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# Trophic segregation of anuran larvae in two temporary tropical ponds in southern Vietnam

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Trophic differentiation of tadpoles of four anuran species (*Hoplobatrachus rugulosus, Microhyla fissipes, M. heymonsi, Polypedates megacephalus*) with different oral morphologies was studied in temporary ponds in a monsoon tropical forest in southern Vietnam. All tadpole species were found to be omnivorous, including filter-feeding microhylids. Both gut contents analysis and stable isotope analysis provided enough evidence of resource partitioning among coexisting species. Gut contents analysis supported the expected partitioning of food resources by tadpoles with different oral morphologies and showed differences in the food spectra of filter-feeding and grazing species. Stable isotope analysis revealed more complex trophic niche segregation among grazers, as well as amongst filter-feeders. Tadpole species differed mainly in $\delta^{13}C$ values, indicating a dependency on carbon sources traceable to either of aquatic or terrestrial origins. Furthermore, tadpoles with generalised grazing oral morphology (*P. megacephalus*) can start feeding as suspension feeders and then shift to the rasping mode. Controlled diet experiment with *P. megacephalus* larvae showed a diet-tissue isotopic fractionation of approximately 1.9‰ and 1.2‰ for $\Delta^{13}C$ and $\Delta^{15}N$, respectively. In natural habitats, the difference in $\delta^{13}C$ and $\delta^{15}N$ values between body tissues and gut contents of four tadpole species averaged 2.8‰ and 1.0‰, respectively.

*Key words:* Amphibia, aquatic food web, Cat Tien National Park, Microhylidae, mouthparts, Southeast Asia, stable isotopes, trophic shift

## INTRODUCTION

Anuran larvae show diverse feeding modes and often play significant roles in aquatic food webs (Wilbur, 1997; Ranvestel et al., 2004; Whiles et al., 2006). Moreover, due to their biphasic life history and high biomass values in riparian zones, anurans carry out a unique ecological function by transferring nutrients and energy between aquatic and terrestrial ecosystems (Seale, 1980; Ballinger & Lake, 2006; Whiles et al., 2006). Deciphering trophic links of anuran larvae and their impact on other members of aquatic food webs is of crucial importance for the elaboration of adequate measures for amphibian conservation and forecasting the ecological consequences of amphibian decline throughout the world (Hunte-Brown, 2006; Verburg et al., 2007; Barnum et al., 2013). Tropical ecosystems harbour the greatest taxonomic and morphological diversity of tadpoles that have a range of feeding modes. Tadpole ecomorphological guilds have been described based on oral morphology, feeding behaviour, and microhabitat use (Altig & Johnston, 1989; Vera Candioti, 2006, 2007). However, studies related to the trophic ecology of tropical tadpoles remain scarce and are mostly confined to the Neotropics. Few studies have been published on tadpole trophic ecology in Southeast Asia (Heyer, 1973, 1974; Inger, 1986; Inger et al., 1986). Meanwhile, this region is characterised by the presence of some morphologically distinct and ecologically specialised forms. Representatives of the Asian lineage of the family Microhylidae, whose larvae form an important element of tropical pond communities in Southeast Asia, are among them (Heyer, 1973, 1974; Inger, 1985; Leong & Chou, 1999). Microhylid tadpoles are characterised by the absence of any keratinised mouthparts and are assigned to the guild of neustonic or suspension feeders; however, data on their diet are fragmentary (Savage, 1952; Heyer, 1973; Inger, 1986).

A variety of methodological constraints summarised by Altig et al. (2007) hamper studies on the diet of tadpoles using traditional methods of the analysis of digestive tract contents. The main limitations of this approach include: (1) ingested substrates are not necessarily the assimilated ones, since some food items may pass through the digestive tract undigested; (2) some substrates may be swallowed incidentally but fail to

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provide energy or nutrients (empty frustules of diatoms, arthropod exuviae, etc.); and (3) grazing tadpoles, especially scavengers or those feeding on macrophytes, often grind the food substrates into very small particles making their identification difficult. Finally, (4) bacteria and dissolved organic matter that may greatly contribute to tadpole feeding are typically overlooked in gut contents analyses (Heyer, 1973). Moreover, new data show that amphibian larvae can use the nutrients from dissolved organic matter as an additional nutrient source (Katayama et al., 2016).

Nevertheless, gut contents analyses provide valuable information on the main feeding modes of tadpoles. The majority of anuran larvae, except for some highly specialised carnivorous or oophagous forms, are usually considered as herbivorous, feeding mainly on microalgae (e.g., Seale & Beckvar, 1980; Lajmanovich, 2000; Sinha et al., 2001; Rossa-Feres et al., 2004) or algal and vascular macrophytes (e.g., Anderson & Kneitel, 2015). However, other studies show that detritus and microbial biofilms, as well as animal material (benthic invertebrates, anuran eggs) play dominant roles in the feeding of certain species (Petranka & Kennedy, 1999; Quammen & Durtsche, 2003; Frauendorf et al., 2013). Other food items such as zooplankton, fungi or pollen are only occasionally mentioned as important components of the diet (e.g., Wagner, 1986; Sengupta et al., 2013). Furthermore, differences in oral morphology and feeding mode, along with temporal and spatial microhabitat partitioning suggest a pronounced separation of trophic niches of coexisting species (Pavignano, 1990; Echeverria et al., 2007; Prado et al., 2009). Even tadpoles with generalised oral morphologies and similar feeding modes display a resource-based trophic differentiation (Rossa-Feres et al., 2004; Zhou et al., 2005).

Stable isotope analysis is a widely accepted tool in trophic ecology (Newsome et al., 2007). As a rule, stable isotope composition cannot reveal particular food items, it does provide information on the main types of resources used and on the organism's trophic level. The content of $^{15}N$ (usually expressed as $\delta^{15}N$ values) increases by 2–3‰ per trophic level (the so-called trophic fractionation, Martinez del Rio et al., 2009) and thus indicates the relative position of an animal within its food chain. Variations in the carbon isotope ratio of body tissues (expressed as $\delta^{13}C$) reflect the major types of carbon sources entering a food web.

Stable isotopes analysis showed that the trophic position of tadpoles in freshwater food webs can vary from primary consumers (Vander Zanden & Rasmussen, 1999) to relatively high trophic levels that imply a large degree of carnivory (Schiesari et al., 2009). Furthermore, isotopic signatures of tadpoles supported the separation of trophic niches among coexisting species (Hunte-Brown, 2006; Verburg et al., 2007; Schiesari et al., 2009), as well as ontogenetic trophic shifts in certain tadpole species (Jefferson & Russell, 2008; Trakimas et al., 2011; Caut et al., 2013).

Monsoon forest ecosystems of southern Vietnam are rich in amphibian species that reproduce in lentic water bodies, including temporary ponds. Among approximately 40 species of frogs inhabiting the Cat Tien National Park, more than half deposit their eggs in rain ponds. Many frog species reproduce simultaneously in the same ponds and form complex communities of coexisting tadpoles that mostly belong to the families Dicroglossidae, Microhylidae and Rhacophoridae (Vassilieva et al., 2016).

In this study, we aimed at revealing the trophic position of coexisting species of tropical anuran larvae. We hypothesised that tadpoles with different types of oral morphology use different types of food. To estimate the trophic position of tadpoles, we compared the gut contents composition and the isotopic composition of the tissues of larvae of four anuran species in two temporary ponds.

Adult anurans are strict predators and occupy top trophic levels in pond systems (Kupfer et al., 2006). Therefore, it is to be ascertained that the isotopic composition of tadpoles depends only upon their food sources and not being influenced by the yolk. In addition, the trophic fractionation of carbon and nitrogen stable isotopes in tadpole tissues varies between published studies (Schiesari et al., 2009; Trakimas et al., 2011). To verify our isotopic data, we therefore compared the stable isotope composition of body tissues and gut contents in tadpoles collected in the field, and estimated isotopic trophic fractionation in anuran larvae reared on a controlled diet.

## MATERIALS AND METHODS

**Study site**

Two temporary ponds were studied in the Cat Tien National Park, southern Vietnam (N 11º 25', E 107º 25'; about 120 m a.s.l.). The climate is tropical monsoon with two distinct seasons: a rainy season from May to November and a dry season from December to April. The mean annual temperature is close to 26.4°C, and the mean annual rainfall is about 2,500 mm. Pond 1 was an open rain pond with an area of about 30 $m^2$, the maximum depth was 60 cm, and the prevalent depths were 15–25 cm. The bottom was slimy, with numerous submerged stones and decaying bamboo stems. The surrounding vegetation included both, C4 and C3 plants (Appendix 1). Pond 2 was a shaded rain pond (canopy closure 80 %) with an area of about 20 $m^2$, a maximal depth of 50 cm and prevalent depths of 20–30 cm. The bottom was litter-covered, with some submerged stones and fallen logs. The surrounding vegetation consisted of C3 plants only. Both ponds were filled from May to December, and dried out during the dry season. Detailed descriptions of the pond microhabitats are given in Appendix 1.

**Sample collection**

Pond 1 was sampled twice, on 20–25 May 2009 and on 5–8 June 2011, and Pond 2 was sampled only once on 5–8 June 2011. Tadpoles were sampled randomly with a dip net (diameter 300 mm, 0.8 mm mesh). The four most abundant species were selected for detailed analyses: *Hoplobatrachus rugulosus* (Wiegmann, 1834) (Dicroglossidae), *Polypedates megacephalus* Hallowell,

1861 (Rhacophoridae), *Microhyla fissipes* Boulenger, 1884 and *Microhyla heymonsi* Vogt, 1911 (Microhylidae). Tadpoles identification and measurements (total length) were performed with a Leica EZ4 dissecting microscope (Germany) and a digital caliper. All tadpoles were staged after Gosner (1960). Among *P. megacephalus* sampled in 2009, early (30–33) and advanced (34–38) larval stages were analysed separately. The number of samples used in different analyses is given in Table 1. Tadpoles were euthanised by freezing that proved to be a suitable method when collecting samples for stable isotope analysis (Atwood, 2013). Sampling procedures were performed according to the regulations of the Bioethics Committee of the Biological Faculty of Moscow State University.

Various organic substrates were sampled in each pond simultaneously with tadpoles. The suspended matter (seston) was sampled with a plankton dip net (75×95 mm, 400 µm mesh). The organisms attached to submerged surfaces (periphyton) were collected by scraping submerged logs, herbaceous macrophytes, bamboo stems, and stones. The bottom slime was collected in a 100 ml rubber bag and filtered through a two-layered 400 µm mesh. Immersed herbaceous macrophytes and leaf litter from the bottom were sampled as whole leaves. Predatory aquatic arthropods, such as water skaters, boatmen (Hemiptera), and dragonfly larvae (Odonata) were collected in 2011 with a dip net.

**Gut contents analysis**

Qualitative analyses of tadpole gut contents were performed in 2009 (Pond 1) and 2011 (Pond 2). From each specimen, the first third of the digestive tract was removed, its contents extracted in a Petri dish were diluted with water and examined with a MBS-10 dissecting microscope (LZOS, Russia). The following categories of food substrates were distinguished: crustaceans (whole specimens or fragments of Copepoda, Ostracoda and Cladocera), filamentous algae (green and blue-green algal filaments), green microalgae (unicellular and polymonad green algae), fungal mycelium, macrophytes (fragments of leaves or stems of higher plants), diatoms, testaceans (tests of Thecamoeba), rotifers, insect eggs, bryozoan statoblasts, pollen grains, seeds of higher plants, terrestrial arthropods (insects and mites), aquatic insects (including aquatic larvae of terrestrial insects), and tadpoles. The amorphous mass of an indefinite origin was designated as "detritus". For each tadpole species, the relative frequency of each substrate category in the gut was estimated as the percentage occurrence (%) of specimens with this substrate in the gut of the total number of specimens examined.

**Laboratory experiment**

A controlled diet experiment was performed in order to estimate the trophic fractionation of stable isotopes in one of the tadpole species. A recently deposited egg clutch of *P. megacephalus* was collected in an artificial pond nearby the study site in May 2009. Tadpoles were reared in aquaria at ambient temperature (ca. 26 ºC) and light regime from hatching to the completion of metamorphosis and fed *ad libitum* with chopped and scalded mustard leaves (*Brassica integrifolia*). As shown in preliminary experiments, this diet supported normal growth and development of tadpoles. Mustard leaves (food), muscle tissue and yolk of tadpoles were used for stable isotope analysis. Samples of muscle tissue of developing tadpoles of different stages (22 at hatching, 27, 32–33, 37–38, 41–42, 43–45) were taken under dissecting microscope from the tail base. Yolk mass was extracted from the gut of newly hatched tadpoles (stage 22). The trophic fractionation was calculated as Δ value ($\Delta=\delta_{consumer} - \delta_{food}$). In addition, we compared $\delta^{13}C$ and $\delta^{15}N$ values in muscle tissues and gut contents in four tadpole species collected in Pond 1 in 2011 (Table 1).

**Stable isotope analysis**

Muscle tissue from the tail base and the gut contents of tadpoles were sampled from freshly collected specimens. Large tadpoles (body length more than 20 mm) were sampled individually. For small tadpoles of early developmental stages (body length less than 20 mm),

**Table 1.** Samples of tadpoles used for stable isotope analysis (SIA) and qualitative analysis of gut contents (AGC).

| Species | Pond/Year | Stages | Body length (mm) | SIA (muscle tissue) | SIA (gut contents) | AGC |
|---|---|---|---|---|---|---|
| *Hoplobatrachus rugulosus* | Pond 1/ 2009 | 35–40 | 42.0–53.0 | 14 | – | 5 |
| | Pond 1/ 2011 | 35–38 | 40.0–48.0 | 10 | 5 | – |
| *Microhyla fissipes* | Pond 1/ 2009 | 35–38 | 20.0–23.5 | 12 | – | 7 |
| | Pond 1/ 2011 | 35–28 | 20.0–23.5 | 11 | 3 | – |
| | Pond 2/ 2011 | 32–33 | 15.5–20.0 | 8 | – | 5 |
| *Microhyla heymonsi* | Pond 1/ 2009 | 35–40 | 22.0–27.0 | 10 | – | 6 |
| | Pond 1/ 2011 | 35–40 | 22.0–27.0 | 10 | 4 | – |
| | Pond 2/ 2011 | 32–33 | 12.5–15.0 | 6 | – | 5 |
| *Polypedates megacephalus* | Pond 1/ 2009 (early stages) | 31–33 | 17.0–20.0 | 14 | – | 5 |
| | Pond 1/ 2009 (advanced stages) | 35–38 | 30.0–34.0 | 15 | – | 5 |
| | Pond 1/ 2011 (early stages) | 30–31 | 17.0–20.0 | 10 | 12 | – |
| | Pond 2/ 2011 (advanced stages) | 34–35 | 25.0–30.0 | 10 | – | 5 |

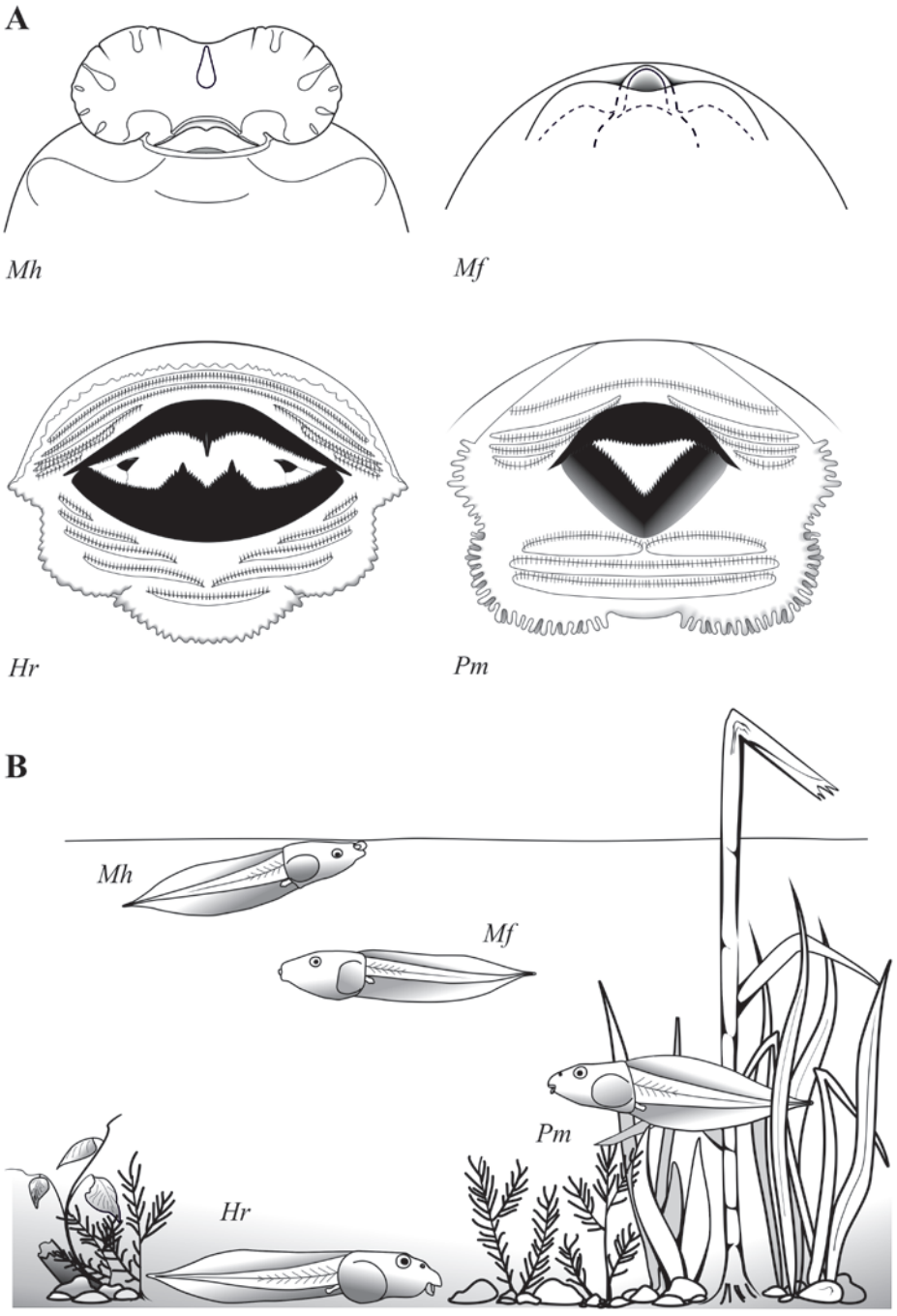


**Fig. 1.** A) Oral morphology of the tadpoles used in the study; *Mh*, *Mf* – dorsal view, *Hr*, *Pm* – ventral view. B) Microhabitats used by tadpoles in the Pond 1. Abbreviations: *Mh – Microhyla heymonsi*; *Mf – Microhyla fissipes*; *Hr – Hoplobatrachus rugulosus*; *Pm – Polypedates megacephalus*.

several individual samples were pooled. All samples were immediately dried at 60ºC. Leaf litter, plants, seston, periphyton, bottom slime and gut contents were ground to a homogeneous powder. In predatory arthropods, legs were analysed. Stable isotope analysis was conducted with a Thermo Delta V Plus continuous-flow IRMS, coupled with an elemental analyser (Flash 1112) equipped with a Thermo No-Blank device at the Joint Usage Centre of the A.N. Severtsov Institute of Ecology and Evolution RAS, Moscow. The isotopic composition of N and C was expressed in a δ-notation relative to the international standard (atmospheric nitrogen and VPDB, respectively): $\delta X(‰)=[(R_{sample}/R_{standard})-1] \times 1000$, where R is the ratio of the heavier isotope to the lighter isotope. Samples were analysed with reference gas calibrated against the IAEA reference materials USGS 40 and USGS 41 (glutamic acid). The accuracy of measurements was better than ± 0.2 δ units.

**Statistical analysis**

Chi-square test of equal or given proportions (prop. test in R, https://stat.ethz.ch/R-manual/R-devel/library/stats/html/prop.test.html) was used to compare the percentage occurrence of certain food categories in the gut of different species. Unequal n Tukey HSD test as performed in Statistica 6.0 (Statsoft, Tulsa) was used for comparing the stable isotope composition of tadpoles. The mean differences in $\delta^{13}C$ or $\delta^{15}N$ values between

**Table 2.** Stable isotope composition of carbon and nitrogen (means and 1 SD) in the muscle tissue of four species of tadpoles collected in two temporary ponds in southern Vietnam; n=6–15. Different letters within a column indicate significant differences in δ values (unequal n Tukey HSD test, $P<0.05$).

| | Pond 1 (2009) | | | | Pond 1 (2011) | | | | Pond 2 (2011) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $\delta^{13}C‰$ | | $\delta^{15}N‰$ | | $\delta^{13}C‰$ | | $\delta^{15}N‰$ | | $\delta^{13}C‰$ | | $\delta^{15}N‰$ | |
| *Polypedates megacephalus,* advanced stage | -29.3 | (1.7) a | 5.8 | (0.4) a | | | | | -27.2 | (0.3) a | 3.0 | (0.3) a |
| *Polypedates megacephalus,* early stage | -27.6 | (0.8) b | 6.3 | (0.4) b | -27.3 | (0.8) a | 5.8 | (0.8) a | | | | |
| *Microhyla fissipes* | -26.8 | (0.9) bc | 5.8 | (0.3) a | -27.4 | (0.3) a | 6.1 | (0.2) a | -28.1 | (0.3) b | 2.2 | (0.2) b |
| *Microhyla heymonsi* | -24.1 | (0.2) d | 6.1 | (0.4) ab | -24.9 | (0.5) b | 6.3 | (0.5) a | -25.3 | (0.3) c | 3.2 | (0.3) a |
| *Hoplobatrachus rugulosus* | -26.1 | (1.1) c | 7.7 | (0.3) c | -24.6 | (0.2) b | 6.4 | (0.1) a | | | | |

muscle tissues and gut contents were estimated using a simple linear regression with the slope forced to 1. Data on the isotopic composition are reported as means ± 1SD.

## RESULTS

**Oral morphology and microhabitat use by tadpoles**

Four species of tadpoles dominated in Pond 1 during the sampling periods: *M. heymonsi, M. fissipes, H. rugulosus,* and *P. megacephalus*. In Pond 2, only three species reached high abundance levels: *M. heymonsi, M. fissipes,* and *P. megacephalus*.

In *M. heymonsi* (Fig. 1A, *Mh*), the mouth is dorsally positioned, edged anteriorly with a wide, dorsally oriented funnel with several prominent papillae; the upper labium is elevated, the oral flaps form rounded lobes at each mouth corner. In *M. fissipes* (Fig. 1A, *Mf*), the mouth is positioned terminally; a small, U-shaped lower labium is almost hidden by the overhanging upper labium. Keratinised elements are absent from both microhylids.

In *H. rugulosus* (Fig. 1A, *Hr*), a wide, elliptical oral disc is oriented anteroventrally; the upper and lower labia are fringed with short flattened papillae. A wide and strong keratinised beak has serrated margins; the upper sheath bears a large medial tooth-like projection, the lower sheath is supplied with two smaller tooth-like projections on both sides of the medial notch. Two large fang-shaped spurs are positioned inside the mouth at the corners of the jaws. Pointed tooth-shaped denticles are arranged in double or triple series along the edge of the upper labium and the horizontal ridges on the upper and lower labia; the labial teeth row formula is 5(3–5)/5(1–4).

In *P. megacephalus* (Fig. 1A, *Pm*), a moderately wide, elliptical oral disc is oriented anteroventrally; the mouth corners and lower labium are fringed with a continuous single (locally double) row of short conical papillae, with a narrow gap in the medial part of the lower labium. Arch-shaped mouth sheaths have finely serrated margins. Spike-like denticles are arranged in a single series along the edge of the upper labium and the horizontal ridges on the upper and lower labia; the labial teeth row formula is 4(2–4)/3(1).

In ponds 1 and 2, *M. heymonsi* tadpoles often formed loose groups of 15–20 specimens. They fed at the water surface, adhering to the surface film with their oral funnels (Fig.1B, *Mh*). The tadpoles of *M. fissipes* usually formed dense groups of several dozen specimens. In the study ponds they were observed to feed while moving slowly in midwater or close to the surface (Fig. 1B, *Mf*). The tadpoles of *P. megacephalus* did not form groups and stayed mostly in midwater among the vegetation (Fig. 1B, *Pm*); they scraped plant and submerged log surfaces or, incidentally, ate tadpoles and floating microhylid eggs. In Pond 1, the tadpoles of *H. rugulosus* stayed mostly at the bottom (Fig. 1B, *Hr*) where they rasped macrophytes and preyed on microhylid tadpoles.

**Gut contents analysis**

Amorphous detritus formed the bulk of all digestive tract contents, but it was coarse and loose in *H. rugulosus* and *P. megacephalus* and thin and homogeneous in *Microhyla* spp. The diversity and occurrence of other food items in the tadpole gut varied between ponds, amphibian species and developmental stages of *P. megacephalus* (Fig. 2).

Significant differences in food spectra were observed between tadpole species, with the diversity of food items being generally higher in Pond 1. The diet of *H. rugulosus* differed from other species through high occurrences of animal food, including microcrustaceans and aquatic insects, such as mayflies larvae, chironomid larvae, notonectids and adult dipterans, as well as tadpoles ingested intact (Fig. 2A). The food spectrum of advanced larvae of *P. megacephalus* was more diverse than that of early larvae of the same species. The food spectra of *Microhyla* species and early larvae of *P. megacephalus* were similar, although *M. heymonsi* had higher occurrences of animal food (microcrustaceans and terrestrial arthropods, such as springtails and nymphs of mites) and floating food items, such as pollen, small seeds and bryozoan statoblasts. The gut of advanced *P. megacephalus* and *H. rugulosus* tadpoles contained large amounts of soil particles mixed with the detritus mass.

In Pond 2 (Fig. 2B), all species retained certain features of their food spectra revealed in Pond 1. Thus,

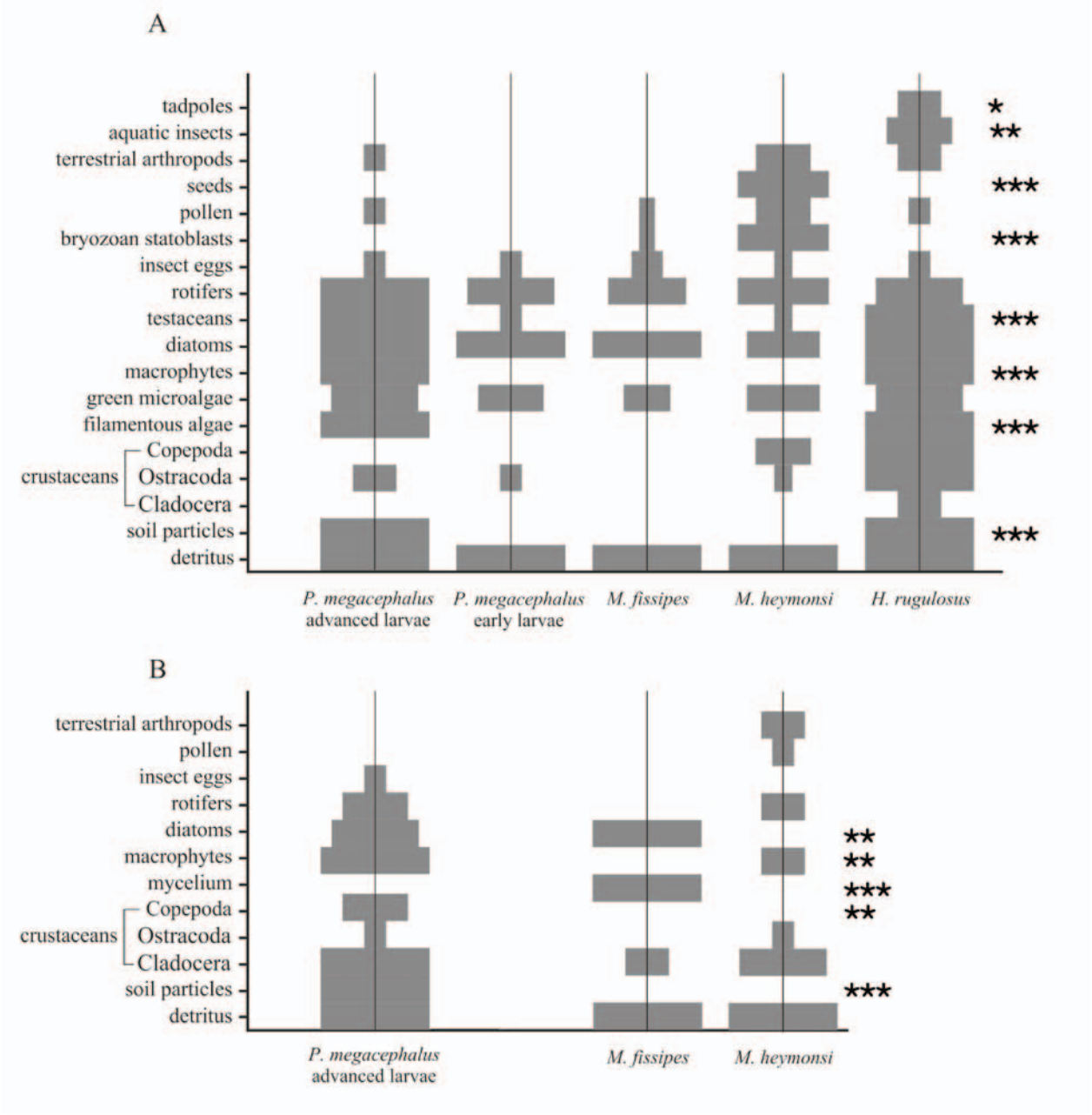


**Fig. 2.** Percentage occurrence of main feeding substrates in the digestive tracts of tadpoles (% of the number of specimens studied; detritus block corresponds to 100%). A: Pond 1, B: Pond 2. Asterisks indicate significant difference in the occurrence of certain substrates in different species (Chi-square test of proportions; * P<0.05, ** P<0.01, *** P<0.001).

*P. megacephalus* differed from *Microhyla* spp. by the presence of soil particles and by largely feeding on macrophytes; the food of *M. heymonsi* included pollen and terrestrial arthropods. In contrast to Pond 1, green algae were absent from all food spectra, although all specimens of *M. fissipes* consumed fungal mycelium. All tadpole species fed on microcrustaceans (mostly cladocerans).

Infusorians and nematodes observed in the tadpole guts in Pond 1 were not included in the food spectra because these organisms were found alive in recently euthanised specimens and could be parasites or symbionts. Infusorians were recorded in all species except early larvae of *P. megacephalus*; nematodes were observed in advanced *P. megacephalus*, *H. rugulosus* and *M. heymonsi* tadpoles.

**Stable isotope composition**

In both ponds, the tadpoles of different species showed a clear differentiation in their "isotopic trophic niches", even though the total variation in mean $\delta^{13}C$ or $\delta^{15}N$ values within each pond was relatively small. In Pond 1 (2009), all species as well as different ontogenetic groups of *P. megacephalus* differed significantly either in $\delta^{13}C$ or $\delta^{15}N$ values (Table 1). Larvae of *H. rugulosus* were strongly enriched in $^{15}N$ compared to other species. *M. heymonsi* were enriched, while advanced larvae of *P. megacephalus* were depleted in $^{13}C$ (Fig. 3A). In 2011, the $\delta^{13}C$ and $\delta^{15}N$ values of *M. fissipes, M. heymonsi* and early larvae of *P. megacephalus* remained nearly

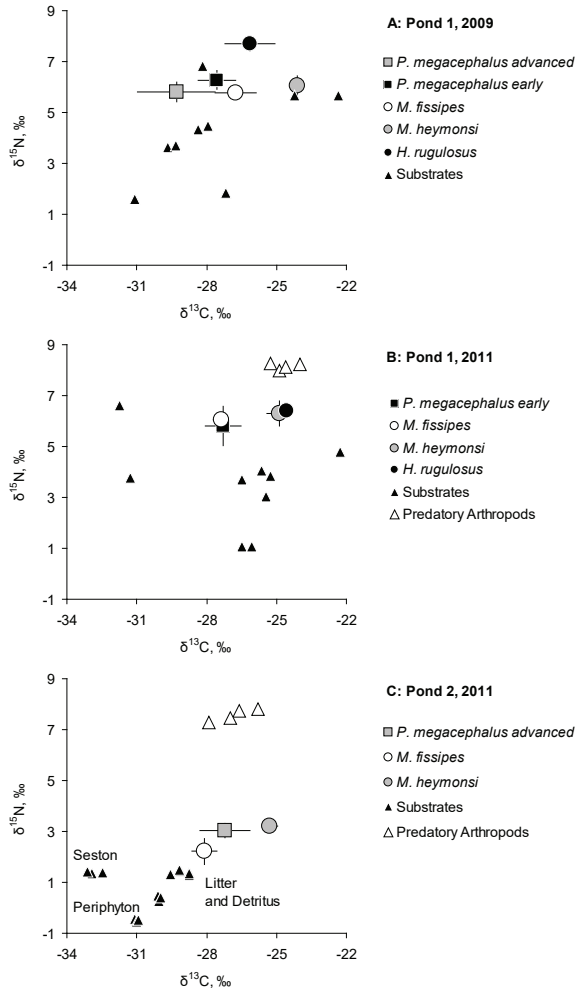


**Fig. 3.** Carbon and nitrogen stable isotope composition ($\delta^{13}C$ and $\delta^{15}N$ values) of tadpoles, their potential food substrates (periphyton, plant litter, macrophytes, detritus and seston) and predatory insects in two temporary ponds. Pond 1 (A, B) was sampled in 2009 and 2011. Points show means ± 1 SD (n=6–15) for tadpoles and individual samples for other materials. In Pond 1, different types of organic substrates did not differ consistently in the isotope composition.

the same as in 2009. In contrast, the $\delta^{15}N$ values of *H. rugulosus* were much smaller and this species failed to differ in this respect from three other species. Overall, the difference in stable isotope composition among species inhabiting Pond 1 was less pronounced in 2011, but *H. rugulosus* and *M. heymonsi* had significantly higher $\delta^{13}C$ values than two other species (Fig. 3B).

In Pond 2, tadpoles of three species differed significantly in $\delta^{13}C$ values, *M. heymonsi* was most enriched, and *M. fissipes* was most depleted in $^{13}C$. In addition, *M. fissipes* tadpoles were depleted in $^{15}N$ compared to other species (Fig. 3C).

There was high variation in $\delta^{15}N$ and, especially $\delta^{13}C$ values both within and between different types of organic substrates in Pond 1 (Fig. 3AB). Most likely, this reflected the high diversity in the isotopic signatures of local vegetation that included plants with C3 and C4 types of photosynthesis. In contrast, several types of organic substrates differed clearly in Pond 2 which was surrounded by C3 vegetation only. Seston samples were depleted in $^{13}C$ ($\delta^{13}C$= –32.8±0.3‰), whereas plant litter and detritus (mainly of terrestrial origin) were enriched in $^{13}C$ ($\delta^{13}C$= –29.4±0.6‰). In both ponds, predatory insects were considerably enriched in $^{15}N$, but not in $^{13}C$, relative to tadpoles.

**Trophic fractionation of stable isotopes (Δ values)**

In the controlled diet experiment, the stable isotope composition of tadpole muscle tissue at hatching ($\delta^{13}C$= – 26.9±0.1 ‰; $\delta^{15}N$=8.9±0.1 ‰) was similar to that of yolk ($\delta^{13}C$= – 26.7±0.1 ‰; $\delta^{15}N$=8.6±0.1 ‰). At stage 27 (seven days after hatching, five days of active feeding) the tadpole isotopic signature shifted towards that of the food, and from stage 33 (10–12 days after hatching) until the end of metamorphosis (stage 45, 30 days after hatching) the tadpole isotope composition remained nearly stable. This pattern indicates an isotopic equilibrium with the food (Fig. 4). Advanced tadpoles were enriched in $^{13}C$ and $^{15}N$ relative to their diet by 1.9 ± 0.2‰ and 1.2 ± 0.1‰, respectively.

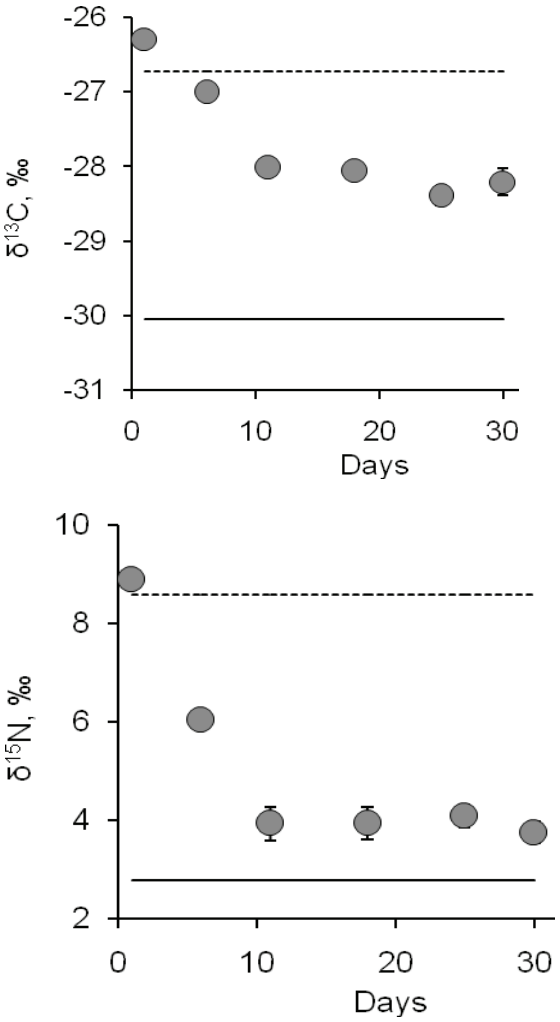


**Fig. 4.** Changes in the carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) stable isotope composition in *Polypedates megacephalus* tadpole muscle tissues under experimental conditions. Means and 1 SD (often not visible), n=4–20. Dotted line: isotope composition of yolk; continuous line: isotope composition of food (scalded mustard).

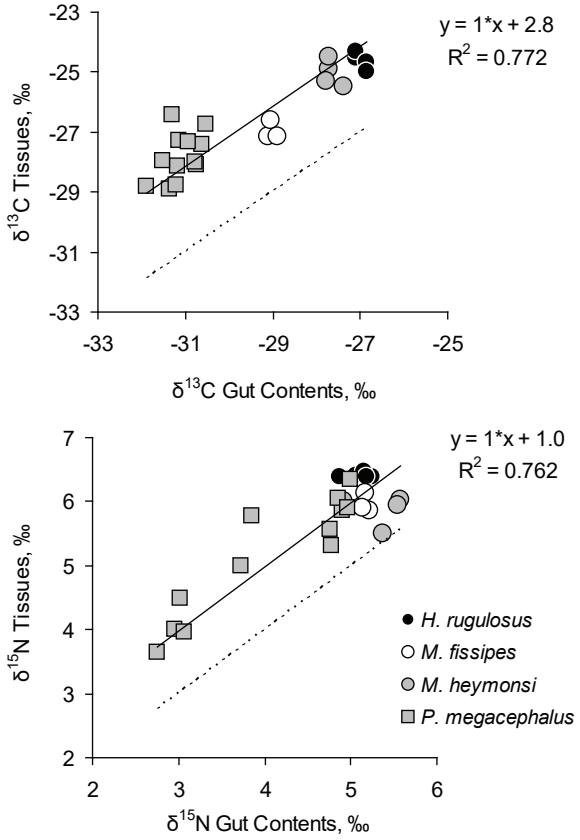


**Fig. 5.** Carbon and nitrogen stable isotope composition ($\delta^{13}C$ and $\delta^{15}N$ values) in muscle tissue (tail) and gut contents of tadpoles from the open temporary pond (Pond 1, 2011). Points show individual samples. Slope of the regression was forced to 1. Dotted lines show 1:1 ratio.

A slightly higher difference in $\delta^{13}C$ values between muscle tissues and gut contents was found in *P. megacephalus* and other tadpoles collected in natural habitats. When averaged across all four species, this difference amounted to 2.8 ± 0.7 ‰. The difference in $\delta^{15}N$ values between muscles and gut contents averaged 1.0 ± 0.4 ‰, thus being nearly identical to the $\Delta^{15}N$ value obtained in the laboratory experiment (Fig. 5).

## DISCUSSION

The tadpole communities in the studied temporary tropical ponds included several species that differed in their oral morphologies and feeding modes; according to Altig & Johnston (1989), they belong to different ecomorphological guilds. The microhylids *M. fissipes* and *M. heymonsi* lack any keratinised mouthparts and filter their food from the water or from the surface film. *M. fissipes* inhabiting the water column and showing a depressed body and tail flagellum (Chou & Lin, 1997; Hendrix et al., 2008) is a typical suspension feeder (guild II.17b in Altig & Johnston, 1989). *M. heymonsi* with its enlarged, upward directed funnel surrounding the mouth is a typical neustonic feeder (guild II.16) that collects food from the water surface (Chou & Lin, 1997). Larvae of frogs belonging to the genus *Hoplobatrachus* possess multiple denticle rows and are known to have carnivorous habits (Grosjean et al., 2004; Altig et al., 2009). Our observations confirmed the predatory feeding habits of *H. rugulosus* tadpoles, which can be attributed to guild II.13a (lentic-benthic, grazer, highly carnivorous). Larvae of *P. megacephalus* with their generalised grazing oral morphology (anteroventral mouth, keratinised jaw sheaths and labial denticles) belong to guild II.15b (nektonic grazer) and mainly feed by rasping surfaces, although advanced larvae were also observed eating anuran eggs or tadpoles (A. Vassilieva, pers. obs.).

Even though pond-dwelling anuran larvae are typically generalist feeders (Petranka & Kennedy, 1999; Prado et al., 2009), the described diversity of tadpole morphologies and feeding modes (Fig. 1) presumes a certain degree of trophic niche segregation between species. This notion was tested using both the traditional method of gut contents analysis and stable isotope analysis.

Qualitative gut contents analyses indicated that all studied species are omnivorous and consume varying fractions of plant and animal components (Fig. 2). The more or less uniform mass defined as detritus was the most frequent item in the guts of all species, even though the composition and origin of this mass might be different in filter-feeding and grazing species. Comparisons of the food spectra of four tadpole species in Pond 1 revealed a similarity between two species with a grazing oral apparatus, *H. rugulosus* and *P. megacephalus* (advanced larvae), and between two filter-feeding species without keratinised mouthparts, *M. fissipes* and *M. heymonsi*. The food spectrum of early larvae of *P. megacephalus* was more similar to that of filter-feeding microhylids, especially of the nektonic *M. fissipes*. The absence of soil particles or large vegetable fragments in the gut of early *P. megacephalus* tadpoles seem to indicate that the first developmental stages are mainly suspension-feeders rather than grazers or macrophages, despite their grazing mouthparts. Several studies demonstrated that tadpoles with generalised oral morphologies are well adapted to feeding on suspended organic particles (Wassersug & Hoff, 1979; Seale, 1982).

The difference between the grazer *P. megacephalus* and the filter-feeding microhylids was also pronounced in the Pond 2, in spite of lower resource diversity and the presence of microcrustaceans in the gut of all species. Although Schiesari et al. (2009) showed that microcrustaceans and insects are frequently consumed by tadpoles with a generalised oral apparatus, the capability of filter-feeders such as *Microhyla* tadpoles for preying on planktonic crustaceans has previously not been recorded. The larvae of *Microhyla* spp. were considered to feed on small suspend organic particles and planktonic microorganisms (Altig & Johnston, 1989; Hoff et al., 1999); only Heyer (1973) reported small mites (presumably taken from the water surface) in the *M. heymonsi* gut contents.

Some substrate categories found in the gut of tadpoles were likely to be of no high nutritional value given their small sizes and low quantities. However,

they can clarify the feeding modes and microhabitat preferences of different species (Echeverria et al., 2007). For instance, high occurrences of testate amoebae in the gut of *H. rugulosus* confirm the larvae of this species as being mostly bottom-dwelling, since testaceans were very abundant in Pond 1 sediments (see Appendix 1). In contrast, floating air-filled bryozoan statoblasts that were common in the gut of *M. heymonsi* tadpoles are confined to the water surface. Pollen can be, at least sometimes, an important food source for tadpoles with generalised mouth morphologies (Diaz-Paniagua, 1985; Wagner, 1986). In our samples, pollen grains were detected in substantial quantities only in the gut of the surface-feeding *M. heymonsi*. The oral morphology of this species facilitates feeding on floating pollen, a highly nutritive food (Roulston & Cane, 2000).

The consumption of certain food categories by tadpoles of the same species varied between ponds. The diversity of food items was reduced in Pond 2 in comparison with Pond 1, in particular reference to filamentous and green unicellular and polymonad algae that were regularly consumed by tadpoles in Pond 1. On the other hand, microcrustaceans and micromycetes were abundant both in seston and periphyton as well as in the digestive tracts of tadpoles in Pond 2. These data presume that not only tadpoles with generalised mouth morphologies, but also larvae with highly specialised oral apparatuses such as microhylids are capable of changing their diet depending on resource availability. Additionally, the intake of different groups of crustaceans varied among ponds and tadpole species. In Pond 1, advanced *P. megacephalus* tadpoles occasionally fed on ostracods, which are mostly substrate-dwelling crustaceans and could be ingested concomitantly with algae or macrophytes, while the filter-feeding *M. heymonsi* mostly ingested planktonic copepods. In Pond 2, the more abundant cladocerans were prevalent among the ingested crustaceans in both of these tadpole species (Fig. 2).

Apparently, the diversity of feeding substrates used by tadpoles correlated with the overall diversity of the pond ecosystem. The seston and periphyton composition, as well as the macrophyte surroundings were markedly more diverse in the open-canopy Pond 1 than in the closed-canopy Pond 2 (see Appendix 1). The relatively low diversity of the Pond 2 food web was assumingly caused by dense canopy closure and intense shading that inhibited the development of autotrophic organisms and, especially, green algae, which formed a substantial part of seston in the open-canopy Pond 1. Previous studies on the impact of canopy closure on tadpole development demonstrated that potential food substrates such as periphyton tended to be more abundant and more diverse in open-canopy ponds (Skelly et al., 2002). Filamentous algae were more frequently consumed in open-canopy ponds than in closed-canopy ones, whereas invertebrates were a more important source of nutrition for tadpoles in closed-canopy ponds (Schiesari, 2006).

Stable isotope analysis provided further information on the mechanisms of trophic differentiation among coexisting anuran larvae. As expected, the highly carnivorous *H. rugulosus* was most enriched in $^{15}N$, indicating that this species occupies the highest trophic level among all anuran species studied. On the other hand, the inter-annual difference in $\delta^{15}N$ values of *H. rugulosus* suggests that its carnivory is of opportunistic nature. In 2009, the $\delta^{15}N$ values of *H. rugulosus* (7.7±0.3‰) were similar to those of predatory arthropods (around 8‰), but in 2011 this species did not differ significantly in $\delta^{15}N$ values from other tadpole species (Fig. 3B). There was a significant difference in $\delta^{13}C$ values of muscle tissues as well as of gut contents between two grazers, *P. megacephalus* and *H. rugulosus* (Figs. 3, 5). This indicates that these two species used resources of different origins. Due to the high variation in $\delta^{13}C$ values of potential food substrates in Pond 1, proximal reasons for this differentiation are not clear. Most likely, *P. megacephalus* used preferentially autochthonous aquatic resources, whereas *H. rugulosus* consumed more carbon of terrestrial origin (see discussion on microhylid species below).

The isotopic signatures of larval *P. megacephalus* confirmed drastic differences in the diet of tadpoles at different developmental stages. Age-related shifts in the feeding mode of tadpoles was previously shown in *Rana catesbeiana, R. chaochiaoensis* (Ranidae) and *Nannophrys ceylonensis* (Dicroglossidae) (Zhou et al., 2005, 2007; Wickramasinghe et al., 2007). The enrichment of early *P. megacephalus* larvae in $^{13}C$ and $^{15}N$ relative to advanced larvae could not be caused by the high isotope values inherited from adult frogs with yolk because the isotopic equilibrium of larval tissues with the diet was likely established soon after the beginning of active feeding. This was shown in our laboratory experiment (Fig. 4) that corroborates the earlier published results (Trakimas et al., 2011). As suggested by the gut contents analysis (Fig. 2), early larvae of *P. megacephalus* are mainly suspension-feeders, very similar to *M. fissipes* in diet composition. This similarity is also seen in the isotopic composition of these two species (Fig. 3 AB).

In both ponds, two species of filter-feeding microhylids, *M. fissipes* and *M. heymonsi*, differed strongly in $\delta^{13}C$ values (Fig. 3). Autotrophic producers in small lentic ponds are often depleted in $^{13}C$ relative to terrestrial producers, mainly due to the re-fixation of $CO_2$ respired by microbial decomposers (France & Schlaepfer, 2000; Post, 2002). This pattern can clearly be seen in Pond 2, where seston was depleted in $^{13}C$ relative to the litter of terrestrial origin (Fig. 3C). The enrichment of *M. heymonsi* in $^{13}C$ may therefore be explained by a higher proportion of 'terrestrial' carbon in the diet of this species. As a surface feeder, *M. heymonsi* often consumed pollen, seeds and terrestrial arthropods, whereas the diet of the suspension feeder *M. fissipes* included more diatoms (Fig. 2). The difference in the diet of the two microhylids is further confirmed by considerably different $\delta^{13}C$ values of their gut contents (Fig. 5). These findings are in agreement with previous stable isotope studies in tropical freshwater ecosystems that revealed a differentiation

of trophic niches among tadpoles with different feeding habits, such as herbivorous grazers, surface feeders and microbial feeders (Whiles et al., 2006; Verburg et al., 2007).

Deciphering isotopic data requires knowledge on several confounding factors, including species-specific variations in trophic fractionation and the chemical composition of animal tissues. For instance, lipids are depleted in $^{13}C$, and more fatty organisms or tissues tend to have relatively low $\delta^{13}C$ values (Post et al., 2007). In our study, the difference in the mass C/N ratio (which reflects the fat content) of muscle tissues of four tadpole species varied little, from 3.4±0.1 in *M. fissipes* to 3.5±0.2 in *H. rugulosus*, suggesting that $\delta^{13}C$ values were not biased.

The trophic fractionation was studied in detail in one species only (Fig. 4). Nevertheless, comparisons of the muscles and gut contents suggest that, in spite of considerable variations in diet composition, the trophic fractionation of carbon isotopes was similar in all species studied (Fig. 5). The $\Delta^{13}C$ value obtained in our laboratory experiment with *P. megacephalus* (+1.9‰) was similar to 1.7‰ reported for *Lithobates sylvaticus* tadpoles (Schiesari et al., 2009) and well within the range of $\Delta^{13}C$ values derived by Caut et al. (2013).

The trophic fractionation of nitrogen stable isotopes in *P. megacephalus* tadpoles (+1.2‰) was similar to the $\Delta^{15}N$ values obtained by Trakimas et al. (2011) for *Rana temporaria* (1.1–1.2‰, depending on developmental stage). Considerably higher $\Delta^{15}N$ values were obtained in most other experiments with tadpoles and aquatic consumers in general (Post, 2002; Schiesari et al., 2009; Caut et al., 2013). In the field-collected tadpoles, the difference in $\delta^{15}N$ values between muscles and gut contents averaged +1.0‰, but varied depending on species. In particular, it was lower in *M. heymonsi* than in other tadpoles (Fig. 5). However, it seems unlikely that species-specific variations in $\Delta^{15}N$ values could affect the main results of our study, as tadpole species differed mainly in $\delta^{13}C$ values (Fig. 3).

To conclude, our data showed that even a small, temporary, tropical pond can support a functionally diverse community of anuran larvae. Gut contents analysis supported the expected partitioning of food resources by tadpoles with different oral morphologies and showed differences in the food spectra of filter-feeding and grazing species. Stable isotope analysis revealed a more complex pattern of trophic niche segregation among grazers and also among filter-feeders. Furthermore, our data suggest that tadpoles with generalized grazing oral morphologies (*P. megacephalus*) can start feeding as suspension feeders and then shift to the rasping mode. Trophic niche segregation among anuran larvae may contribute to maintaining the high species diversity in tropical ecosystems. This segregation is achieved not only by the use of different microhabitats and selective feeding on certain food types, but also through the dependency on different carbon sources traceable as either of aquatic or terrestrial origins.

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## APPENDIX

**Appendix 1.** Description of the study sites in the Cat Tien National Park, southern Vietnam.

**Pond 1.** Open rain pond with an area of about 30 $m^2$ and the maximum depth of 60 cm; prevalent depths were of 15–25 cm. Bottom was slimy, with submerged stones and decaying bamboo stems. Surrounding vegetation was represented by the C4 grass *Saccharum* sp. (Poaceae), and various C3 plants, including *Donax canniformis* (Marantaceae), monocotyledon (Cyperaceae, Poaceae) and dicotyledon herbs, bushes (Mimosaceae), trees *Streblus* sp. (Moraceae), *Leea rubra* (Leeacea), *Grewia* sp. (Tiliaceae), *Mangifera* sp. (Anacardiaceae), lianas *Acacia* sp. (Mimosaceae), *Combretum* sp. (Combretaceae), and *Dendrocalamus* sp. bamboo (Poaceae).

Seston was mainly formed by filamentous algae with the predominance of *Oedogonium* (Chlorophyta) and *Spirogyra* (Charophyta). *Volvox* colonies and unicellular desmids *Closterium* (Chlorophyta), as well as flagellate protists Euglenozoa (*Euglena*) and Dinoflagellata were most abundant in the water column. Among plankton crustaceans, calanoid copepods and the cladoceran *Diaphanosoma* were abundant; large ostracods and several species of cladocerans (*Karualona*, *Mainodaphnia, Alona*) were observed as few individuals. The periphyton on submerged stones, logs, bamboo stems and herbaceous macrophytes was mainly composed of filamentous algae *Oedogonium* (Chlorophyta) and *Hapalosiphon* (Cyanophyta), hyphae of unidentified fungi were also observed. Other algae in the periphyton were represented by unicellular and colonial forms of desmid green algae (*Closterium*, *Desmidium*, *Cosmarium*, *Pleurotaenium*) and diatoms Bacillariophyta (*Navicula*, *Pinnularia*, *Stauroneis*). Among protists, flagellates (*Euglena*) and testate amoebae (*Arcella*, *Difflugia*) were abundant. Bryozoans, sedentary rotifers and infusorians, as well as small (up to 1 mm) nematodes and oligochaetes were also recorded. Macroinvertebrates were represented by crabs (Decapoda), dragonfly larvae (Odonata), diving beetles (Coleoptera), boat bugs, water scorpions, and water skaters (Hemiptera).

Nine anuran species (Microhylidae, Rhacophoridae, Dicroglossidae) were observed to spawn in the pond. Larvae of six species (*Hoplobatrachus rugulosus, Microhyla butleri* (Boulenger, 1900)*, M. fissipes, M. heymonsi, Occidozyga lima* (Gravenhorst, 1829)*, Polypedates megacephalus*) were recorded in May–June 2009.

**Pond 2.** Shaded rain pond (canopy closure 80 %) with an area of about 20 $m^2$ and the maximum depth of 50 cm; prevalent depths were of 20–30 cm. Bottom was litter-covered, with some submerged stones and fallen logs. Surrounding vegetation included C3 plants: bushes *Phyllanthus* sp. (Euphorbiaceae), trees *Syzygium* sp. (Myrtaceae), *Ochrocarpus* sp. (Guttiferae) and *Mangifera* sp. (Anacardiaceae), lianas Connaraceae and Menispermaceae, and *Dendrocalamus* sp. bamboo (Poaceae); herbaceous plants on banks or in the water were absent.

Important part of seston was formed by mycelium and spores of micromycetes; both the perfect and imperfect fungi were present. Among algae, individual *Closterium* (Chlorophyta) and *Pinnularia* (Bacillariophyta) were recorded. Crustaceans *Maina* (Cladocera) and calanoids (Copepoda) were abundant in the plankton. Periphyton on submerged stones and logs was represented by diverse kinds of mycelium, inhabited by protists

(*Euglena* and various infusorians), small oligochaetes and rotifers. Single colonies of *Dinobryon* (Chrysophyta) were recorded. Macroinvertebrates were represented by crabs (Decapoda), dragonfly larvae (Odonata), and water skaters (Hemiptera).

Five anuran species (Microhylidae, Rhacophoridae, Dicroglossidae) were observed to spawn in the pond. Larvae of four species (*Occidozyga martensii* (Peters, 1867), *M. fissipes, M. heymonsi, P. megacephalus*) were recorded in May–June 2011.