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1 TITLE: Flexible feeding kinematics of a tropical carnivorous anuran tadpole

3 RUNNING TITLE: Feeding behavior of tadpoles

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ABSTRACT

Leptodactylus labyrinthicus tadpoles are known predators of anuran eggs and hatchlings, but they are also able to filter-feed in the water column and scrape food off of firm substrates. We evaluated and compared these alternative feeding behaviors in relation to feeding kinematics and the shape of the mouth with high-speed digital imaging. We tested the hypotheses that (i) *L. labyrinthicus* tadpoles use functionally different feeding kinematics when feeding on alternative food sources and (ii) that the jaw sheaths of *L. labyrinthicus* tadpoles deform less during filter-feeding and substrate grazing compared to more common tadpoles not so specialized for macrophagous carnivory. Our results show that filtering and scraping feeding behaviors differ significantly in both kinematics and shape of the mouth. During filter-feeding, tadpoles display longer gape cycles and attain a narrower maximum gape earlier in the cycle compared to substrate grazing. Jaw deformation during opening and closing phases of the gape cycle is more pronounced during grazing on firm substrates. This deformation contributes to the achievement of a wider maximum gape during feeding. These differences appear to reflect behavioral adjustments by the tadpoles to maximize food intake. Feeding in tadpoles of *L. labyrinthicus* is not restrained by their typical carnivorous morphology. On the contrary, *L. labyrinthicus* tadpoles seem to be opportunistic feeders able to obtain nutrients from a variety of food sources by using different feeding strategies.

Key words: Anura, behavioral plasticity, biomechanics, geometric morphometrics, food source.

INTRODUCTION

Tadpole feeding is influenced by morphological, physiological and behavioral traits (e.g. Seale & Wassersug, 1979; Vera Candioti, Lavilla & Echeverría, 2004; Pryor & Bjorndal, 2005) as well as microhabitat use (e.g. Rossa-Feres, Jim & Fonseca, 2004; Sousa Filho et al., 2007) and seasonal activity (e.g. Peterson & Boulton, 1999; Echeverría, Volpedo & Mascitti et al., 2007). This has led to a variety of trophic specializations in tadpoles, although the larvae of most species are considered opportunistic omnivorous feeders (Altig, Whiles & Taylor, 2007). As such, anuran tadpoles can consume a diversity of food items, including algae, bacteria, fungi, protozoans, detritus, invertebrates, anuran eggs, and other tadpoles (reviewed by Altig et al., 2007). Depending on the quality and quantity of available food sources, many tadpoles can alternate between being omnivorous, microphagous suspension feeders, and opportunistic macrophagous predators (Petranka & Kennedy, 1999; Richter-Boix et al., 2007). To date, tadpole feeding behavior has only been described as tadpoles forage on a single type of food (e.g. Venesky et al., 2011; Venesky et al., 2013; Wassersug and Yamashita, 2001). Therefore, it is still unclear how tadpoles adjust their feeding mechanics to a diversity of food items and, consequently, to different substrates.

Herein, we explore the biomechanics of alternative feeding strategies of the macrophagous and carnivorous tadpole of *Leptodactylus labyrinthicus* (Spix, 1824) when provided with suspended and attached food sources. This species occurs in temporary aquatic habitats in Brazil and Paraguay, including Cerrado and Cerrado-like enclaves within Amazonia and the Atlantic rain forests (Heyer, 2005). Tadpoles of *L. labyrinthicus* present distinct morphological adaptations associated with macrophagy, including prominent jaw sheaths, reduced labial tooth rows (one anterior and two posterior tooth rows, Figure 1), and a slender body (Rossa-Feres & Nomura, 2006).

They prey upon con- and heterospecific tadpole eggs and hatchlings (Prado et al., 2005; Shepard & Caldwell, 2005; Silva, Giaretta & Facure, 2005). However, in captivity, tadpoles actively feed upon, and can survive through metamorphosis on, suspended particles of food as well as food attached to submerged surfaces (Rossa-Feres personal observation). Given this dietary breadth, *L. labyrinthicus* larvae can serve as a model tadpole for studying how anuran larvae adjust their feeding kinematics to different food sources.

In a laboratory experiment, we provided tadpoles of *L. labyrinthicus* with an algal-based powdered food that was either suspended on the water surface or attached to a planar glass substrate, and used high-speed digital videography to film tadpoles as they consumed the food. This methodology has allowed researchers to investigate functionally complex behaviors, such as feeding, with enough precision to visualize subtle but important differences in those behaviors (e.g. Wassersug & Yamashita, 2001; Venesky et al., 2013). Given the unpredictable hydroperiod in tropical pond systems (e.g. Rossa-Feres & Jim, 2001) and the selective pressures to reach a critical size before metamorphosis (e.g. Wilbur & Collins, 1973), we hypothesized that the carnivorous tadpoles *L. labyrinthicus* would adjust their feeding kinematics to maximize food consumption when feeding on alternative food sources. In addition, we predicted that the relatively robust jaw sheaths of *L. labyrinthicus*, which are understood to be an adaptation for macrophagous carnivory, would be less flexible and show little or no deformation (i.e., adjustment in shape) when the tadpoles were suspension feeding on finer, particulate material (Venesky et al., 2011; Venesky et al., 2013).

MATERIAL AND METHODS

Collection of tadpoles

Tadpoles of *L. labyrinthicus* were collected from marshes on the banks of ponds in Icém (20°21'47.66"S, 49°14'13.08"W), northwestern of São Paulo State, and in Buenópolis (17°54'45.50"S, 44°14'45.92"W), Minas Gerais State, Brazil. Tadpoles were transported in plastic bags filled with water from the pond to the laboratory at the Universidade Estadual Paulista – UNESP, campus of São José do Rio Preto, São Paulo State, Brazil. In the laboratory, tadpoles were maintained in polyethylene aquaria (37 x 30 x 10 cm) filled with dechlorinated and continuously aerated tap water. Tadpoles were maintained at 22°C on a natural photoperiod and were fed *ad libitum* once a day with a mixture of flocculated (Alcon BASIC®, Alcon, Brazil) and powdered (Sera Micron®, SERA, Germany) commercial fish food until the beginning of the experiments. Tadpoles were acclimated to laboratorial conditions for at least 5 days before trials. Tadpoles were collected under permit from the Brazilian Institute of Environment and Natural Resources (IBAMA) and ICMBio – Ministério do Meio Ambiente, Brazil (SISBIO no. 18163-1 to D.C.R.-F.) and maintained under approval from the Ethics Committee on the use of Animals (CEUA-IBILCE/UNESP), in accordance with the National Council for Control of Animal Experimentation (CONCEA).

Feeding Trials

In our first experiment, we filmed four tadpoles (mean $\pm$ SD, total length: 58.45 $\pm$ 4.37 mm; range of Gosner developmental stage: 35 - 39) at 500 frames/s as they fed on suspended particles of food on the surface of the water. Prior to filming, we placed individual tadpoles in a glass container (15 cm tall and 5 cm in diameter) that contained about 250 mL of aged tap water. We then prefocused the camera (Fastec TroubleShooter LE 250; Fastec Imaging, California USA) on the surface of the water and sprinkled a fine particulate food (Sera Micron®) on the surface of the water until it

formed a thin and uniform film. The amount of food added to the container was not standardized but appeared uniformly distributed on the surface of the water.

In our second experiment, we filmed three tadpoles (total length: 61.90 ± 4.11 mm; developmental stage: 39) as they fed on particles attached to a planar glass surface. Prior to filming, we made of a mixture of Sera Micron® and water, brushed it on one side of glass microscope slides (7.5 x 2.5 cm), and allowed it to air dry. We then mounted the clean side of one food-covered slide against the inside wall of a glass aquarium (8.5 x 8.5 x 8.5 cm), filled it with aged tap water, and filmed tadpoles in individual trials while they grazed on the food-covered surface. Because the kinematics of tadpole feeding is influenced by the resistance they encounter (Wassersug & Yamashita, 2001), all slides were made with the same concentration of Sera Micron® and we used a fresh food-covered slide for each videography trial to ensure that tadpoles had access to a substrate of similar density and food concentration.

The camera was prefocused on the food-covered surface prior to each trial following the protocol described in Venesky et al. (2011). During filming, the camera's vertical field of view was adjusted as necessary. As in the first experiment, we filmed tadpoles at 500 frames/s using the Fastec camera. In these trials, we recorded single feeding bouts with a "feeding bout" defined as beginning when a tadpole contacted the food covered surface and opened its mouth to the time when the tadpole completely closed its mouth and swam away. Each feeding bout consisted of a continuous series of "gape cycles", during which the tadpoles scraped food from the slide. As per Venesky et al. (2011), a "gape cycle" (1) starts with the jaw sheaths fully closed and the anterior and posterior tooth rows in closest proximity; (2) proceeds to the point where the mouth is fully open and the labial tooth rows reached maximum gape; and (3) ends with full

closure of the jaw sheaths and anterior and posterior tooth rows again in closest proximity to each other.

After each feeding trial, we anesthetized the tadpoles with 2% lidocaine hydrochloride and then fixed it in a 1:1 solution mixture of 70% alcohol and 15% formalin. The developmental stage was determined according to Gosner (1960). Tadpoles were deposited in the amphibian collection DZSJRP-Tadpoles of the Department of Zoology and Botany, UNESP, São José do Rio Preto, SP, Brazil.

Feeding kinematics

In order to compare the kinematics of filter-feeding at the water's surface with feeding while grazing on (i.e., scraping material off) a solid substrate, we quantified four kinematic variables that were common to feeding in both settings: (i) *Duration of the full gape cycle* – the duration of time from when the jaws begin to open until they are fully closed; (ii) *Time to maximum gape* – the duration of time from when the mouth starts to open to when maximum gape is achieved; (iii) *Percentage of time to maximum gape* – the duration of time, as percentage of the total time of the gape cycle, when maximum gape is achieved; and (iv) *Maximum gape* – the length (in millimeters) from the internal border of upper jaw sheath to the internal border of the lower jaw sheath. We extracted data from five randomly selected gape cycles from each tadpole (n=7). All measurements were obtained using ImageJ 1.47m (Rasband, 2012).

Plasticity in jaw sheath shape

In our next set of analyses, we assessed patterns of shape variation in tadpoles' mouth between filtering and substrate grazing behavior. When tadpoles graze upon a substrate, they generate a suspension of material that is drawn into their mouths and

then filtered out of the water by internal oral structures. Thus, whether a tadpole is ingesting particulate matter already in the water column or grazing on a substrate to produce a suspension, the tadpole in both situations is filter-feeding. However, to keep the wording simple here, we refer throughout to the first situation as "filter-feeding" and the second as "grazing." We obtained digital images of the tadpoles' mouth by extracting frames of high-speed video files with Midas OS (Xcitex Inc. 2012) and used the landmark-based geometric morphometrics method (Rohlf & Marcus, 1993; Zelditch et al., 2004) to evaluate shape variation in mouth. We focused our analysis to three different phases of the gape cycle: (i) opening, which is the point in the gape cycle where the anterior and posterior jaw sheaths are beginning to open but are still in contact with each other; (ii) maximum gape, which is the point in the gape cycle where the anterior and posterior jaw sheaths are furthest apart from each other; and (iii) closing, which is the point in the gape cycle where the anterior and posterior jaw sheaths are closing and regain contact with each other. For each phase, we placed five digital landmarks on each jaw sheath using TpsDIG 2.16 (Rohlf, 2010). For each jaw sheath, landmarks were placed in the left and right extremes, the center, and a point equidistant between the lateral extremes and center of jaw sheath. Selection of landmarks were based on the ability to easily and consistently identify the same position on the anatomical structure, their visibility throughout the entire gape cycle, and their ability to represent the change in shape of the structure during feeding (Venesky et al., 2013).

A Generalized Procrustes Analysis (Gower, 1975; Rohlf & Slice, 1990) was used to superimpose the specimens through the alignment of their landmarks configurations to a Cartesian plane after accounting for differences in position, orientation, and scale. Generalized Procrustes Analysis was performed with the use of the function `gpagen` in the `geomorph` package (Adams & Otárola-Castillo, 2013) of R

software (R Core Team, 2013). The resulting Procrustes-aligned coordinates represent the shape of each specimen and were used in further statistical analysis.

Statistical analysis

To determine whether the kinematics of the jaw movements differed between filter-feeding and substrate grazing behaviors, we used repeated measures multivariate analysis of covariance (MANCOVA). The kinematic variables were defined as the response variables, feeding mode (i.e., filter-feeding versus substrate grazing) was used as the independent variable, and total length of tadpoles was used as covariate to control for size differences. As measures of kinematic variables were obtained for five gape cycles of each tadpole, gape cycle was used as repeated measure. Additionally, we used repeated measures univariate analysis of covariance (ANCOVA) on each response variable to assess which variables were responsible for the significant main effects. Analysis was performed using the function Anova in car package of R software (R Core Team, 2013).

To visualize shape variation in shape space, a Principal Components Analysis based on a covariance matrix of the Procrustes-aligned coordinates was performed using MorphoJ (Klingenberg, 2011). Also, to verify whether the shape of tadpoles' mouth differs between filter-feeding and scraping a surface for food, we performed a Distance-based Permutational Multivariate Analysis of Variance (PERMANOVA, Anderson, 2001) based on Euclidean distance with Procrustes-aligned coordinates as dependent variables and feeding behavior, cycle phase (opening and closing) and an interaction term between mode of feeding and cycle phase as independent variables. PERMANOVA was performed using adonis function in vegan package (Oksanen et al., 2013) of R software (R Core Team, 2013).

RESULTS

The feeding kinematics of *L. labyrinthicus* tadpoles differ in relation to the type of surface they are feeding from (repeated measures MANCOVA: $F_{1,4} = 16.55$, $p = 0.01$), except the duration of gape cycle (Table 1). Although filter-feeding tadpoles had a longer gape cycle relative to tadpoles scraping food from a firm, planar substrate (Table 2), this difference was not significant (Table 1). Filter-feeding tadpoles achieved maximum gape during the first 1/3 of the cycle, whereas tadpoles that scraped food off a firm substrate reached maximum gape during the second half of the gape cycle (Table 2). In addition, even after controlling for size differences among individuals, tadpoles that scraped the substrate for food had a larger maximum gape compared to the filter-feeding tadpoles (Table 2). Furthermore, in relation to grazing behavior, it is also noteworthy that the upper jaw does not hold to the substrate during closing phase: as the mouth starts to close, this jaw slips across the surface.

In terms of plasticity in the jaw sheaths of tadpoles, the first principal component explained 68.3% of the shape variation between feeding modes and reflects the shape of the jaw sheaths as tadpoles close their mouths. In contrast, the second principal component explained 10.6% of shape variation between the two feeding modes and is related to the opening phase of the gape cycle (Figure 2). Together the first two components explained 78.9% of the total shape variation. The only factor that influenced how *L. labyrinthicus* tadpoles move their jaw sheaths during feeding is the behavior itself (Table 3). In other words, even after controlling for differences in the kinematics of the jaw as the mouth opened and closed, we found significant differences in the plasticity of the jaw sheaths related to feeding mode. As the tadpoles opened and closed their jaws to scrape food, the lower jaw elongated and narrowed along its

transverse axis (Figure 2). In contrast, as tadpoles opened and closed their jaws while filter-feeding, the lower jaw got wider (Figure 2). Although we did not detect a significant feeding mode x gape cycle phase interaction in the jaw sheath plasticity (Table 3), changes in oral shape between feeding behaviors appeared to reflect the shape changes of the lower jaw during mouth closure.

DISCUSSION

Species that live in heterogeneous and/or temporary habitats often face unfavorable environmental conditions that can then drive the evolution of phenotypic plasticity in ecological generalists (Ghalambor et al., 2007). Ecological specialists can also persist in unstable environments, especially when the costs of generalist/plastic phenotypes outweigh those associated with specialist phenotypes (e.g. Van Tienderen, 1991; Agrawal, 2001). Behavioral flexibility might provide an intermediate strategy (Futuyma & Moreno, 1988), whereby specialists can use alternative tactics to increase fitness during unfavorable conditions. Prior to the present study, tadpoles of *L. labyrinthicus* were considered strictly carnivorous and dietary specialists (Shepard & Caldwell, 2005; Silva, Giaretta & Facure, 2005). Contrary to that perspective, our results demonstrate that tadpoles of *L. labyrinthicus* can perform as a dietary generalist and can use at least two alternative, functionally different, feeding strategies. As well as being macrophagous carnivores, they can be suspension feeders and scrape food off a firm substrate to generate a suspension of material subsequently extracted from the water once drawn into their mouths. The feeding diversity we observed in *L. labyrinthicus* reveals how tadpoles with specialized feeding anatomy can use flexible behavioral strategies for feeding on different substrates.

Our videographic data show clear differences in the oral kinematics between filter-feeding and substrate grazing by *L. labyrinthicus* tadpoles. For instance, when the tadpoles solely filter-feed, they have relatively long gape cycles and they reach maximum gape proportionally sooner within the gape cycle than when they scrape upon a substrate. In addition, filter-feeding tadpoles of *L. labyrinthicus* achieve maximum gape proportionally earlier in the gape cycle and widen their jaws along the horizontal axis more so than when they scrape food off a solid substrate. Although we did not quantify food consumption between these two feeding strategies, these results suggest that behavioral adjustments used by the tadpoles may help maximize food intake in heterogeneous environments. Future studies could compare buccal pumping rates and capture efficiency (e.g. Seale & Wassersug, 1979; Wassersug & Hoff, 1979) between tadpoles with mixed feeding strategies to test for differences in feeding efficiency between these, and other, feeding strategies.

In terms of substrate-scraping behavior, we found similarities and differences between how this is accomplished by common grazing tadpoles with a more generalized oral morphology and *L. labyrinthicus*. In a previous study, Wassersug & Yamashita (2001) found that the dietary generalist larvae of *Lithobates catesbeianus* increase the speed in which they open and close their mouths when they meet resistance as they scrape an algal covered surface. Our result showing that *L. labyrinthicus* larvae vary the speed of their gape cycle, depending on whether they are filter feeding or foraged on an algal-covered substrate, corroborates the Wassersug & Yamashita findings. Our results and theirs collectively suggest that kinematic adjustment in the speed that tadpoles open and close their mouths is common across diverse taxa.

In contrast to what has been described for other grazer tadpoles (Wassersug & Yamashita, 2001; Venesky et al., 2010; Venesky et al., 2013), it does not appear that

tadpoles of *L. labyrinthicus* can effectively use their keratinized teeth to anchor their oral disc to the substrate when grazing on a planar surface. Instead, as the mouth starts to close, the upper jaw slips across the surface. In the present study, we focused only on feeding kinematics that were shared by the *L. labyrinthicus* tadpoles during filter-feeding and grazing, and did not quantify the extent in which the oral structures slip on the surface during grazing. As tooth rows help anchor the oral disc to the substrate (e.g. Wassersug & Yamashita, 2001, Venesky et al., 2010, Venesky et al., 2013), it is likely that poor anchoring of the mouth to the substrate is a consequence of the reduced number of labial tooth rows of *L. labyrinthicus* tadpoles (i.e., one anterior and two posterior tooth rows) compared to the common configuration in anuran tadpoles (i.e., two anterior and three posterior rows; Altig et al., 2007).

It is well known that both protein and lipid nutrients obtained from animal matter promote rapid tadpole growth and development (e.g. Kupferberg, 1997; Richter-Boix et al., 2007). The distribution of macroscopic animal prey is sometimes patchy and an ostensibly macrophagous carnivore may need to augment caloric and/or nutritional intake by consuming alternative food sources (e.g. Petranka & Kennedy, 1999; Schiesari, Werner & Kling, 2009). Suspension-feeding and substrate grazing by *L. labyrinthicus* tadpoles in natural environments could supplement their nutritional requirements through the consumption of alternative food sources available on the water surface films (Goldacre, 1949; Wotton & Preston, 2005) and on other surfaces in their aquatic habitat (McMahon, Hunter & Russel-Hunter et al., 1974; Weitzel, 1979). Future studies that examine *L. labyrinthicus* growth rates and development raised on different larval diets, may help determine how important alternative tadpole feeding strategies are for the overall fitness of the species.

Previous studies on the feeding mechanics of tadpoles have largely focused on the buccal pumping mechanism of filter-feeding tadpoles (e.g. Wassersug & Hoff, 1979) or scraping behavior (e.g. Venesky et al., 2011). Our study shows that tadpoles of *L. labyrinthicus* are more plastic in their feeding behaviors than their external oral morphology suggests. Such plasticity appears advantageous for tadpoles of species, like *L. labyrinthicus*, that occupy temporary habitats and have to grow fast in order to metamorphose before pond drying (e.g. Richter-Boix et al., 2007). Our results demonstrate that tadpoles can adjust their feeding kinematics in response to different food and support the idea that the range of feeding strategies cannot always be inferred from external morphological features (e.g. Vera Candioti, 2006; Schiesari, Werner & Kling, 2009). These results highlight the need for studies that link behavioral research with functional morphology.

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TABLES

Table 1. The results from repeated measures ANCOVA documenting the variation in feeding kinematics of tadpoles of *Leptodactylus labyrinthicus* as a function of their feeding behavior (filter-feeding versus grazing). We used the total length of each tadpole as a covariate in each statistical test. df: degrees of freedom, F: F-ratio (value of observed F statistic), P: probability value.

Kinematics variables (A-D)			
Factors	df	F	P
A. Duration of Gape Cycle			
Behavior	1	6.069	0.069
Total length	1	0.569	0.492
Gape Cycle	4	1.429	0.269
B. Maximum Gape			
Behavior	1	264.334	8.38E-05
Total length	1	0.294	0.616
Gape Cycle	4	0.171	0.949
C. Time to Maximum Gape			
Behavior	1	14.618	0.018
Total length	1	1.697	0.262
Gape Cycle	4	1.491	0.251
D. Percentage of time to maximum gape			
Behavior	1	101.028	5.51E-04
Total length	1	1.026	0.368
Gape Cycle	4	1.814	0.175

Table 2. Mean $\pm$ Standard Deviation of kinematic variables measured during the display of two feeding behaviors; i.e., filter-feeding versus grazing.

Kinematics variables	Feeding Behavior	
	Filtering	Scraping
Duration of Gape Cycle (ms)	87.80 $\pm$ 9.69	73.47 $\pm$ 9.78
Maximum Gape (mm)	0.79 $\pm$ 0.04	1.30 $\pm$ 0.07
Time to maximum gape (ms)	29.20 $\pm$ 4.56	41.07 $\pm$ 9.91
Percentage of time to maximum gape (as % of gape cycle)	0.33 $\pm$ 0.05	0.55 $\pm$ 0.06

Table 3. Effects of feeding behaviors (filter-feeding versus grazing), gape cycle phase (opening and closing) and the interaction term between the two factors on the shape of the mouth *Leptodacylus labyrinthicus*.

Factors	df	F	R ²	P
Behavior	1	80.536	0.546	0.001
Cycle Phase	1	0.394	0.003	0.729
Behavior x Cycle Phase	1	0.474	0.003	0.645
Residuals	66		0.447	
Total	69		1	

FIGURE LEGENDS

Figure 1. A representative tadpole of *Leptodactylus labyrinthicus* at Gosner developmental state 36. The tadpole was collected in northwestern region of São Paulo State, Brazil. Scale: 1 mm.

Figure 2. A plot of the Procrustes-aligned specimens of *Leptodacylus labyrinthicus* along first two dimensions of tangent space showing the plasticity of the jaw sheaths. The first axis represents closing phase, whereas the second axis represents opening phase of gape cycle. Squares represent data from filter-feeding tadpoles and circles represent data from tadpoles that fed by scraping a planar substrate. Patterns of shape variation along each component are represented by the warped outlines; black is the average shape and grey represents individuals with high scores. When filter-feeding, the posterior jaw sheath of tadpoles got wider whereas the jaw elongated and narrowed along its transverse axis when scraping for food.