melastomataceae, Poaceae, Anacardiaceae, Myrtaceae, and Urticaceae) in the guts and feces of the tadpoles of *Scinax curicica*, *Phasmahyla jandaia*, and *Odontophrynus juquinha*, representing three tadpole ecomorphotypes (nektonic, neustonic, and benthic, respectively; Fig. 3). The diversity of pollen consumption did not differ among tadpole ecomorphological types, developmental stage, or season. The null model had a better performance than all other models (Table 1).

Both total number of pollen grains and proportion of digested grains differed among species and among digestion phases. For total number of pollen grains there was no significant interaction between explanatory variables. *Phasmahyla jandaia* consumed more pollen grains than both *Scinax curicica* (estimate = 0.768, SE = 0.175, z = 4.376, $p < 0.001$) and *Odontophrynus juquinha* (estimate = -0.510, SE = 0.167, z = -3.055, p = 0.006), that did not differ between each other (estimate = 0.258, SE = 0.188, z = 1.371, p = 0.356; Fig. 4A). All digestion phases differed among each other regarding total number of pollen grains except for the third section of the gut and feces (Table 2, Fig. 4A).

Species also differed among each other regarding proportion of digested grains, with *P. jandaia* digesting more efficiently than both *S. curicica* (estimate = 0.586, SE = 0.172, z = 3.407, p = 0.002) and *O. juquinha* (estimate = -0.474, SE = 0.168, z = -2.819, p

= 0.013), that did not differ between each other (estimate = 0.111, SE = 0.184, z = 0.605, p = 0.818; Fig. 4B). Proportion of digested grains was greater in the second portion of the gut, that differed from all other portions which, in their turn, did not differ from each other (Table 2, Fig. 4B). There was no difference in neither total number of pollen grains nor proportion of digested grains among developmental stage ranges or between seasons.

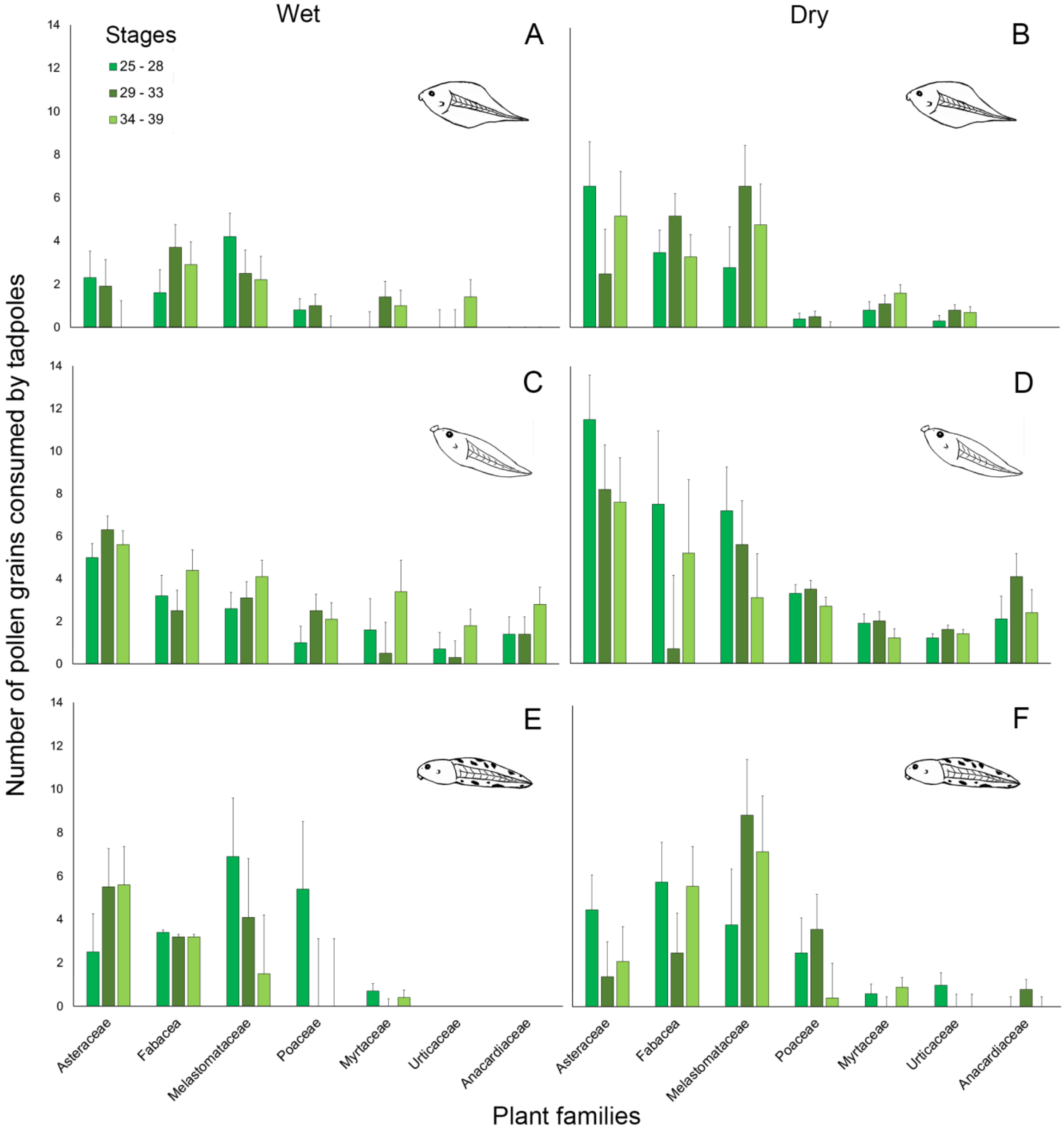


Figure. 3. Numbers of pollen grains from different plant families ingested by tadpoles of *Scinax curicica*, (A, B) *Phasmahyla jandaia* (C, D), and *Odontophrynus juquinha* (E, F) in three developmental stage ranges (25–28, 29–33, and 34–39; *sensu* GOSNER, 1960) during wet (A, C, E) and dry (B, D, F) seasons.

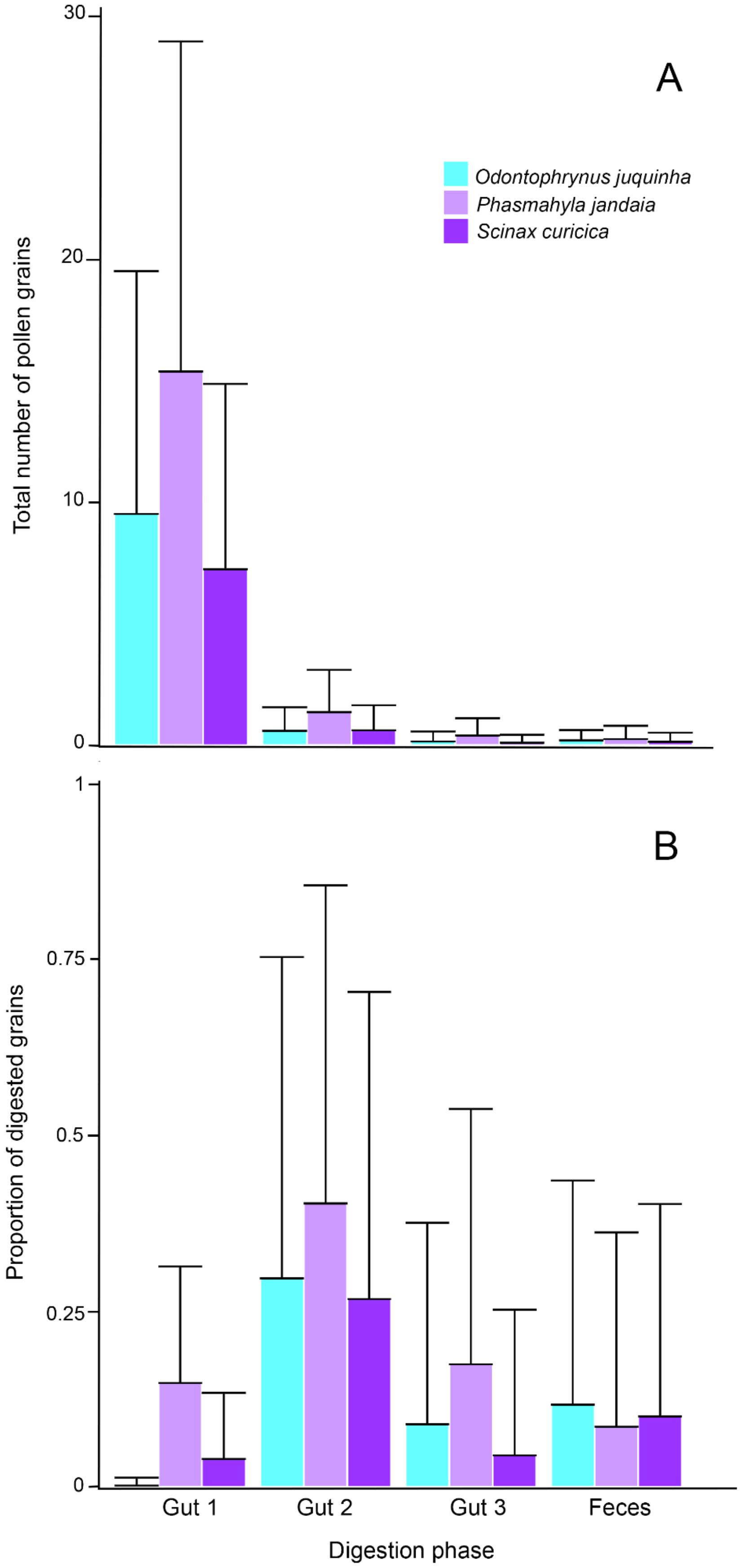
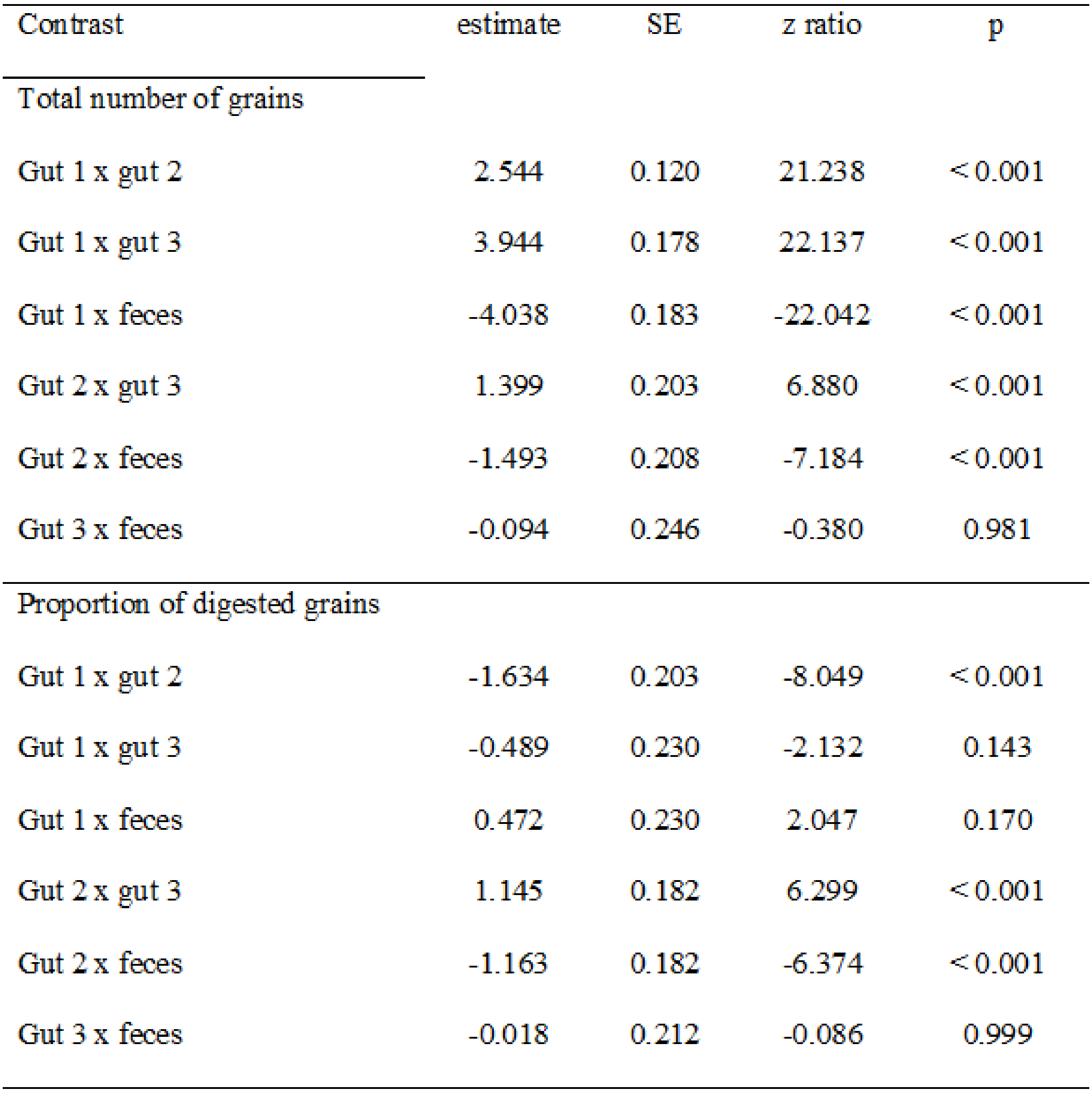


Figure. 4. Total number of pollen grains and proportion of digested pollen grains in three digestion phases represented by three gut sections and feces of tadpoles of *Phasmahyla jandaia*, *Scinax curicica*, and *Odontophrynus juquinha*.

	df	Deviance	Residual df	Residual deviance	P
Species	2	2.04	96	68.31	0.263
Stage	2	0.12	94	68.19	0.942
Season	1	0.23	93	67.96	0.584
Species x stage	4	2.22	89	65.74	0.573
Species x season	2	1.67	87	64.07	0.334
Stage x season	2	0.06	85	64.01	0.961
Species x stage x season	4	2.21	81	61.79	0.574

Table 1. Results of the complete GLM (AICc = 281.91) investigating the effects of tadpole ecomorphological types (represented by species), developmental stage, season, and/or their potential interactions on pollen consumption by tadpoles (given by number of grains from different plant families). The best model was the null model (AICc = 251.25).



Contrast	estimate	SE	z ratio	p
Total number of grains				
Gut 1 x gut 2	2.544	0.120	21.238	< 0.001
Gut 1 x gut 3	3.944	0.178	22.137	< 0.001
Gut 1 x feces	-4.038	0.183	-22.042	< 0.001
Gut 2 x gut 3	1.399	0.203	6.880	< 0.001
Gut 2 x feces	-1.493	0.208	-7.184	< 0.001
Gut 3 x feces	-0.094	0.246	-0.380	0.981
Proportion of digested grains				
Gut 1 x gut 2	-1.634	0.203	-8.049	< 0.001
Gut 1 x gut 3	-0.489	0.230	-2.132	0.143
Gut 1 x feces	0.472	0.230	2.047	0.170
Gut 2 x gut 3	1.145	0.182	6.299	< 0.001
Gut 2 x feces	-1.163	0.182	-6.374	< 0.001
Gut 3 x feces	-0.018	0.212	-0.086	0.999

Table 2. Comparison of pollen digestion levels represented by (1) total number of pollen grains and (2) proportion of digested pollen grains among four different digestion phases of tadpoles represented by three consecutive sections of the gut and feces.

5. DISCUSSION

We showed that tadpoles of different ecomorphotypes consumed different amounts of pollen but did not differ in diversity of plant families represented. As expected, the neustonic tadpoles of *Phasmahyla jandaia* were the ones that consumed the greatest amounts of pollen, however the nektonic *Scinax curicica* and the benthic *Odontophrynus juquinha* did not differ from each other. *Phasmahyla jandaia* tadpoles show adaptations to feed at the water surface. They have umbelliform oral discs above the head and they produce a subtle water flow with the tip of the tail that may help capture suspended particles (LEITE et al., 2008). Although we expected benthic tadpoles to have more difficult access to pollen at the water surface, *Odontophrynus juquinha* consumed an amount of pollen comparable to *S. curicica*, corroborating the ability of benthic tadpoles to reach the water surface for pollen, as also reported for *S. machadoi* (KLOH et al., 2021a). Once in the aquatic habitat, pollen is available for a short time interval and its nutritional value rapidly decreases (WAGNER, 1986). Thus, aquatic pollen consumers, such as these tadpoles, are expected to behaviorally adapt to benefit from this resource (KLOH et al., 2021a).

Most consumed pollen grains were from the family Melastomataceae (Order Myrtales). This is an important plant family in Brazil, with 73 genera, 19 of which endemic. Most of them occur at montane meadows, where they show ecological interactions with bees, wasps, hummingbirds, and bats (OLIVEIRA & ROMERO, 2008; SANTOS et al., 2011). Here we report the importance of pollen from this plant family also for tadpoles. At montane meadows many plants families flower during the dry season (COELHO et al., 2006; CERQUEIRA et al., 2008). Additionally, winds are stronger during the dry season (NASCIMENTO, 1998), and we suppose this could facilitate pollen dispersion and, consequently, its availability in aquatic ecosystems. In this context, pollen should be an important food source for tadpole growth and development to metamorphosis during the dry season, when periphyton is likely to have reduced growth (CAMARGO & FERRAGUT, 2014). Besides, the smaller water volume and reduced current of the streams during the dry season (JSK and PCE, pers. obs.) may allow pollen to remain available for longer periods at the water surface over specific microhabitats. However, pollen consumption did not differ between wet and dry seasons for any of the studied species, neither among the developmental stage ranges compared, reinforcing the importance of pollen as a nutrient during the whole year. These results indicate that tadpoles are likely to actively consume pollen at all developmental stages, as already demonstrated for *P. jandaia* (KLOH et al., 2021b), and whenever available, despite likely differences in availability between seasons (e.g., MODRO et al., 2007; SILVA et al., 2020a).

As expected, we found a greater number of pollen grains in the first section of the gut, with numbers decreasing towards the end, after progressive digestion. The greatest proportions of digested pollen grains occurred in the second section of the gut, where many grains were already broken but their remains could still be observed. From this section on, the number of grains decreased even more, the proportion digested did not change, and the digestion process was likely over (see Fig. 4). These results indicate an efficient digestion of pollen grains despite their resistant external layers composed by cellulose and sporopollenin, a very resistant polymer that protects pollen grains from mechanical impacts and desiccation (ROULSTON & CANE, 2000). The ability of tadpoles to digest pollen is certainly important, as it provides access to pollen internal contents rich in lipids, vitamins, mineral salts, and proteins (ROULSTON & CANE, 2000). Tadpoles need great nutrient consumption to reach metamorphosis in time constrained habitats (ALTIG et al., 2007), and pollen is likely to booster development, as observed for *Scinax machadoi* (KLOH et al., submitted manuscript).

Besides consuming more pollen, *Phasmahyla jandaia* digested pollen more efficiently than the other species, indicating a greater adaptation to use pollen as food. Pollen is not easy to digest, and its digestion by consumers may occur through (1) mechanical breakage, (2) pollen perforation with mouthparts, (3) pollen wall dissolution by enzymes, (4) induction of germination or pseudo-germination, (5) pollen breakage through osmotic chock, or (6) penetration of pollen wall by digestive enzymes (ROULSTON & CANE, 2000). Tadpoles have long guts, already interpreted as an adaptive compensation for reduced peristaltic movements and lack of glands that produce efficient digestive enzymes (ALTIG et al., 2007). However, the presence of sand grains in tadpole guts have already been recorded and their ingestion could be intentional as a strategy to break resistant food items, such as pollen grains and diatoms (Bacillariophyta) with rigid siliceous cell walls (ALTIG et al., 2007; KLOH et al., 2018b). Tadpoles may break pollen grains mechanically with the aid of sand grains, through osmotic pressure in their guts, or with some acid proteases (SANTOS et al., 2016), which are considered as rare digestion methods (ROULSTON & CANE, 2000). The presence of broken grains in the guts of all studied species corroborates pollen digestion by the studied tadpoles. On the other hand, no signs of germination or pseudo-germination were detected. Breakage of pollen grains by mouthparts is also unlikely in these tadpoles, as most pollen grains were intact in the first section of the gut.

Here we report consistent ingestion and digestion of pollen grains by tadpoles of three anuran species, representing three tadpole ecomorphotypes. Our results indicate tadpole adaptation for pollen ingestion even in ecomorphotypes that do not usually feed at the water surface, suggesting an important role of pollen for tadpole nutrition. Riparian vegetation is key to sustain species in riparian and aquatic habitats (HUNT et al., 2020). The likely importance of pollen to aquatic food webs should not the neglected and reinforces the benefits of riparian vegetation conservation for the integrity of aquatic ecosystems.

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CONCLUSÃO GERAL

O que aprendemos sobre dieta de girinos até aqui.

Como é possível observar, os resultados mostram que a dieta de girinos Neotropicais possui aspectos interessantes do ponto de vista ecológico e de conservação. Porém, esses aspectos ainda são muito pouco explorados e, com isso ainda temos uma visão limitada sobre a influência dos alimentos consumidos na história natural das larvas dos anuros. E possível detectar a existência de uma plasticidade comportamental por parte dos girinos para o comportamento de forrageio, evidenciando que existe uma adaptação à disponibilidade de alimentos nos ambientes. A partir desse ponto, é possível destacar como a evolução moldou esses organismos para serem eficientes na busca por alimentos, não somente no aspecto morfológico, mas também no comportamental.

A Teoria do Forrageamento Ótimo (TFO) encontra nos girinos excelentes modelos de estudo, uma vez que os resultados direcionam esses organismos para o centro dessa teoria, que até então e mais estudada e evidente em vertebrados endotérmicos (Aves e Mamíferos). Encontramos evidências importantes de que é necessário estudar com cautela os diferentes estágios de desenvolvimento das larvas dos anuros, pois cada etapa corresponde a uma série de transformações importantes, que carregam em si mudanças morfológicas, fisiológicas e comportamentais específicas.

Fica evidente a necessidade de mais estudos que abordem dieta de girinos, que não somente descrevam estruturas de comunidades as quais esses organismos componham, mas que testem hipóteses e teorias ecológicas. Com esses novos *insights* é possível ter um melhor entendimento dos fatores que atuam na ecologia nutricional das espécies, aumentando os níveis de conhecimento sobre o assunto. Assim, será possível produzir melhores protocolos e estratégias de conservação, visto que os anfíbios em geral são um dos grupos de vertebrados mais ameaçados em perdas populacionais e extinções locais na atualidade, seja pela perda crescente de habitats, disseminação de doenças ou pelas mudanças climáticas.

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