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POPULATION ECOLOGY - ORIGINAL RESEARCH

Plastic changes in tadpole trophic ecology revealed by stable isotope analysis

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Abstract Amphibian larvae constitute a large fraction of the biomass of wetlands and play important roles in their energy flux and nutrient cycling. Interactions with predators and competitors affect their abundance but also their foraging behaviour, potentially leading to non-consumptive cascading effects on the whole trophic web. We experimentally tested for plastic changes in larval trophic ecology of two anuran species in response to competitors and the non-lethal presence of native and non-native predators, using stable isotope analysis. We hypothesized that tadpoles would alter their diet in the presence of competitors and native predators, and to a lesser extent or not at all in the presence of non-native predators. First, we conducted a controlled diet experiment to estimate tadpole turnover rates and discrimination factors using *Pelobates cultripes* and *Bufo calamita*. Turnover rates yielded a half-life of 15–20 days (attaining a quasi-isotopic equilibrium after 2 months), whereas discrimination factors for natural controlled diets resulted in different isotopic values essential for calibration. Second, we did an experiment with *P. cultripes* and *Rana perezi* (=*Pelophylax perezi*) where we manipulated the presence/absence of predators and heterospecific tadpoles using microcosms in the laboratory. We detected a significant shift in trophic status of both amphibian species in the presence of non-native crayfish: the δ^{15}N values and macrophyte consumption of tadpoles increased, whereas their detritus consumption decreased. This suggests that tadpoles could have perceived crayfish as a predatory risk or that crayfish acted as competitors for algae and zooplankton. No dietary changes were observed in the presence of native dragonflies or when both tadpole species co-occurred. Stable isotopic analysis is an efficient way to assess variation in tadpoles' tropic status and hence understand their role in freshwater ecosystems. Here we provide baseline isotopic information for future trophic studies and show evidence for plastic changes in tadpoles' use of food resources under different ecological scenarios.

Keywords Discrimination factor · Turnover · Isotopic incorporation · Invasive species · Diet shift

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Introduction

Species interactions can have large effects on community diversity, stability and productivity. In freshwater systems, loss of consumer diversity is occurring at an alarming rate, with profound ecological consequences (Whiles et al. 2010). Amphibian larvae are key consumers in a variety of freshwater systems, both lotic and lentic, where they can reach high densities and biomass, and also serve as important prey for a number of other species (Wells 2007; Schiesari et al. 2009). Larvae of most anuran species are herbivores that complement their diets with additional types of resources such as detritus, bacteria, plankton or fungi, and hence can be classified as opportunistic

omnivores or detritivores (Hoff et al. 1999; Altig et al. 2007). Although omnivory may impose costs in terms of reduced efficacy of detecting, handling, and assimilating prey, it may provide greater flexibility in diet choice, which could prove advantageous in response to variation in the type and availability of food resources, or the presence of competitors and/or predators (Coll and Guershon 2002).

Interactions with predators and competitors, however, not only affect the abundance of amphibian larvae but can also induce changes in the amount or type of resources consumed by tadpoles, leading to non-consumptive cascading effects on the whole community structure across various trophic levels (Miner et al. 2005). Tadpoles are very perceptive of their environment. They are capable of detecting water-borne predator cues and alarm cues from attacked conspecific tadpoles and consequently alter their behaviour and physiology by increasing refuge use, reducing activity levels and/or increasing tail depth (Werner and Anholt 1993; Van Buskirk 2001). Similarly, tadpoles respond to competitors by increasing foraging activity and altering their morphology (Relyea 2002). Environmentally induced changes in tadpoles' overall activity level, presence in the water column and use of refugia are thus widespread (Anholt and Werner 1995; Richardson 2001) and could cause changes in their diet as a consequence of shifts in microhabitat (e.g. shifts from feeding on macrophytes to increased filtration in the water column or grazing periphyton). Moreover, competitor- and predator-induced developmental shifts affect tadpoles' anatomy in ways that could be associated with changes in their diet, as gut length, oral disc size, beak width, and tooth rows vary in response to predators and competitors (Relyea and Auld 2004, 2005). Few studies, however, have studied differences in diet within local amphibian guilds (Wells 2007), and fewer still have analysed within-species variation in diet according to microhabitat variation (Schiesari et al. 2009; Whiles et al. 2010), with the exception of the dramatic polyphenism between omnivorous and carnivorous morphs in spadefoot toads (Frankino and Pfennig 2001; Martin and Pfennig 2010). Predator- or competitor-induced plastic changes in tadpole trophic ecology may be of high relevance given their prominent role in aquatic systems (Peacor and Werner 1997; Peacor and Werner 2000; Pfennig et al. 2006).

Trophic shifts in omnivorous species are known to represent a major advantage in predator–prey interactions, as they allow prey to alter their resource use to avoid encounters with predators while continuing to feed (e.g. Ruehl and DeWitt 2007; Caut et al. 2008b). Accurate assessments of the trophic relations of omnivorous species, however, require detailed information on diets and assimilation (Altig et al. 2007; Schiesari et al. 2009), which is methodologically complicated in aquatic systems. Standard methods used to study tadpole diets (e.g. foraging observations, gut contents and faecal analysis) provide detailed accounts of what tadpoles have ingested as a snapshot of a species' diet (Díaz-Paniagua 1985) but may not be reliable indicators of real assimilation over longer time periods (Martínez del Rio et al. 2009). These limitations of standard techniques can be overcome using stable isotopes as tracers of nutrient flow in food webs (Gannes et al. 1997).

Interpretations of stable isotope signatures depend on a series of assumptions about the relationship between isotope ratios of consumers and their food resources (Gannes et al. 1997; Caut et al. 2008a). Isotopic signatures of C (δ^{13}C) are preserved from the source through the food chain (e.g. origin), whereas N signatures (δ^{15}N) become enriched following transfer to a higher trophic level (Martínez del Rio et al. 2009). Isotope ratios account for temporal variance in resource assimilation over time because the diet isotopes require time to become assimilated into the consumer's tissue (turnover rate).

A difficulty in using isotopic models for evaluating incorporation of resources from different diets is that consumer metabolic processes may discriminate between different isotopes; their isotopic ratio may not exactly correspond to the isotopic ratio of the food resource. The difference in isotopic composition between any tissue of an animal (e.g. muscle and liver) and its diet is represented by a discrimination factor (Δ). Isotopic discrimination factors may differ widely among consumer classes (Caut et al. 2008c, 2009, 2010). Diet reconstructions rely on the use of isotopic models to derive quantitative estimates of dietary contributions from isotopically distinct components, and require precise estimates of such discrimination factors (Phillips and Gregg 2001; Caut et al. 2009). Small variations in discrimination factors can lead to large errors or meaningless results from isotopic models (Ben-David and Schell 2001; Caut et al. 2008a). A handful of studies have analysed amphibian trophic ecology through stable isotopes, assessing the trophic status of terrestrial adults and aquatic tadpoles, and even reporting ontogenetic changes in diet (Kupfer et al. 2006; Verburg et al. 2007; Jefferson and Russell 2008; Schiesari et al. 2009), but have not taken into account turnover rates and discrimination factors.

Here we tested for plastic changes in larval trophic ecology of two anuran species from southwestern Spain (Iberian green frog, *Rana perezi*, and Western spadefoot toad, *Pelobates cultripes*) in response to competitors and two types of predator using stable isotope analysis. It is key in designing sampling periodicity and interpreting isotope analyses to have estimates of discrimination factors and turnover rates specific to the study system, so we first conducted an experiment where we fed controlled diets to *P. cultripes* and Natterjack toad (*Bufo calamita*) tadpoles, hence obtaining discrimination factors and turnover rates for anuran larvae for the first time.

Then, we conducted a microcosm experiment where we exposed tadpoles to the non-lethal presence of native dragonfly nymphs (*Anax imperator*) or non-native red swamp crayfish (*Procambarus clarkii*). Red swamp crayfish have a huge impact on aquatic systems, affecting several trophic levels as they are both primary consumers (filter-feeders and macrophyte consumers) and important predators of amphibian eggs and larvae (Geiger et al. 2005; Gherardi 2007; Cruz et al. 2008; Ficetola et al. 2011). Because anti-predator responses critically depend upon predator cue recognition, they may fail against novel invasive species (Cox and Lima 2006; Freeman and Byers 2006; Gomez-Mestre and Díaz-Paniagua 2011). We therefore hypothesized that tadpoles would alter their diet in the presence of competitors and native predators, and to a lesser extent in the presence of non-native predators.

Materials and methods

Controlled diet experiment

We conducted a controlled diet experiment on larvae of two anuran species (*P. cultripes* and *B. calamita*) to determine our capacity to distinguish among possible alternative tadpole diets, the timeframe required for the diet to leave a detectable isotopic signature, and a range of discrimination factors for different diets. We then used this range of discrimination factors and diets as a baseline to calculate diet-dependent discrimination factors in tadpoles (for any tadpole species and diet, following Caut et al. 2009). We used four controlled diets, all based on natural resources that appear in tadpole diets in varying proportions (Díaz-Paniagua 1985). The macrophyte diet (diet M) consisted of a mixture of equal parts of three abundant aquatic plant species in the temporary ponds of Doñana Biological Reserve (36°59′N/6°27′W) in southwestern Spain, where tadpoles were collected: *Myriophyllum alterniflorum*, *Ranunculus peltatus* and *Callitriche obtusangula* (García Murillo et al. 2006). The algal diet (diet A) was obtained from a culture of *Scenedesmus intermedius* inoculated with pond water from Doñana. The resulting mixed algal culture was grown and kept in a bioreactor, a plexiglass cylinder filled with filtered pond water aerated with a constant flow and constantly illuminated. The final culture was a mixture of different algal species, including *Scenedesmus intermedius*, *Scenedesmus securiformis*, *Nitzschia* sp., *Navicula* sp., *Chlamidomonas* sp., *Oscillatoria* sp., *Chlorella* sp., *Dyctiosphaerium* sp., *Volvox* sp., *Peridium* sp. *and Microcystis aeruginosa*, all common in the ponds in Doñana (López et al. 1991). We obtained zooplankton samples (diet Z) by dip-netting, and these consisted of 99.8 % *Daphnia magna*. Dead tadpoles (diet T) were all laboratory-reared *P. cultripes* fed macrophytes from a prior experiment that could not be released back in the field and were sacrificed and frozen the year before. All constituents of our experimental diets were obtained from ponds within the Doñana Biological Reserve. The diets were dried to constant volume (at least 4 days at 55 °C) and ground to a fine powder.

In February 2010, we kept portions of three different clutches per species (all captured at the same stage of development and from the same pond within the Reserve) inside a climatic walk-in chamber at Estación Biológica de Doñana where they were kept in a 12 h light: 12 h dark photoperiod at 20 °C to enhance tadpole growth. Upon reaching the free-feeding stage (Gosner stage 25; Gosner 1960), tadpoles were transferred to each of the pure diet treatments. We collected tadpoles for isotopic analysis at four different time points: at days 21, 40, 62 and 87 (day 70 in the zooplankton diet). Our design consisted of two replicates per clutch in each treatment at each sampling time, resulting in 6 replicates × 4 diets × 4 sampling times per species. Each replicate consisted of ten *B. calamita* tadpoles or five *P. cultripes* tadpoles, [except for the first sampling (T_{21}), when given the small tadpole sizes we had to use 15 and ten individuals for *B. calamita* and *P. cultripes*, respectively, to ensure enough sample for isotopic analyses]. Due to species-specific differences in size and growth rate, we raised *B. calamita* tadpoles in 1-L containers, whereas *P. cultripes* tadpoles were raised in 3-L ones. At the beginning of the experiment (T_0), we took a sample of 20 hatchlings of each clutch to determine the starting isotopic values. Up to that point, tadpoles had relied on yolk to grow and develop, so variation in isotopic compositions would likely reflect differences in maternal diet. Tadpoles were fed 0.5 g of a powdered diet every second day, as water was renewed. The containers were thoroughly cleaned to prevent algal blooming, and dead tadpoles were removed upon sight to prevent scavenging. At each sampling time, tadpoles were sacrificed by freezing and developmental stage was determined. All samples were kept at −20 °C until analysis. The consistency of the dietary isotopic composition was investigated by measuring the isotopic signature of different diets randomly taken from the initial powder stock 5 times throughout the experiment (Table 1).

Microcosm experiment

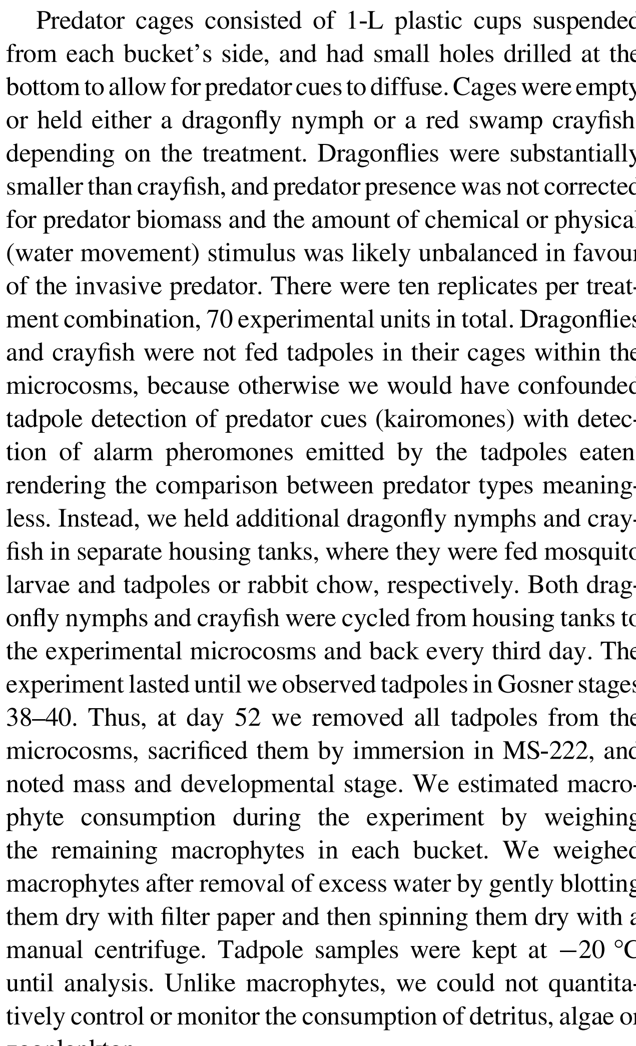
We used a microcosm experiment to raise tadpoles of two anuran species (*P. cultripes* and *R. perezi*) in different scenarios of competition and predator presence that could alter their trophic ecology. Due to the breeding phenology of amphibians in Doñana, *B. calamita* tadpoles were no longer available for this experiment and we used *R. perezi*

Table 1 Isotopic values (‰ ± SE) of the resources in the controlled diet experiment and the microcosm experiment

Resource	δ^{13}C	δ^{15}N
Controlled diet		
Macrophytes	−26.16 ± 0.04	8.43 ± 0.08
Zooplankton	−21.50 ± 0.05	8.72 ± 0.07
Algae	−19.21 ± 1.01	−4.27 ± 0.44
Dead tadpoles	−26.66 ± 0.07	6.44 ± 0.10
Microcosm		
Macrophytes	−26.62 ± 0.41	5.05 ± 0.40
Zooplankton	−18.57 ± 0.12	8.72 ± 0.16
Algae	−18.41 ± 0.94	−4.25 ± 0.76
Detritus	−27.97 ± 0.39	4.25 ± 0.37

tadpoles instead. Tadpoles from this species are more likely to compete with *P. cultripes*. Moreover, using a different species also allowed us to apply the method developed in the previous experiment, which was designed to obtain discrimination factors for any tadpole species and diet (as reviewed by Caut et al. 2009). For both amphibian species, our treatments consisted of: (1) no predator, (2) the non-lethal presence of dragonfly nymphs (*Anax imperator*, native predators), or (3) the non-lethal presence of red swamp crayfish (*Procambarus clarkii*, invasive predators). An additional treatment was made with the two species together with no predator to examine the effect of competition. Microcosms were 10-L plastic buckets filled with a 2:8 mixture of pond and carbon-filtered tap water, aerated and maintained at 20 °C and 12 h:12 h light:dark photoperiod in a climatic chamber. Pond water was added as inoculum for zoo- and phytoplankton. Each bucket was also provided with 10 mL of a well-mixed water sample with high density of *D. magna* and 160 g of *Myriophyllum alterniflorum* (80 g/monthly to maintain this resource throughout the experiment), planted in small plastic cups with a spoonful of pond sediment covered with small inert aquarium pebbles.

Other than tap water, all components came from Doñana Biological Reserve. Microcosms were set up 3 weeks prior to the onset of the experiment; 500 g of pond sediment was added to each bucket in addition to the pond water. Macrophytes and detritus in the microcosms were sampled for isotopic analyses. We collected tadpoles of a similar stage (Gosner stage 25) in April 2010 and raised them in the laboratory for 2 months prior to the beginning of the experiment and fed them a control diet (rabbit chow) in order to establish a common dietary baseline. We then randomly assigned individual tadpoles to each bucket according to treatments. Chamber shelves were treated as experimental blocks, and all treatments were represented once on each shelf, randomly positioned upon it.



Predator cages consisted of 1-L plastic cups suspended from each bucket's side, and had small holes drilled at the bottom to allow for predator cues to diffuse. Cages were empty or held either a dragonfly nymph or a red swamp crayfish, depending on the treatment. Dragonflies were substantially smaller than crayfish, and predator presence was not corrected for predator biomass and the amount of chemical or physical (water movement) stimulus was likely unbalanced in favour of the invasive predator. There were ten replicates per treatment combination, 70 experimental units in total. Dragonflies and crayfish were not fed tadpoles in their cages within the microcosms, because otherwise we would have confounded tadpole detection of predator cues (kairomones) with detection of alarm pheromones emitted by the tadpoles eaten, rendering the comparison between predator types meaningless. Instead, we held additional dragonfly nymphs and crayfish in separate housing tanks, where they were fed mosquito larvae and tadpoles or rabbit chow, respectively. Both dragonfly nymphs and crayfish were cycled from housing tanks to the experimental microcosms and back every third day. The experiment lasted until we observed tadpoles in Gosner stages 38–40. Thus, at day 52 we removed all tadpoles from the microcosms, sacrificed them by immersion in MS-222, and noted mass and developmental stage. We estimated macrophyte consumption during the experiment by weighing the remaining macrophytes in each bucket. We weighed macrophytes after removal of excess water by gently blotting them dry with filter paper and then spinning them dry with a manual centrifuge. Tadpole samples were kept at −20 °C until analysis. Unlike macrophytes, we could not quantitatively control or monitor the consumption of detritus, algae or zooplankton.

Isotopic analysis

In the controlled diet experiment, where tadpoles were small, we were forced to use the whole body (gastrointestinal tract removed) for analysis and pooled all individuals within each bucket. When mortality was high (e.g. algal diet), we had to pool samples by clutch or treatment for a given sampling time. Given the tadpole sizes in the microcosm experiment, we analysed tail muscle and liver for *P. cultripes* but only tail muscle for *R. perezi*, since *R. perezi* tadpoles were too small for the liver to be sampled. We selected liver and muscle because their turnover rates of stable isotopes are different, which reflects a faster assimilation time for liver than for muscle (Martínez del Rio et al. 2009). Knowing the turnover rates is important in deciding a sampling scheme in the field to determine trophic shifts that may have occurred at different times. All samples were dried and ground to a fine powder. Isotopic analyses were performed using a mass spectrometer (Optima, Micromass, UK) coupled to a C–N–S elemental

analyser (Carlo Erba, Italy). Ratios are presented as δ values (‰), expressed relative to the Vienna Pee Dee belemnite standard and to atmospheric N_2 for C and N, respectively. Reference materials were IAEA-CH-6 (−10.4 ‰) and IAEA-N1 (+0.4 ‰) for δ^{13}C and δ^{15}N, respectively. One hundred replicate assays of internal laboratory standards indicate measurement maximum errors (SD) of ±0.2 ‰ and ±0.15 ‰ for stable C and N isotope measurements, respectively.

Caution must be used when applying stable isotopes to tissue with high lipid contents because the low δ^{13}C values in lipids compared to other tissue may bias interpretation. Although this problem may be overcome by removing lipids from tissue samples, we did not do so due to the small size of the tissue samples coupled with the anticipated loss of tissue during the lipid extraction process. Post et al. (2007) proposes a general correction for lipid content when the C:N ratio of the tissue being sampled is >3.5 for aquatic animals. Following their equation (δ^{13}Cnormalized = δ^{13}Cuntreated − 3.32 + 0.99 C:N) lipid contents of the zooplankton diet were normalized. However, when working with a group of organisms that are not represented in Post et al. (2007) such as *R. perezi* and *P. cultripes*, lipid extraction should be used to estimate their relationship between delipidated δ^{13}C and C:N. This is particularly important due to the high variation among taxonomic groups in the C:N ratio of lipid-free tissue (Sweeting et al. 2006). Hence we conducted a preliminary analysis on tadpoles (nine *R. perezi* and nine *P. cultripes*) exclusively fed on rabbit chow for 62 days, and this allowed us to compare isotopic values before and after lipid extraction (using the Folch method for lipid extraction; Folch et al. 1957). We found a significant linear relationship between delipidated δ^{13}C (of the tadpole whole body without the stomach) and the C:N ratio ($F_{1,16} = 41.83$, $P > 0.001$, $R^2 = 0.72$) and we used the equation δ^{13}Cnormalized = δ^{13}Cuntreated − 1.11 + 0.37C:N to normalize all tadpole C isotopic values.

Statistical analyses

For the controlled diet experiment, isotopic incorporation data were fitted using a Marquardt non-linear fitting routine (NLIN, SAS) using the following equation:

$$y = a + be^{ct}$$

where y is δX (^{13}C or ^{15}N), a is the value ($\delta X_{(\infty)}$) approached asymptotically, b is the total change in the value following the switch in diet [$\delta X_{(\infty)} - \delta X_{(t)}$], c is the turnover rate and t is the time (days) since the switch. Turnover rate was also expressed in terms of half-life ($t_{1/2}$), i.e. the time it takes for the isotopic composition of the tissue to reach a midpoint between the initial and final values: $t_{1/2} = (\ln 0.5)/c$. Discrimination factors between a food resource (W) and a consumer (Y) are described in terms of the difference in delta (δ) values using the Δ notation, where $\Delta = \delta Y - \delta W$. In the only three cases where models did not converge for isotopic C, we estimated Δ by the means of isotopic values after 60 days of experiment (Table 2).

To test the effects of treatment on the isotopic values of muscle of the two tadpole species we performed a multivariate ANOVA on both isotopic elements together (δ^{13}C and δ^{15}N), including species (*Pelobates* or *Rana*) and treatment [control, predator presence (native or invasive), and presence of heterospecific tadpoles] as main factors. We also included mass at the end of the experiment as a covariate and tested all possible interactions between the covariate and the main factors. The interactions with the covariate were not significant and hence we removed them from the model, keeping mass as the only covariate. Both main factors had significant effects on the dependent variables and therefore we conducted univariate analyses of covariance (ANCOVAs) for each isotopic element separately. Fisher's least significant difference (LSD) test was conducted to test for post hoc differences among groups. An additional ANCOVA was performed for *Pelobates* to test for differences between tissues (muscle and liver) in their response to treatments. We applied a Dunn-Sidak correction of the significance level since the muscle data for *Pelobates* were used twice. To test the effect of treatments on tadpole mass or developmental stage and on the amount of macrophytes consumed, we used ANOVA followed by a Fisher LSD for post hoc differences among the seven treatments. All these analyses were performed using STATISTICA 8.0 (StatSoft 2007). All tests fulfilled parametric assumptions and we applied no data transformations.

The relative isotopic contribution of the diet in microcosms was calculated using the SIAR package (Parnell et al. 2010). This model uses Bayesian inference to solve for the most likely set of dietary proportions given the isotopic ratios in a set of possible food sources and a set of consumers. As discrimination factors depend on several sources of variation (e.g. taxon, environment and tissue), diet-dependent discrimination factors (Δ^{13}C and Δ^{15}N) for tadpoles were calculated from the controlled diet experiment by using regression equations between tadpole Δ^{13}C and Δ^{15}N and their corresponding diet isotopic ratios following Caut et al. (2009).

Results

Controlled diet experiment

Mortality of tadpoles fed control diets was high, and was higher for *B. calamita* than for *P. cultripes* (mortalities for

Table 2 Exponential equations (with R^2), half-life ($t_{1/2}$) in days and discrimination factor (Δ, ‰) for different control diets in *Bufo calamita* and *Pelobates cultripes*

Diet	Equation (R^2)	df_n, df_d	F	P	$t_{1/2}$	Δ
Nitrogen						
B. calamita						
Z[a]	$y = 13.07 - 13.01e - 0.0423x$ (0.95)	2, 16	123.86	<0.001	16.39	4.35
M[a]	$y = 13.54 - 13.08e - 0.0289x$ (0.95)	2, 15	121.95	<0.001	23.98	5.11
T[a]	$y = 8.38 - 7.59e - 0.0479x$ (0.93)	2, 13	79.07	<0.001	14.47	1.95
Z[b]	$y = 12.90 - 4.92e - 0.0552x$ (0.74)	2, 6	5.58	0.070	12.56	4.18
M[b]	$y = 12.37 - 4.15e - 0.0392x$ (0.96)	2, 6	43.28	0.002	17.68	3.94
T[b]	$y = 10.36 - 2.34e - 0.0446x$ (0.63)	2, 6	3.43	0.136	15.54	3.93
P. cultripes						
Z	$y = 12.90 - 11.39e - 0.0422x$ (0.95)	2, 26	208.56	<0.001	16.43	4.18
M	$y = 13.83 - 12.55e - 0.0150x$ (0.90)	2, 23	92.97	<0.001	46.21	5.40
T	$y = 7.57 - 5.90e - 0.0577x$ (0.79)	2, 24	40.23	<0.001	12.01	1.14
A	$y = -6.58 + 8.58e - 0.0108x$ (0.70)	2, 13	12.78	0.001	64.18	−2.31
Carbon						
B. calamita						
Z[a]	$y^c = -20.30 - 3.64e - 0.0754x$ (0.85)	2, 22	59.45	<0.001	9.19	−1.73
M						2.33*
T						0.66*
P. cultripes						
Z	$y = -19.87 - 3.45e - 0.0282x$ (0.82)	2, 26	55.28	<0.001	24.58	−1.3
M	$y = -24.66 + 1.51e - 0.0298x$ (0.72)	2, 23	26.61	<0.001	23.25	1.5
T						0.64*
A	$y = -19.08 - 4.05e - 0.0552x$ (0.95)	2, 13	97.26	<0.001	12.56	0.13

We only calculated and represented parameters when the exponential/NLIN model converged. However, when the equation model did not significantly converge for isotopic C (*), we estimated Δ by means of isotopic values after 60 days of the experiment

Z zooplankton, *M* macrophytes, *T* dead tadpoles, *A* algae

[a] For two clutches with initial $\delta^{15}N = 0.87 \pm 0.21$ ‰

[b] For one clutch with initial $\delta^{15}N = 8.14$ ‰

[c] For all three clutches together

Bufo and *Pelobates*, respectively: 63.3 and 49.3 % for diet A; 34.4 and 24.7 % for diet M; 34.8 and 8.7 % for diet Z; and 34.8 and 13.3 % in diet T). There was no mortality due to the direct handling. Mortality of diet Z included what we suspect was an episode of toxic algal bloom in the last block of *B. calamita*, but there were enough survivors for isotopic analyses. The small size of *B. calamita* coupled with the high mortalities in treatment A prevented an isotopic analysis for that diet.

We analysed the differences in initial isotopic values among clutches to know whether we should do a fit for every clutch or if we could group similar clutches together. Overall, there were few differences in N and C isotopic values among clutches within species (<0.5 ‰), with the exception of N in *B. calamita*, where we found an eightfold difference for one clutch (clutch$_1$ $\delta^{15}N = 1.1$ ‰, clutch$_2$ $\delta^{15}N = 0.7$ ‰ and clutch$_3$ $\delta^{15}N = 8.1$ ‰). Given this difference, we fitted the data from this clutch separately from the data for the other two clutches before performing the analysis of isotopic incorporation (see Fig. 1).

We fitted an exponential model for the whole body $\delta^{15}N$ for all diets and species. For $\delta^{13}C$, the exponential model fitted significantly for all diets in *P. cultripes* but only for diet Z in *B. calamita*, probably due to the low C isotopic difference between the initial clutch values and diet-induced ones (Table 2; Fig. 1). N and C half-lives were in the same range for the two species and diets (except for the N half-life in diet A), averaging ~15–20 days (Table 2; Fig. 1). All tadpoles were enriched in ^{15}N and ^{13}C relative to their diet (excluding diet A for N and diet Z for C; Table 2). $\Delta^{15}N$ and $\Delta^{13}C$ was more similar between species (or among clutches) than among diets. $\Delta^{15}N$ mean in tadpoles was 3.80 ± 0.46 ‰, with 3.91 ± 0.61 ‰ for *B. calamita* and 3.57 ± 1.26 ‰ for *P. cultripes*. Mean $\Delta^{13}C$ in tadpoles was 1.19 ± 0.31 ‰.

Microcosm experiment

Treatments had no effect on tadpole mass ($F_{3,36} = 1.84$, $P = 0.157$ and $F_{3,33} = 1.79$, $P = 0.169$, for *Pelobates* and *Rana*, respectively), or developmental stage ($F_{3,36} = 1.13$, $P = 0.350$ and $F_{3,33} = 0.84$, $P = 0.484$, for *Pelobates* and *Rana*, respectively).

N isotopic values in *Pelobates* differed between tissues ($F_{1,68} = 14.89$, $P < 0.001$) and treatments ($F_{3,68} = 19.91$, $P < 0.001$) but the interaction was not significant ($F_{3,68} = 0.02$, $P = 0.997$). Muscle values were lower than liver values for N (Fig. 2). Differences in N isotopic values among treatments were due to higher values of the crayfish treatment with respect to the other treatments ($P < 0.001$

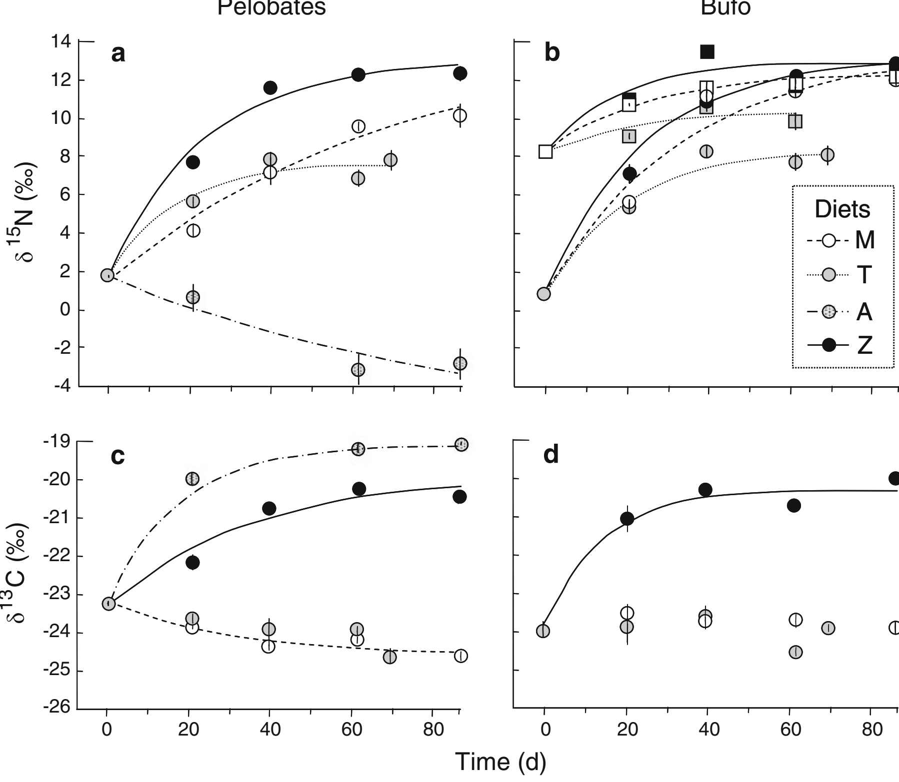


Fig. 1 Trends in δ^{15}N (**a**, **b**) and δ^{13}C values (**c**, **d**; mean ± SE) for *Pelobates cultripes* and *Bufo calamita* (three clutches each) fed four different diets for 80 days: macrophytes (M), dead tadpoles (T), algae (A) and zooplankton (Z). One clutch of *B. calamita* (*squares*) was separated because it differed in initial N isotopic values compared to the other two clutches, but not in initial C isotopic values. The small size of *B. calamita* coupled with the high mortalities in treatment A prevented an isotopic analysis for that diet. Exponential fits are only shown when significant

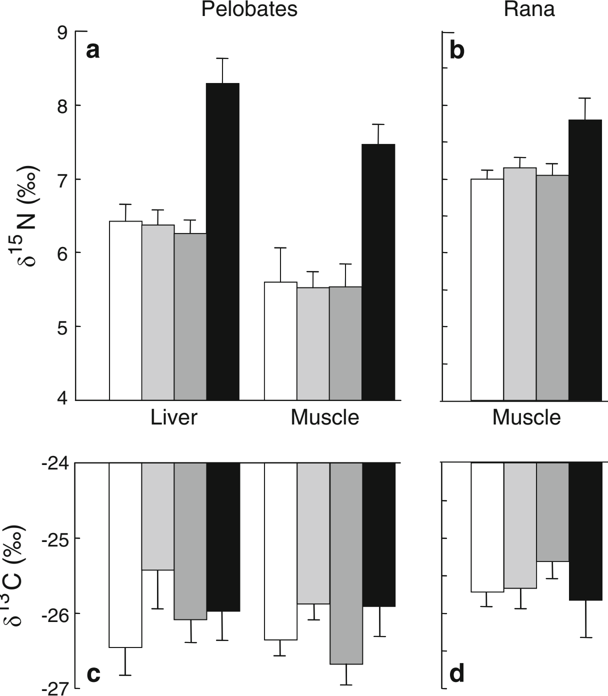


Fig. 2 **a**, **b** δ^{15}N and **c**, **d** δ^{13}C values (mean + SE) of *Pelobates cultripes* liver and muscle and *Rana perezi* muscle in the presence or absence of native dragonfly nymphs (*Anax imperator*) or the invasive predator red swamp crayfish (*Procambarus clarkii*), resulting in four treatments: no predator with one tadpole species (control, *white bars*), no predator—two species *P. cultripes* and *R. perezi* together (*light grey bars*), dragonfly predator (*dark grey bars*) or crayfish predator (*black bars*)

in all cases; Fig. 2). C isotopic values in *Pelobates* were similar between tissues and treatments and their interaction was not significant ($P > 0.08$ in all cases).

Muscle N isotopic values did not vary with tadpole mass ($F_{1, 68} = 1.267$, $P = 0.264$), but differed between species (*Pelobates* yielded lower values than *Rana*; $F_{1, 68} = 19.23$, $P < 0.001$) and between treatments ($F_{3, 68} = 10.88$, $P < 0.001$) (Fig. 2). The species-by-treatment interaction, however, was non significant ($F_{3, 68} = 1.975$, $P = 0.126$). Both species showed higher N values in the presence of crayfish (*Pelobates*, all $P < 0.001$; *Rana* control versus crayfish $P = 0.037$; Fig. 2). Muscle C isotopic values did not vary with tadpole mass ($F_{1, 68} = 0.06$, $P = 0.806$). Unlike N values, there was no difference in C values between species or treatments, and there was no interaction effect.

We obtained regression equations between tadpole Δ^{13}C and Δ^{15}N and their corresponding dietary isotopic ratios from the controlled diet experiment $\Delta^{13}C = -0.35\delta^{13}C - 7.70$ ($F_{1, 5} = 20.21$, $P = 0.006$, $R^2 = 0.80$); and $\Delta^{15}N = 0.53\delta^{15}N - 0.37$ ($F_{1, 8} = 38.32$, $P < 0.001$, $R^2 = 0.83$). These equations allowed the reliable estimation of discrimination factors (Δ^{13}C and Δ^{15}N) of tadpoles for the various resources available in the microcosms, to inform the isotopic model *SIAR*. Isotopic values of the resources in microcosms are shown in Table 1.

For the two species and treatments the SIAR model (to calculate the ranges of feasible contributions for each food source) suggested that macrophytes and detritus constituted

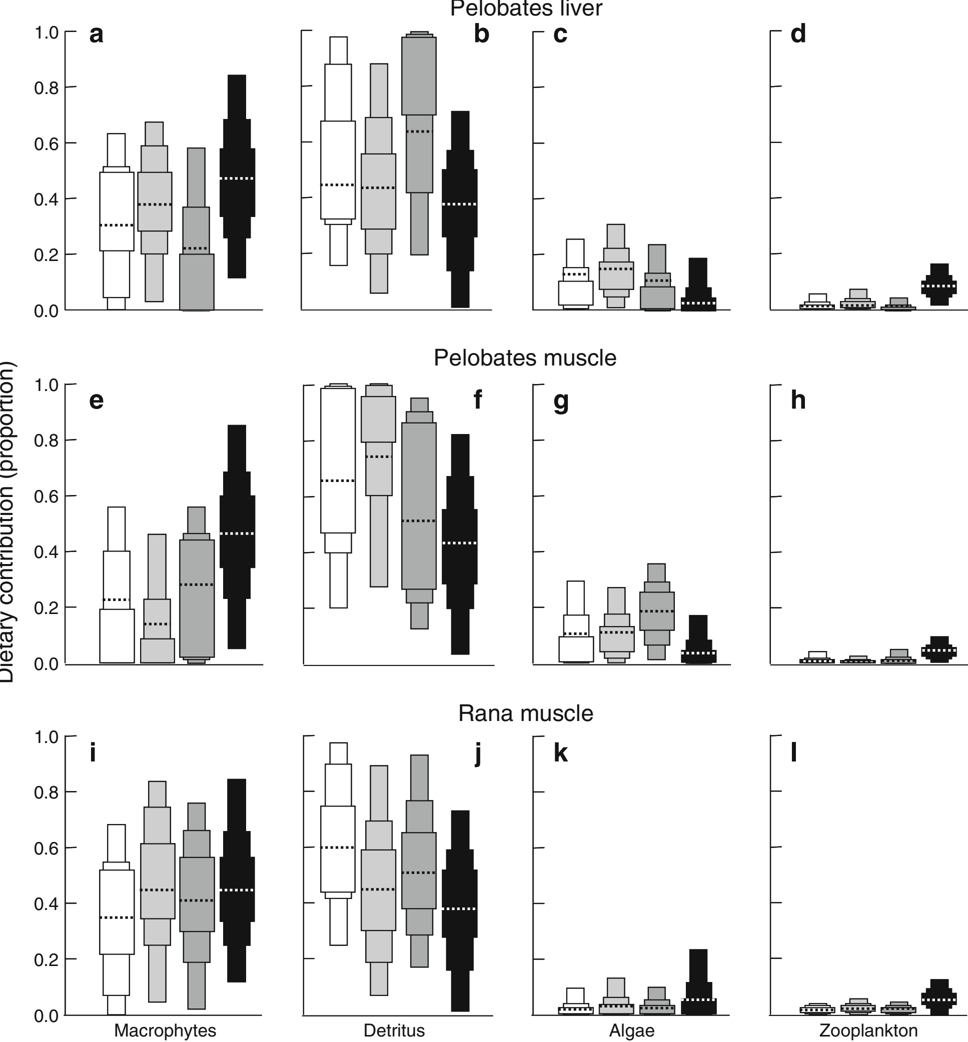


Fig. 3 Dietary contributions (proportion) of the four potential food sources (macrophytes, detritus, algae and zooplankton) for *P. cultripes* (**a**–**d** liver; **e**–**h** muscle) and *R. perezi* (**i**–**l** muscle) resulting in four treatments: no predator with one tadpole species (control, *white bars*); no predator—two species *P. cultripes* and *R. perezi* together (*light grey bars*); dragonfly predator (*dark grey bars*); or crayfish predator (*black bars*). *Histograms* show the distribution of feasible contributions from each food source to the two species' diets resulting from the application of the SIAR isotopic model. Values shown are 5–95 percentile ranges for these distributions. *Dashed line* represents the mean of the distribution

the majority of the diet, followed by algae with little contribution from zooplankton (Fig. 3). Overall, *Rana* showed a higher proportion of macrophytes in their diet than *Pelobates*, who consumed proportionately more detritus than macrophytes. In contrast, *Pelobates* had a greater algal contribution to its diet than *Rana*. Within species, tadpoles in different treatments varied in their dietary composition. In single species, combined species and dragonfly treatments, diet mostly comprised detritus (average values ranged between 45 and 70 %), macrophytes (~15–40 %) and algae (~3–19 %). However, in the crayfish treatment, diets of both tadpole species had a more even contribution of macrophytes (~45–50 %) and detritus (~35–40 %). The algal contribution tended to decrease in *Pelobates* and the zooplankton contribution increased in both species (Fig. 3).

The SIAR model indicated a smaller relative contribution of macrophytes to *Pelobates*' diet than to *Rana*'s diet, although the actual amount of macrophyte biomass consumed was higher for *Pelobates* than for *Rana* ($P < 0.016$; Fig. 4). Moreover, macrophyte consumption differed among treatments ($F_{6,\ 61} = 18.42$, $P < 0.001$, Fig. 4). For each species, consumption was higher when crayfish were present (136.5 ± 5.2 g for *Pelobates* and 78.9 ± 9.8 g for *Rana*, respectively) than when tadpoles were alone (106.5 ± 7.0 g, $P = 0.008$ and 47.0 ± 6.5 g, $P = 0.006$, for *Pelobates* and *Rana*, respectively) or with a native predator (109.0 ± 10.0 g, $P = 0.014$ and 48.3 ± 8.2 g, $P = 0.009$, for *Pelobates* and *Rana*, respectively). There were no significant differences in macrophyte consumption between single tadpole species and native predator treatments ($P = 0.819$ and $P = 0.905$, for *Pelobates* and *Rana*, respectively). Consumption when both tadpole species were together (105.5 ± 7.3 g) was not different to that of *Pelobates* alone ($P = 0.927$) or with a native predator ($P = 0.749$) but was significantly higher than in all *Rana* treatments ($P < 0.001$).

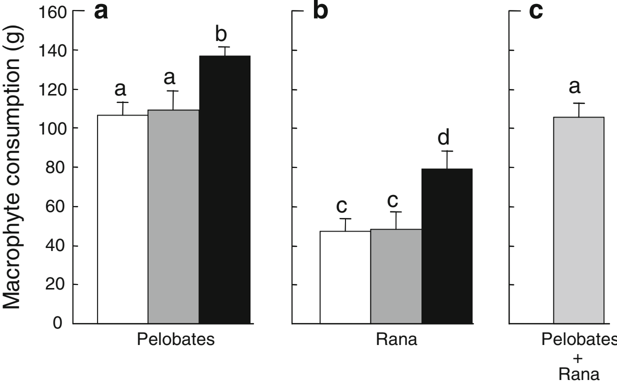


Fig. 4 Macrophyte consumption (mean + SE, difference between macrophyte mass at the beginning and at the end of the experiment) by **a** *P. cultripes* and **b** *R. perezi* in the presence or absence of native dragonfly nymph (*Anax imperator*) or invasive predator red swamp crayfish (*Procambarus clarkii*). Three treatments were no predator with only one tadpole species (control, *white bars*), with dragonfly predator (*dark grey bars*) or with crayfish predator (*black bars*). **c** An additional treatment included the two tadpole species together in the absence of predators (*light grey bars*) to examine the potential effect of competition. *Different letters* indicate significant differences (Fisher's least significant difference for post hoc differences: $P < 0.05$)

Discussion

Isotopic incorporation: discrimination factors and turnover rate estimation

For the first time, we quantified the dynamics of N and C isotopic incorporation in tadpoles (*P. cultripes* and *B. calamita*) via controlled feeding on pure diets. The turnover rate showed a half-life of ~15–20 days and tadpoles attained a quasi-isotopic equilibrium [nearly asymptotical values, $\delta X_{(\infty)}$] after 2 months. This result confirms the possibility of using stable isotopes for a comparison of the trophic ecology of tadpoles in different habitats or ecological scenarios, because the isotopic equilibrium can be reached before the completion of tadpole development in a large number of species (Wells 2007).

We obtained mean N and C isotopic values for different diets that were within the range reported previously for a large array of taxa from arthropods to mammals, including tadpoles (Caut et al. 2009; Schiesari et al. 2009). Nonetheless, the range of discrimination factors was wide, especially for N, due to differences among diets (caused by differences in protein quality, type of food and diet isotopic ratio; see Caut et al. 2008a, 2009). Hence we found that diet-dependent variation in isotopic values within amphibian species can be greater than differences across species. Therefore, assessment of the trophic status of tadpoles by comparison of their isotopic values with those of their potential food resources (e.g. Verburg et al. 2007; Jefferson and Russell 2008; Schiesari et al. 2009) is better informed by calibrating the models with respect to estimated discrimination factors (Caut et al. 2009). The wide range of discrimination factors obtained here, combined with the use of diet-dependent discrimination factors, will be a useful baseline for future research on tadpoles' trophic status in the field.

Trophic plasticity in the presence of predators or competitors

P. cultripes tadpoles are larger than those of *R. perezi* and had a greater overall herbivorous impact on macrophyte biomass (they consumed on average twice as much macrophyte biomass as *R. perezi*; Fig. 4), even though detritus constituted a bigger portion of their diet. Interestingly, macrophyte consumption when both tadpole species were present was no different from that of *P. cultripes* alone. This lack of additive effect on plant biomass consumption could be indicative of reduced foraging in either one of the species or both, possibly due to competition, although it was not severe enough to cause reduced growth in any of the species over the short timescale of the experiment.

Trophic status changed considerably among treatments in evidence of plasticity in the use of food resources. In contrast to our predictions, we detected a significant trophic shift (higher δ^{15}N) in both *P. cultripes* and *R. perezi* in the presence of the non-indigenous species, the red swamp crayfish, while no changes in the diet of any of the species were observed in the presence of native dragonflies. Macrophytes, together with zooplankton, had the highest N isotopic values observed, suggesting that the changes in isotopic values of tadpoles were due to an increase in the relative contribution of macrophytes to the tadpoles' diet in the presence of crayfish, especially given the scarcity of zooplankton relative to macrophyte biomass, which declined throughout the experiment. This interpretation was strongly supported by actual differences among treatments in macrophyte consumption and confirmed with isotopic models.

Increased macrophyte consumption in the presence of crayfish could be interpreted as a response to perceived predation risk, as tadpoles could have sought refuge amid macrophytes and avoided the water column and open areas, hence increasing macrophyte consumption as a consequence of behavioural plasticity. However, anti-predator responses are unlikely to be the reason for increased macrophyte consumption in the crayfish treatment because *R. perezi* tadpoles from Doñana do not respond to chemical or visual cues from non-indigenous crayfish, while readily responding to dragonfly nymphs (Gomez-Mestre and Díaz-Paniagua 2011). Likewise, red swamp crayfish chemical cues do not trigger antipredator defences in *P. cultripes* tadpoles (Online Resource 1), and neither dragonfly nor crayfish chemical cues alone are sufficient to induce increased macrophyte consumption in *P. cultripes* (Online Resource 1).

Alternatively, the observed increase in macrophyte consumption could be a response to competition from crayfish. Red swamp crayfish are important primary consumers and efficient filter feeders (Geiger et al. 2005), to the extent that a large part of their diet is composed of algae and zooplankton (Gutiérrez-Yurrita et al. 1998; Geiger et al. 2005). Although *P. clarkii* has a big predatory impact on amphibian guilds (Cruz et al. 2006, 2008), it is also likely that they have a dual effect as competitors too since they also feed on many of the same sources that tadpoles use (Gutiérrez-Yurrita et al. 1998; Geiger et al. 2005). The cages used in the microcosms allowed water flow, and while crayfish were prevented from accessing tadpoles or macrophytes, they were able to filter algae and zooplankton from the water column, which are important components of their diet (Geiger et al. 2005). This would be congruent with decreased algal consumption of *P. cultripes* in the crayfish treatment, although we found no such effect in *R. perezi*. Co-occurrence of both tadpole species modified the overall macrophyte consumption, but did not have an effect on the relative importance of food resources, suggesting that if competition between tadpoles occurred, it did not result in trophic segregation.

The trophic shifts observed, presumably due to competition from crayfish, may have relevant consequences for ecosystem functioning. Tadpoles constitute an important but imperiled group of consumers in freshwater habitats (Altig et al. 2007) and their feeding behaviour is often linked to functional roles, e.g. altering resource availability or quality for other consumers, (Altig et al. 2007; Whiles et al. 2010). Tadpole trophic shifts may have indirect cascading effects on the trophic structure of freshwater ecosystems. Stable isotopic analyses will help us better understand the trophic ecology of tadpoles, their role in aquatic systems and their dietary shifts in response to ecological disturbances such as the introduction of invasive species.

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Oecologia

Plastic changes in tadpole trophic ecology revealed by stable isotopes analysis

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Online Resource 1.

Effects of exposure to chemical cues from indigenous dragonfly nymph and invasive crayfish on macrophyte consumption.

RATIONALE

Description of methods and results of a companion experiment to Caut et al., designed to test whether the chemical cues from invasive crayfish (*Procambarus clarkii*) trigger *per se* a shift in the trophic ecology of larval spadefoot toads (*Pelobates cultripes*). Results from the microcosms experiment described in the main paper indicate that *P. cultripes* and *Rana perezi* tadpoles increased their consumption of aquatic plants of the species *Myriophyllum alterniflorum*. Crayfish could have triggered an anti-predator response in tadpoles, inducing them to seek refuge amid the aquatic vegetation and increase their macrophyte consumption. A previous study, however, showed that *P. perezi* tadpoles from the populations here studied do not respond to chemical or visual cues from invasive crayfish (Gomez-Mestre & Diaz-Paniagua 2011). However, crayfish are efficient filter feeders (Gutierrez-Yurrita et al. 1998; Geiger et al. 2005) and they could alternatively competed with tadpoles in the microcosms for plankton, inducing in them a shift towards increased macrophyte consumption, and hence reducing competition. To help clarifying this point, we set out to test whether water borne chemical cues alone from either native predators (*Anax imperator*) or invasive crayfish (*P. clarkii*) collected from the same study area (Doñana Biological Reserve in Southwestern Spain, 36°59'N / 6°27' W) induced increased macrophyte consumption in spadefoot toad tadpoles. To verify whether tadpoles were responding to the different types of predators, we also tested for induced changes in tadpole morphology using landmark-based geometric morphometric analysis.

METHODS

On April 16th 2012 we dip-netted *P. cultripes* tadpoles from a pond within the Doñana Biological Reserve, and also collected the macrophyte species *Myriophyllum alterniflorum*, dragonfly nymphs and crayfish from nearby ponds. Brought back to climatic chambers at Estación Biológica de Doñana (Seville), 30 tadpoles were placed individually in 4 L round plastic containers. The containers had been set up a week in advance with a layer of pond sediment (30 g dry weight), potted macrophytes, 3 L of carbon-filtered tap water and 500 mL of Doñana pond water from the same pond where macrophytes were collected. We weighed 15 g of macrophytes after removing excess water with a manual spinner and planted them in small plastic pots with a spoonful of pond sediment covered in aquarium gravel. We established three levels of a predator cue factor: no predator, dragonfly cues, crayfish cues, each replicated 10 times. To obtain predator cues, we kept each predator type in individual containers with macrophytes, to provide support and food (for crayfish). Dragonfly nymphs were kept in 500 mL water, whereas crayfish were kept in 1 L (due to inherent differences in size between the two predators) for 48h prior to use of the water as vehicle of chemical cues. Neither dragonfly

nymphs nor crayfish were fed tadpoles during the experiment, so that cues would correspond to starved predators, to avoid confounding effects from tadpole alarm cues. Twice a day we collected water from at least three individual predators of each type, mixing them well within predator type and added 10 mL to the corresponding containers. The same amount of dechlorinated tap water was also added twice a day to the no predator containers. Tadpoles were kept in the experimental containers for a week, and then macrophytes from each container were harvested, removed the excess water, and weighed to the nearest 0.01 g. Tadpoles were then placed in plexiglass photobooth and photographed for morphological analysis. We then used a geometric morphometric approach to analyse variation in shape among treatments. We scaled each photograph and digitized 10 landmarks on each individual (Fig. ESM1) using tpsDig software (Rohlf 2010), and then obtaining a matrix of relative warps in tpsRelw (Rohlf 2010). The first relative warp explained 47% of the variance in shape and we used it as explanatory variable for tadpole morphology in further statistical tests.

To test the effects of the predator cue treatment on macrophyte consumption and tadpole morphology we fitted a simple general linear model for each variable, and when necessary Tukey HSD tests were conducted to test for post-hoc differences among treatments. Inclusion of tadpole length as a covariate in the analysis of macrophyte consumption did not change the results and was finally not included.

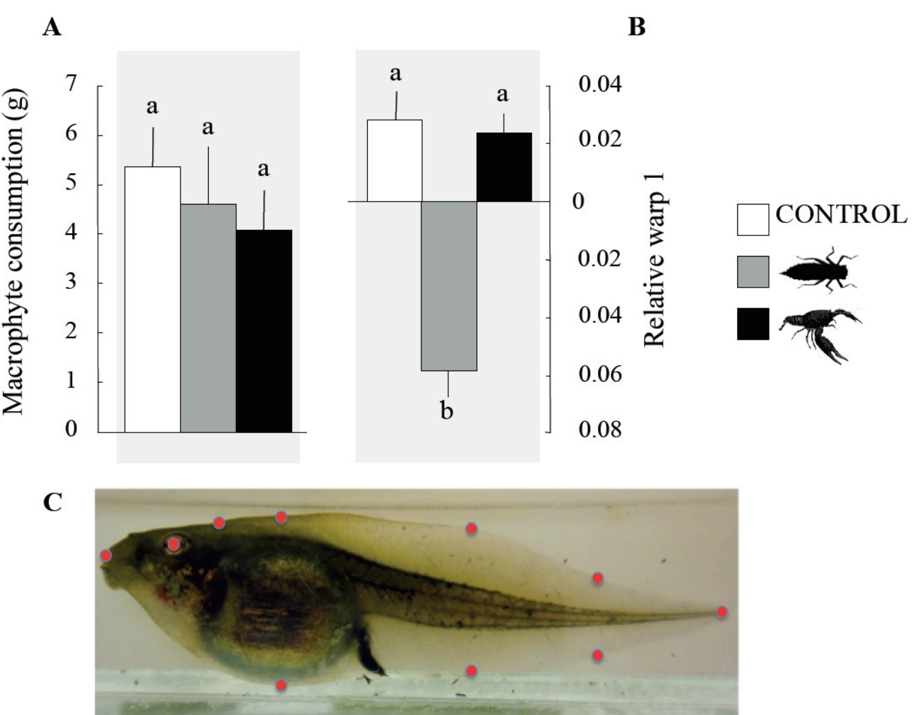

Fig. ESM1. Effects of exposure to presence/absence of chemical cues from either indigenous dragonfly nymphs (*Anax imperator*, gray,) or invasive red swamp crayfish (*Procambarus clarkii*, black,) on (A) mean (+ SE) macrophyte consumption, and (B) mean (+ SE) tadpole shape represented by the first relative warp from a landmark-based geometric morphometrics analysis. Different letters indicate significant post-hoc differences ($P < 0.05$). (C) Landmarks used in the geometric morphometric analysis.

RESULTS

Exposure to chemical cues from dragonfly predators resulted in changes in tadpole morphology, whereas no morphological response was detected in response to crayfish ($F_{2,26} = 29.10$, $P < 0.0001$; Fig ESM1). Hence, *P. cultripes* expressed anti-predator defenses against dragonfly nymphs but not against invasive crayfish. We observed no differences among treatments in macrophyte consumption ($F_{2,28} = 0.519$, $P = 0.66$), indicating that neither native dragonfly cues nor invasive crayfish cues lead to increased macrophyte consumption in spadefoot toad tadpoles.

These results confirm that, similarly to *Rana perezi* tadpoles (Gomez-Mestre and Díaz-Paniagua 2011), *P. cultripes* tadpoles from the Doñana National Park population tested do not recognize crayfish as a predator, since they did not respond morphologically to their chemical cues. Thus, increased macrophyte consumption of tadpoles in presence of caged crayfish reported in the microcosms experiment in Caut et al. are unlikely to be the consequence of tadpole antipredator responses against crayfish.

Instead, crayfish likely acted acted as a competitor for resources. Because crayfish were caged in that experiment and had no access to macrophytes it could have acted filtering the other available resources, algae and zooplacton, causing the trophic shift to macrophytes in tadpoles.

LITERATURE CITED

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