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## Research Article

# Variation of the stable isotope niches of native amphibians in ponds invaded by the red swamp crayfish

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## Abstract

Relationships between native and invasive species can modify trophic interactions in food webs and the diet of native species, leading to substantial changes in their trophic niches. We quantified the stable isotope niche of native amphibians (two species of tadpoles and two species of newts) and the invasive red swamp crayfish (*Procambarus clarkii*) in 18 ponds of an area invaded for more than 30 years. We tested whether crayfish presence and abundance explained variation in the size and position of the amphibians' stable isotope niches compared with proxies of pond resource availability and competition levels. Agile frog tadpoles (*Rana dalmatina*) had consistently low trophic positions, while tree frog tadpoles' niches (*Hyla arborea*) showed signs of an opportunistic diet. Newts (palmate newt (*Lissotriton helveticus*) and marbled newt (*Triturus marmoratus*)) had high trophic positions consistent with a predatory diet. Crayfish showed a high level of trophic variability, but their trophic niche never overlapped with the trophic niche of amphibians. Amphibian niche size and position were associated with amphibian density and pond canopy cover rather than with crayfish presence or abundance. This study suggests limited changes in amphibian diets in the presence of red swamp crayfish in ponds compared with results from experimental studies, suggesting that complex environmental conditions and the long time since invasion might limit trophic interactions between these native and invasive species.

**Key words:** Biological invasion, environmental variable, newt, ontogenetic shift, pond, stable isotope analysis, tadpole



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## Introduction

Biological invasions represent a major threat to biodiversity because they induce considerable impacts on native species in recipient ecosystems (Elton 1958; Shea and Chesson 2002; Ricciardi et al. 2013), often measured as changes in abundance or distribution of native species (Nentwig 2008; Davis 2009; Simberloff et al. 2013). Analyses of realised ecological niches, and trophic niches in particular, contribute to identifying proximate responses of native species that may be associated with changes in population dynamics during biological invasions (ecological displacement *sensu* David et al. (2017)). Alterations of the trophic niches of native species have been evidenced in empirical studies in the form of diet shifts

in native species in invaded ecosystems (Vander Zanden et al. 1999; Nilsson et al. 2012; Jackson et al. 2016). Successful invasive species often establish several types of relationships with native species (e.g. predation and competition) and analyses of stable isotope niches, as proxies of trophic niches (Newsome et al. 2007), are integrative of the additive consequences that these relationships may have on the diet of native species (David et al. 2017). In addition, natural variation in resource levels affects patterns of realised trophic niches in both native and invasive species (Evangelista et al. 2014; Carreira et al. 2017; McCue et al. 2020). Quantifying changes in stable isotope niches of native species in multiple ecosystems is, thus, expected to help gain insights into the dietary responses of native species interacting with invasive species in ecosystems (Strauss et al. 2006; David et al. 2017).

The red swamp crayfish (*Procambarus clarkii*) is one of the most widespread and harmful aquatic invasive species worldwide, notably because it modifies habitats (e.g. destruction of aquatic vegetation) and is an omnivorous species that opportunistically feeds on aquatic plants, detritus, invertebrates, fish, amphibian eggs and larvae (Correia 2003; Alcorlo et al. 2004; Twardochleb et al. 2013). There is a general agreement that the red swamp crayfish is responsible for major impacts on amphibian populations in multiple ways, with predation being at the top of the list (Beebee and Griffiths 2005; Cruz et al. 2006a; Ficetola et al. 2011). With its broad diet, this crayfish may also compete with both herbivorous-omnivorous tadpoles (Montaña et al. 2019) and predatory newts (Sánchez-Hernández 2020). Predation, competition and non-trophic interactions with the red swamp crayfish, including habitat modification and antagonistic interactions (Gamradt et al. 1997; Rebelo and Cruz 2005; Arribas et al. 2014), may all induce dietary changes in native amphibians. Two mesocosm studies found evidence for diet changes in amphibians in sympatry with red swamp crayfish using stable isotope analyses (SIA), in the form of increased trophic positions and decreased  $\delta^{13}\text{C}$  values (Caut et al. 2013; Arribas et al. 2015). However, knowledge on the dietary response of amphibians to the red swamp crayfish in natural environments is limited.

In the present study, we quantified the variation in stable isotope niches of four native amphibians using carbon and nitrogen isotopes in 18 ponds of a region that has been colonised by the invasive red swamp crayfish for three decades. The stable isotope niches of these amphibians - tadpoles of agile frogs (*Rana dalmatina*) and of European tree frogs (*Hyla arborea*), adult palmate newts (*Lissotriton helveticus*) and adult marbled newts (*Triturus marmoratus*) - remain undescribed in natural ecosystems. We first described the position of their stable isotope niches relative to that of the red swamp crayfish. The invasive red swamp crayfish is expected to have intermediate trophic positions between tadpoles and newts with possible partial diet overlap due to the crayfish diet being made up of food items from all trophic positions (Alcorlo et al. 2004). The simple presence of crayfish may have dietary impacts on native amphibians if chemical cues trigger anti-predatory strategies (Nunes et al. 2013; Arribas et al. 2015) that modify foraging behaviour and the choice of foraging areas, for example. Besides, dietary impacts from competition and non-trophic interactions likely increase with crayfish abundance according to the abundance-impact hypothesis (Bradley et al. 2019). Finally, diet variation is expected to be linked with food availability in ponds. Thus, we tested if variation in size or in position of amphibians' stable isotope niches was linked to crayfish presence or abundance, to proxies of pond productivity (e.g. canopy cover, pond area and aquatic vegetation cover) and to proxies of intra- and interspecific competition (i.e. amphibian abundances).

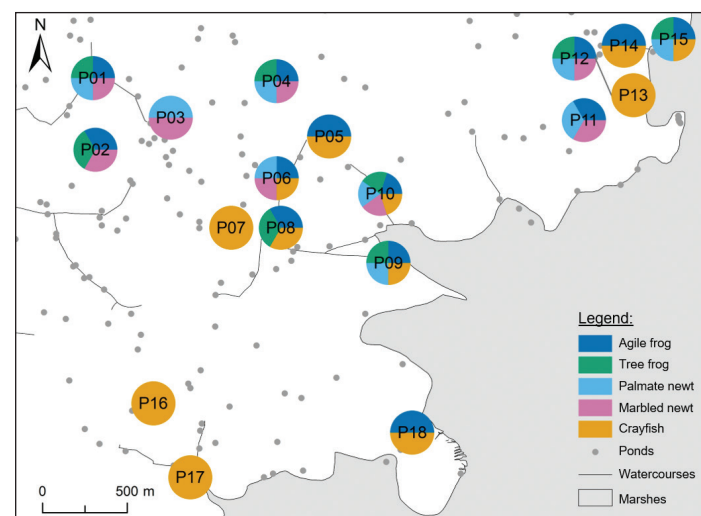
Changes in the stable isotope niche of amphibians predominantly consistent with red swamp crayfish presence or abundance rather than with other variables would indicate disproportionate effects of the invasion on the diet of native amphibians.

## Materials and methods

### Study populations and pond characteristics

The present study focused on tadpoles of agile frogs, tadpoles of European tree frogs, adult palmate newts and adult marbled newts in a large network of ponds in the Regional Natural Park of Brière (northwest France, ~ 15 ponds/km<sup>2</sup>, see Belouard et al. (2019a) for a detailed description of the study area). In ponds, herbivorous-omnivorous tadpoles develop from March to June and adult predatory newts are present from January-March to June-July (Montaña et al. 2019; Sánchez-Hernández 2020). Larval newts, which were too small to be non-lethally sampled, and adult agile frogs and tree frogs, which only spend a few days in ponds to lay eggs, were not included in the present study. The red swamp crayfish was introduced in the Brière marsh in the early 1980s. It colonised half the ponds of the study area, those at short aquatic distances from the marsh, regardless of their surface, depth, hydrology, usage, chemistry or landscape density (Tréguier et al. 2018).

The stable isotope niches of amphibians and crayfish were studied in 18 ponds (P01–P18) in May–June 2016 or 2017 depending on ponds (Fig. 1, Table 1). Two ponds initially included in our sampling scheme were not considered here, due to the lack of molluscs to standardise nitrogen values (see subsection - Treatment of stable isotope data). The pond selection was based on the possibility to trap multiple specimens of the species of interest (primary consumers for stable isotope data standardisation, amphibians and/or crayfish). For reference, amongst the 157 ponds of the study area sampled in May 2012 (Tréguier et al. 2018), only 26 (17%) yielded at least 10 crayfish. Therefore, a limited fraction of ponds was appropriate to obtain enough specimens for SIA. The selection of 18 ponds was not intended to be representative of the co-existence rates between species, a topic addressed elsewhere (Belouard et al. 2019a). The ponds under study include natu-



**Figure 1.** Map of the area and study ponds (numbered geographically). The spatial distribution and assemblages of amphibian and crayfish populations used in SIA are represented for each study pond.

**Table 1.** Environmental characteristics and abundances of target taxa in the 18 ponds studied. The number of samples processed in SIA are reported. In a few cases, no individuals were trapped during standardised trapping sessions due to low densities, but we captured them during additional trapping and used them for SIA. Populations (numbers) with an asterisk were not used for the calculation of population niche metrics due to low sample sizes (see text for more details).

Pond	Year of sampling	Habitat descriptors								Number of samples processed in SIA				
		Area (m <sup>2</sup> )	Canopy cover (%)	Aquatic vegetation cover (%)	Crayfish abundance (ind/trap/12h)	Agile frog (ind/m <sup>2</sup> )	Tree frog (ind/m <sup>2</sup> )	Palmate newt (ind/m <sup>2</sup> )	Marbled newt abundance (ind/trap/12h)	Crayfish (juveniles/adults)	Agile frog tadpoles	Tree frog tadpoles	Palmate newt	Marbled newt
P01	2017	143	0	90	0	12	30	8	0.58	0	20	19	20	19
P02	2016	536	10	100	0	2	2	0	0.55	0	9*	19	13	19
P03	2016	166	0	90	0	0	0	7	0.7	0	20	15	20	16
P04	2017	69	10	95	0	48	95	3	0.63	0	20	20	19	7
P05	2017	1079	10	0	0.8	2	0	0	0	11/20	20	0	0	0
P06	2016	525	75	0	0.11	3	0	2	0.44	1/3*	10	0	19	20
P07	2017	460	70	0	1.26	0	0	0	0	9/19	15	0	1*	0
P08	2017	34	30	1	1.5	8	5	0	0	26/10	20	0	20	2*
P09	2016	144	30	30	0.22	10	106	1	0	5/20	19	20	12	0
P10	2016	179	30	80	1.1	4	3	1	0.2	12/18	20	0	9	6*
P11	2017	300	15	10	0	1	0	4	0.5	0	20	20	20	9
P12	2017	105	70	80	0	13	23	5	0.43	0	20	20	20	11
P13	2016	146	25	0	1.78	0	0	0	0	10/20	0	0	1*	0
P14	2017	90	25	5	1.29	22	0	0	0	20/20	20	20	1*	0
P15	2016	138	50	70	0.44	8	11	3	0	17/20	20	14	20	0
P16	2017	780	50	0	0.78	0	0	0	0	2/20	10	0	1*	0
P17	2016	34	90	0	1.07	0	0	0	0	6/19	8*	0	4*	0
P18	2017	278	75	0	1.77	1	0	0	0	23/20	0	0	0	0

ral variation in amphibian and crayfish abundances and environmental conditions across the study area (see subsection - Sample collection and Table 1).

Given that ponds harbour a diversity of food resources that is difficult to sample exhaustively, we used three pond characteristics as proxies for food resource availability: (1) canopy cover (0–90% of forested shoreline, estimated as the proportion of shoreline with trees by a single fieldworker; Table 1) known to negatively influence pond productivity (Skelly et al. 2002; Schiesari 2006); (2) pond area (34–1079 m<sup>2</sup>, BD ORTHO map) as an indirect proxy of the hydrology and stability of ponds and of the resulting composition of resident communities (Post et al. 2000; Williams 2006, Table 1); and (3) aquatic vegetation cover (floating and submerged macrophytes, 0–100%, visual estimation during fieldwork; Table 1) playing a significant role in food availability (Scheffer 1998; Williams 2006; Oertli and Frossard 2013).

### Sample collection

In each pond, sampling occurred when tadpoles had grown hind limbs (Gosner stage > 38, Gosner (1960)), to ensure that their stable isotope values reflected pond resources and not their maternally-inherited terrestrial stable isotope values

(Trakimas et al. 2011; Caut et al. 2013). We assumed that, at this time, adult newts had also assimilated the stable isotope values of resources they consumed in ponds.

Amphibians and crayfish were sampled using pipe exhaustion sampling or unbaited wire minnow traps, depending on their body size. Pipe sampling consists in quickly plunging a 0.25-m<sup>2</sup> hollow cylinder through the water column into the sediments, thus providing a closed unit to sample swimming animals (see Skelly and Richardson (2010)). Amphibians were caught in the pipe with a dip net of 4-mm mesh until depletion. Four pipe samples were spaced on the banks of each pond to estimate tadpoles and adult palmate newt densities (individuals/m<sup>2</sup>) and to sample specimens in diverse microhabitats. Larger species (adult marbled newts and red swamp crayfish) were better caught using a 12-h trapping session conducted overnight (trap characteristics: length × width × height: 50 × 29 × 21 cm, 5.5-mm mesh size with two side entrances, 4-cm inner opening diameter). Traps were set roughly every 5 m along the shoreline, with a maximum of 20 traps per pond (mean pond perimeter: 66 m, range: 22–172 m). Abundances were expressed in catch-per-unit-effort (CPUE, mean number of individuals/trap/12h). The crayfish abundances recorded were lower than in previous years in these ponds, but similar to those recorded in most other ponds of the region that year (Belouard et al. (2019a, 2019c) for crayfish abundances in 2012–2016). In each pond, we aimed to sample 20 individuals of each target species to capture intra-specific variation in stable isotope values. Additional trapping was, therefore, conducted when necessary. In a few cases, we did not reach this objective (Table 1) as amphibian populations are sometimes low in individual ponds. Ontogenetic dietary changes have been demonstrated in red swamp crayfish (Correia 2003; Paillisson et al. 2011; Alcorlo and Baltanás 2013), so we aimed at sampling 20 juveniles and 20 adults per pond to confirm the ontogenetic diet shift and better capture the extent of the trophic space of crayfish populations. Overall, red swamp crayfish were found in 12 of 18 ponds and amphibians in 9 to 16 ponds depending on species (Table 1). The fin of amphibians was sampled using a biopsy punch (one to two 2.5- or 3-mm-diameter samples per amphibian, see details in Belouard et al. (2019b)) and individuals were immediately released. Crayfish were euthanised by freezing during the field campaign.

Primary consumers were collected to standardise isotope values between ponds. Macroinvertebrates (*Physa acuta*, *Corbiculidae* spp., *Gammarus gammarus*, *Asellus aquaticus*, *Corixidae* spp.) were collected during pipe sampling and three zooplankton samples were obtained by filtering 50 l of water each. Two to five taxa were found per pond and up to three samples were processed per taxa per pond for a total of 205 samples (Suppl. material 1: table S1). Each macroinvertebrate sample was made of an average of  $7 \pm 3$  SD individuals. All samples were kept on ice during field collection and frozen at -20 °C until further processing.

### Sample preparation and stable isotope analysis

Crayfish were measured from the tip of the rostrum to the end of the telson to the nearest millimetre before sampling the abdominal muscle. Length-frequency histograms showed a bimodal distribution with juveniles (mean total length of  $40.1 \text{ mm} \pm 8.6 \text{ SD}$ , range: 19–60 mm) and adults ( $92.0 \text{ mm} \pm 9.2 \text{ SD}$ , range: 75–122 mm). Other macroinvertebrates were processed whole, molluscs without shells. All samples were rinsed with deionised water, then freeze-dried for 48 hours.



They were ground to homogenise tissues, except amphibian fins and zooplankton due to the small amount of material available. Samples were packed in tin capsules, with  $378 \pm 57 \mu\text{g}$  per sample for amphibians,  $1,012 \pm 64 \mu\text{g}$  for crayfish,  $925 \pm 230 \mu\text{g}$  for other macroinvertebrates. Low-mass samples for amphibians (0.1–1 mg) comply with EU ethical regulations in vertebrates while providing accurate measures for SIA due to their high nitrogen content (see Belouard et al. (2019b), Cornell Stable Isotope Laboratory communication). Carbon and nitrogen stable isotope ratios ( $\delta^{13}\text{C}$  as  $^{13}\text{C}/^{12}\text{C}$  and  $\delta^{15}\text{N}$  as  $^{15}\text{N}/^{14}\text{N}$ ) were measured at the Cornell Stable Isotope Laboratory (Cornell University, Ithaca, NY). Stable isotope ratios were expressed using conventional delta notations  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  relative to international standards, Vienna Pee Dee Belemnite for carbon and atmospheric air for nitrogen. In-house standards resulted in a measurement precision (SD) of 0.14‰ for carbon and 0.07‰ for nitrogen.

### Treatment of stable isotope data

$\delta^{13}\text{C}$  values were lipid-corrected following the equation of Post et al. (2007):

$$\delta^{13}\text{C} = \delta^{13}\text{C}_{\text{untreated}} - 3.32 + 0.99 \times \text{C:N},$$

where  $\delta^{13}\text{C}$  and  $\delta^{13}\text{C}_{\text{untreated}}$  are the lipid-corrected and raw  $\delta^{13}\text{C}$  values of the sample, respectively, and C:N is the carbon-to-nitrogen ratio of the sample.

Stable isotope values of amphibian muscle were derived from the values of the fin samples following the specific mathematical equations established in Belouard et al. (2019b). Corrected  $\delta^{13}\text{C}$  ( $\delta^{13}\text{C}_{\text{cor}}$ ) values between ponds were calculated using the following equation:

$$\delta^{13}\text{C}_{\text{cor}} = (\delta^{13}\text{C}_c - \delta^{13}\text{C}_{\text{C1}}) / (\text{CR}_{\text{C1}}),$$

where  $\delta^{13}\text{C}_c$  is the  $\delta^{13}\text{C}$  value of the sample of interest (crayfish or amphibian),  $\delta^{13}\text{C}_{\text{C1}}$  and  $\text{CR}_{\text{C1}}$  are the mean and range of the mean  $\delta^{13}\text{C}$  values per primary consumer (C1) available in the pond considered (Suppl. material 1: table S1; *Physa acuta*, *Corbiculidae* spp., *Gammarus gammarus*, *Asellus aquaticus*, *Corixidae* spp. and zooplankton; following Olsson et al. (2009)). All C1 were used in this equation because the standardisation of  $\delta^{13}\text{C}$  values consists of including the entire variability of communities. In addition, there is a strong littoral influence on the entirety of pond ecosystems due to their small size (see, for example, Benetti et al. (2014)).

Individual trophic positions (TP) were calculated using the following equation:

$$\text{TP} = \lambda + (\delta^{15}\text{N}_c - \delta^{15}\text{N}_m) / \Delta^{15}\text{N},$$

where  $\lambda = 2$  is the trophic position attributed to the primary consumers present in all study ponds (here, molluscs *Physa acuta* and *Corbiculidae* spp.),  $\delta^{15}\text{N}_c$  is the  $\delta^{15}\text{N}$  value of the sample of interest (crayfish or amphibian),  $\delta^{15}\text{N}_m$  is the mean of the mean  $\delta^{15}\text{N}$  values of each mollusc species available in the pond considered and  $\Delta^{15}\text{N}$  the trophic discrimination factor (set to 3.4, following Olsson et al. (2009)). Only molluscs were considered amongst C1, because molluscs are long-

lived, stable, exclusive herbivores and are, therefore, the most reliable consumers to establish the baseline of the trophic position (Post 2002).

Stable isotope niche metrics were calculated for populations in which more than 10 individuals were sampled (following recommendations in Jackson et al. (2011)). For three newt populations, we sampled only 7–9 individuals (Table 1) despite repeated trapping. High rates of recapture observed thanks to the mark left by the biopsy attested that virtually all the population had been sampled. Therefore, we calculated niche metrics for these populations as well, but made sure that their inclusion did not change trends in the model results. Niche metrics were calculated in a total of 41 amphibian populations (in 15 ponds) and 11 crayfish populations (Table 1). Standard ellipse areas corrected for small sample sizes ( $SEA_c$ ) were calculated as a measure of the stable isotope niche size for each population using the SIBER R package (Jackson et al. 2011). The average position of the niche was calculated as the average TP and the average  $\delta^{13}C_{cor}$  per population. The average position of the niche is based on all individuals, while standard ellipses contain ~ 40% of the individuals in the stable isotope space (Jackson et al. 2011). The degree of stable isotope niche overlap between two species (percentage of species 1's standard ellipse that is overlapped by species 2) was calculated as the ratio between the area of overlap of the two standard ellipses and species 1's ellipse area.

## Statistical analyses

The existence of an ontogenetic shift in resource use in crayfish was tested in each pond (except P06 that had only four crayfish sampled) using linear models. For this specific issue, either crayfish TP or  $\delta^{13}C_{cor}$  were used as independent variables and crayfish length as the explanatory variable.

In addition, we tested if niche metrics ( $SEA_c$ , TP and  $\delta^{13}C_{cor}$ ) of amphibians were associated with crayfish presence or abundance, amphibian abundances or proxies of pond productivity (pond area, canopy cover, aquatic vegetation cover) using Linear Mixed Models with pond identity as a random factor (R package lme4; Bates et al. (2015)). Tadpoles and newts may show qualitatively different variation in niche metrics due to their different diets, so we also included group (tadpoles or newts) as a candidate predictor.  $SEA_c$  was log-transformed to meet assumptions of normality of the model residuals. We computed models with combinations of up to two variables (scaled), including a null model, to avoid overfitting. When considering crayfish, we tested either presence/absence or abundance. Marbled newt and crayfish abundance were highly negatively correlated in this dataset (Spearman's rank correlation  $\rho < -0.8$ ). As this correlation may not be causal (Belouard et al. 2019a), we kept both variables, but they were not included in the same models. Spearman's rank correlations were lower than  $|0.8|$  for all other pairs of variables.

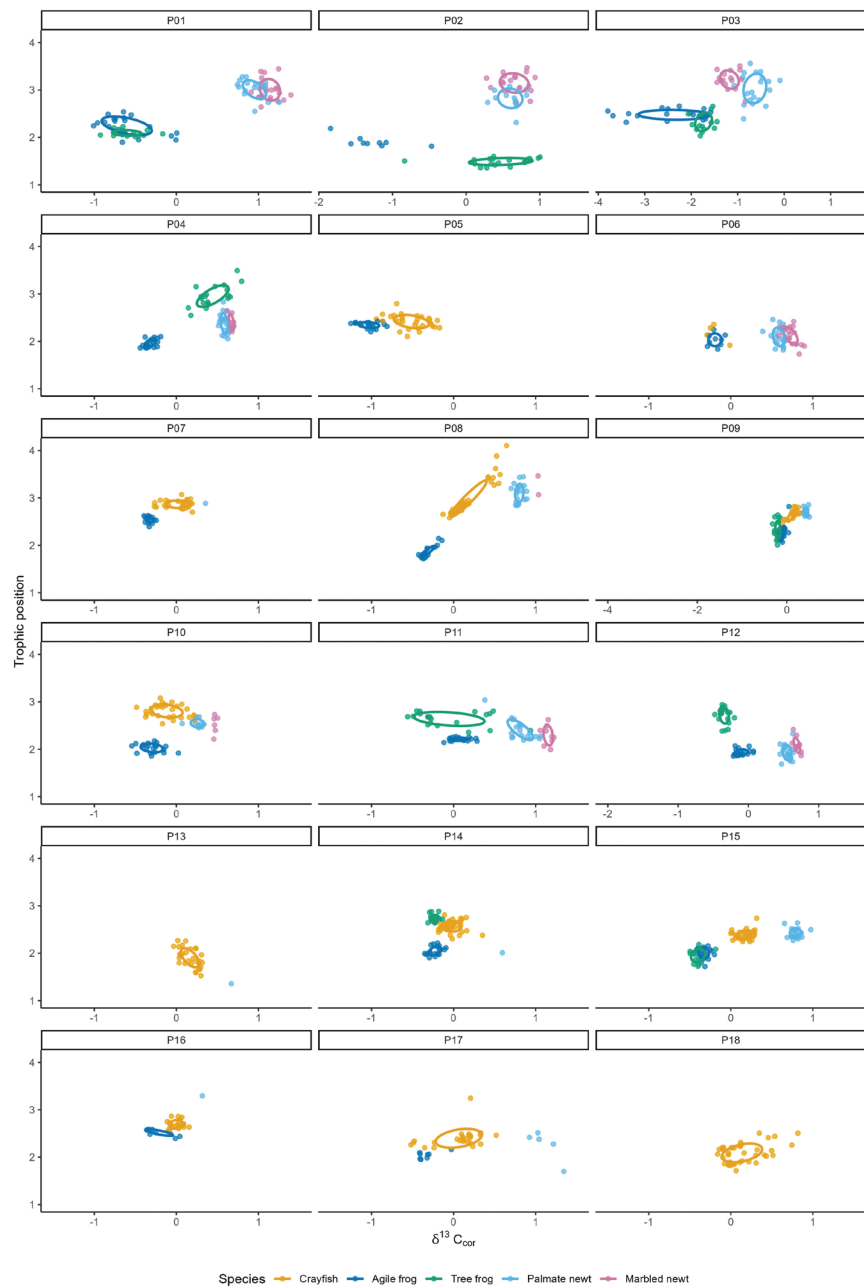
Models were ranked by the Akaike Information Criterion, corrected for small sample sizes (AICc; Burnham and Anderson (2003)). All models within  $\Delta AICc < 2$  of the best model were kept, as they are equivalent in their probability of best explaining the data (Burnham and Anderson 2003). The ranking of the first model including crayfish presence or abundance was noted, as well as the ranking of the null model, for comparison. Statistical analyses were done in R version 4.0.3 (R Core Team 2023).



## Results

### Amphibian and crayfish relative isotope positions

The trophic niche of newts and tadpoles strongly differ on both the carbon and nitrogen stable isotope axes (Fig. 2). Populations of agile frog tadpoles, marbled newts and palmate newts displayed relatively consistent trophic niche between ponds. Both newts consistently showed enriched  $\delta^{13}\text{C}_{\text{cor}}$  compared with agile frog tadpoles (see niche metrics in Suppl. material 1: table S2). Both newts also showed higher TP values (1.93–3.14) than agile frog tadpoles (1.86–2.54), except in a few



**Figure 2.** Stable isotope values of individuals with the associated standard ellipses, where sample size  $\geq 10$ . Note that P09 and P12 have a different x-scale compared to the other ponds due to larger variation in  $\delta^{13}\text{C}_{\text{cor}}$  values.

ponds where the overall range of TP was very small (P06, P11, P17). Overall, marbled newts had higher  $\delta^{13}\text{C}_{\text{cor}}$  than palmate newts, except in P03, and similar TP, except in P02. By contrast, the position of tree frog tadpoles varied greatly, from low (P01, P11, P12, P14, P15) to high  $\delta^{13}\text{C}_{\text{cor}}$  (P02, P04) as well as low (1.49, P01–P03, P15) to high TP (2.95, P04, P11, P12, P14). Niche size varied by a factor of 20 across ponds, with  $\text{SEA}_c$  ranging from  $\sim 0.01$  to  $\sim 0.20$  for each species (0.12 maximum in the marbled newt, Suppl. material 1: table S2).  $\text{SEA}_c$  was correlated between marbled and palmate newts (Spearman's rank correlation  $\rho = 0.93$ ,  $p < 0.01$ ), but not between agile and tree frog tadpoles ( $\rho = -0.29$ ,  $p > 0.05$ ). In a few cases, an overlap was observed between the trophic niche of the two tadpole species (8 to 59% overlap in 3/8 ponds, Suppl. material 1: table S3) and between the two newt species (8 to 15% overlap in 2/7 ponds, Suppl. material 1: table S3).

On the carbon stable isotope axis, crayfish occupied a central position between tadpoles and newts (Fig. 2), except in P06 where the four crayfish specimens had comparably low values as tadpoles of agile frogs. The crayfish displayed a highly variable TP: as low as tadpoles in P05 (2.41), but most often higher and similar to newts (2.39–2.98) in P06–P09, P15 and P17 and at least some individuals higher than newts in P08, P10, P14 and P17 (2.39–2.98). The crayfish niche never overlapped with the amphibian niches (Suppl. material 1: table S3). Crayfish niche size varied by a factor of 10, ranging from 0.02 to 0.16 between ponds (Suppl. material 1: table S2) and was not correlated with tadpoles' or palmate newts'  $\text{SEA}_c$  (Spearman's rank correlation tests, all  $p > 0.05$ ).

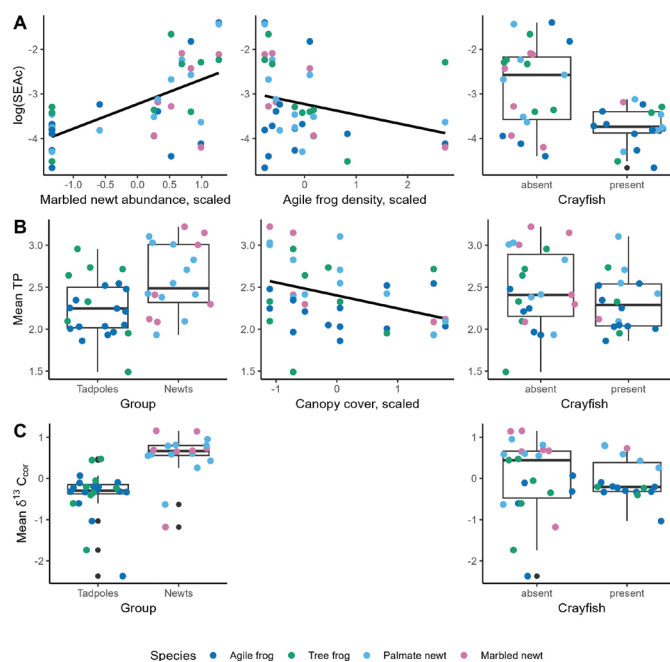
Crayfish  $\delta^{13}\text{C}_{\text{cor}}$  increased with crayfish length in all ponds ( $p < 0.05$ , Suppl. material 1: fig. S1, table S5), except in P10 and P16 where it decreased with crayfish length ( $p < 0.05$ ) and there was no effect of crayfish length in P14. Crayfish TP increased with crayfish length in P08–P10, P14, P18 ( $p < 0.05$ , Suppl. material 1: fig. S2, table S5), decreased with crayfish length in P05 and P13 ( $p < 0.05$ ) and there was no effect of crayfish length in P07 and P15–P17.

### Drivers of amphibian niche metrics

Variation in the niche size and niche position of amphibians was best explained by amphibian abundances and pond productivity proxies (Table 2, Fig. 3). Model selections kept two models for  $\text{SEA}_c$  and TP and a single top model for  $\delta^{13}\text{C}_{\text{cor}}$ . The best models each contained one to two variables and each model explained 20 to 84% of variance (Table 2). The best model including crayfish presence or abundance was ranked 3<sup>rd</sup> to 10<sup>th</sup> depending on niche metrics, with consistently high  $\Delta\text{AICc}$  ( $> 3.3$ ) and low likelihood of being the best model ( $w\text{AICc} < 8\%$ ). Results were qualitatively similar when tree frog populations, that had a variable position in the stable isotope space, were excluded from the analyses (Suppl. material 1: table S4). Amphibian  $\text{SEA}_c$  were larger in ponds with higher marbled newt abundance (averaged estimate: 0.58) and lower agile frog density (Table 2, Fig. 3A). TP decreased with canopy cover (average estimate: 0.35) and was influenced by the amphibian taxa, with newts having higher TP than tadpoles (Fig. 3B, Table 2).  $\delta^{13}\text{C}_{\text{cor}}$  was influenced only by the amphibian taxa, with newts having higher  $\delta^{13}\text{C}_{\text{cor}}$  than tadpoles (Fig. 3C, Table 2).

**Table 2.** Selection of models with  $\Delta AICc < 2$  explaining the variation in niche metrics of amphibian populations (in bold font). All models included pond identity as a random factor. For  $\delta^{13}C_{cor}$ , no additional model was within  $\Delta AICc < 2$  of the top model. Best-ranked model including crayfish presence/absence or abundance and null models added for comparison of AIC criteria. Estimates  $\pm$  SE are provided for each fixed variable. Variance explained (marginal and conditional  $R^2$ ), number of parameters (K) and AIC selection criteria are provided for each model.

Variable	Model rank	Intercept	Agile frog density	Marbled newt abundance	Canopy cover	Crayfish presence	Crayfish abundance	Group	Marginal $R^2$	Conditional $R^2$	K	AICc	$\Delta AICc$	wAICc
log(SEAc)	1	<b>-3.23 <math>\pm</math> 0.11</b>	<b>-0.34 <math>\pm</math> 0.11</b>	<b>0.61 <math>\pm</math> 0.11</b>					<b>0.52</b>	<b>0.56</b>	4	<b>93.6</b>	<b>0</b>	<b>0.35</b>
	2	<b>-3.22 <math>\pm</math> 0.13</b>		<b>0.56 <math>\pm</math> 0.13</b>					<b>0.39</b>	<b>0.56</b>	3	<b>95.3</b>	<b>1.7</b>	<b>0.15</b>
	4	-2.76 $\pm$ 0.20	-0.34 $\pm$ 0.15			-1.10 $\pm$ 0.28			0.41	0.57	4	97.9	4.3	0.04
	10	-3.22 $\pm$ 0.14			-0.35 $\pm$ 0.15		-0.38 $\pm$ 0.13		0.39	0.57	4	99.6	6.0	0.02
	29	-3.35 $\pm$ 0.20							0	0.53	2	102.9	9.3	< 0.01
TP	1	<b>2.59 <math>\pm</math> 0.08</b>			<b>-0.15 <math>\pm</math> 0.06</b>			<b>-0.34 <math>\pm</math> 0.11</b>	<b>0.30</b>	<b>0.30</b>	4	<b>49.2</b>	<b>0</b>	<b>0.36</b>
	2	<b>2.60 <math>\pm</math> 0.09</b>						<b>-0.35 <math>\pm</math> 0.12</b>	<b>0.18</b>	<b>0.20</b>	4	<b>49.5</b>	<b>0.3</b>	<b>0.31</b>
	3	2.40 $\pm$ 0.06							0	0	2	52.6	3.4	0.06
	5	2.63 $\pm$ 0.10				-0.09 $\pm$ 0.12		-0.33 $\pm$ 0.12	0.19	0.20	4	53.9	4.7	0.03
	7	2.61 $\pm$ 0.09					0.03 $\pm$ 0.06	-0.37 $\pm$ 0.12	0.18	0.22	4	55.6	6.4	0.01
$\delta^{13}C_{cor}$	1	<b>0.53 <math>\pm</math> 0.14</b>						<b>-0.91 <math>\pm</math> 0.10</b>	<b>0.41</b>	<b>0.84</b>	3	<b>56.8</b>	<b>0</b>	<b>0.44</b>
	3	0.49 $\pm$ 0.21				0.07 $\pm$ 0.28		-0.91 $\pm$ 0.10	0.39	0.84	4	60.1	3.3	0.08
	9	0.52 $\pm$ 0.15					0.02 $\pm$ 0.12	-0.91 $\pm$ 0.10	0.39	0.84	4	61.8	4.9	0.04
	11	0.01 $\pm$ 0.15							0	0.38	2	93.9	37.1	< 0.01



**Figure 3.** Variables best explaining amphibian niche metrics according to the model selection. X-axis scaled and with y-x models traced for quantitative variables, otherwise boxplots **A** niche size **B** and **C** niche position. Boxplots with crayfish presence/absence added for comparison.

## Discussion

Studying trophic niches is a valuable way to quantify the possible consequences of invasive species on native species. We provided unique results on the stable isotope niches of four native amphibians and factors explaining their variation in ponds. A key finding is that no niche overlap occurred between amphibians and the invasive red swamp crayfish and variation in amphibian niche size and position was associated with amphibian density and a proxy of pond productivity.

The position and segregation of the stable isotope niches of tadpoles and newts were, in most cases, consistent with their documented feeding habits: low TP for herbivorous-omnivorous tadpoles of agile frogs (reviewed in Montaña et al. (2019)) and higher TP for predatory newts (Sánchez-Hernández (2020) and references therein). Tree frog tadpoles showed more inconsistent relative positions, sometimes with high trophic positions. It suggests that tree frog tadpoles may be more opportunistic feeders than agile frogs and they may occasionally prey on small macroinvertebrates (e.g. Chironomids larvae) or forage on carcasses, concordant with a mesocosm study showing a high level of omnivory in tree frog tadpoles (Carreira et al. 2016). The partial niche segregation between newts and between tadpoles likely promotes co-existence between species from the same trophic level (Chesson 2000).

The variation in stable isotope niche metrics of the red swamp crayfish reflects its opportunistic feeding behaviour. Trophic flexibility is typically one of the ecological traits that makes invasive species successful and possibly harmful, because it makes them likely to thrive in diverse environments, be resilient to changes in food availability and compete with many co-occurring species (Davidson et al. (2011) on phenotypic plasticity; Rader et al. 2017). In addition, ontogenetic dietary shifts in red swamp crayfish diet were confirmed here in most ponds. We showed shifts in  $\delta^{13}\text{C}_{\text{cor}}$  values showing differences in food sources between juveniles and adults. In

some ponds, we showed an increase in crayfish trophic position compatible with a more predatory diet at the adult stage, even though some opposed trends were also found in other ponds (see also standard ellipses separated for adult and juvenile crayfish in Suppl. material 1: fig. S3). The flexibility in the trophic ecology of the red swamp crayfish indicates that crayfish, specifically predatory adults, have the potential to impact amphibians through predation and competition for some food resources (Beebee and Griffiths 2005; Twardochleb et al. 2013). Testing whether the age structure of crayfish populations influences the severity of crayfish impacts would be an interesting avenue to explore using, for instance, experimental studies.

Our findings are noteworthy given the rarity of stable isotope studies on amphibians in natural ecosystems, as well as for investigating the potential effect of red swamp crayfish on diets of our study species. We showed that the stable isotope niche of the red swamp crayfish never overlapped with the stable isotope niche of amphibians. This trophic partition may be constitutive, i.e. species never competed for food resources, or induced, i.e. individuals changed their diet to limit competition for a shared resource. Although determining trophic relationships is out of the scope of this work and requires more investigations, predation of crayfish on tadpoles can be suspected in most ponds, while predation is unlikely between adult newts and crayfish, as they occupy similar trophic positions. In cases of intraguild predation (Vieira Marques et al. 2018), such as crayfish feeding on larval newts, or reciprocal intraguild predation if newts also feed on juvenile crayfish, spatial segregation of species in the water column and, therefore, the use of different food resources, is believed to limit the exposure of amphibians to crayfish and vice versa (Belouard et al. 2019a). The inverse question of whether the crayfish niche changes in the presence of amphibians cannot be addressed with our sampling design ( $n = 2$  ponds without amphibians).

Although these potential relationships between the invasive red swamp crayfish and native amphibians could cause dietary changes in amphibians, the stable isotope niches of amphibians were not dictated by the red swamp crayfish. Indeed, amphibian densities and pond canopy cover were better explanatory variables for the variation in all amphibian niche metrics than crayfish. Our result is inconsistent with mesocosm studies that documented significant effects of crayfish on food availability for tadpoles (e.g. Geiger et al. (2005); Rodríguez-Pérez et al. (2016)), causing a diet shift towards resources depleted in  $\delta^{13}\text{C}$  in mesocosms (Arribas et al. 2015). The lack of support for a direct link between the crayfish invasion and amphibian niche metrics that we found could stem from the relatively low crayfish abundances in the ponds studied. As a result, the exposure of amphibians to crayfish may not be high enough to trigger major dietary changes in native amphibians. The difference in findings between other studies and the present study could also come from the simplified environmental conditions and shorter time since introduction in mesocosms compared with natural ecosystems.

First, resource availability likely shapes species realised trophic niches in natural conditions, as shown by the negative effects of canopy cover or amphibian abundances on all niche metrics, a complexity that can only partially be incorporated in experimental studies. Additional investigations are needed to better understand the observed relationships between amphibian niche metrics and a proxy of pond productivity, as well as the effects of intra- and interspecific competition.

Second, observations in natural ecosystems occur in a time window that is rarely equivalent to what can be achieved in experimental studies. As impacts of invasive

species often decrease with time since invasion, mesocosm studies likely unveil exacerbated effects in comparison with some observational studies (Lodge et al. 1998; Strayer et al. 2006; Závorka et al. 2018). Our observational study considers a 35-year-old invasion, which may explain why our results imply milder consequences of the invasion compared to experimental settings. The long-term perspective offered by our study is, indeed, likely the product of changes in habitat and resource use in space and time. For example, at the population scale, behavioural changes in the form of anti-predatory strategies have been observed in a diversity of tadpole species in response to the presence of the red swamp crayfish using laboratory experiments (Nunes et al. 2013; Polo-Cavia and Gomez-Mestre 2014). At the metapopulation scale, immigration from adjacent subpopulations may have rescued declining amphibian populations during the early stages of the invasion, providing time for such changes to take place, especially as crayfish populations themselves likely experienced a decrease in abundances over time (Belouard et al. 2019c).

## Conclusion

Proximate consequences of biological invasions may manifest as dietary changes in native species. Such investigation can be viewed as complementary with analyses of large-scale, ultimate impacts of biological invasions, such as population declines or even extinctions. Overall, our study is a first step towards documenting the proximate effects of the red swamp crayfish on amphibian trophic niches, a topic that would require more investigations. Comparing ponds that are similar in canopy cover and amphibian densities and that either lack or harbour crayfish in a paired sampling design are tasks for a future study. Further challenges for understanding and predicting the fate of invasions in different environmental settings include measuring low-density growth rates (*sensu* Chesson 2000) and combining species niches, fitness and species relative dispersal abilities within the metacommunity framework (Schlägel et al. 2020; Thompson et al. 2020).

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## Additional information

### Conflict of interest

The authors have declared that no competing interests exist.

### Ethical statement

Red swamp crayfish and amphibians were collected under the permits n°07/2016, 12/2017, 2016/SEE-Biodiversité/070, 2017/SEE-Biodiversité/1145 approved by the Préfecture de Loire-Atlantique. Fin biopsies of amphibians were conducted in accordance with ethics on animal welfare and permit n°APAFIS#3125-20152071140177v2. All applicable institutional and/or national guidelines for the care and use of animals were followed.



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## Author contributions

N.B. Conceptualisation, Writing – original draft, Writing – review and editing, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualisation. E.P. Conceptualisation, Writing – review and editing, Investigation, Methodology, Supervision, Validation. J.C. Conceptualisation, Writing – review and editing, Investigation, Methodology, Supervision, Validation. J.M.P. Conceptualisation, Writing – review and editing, Funding acquisition, Investigation, Methodology, Resources, Supervision, Validation.

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## Data availability

Isotope data for the species of interest and taxa used in the baseline calculation are available as Suppl. material 2. R scripts to reproduce the analysis and figures are available on GitHub: <https://github.com/nbelouard/AmphibianIsotopicNiches>.

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## Supplementary material 1

### Detailed sample sizes and stable isotope niche metrics and detailed description of the ontogenetic shift in the stable isotope values in the red swamp crayfish

Authors: Nadège Belouard, Eric J. Petit, Julien Cucherousset, Jean-Marc Paillisson

Data type: docx

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## Supplementary material 2

### Stable isotope data

Authors: Nadège Belouard, Eric J. Petit, Julien Cucherousset, Jean-Marc Paillisson

Data type: csv

Explanation note: Dataset used for the analyses done in the manuscript.

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