



Seasonal changes in trophic ecology of co-occurring freshwater invasive species at a thermal locality

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Abstract We investigated the trophic ecology of three non-native crayfish species (the marbled crayfish *Procambarus virginalis*, the red swamp crayfish *Procambarus clarkii* and the spiny-cheek crayfish *Faxonius limosus*) and two non-native fishes (the eastern mosquitofish *Gambusia holbrooki* and the three-spined stickleback *Gasterosteus aculeatus*)

from two sections of a thermal tributary of the Barát brook in Budapest (Hungary) over the four seasons using carbon and nitrogen stable isotope analysis. All crayfish species occupied an omnivore trophic position in almost all sections and seasons. On the other hand, both fishes had a predatory trophic position, with *G. aculeatus* occupying a higher position than *G. holbrooki*. These patterns are confirmed by the results of the mixing models, showing spatial and temporal changes in the diet composition. We also found interspecific differences in the crayfish trophic niche in both sections during summer and overlapped niches in the other occasions, while fish had always segregated niches. Crayfish

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showed interseasonal differences in the trophic niche in both sections, while fish showed seasonal niche shifts only in the upper section. Our results show a considerable plasticity in the trophic ecology of the studied non-native populations, suggesting that the temperature variability gradient has no important role in determining their trophic niche.

Keywords Alien species · Invasive species · Stable isotopes · Freshwater ecology · Competition · Predation

Introduction

The introduction of non-native species (i.e., those species transported outside their natural biogeographic range; Soto et al., 2024) is an increasing ecological issue (Seebens et al., 2017; Haubrock et al., 2023a,b), being one of the main threats to biodiversity (David et al., 2017; Ricciardi et al., 2017; Roy et al., 2024). Once established in a new area, some non-native species become invasive, spreading in the new environment and exerting ecological, economic or health impacts (Mazza & Tricarico, 2018; Henry et al., 2023; Soto et al., 2023). Non-native species can have strong negative ecological impacts on communities, by modifying their taxonomic and functional composition (Le Hen et al., 2023; Toutain et al., 2024) and altering trophic web structures (Haubrock et al., 2019; Bissattini et al., 2021). Non-native species can compete with or predate on native species (Balzani et al., 2016; Duenas et al., 2021; Ercoli et al., 2025), as well as becoming new prey for native predators (Stellati et al., 2019). As the number and distribution of non-native species increase, understanding the trophic relationships between co-occurring native and non-native, but also among non-native species is particularly important, as these contribute to

determining the success of non-native populations (Bissattini et al., 2018). For example, competition among non-native species (Balzani et al., 2020; Haubrock et al., 2020) can result in their reciprocal limitation (Aksu et al., 2025). Freshwater ecosystems are particularly susceptible to non-native species' introductions and impacts (Poulin et al., 2011; Strayer, 2010; Bernery et al., 2022; Muñoz-Mas et al., 2023).

Hungary is a hot spot of freshwater species introductions (Weiperth et al., 2020; Haraszthy 2022) for fishing or aquaculture purposes as well as for ornamental reasons (Takács et al., 2017). The high number of artificial and natural thermal waters in this country (> 600; Bélteky, 1972; Haraszthy 2022) has been exploited to release many subtropical and tropical plant and animal species, which can survive harsh winter in these conditions (Šajna et al., 2023; Lente et al., 2024; Blaha et al., 2025). This is also the case of freshwater crayfish, which have become popular in the aquarium pet trade (Bláha et al., 2022; Lipták et al., 2023) and have been irresponsibly introduced or naturally dispersed from previously occupied sites to such localities (Weiperth et al., 2019a). Indeed, three invasive species of European Union concern occur at sites with elevated temperatures in Hungary, namely the North American spiny-cheek crayfish *Faxonius limosus* (Rafinesque, 1817), the red swamp crayfish *Procambarus clarkii* (Girard, 1852) and the marbled crayfish *Procambarus virginalis* Lyko, 2017 (Lókkös et al., 2016; Szendőfi et al., 2018). Further already established species with so far restricted distributions are the Mexican dwarf crayfish *Cambarellus patzcuarensis* Villalobos, 1943, and the Florida crayfish *Procambarus alleni* (Faxon, 1884), while the population status of the Australasian red-claw crayfish *Cherax quadricarinatus* (von Martens, 1868) and several other New Guinean *Cherax* species remains uncertain (Weiperth et al., 2017, 2019a, b, 2020; Bláha et al., 2022; Patoka et al., 2025). Similarly, a multitude of non-native fish originating from North America, South America, Asia, Africa and the Ponto-Caspian region are now established in Hungarian freshwater bodies (Takács et al., 2017). Among other species, the thermal waters allowed also several tropical and subtropical ornamental fishes, such as cichlids, to thrive in some places (Takács et al., 2017; Lente et al., 2024). Therefore, these thermal streams can act as winter refugia, from where non-native species can eventually spread in the warmer seasons

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(Weiperth et al. 2019b). However, it is unknown how these peculiar thermal conditions affect the trophic relationships of the co-occurring non-native species.

Stable isotope analysis (SIA) of carbon and nitrogen is a commonly used tool to study trophic relationships and trophic niches (Boecklen et al., 2011; Shipley & Matich, 2020). SIA is based on a predictable increase in stable isotopes signatures of the consumer compared to that of its prey (Layman et al., 2012; Balzani & Haubrock, 2022). While carbon signatures provide information about the carbon source of a consumer, nitrogen signatures are a proxy for the consumer's trophic position (Layman et al., 2012). Therefore, SIA allows, among others, to quantify isotopic niches (proxying trophic niches; Newsome et al., 2007) and to assess the degree of overlap among species' isotopic niches to infer potential interspecific competition in case of limited resources (Balzani et al., 2016, 2020) or niche partitioning (Balzani et al., 2024). Moreover, SIA can be used to reconstruct the diet of a species over longer periods of time compared to dietary analysis, through the use of mixing models (Boecklen et al., 2011). These models estimate the relative proportion of each prey in the diet of a consumer (Parnell et al., 2013). Consequently, SIA has been largely applied to the study of non-native species (Vander Zanden et al., 1999), also to investigate the spatiotemporal variations in their diet and trophic niche (Balzani et al., 2021; Haubrock et al., 2021; Cicala et al., 2023; Westrelin et al., 2023).

In this study, we investigated the trophic relationships of five co-occurring non-native species: three crayfish (*F. limosus*, *P. clarkii* and *P. virginalis*) and two fishes (the eastern mosquitofish *Gambusia holbrooki* Girard, 1859 and the three-spined stickleback *Gasterosteus aculeatus* Linnaeus, 1758). Two sections of a thermal tributary representing a gradient of annual temperature variation (Fig. S1) were sampled across the four seasons. For each species, we assessed the interseasonal changes in the (1) occupied trophic position and (2) isotopic niche characteristics. Moreover, we investigated (3) the degree of interspecific niche overlap (a proxy for potential interspecific feeding competition) within crayfish and fish, and (4) the intraspecific interseasonal niche shifts. Finally, we (5) estimated the diet of each species in each season. We hypothesized that in the section with a more constant water temperature, all species would have a more similar trophic position, isotopic niche and diet

throughout the year, as we expected a similar prey availability across all seasons. On the other hand, in the section with more variable water temperatures, we expected different patterns across seasons for all species and a more variable degree of potential interspecific competition.

Materials and methods

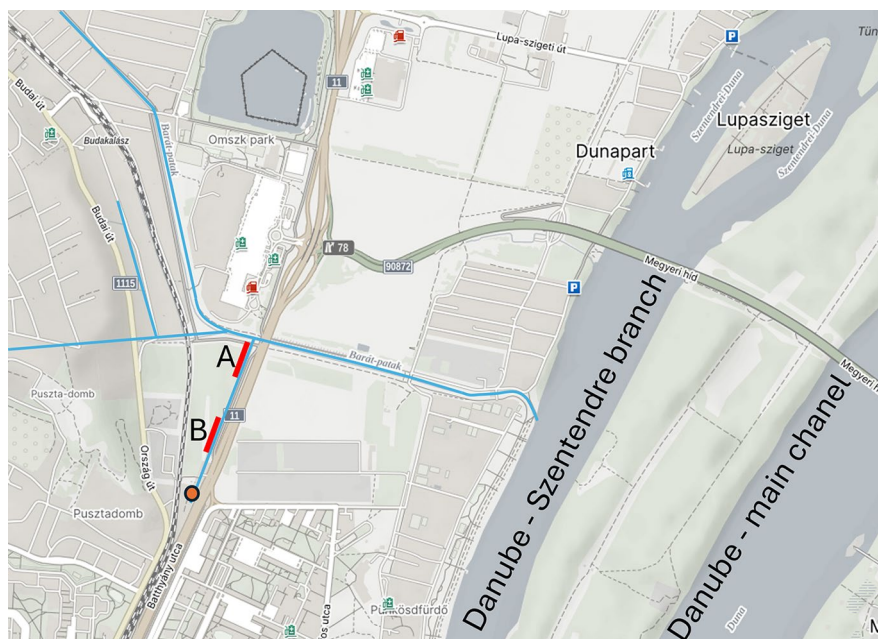
Study site and sampling

The study site (47.6091 N, 19.0618 E) is a small, narrow man-made thermal tributary of the Barát brook, situated between Budapest and Budakalász, Hungary (Fig. 1). This tributary is ~400 m long and ~1 m wide, with a maximum depth of 30 cm. It can be divided into three sections: the upper and lower sections are very similar and have a sandy–muddy bottom and few aquatic plants, while the middle section has a soft, muddy bottom often overgrown by the aquatic macrophyte branched bur-reed *Sparganium erectum* L. In all sections, the tributary banks are paved with concrete panels. In this study, we compared the upper and lower sections, both being represented by a transect of 100 m (Fig. 1). These sections represent a gradient of annual temperature variation, with the upper section having a more constant temperature throughout the year (warmer in winter with ~13 °C and relatively cool in summer with ~19 °C) and the lower section being more variable across seasons (ranging from ~6 °C in winter up to a maximum of 26 °C in summer; Fig. S1).

Sampling was performed four times, over all seasons, in January, April, July and October 2019. Fish and crayfish were collected by electrofishing from the shore (Hans Grassl IG600) or with hand nets. Zoobenthos (i.e., aquatic insects, crustaceans, annelids and mollusks) and juvenile fish and crayfish were collected using a hand net (mesh size 500 µm). Samples of macrophytes, aquatic moss, detritus and algae were also collected by hand from the shoreline and streambed.

In both sections, *P. virginalis* and *F. limosus* co-occurred, while *P. clarkii* was found only in the lower section during autumn. The most abundant crayfish was *P. virginalis*. As for fish, *G. aculeatus* and *G. holbrooki* co-occurred in both sections, although we found no adult individuals of *G. holbrooki* during

Fig. 1 Map of the study site. In red the sampling sections: A (lower section) and B (upper section). The orange point shows the thermal spring. This map was obtained from www.mapy.cz



winter in the lower section and no adult fish in the upper section in autumn.

All fish and crayfish were measured for their standard length (from the rostrum to the last scale of the caudal peduncle) and cephalothorax length (from the tip of the rostrum to the end of carapace), respectively, to the nearest 0.1 mm and weighed to the nearest 0.1 g. All samples were placed on dry ice after collection and then transferred to a laboratory freezer ($-30\text{ }^{\circ}\text{C}$) until further processing for SIA.

Stable isotope analyses

A sample of dorsal and abdominal muscle was taken for each fish and crayfish, respectively. All other samples were processed whole. All samples were dehydrated at $50\text{ }^{\circ}\text{C}$ for 48 h and ground to a fine homogeneous powder, of which approximately 0.6 mg for animal samples and 1.5 mg for plant and detritus samples were weighed into tin capsules. SIA was performed with a Carlo Erba Flash EA 1112 elemental analyzer connected to Thermo Finnigan DELTA-plus and Advantage continuous-flow isotope ratio mass spectrometer (Thermo Electron Corporation, Waltham, MA, USA). The isotope compositions were expressed in the δ notation (‰), calculated based on $\delta^{13}\text{C}$ or $\delta^{15}\text{N} = ((R_{\text{sample}}/R_{\text{standard}}) - 1) \times 1000$, where R represents the $^{13}\text{C}:^{12}\text{C}$ or $^{15}\text{N}:^{14}\text{N}$ ratios, and

the standards were Vienna Pee Dee Belemnite for carbon and atmospheric N_2 for nitrogen. To control for instrument stability, samples of northern pike *Esox lucius* Linnaeus, 1758, muscle and European white birch *Betula pendula* Roth leaves of known isotopic compositions were run after every six samples as internal working standards for animal and plant samples. The analytical precision was $<0.1\text{ }‰$ for $\delta^{13}\text{C}$ and $<0.3\text{ }‰$ for $\delta^{15}\text{N}$.

Statistical analyses

The trophic position of each species was estimated for each season and section separately, using both mollusks and other primary consumers in two-baseline Bayesian models (5 chains, 10,000 iterations) within the R package tRophicPosition (Quezada-Romegialli et al., 2018), using as trophic discrimination factors (TDFs) 2.36 ± 1.08 (SE) ‰ for $\delta^{15}\text{N}$ and 1.18 ± 0.54 ‰ for $\delta^{13}\text{C}$ for crayfish (Glon et al., 2016) and 2.9 ± 1.24 ‰ for $\delta^{15}\text{N}$ and 1.3 ± 1.27 ‰ for $\delta^{13}\text{C}$ for fish (McCutchan et al., 2003).

To make isotopic niches comparable, we standardized stable isotopes values using the following formulas:

$$\delta^{13}\text{C}_{\text{cor}} = (\delta^{13}\text{C}_i - \text{mean}\delta^{13}\text{C}_{\text{cons}}) / \text{range}(\delta^{13}\text{C}_{\text{cons}});$$

$$\delta^{15}\text{N}_{\text{cor}} = \delta^{15}\text{N}_i - \delta^{15}\text{N}_{\text{baseline}} / \text{TDF} + 2,$$

where $\delta^{13}\text{C}_i$ and $\delta^{15}\text{N}_i$ are the individual values, $\text{mean}\delta^{13}\text{C}_{\text{cons}}$ is the mean $\delta^{13}\text{C}$ value across consumers in each season and section, $\text{range}(\delta^{13}\text{C}_{\text{cons}})$ is the difference between the maximum and minimum $\delta^{13}\text{C}$ values across consumers in each season and section, $\delta^{15}\text{N}_{\text{baseline}}$ is the mean $\delta^{15}\text{N}$ of mollusks and other primary consumers in each season and section, and TDF is set as 2.36 for crayfish and 2.9 for fish.

To describe the isotopic niches of each species, for each sampling event, Layman's metrics (Layman et al., 2007) were calculated on the standardized values using the R package SIBER (Jackson et al., 2011). These metrics were: the carbon range (CR) and nitrogen range (NR), which measure the distance between the lowest and the highest $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values, indicating, respectively, the range of primary resources consumed by the species and the range of trophic levels involved; the mean distance to the centroid (CD), which measures the trophic diversity among individuals as the mean Euclidean distance of each individual from the centroid in the isotopic bivariate space; the mean nearest neighbor distance (MNND) and associated standard deviation (SDNND), which provide a measure of trophic redundancy as they estimate the density of individual's isotope ratios and the spatial variation of this packaging, respectively; and the total area (TA), namely the minimum convex area encompassing all individuals' points in the isotopic space. Moreover, the Bayesian standard ellipse area (SEAB) and the corrected standard ellipse area (SEAc; considering 40% of central data points), the latter less sensitive to small sample sizes (Jackson et al., 2011), were calculated for each sampling event using the R package SIBER.

The standardized isotopic niches were graphically represented with the maximum likelihood standard ellipse areas (SEA) encompassing 95% of the data (Jackson et al., 2011). Differences among the niches were assessed separately for crayfish and fish using a permutational multivariate analysis of variance (PERMANOVA) with Euclidean distance and 9999 permutations on $\delta^{15}\text{N}_{\text{cor}}$ and $\delta^{13}\text{C}_{\text{cor}}$ values, with species, section and season (in interaction) as predictors using the function *adonis2* of the R package *vegan* (Oksanen et al., 2022). Additionally, to assess for interspecific differences in the isotopic niche, separate PERMANOVAs, one per sampling event, were run using the same approach but including only the

species as predictor. Similarly, to assess interseasonal differences in the isotopic niche of each species, separate PERMANOVAs, one per species and section, were run using the same approach but including only the season as predictor. Where significant, PERMANOVAs were followed by permutational analyses of variance (PERANOVA) on either $\delta^{15}\text{N}_{\text{cor}}$ or $\delta^{13}\text{C}_{\text{cor}}$ values (for interspecific PERMANOVAs, to identify which stable isotope discriminated the niches), or pairwise tests between seasons (for interseasonal PERMANOVAs, to assess which seasons presented separated niches) with Benjamini–Hochberg *P*-value correction (Benjamini & Hochberg, 1995) using the wrapper function *pairwise.adonis2* (Martinez Arbizu, 2020).

Finally, to estimate the proportions of food sources in species' diets, we performed stable isotopes mixing models on each species for each sampling event using the package MixSIAR (Stock et al., 2018). All mixing models were run using the 'normal' Monte Carlo Markov Chain option (3 chains, chain length: 100,000, burn-in: 50,000, thin: 50) and including C and N concentration dependence. Both residual and process errors were included (Stock & Semmens, 2016). The potential preys were selected for each consumer, season and section separately, as not all preys were available at all sampling events. The choice was based on the species' known biology (Saç, 2023; Musil et al., 2024) as well as on the topology of the consumer's data compared to the preys, running some preliminary mixing models when necessary. For all crayfish species, the final preys selected (when available) were: zoobenthos, plants, aquatic moss, algae, detritus, juvenile crayfish (to account for cannibalism) and juvenile *G. holbrooki*. For *G. holbrooki*, the final preys selected (when available) were: zoobenthos, detritus and algae. For *G. aculeatus*, the final preys selected (when available) were: zoobenthos, *P. virginialis*, *F. limosus*, *G. holbrooki* (both adults and juveniles) and juvenile crayfish. Generalist priors were used in all models. Different TDFs were applied after visually checking their reliability (i.e., after checking that the consumers' data points fell in the polygon defined by the sources). The TDFs obtained by Glon et al. (2016), namely 0.80 ± 0.99 (SD) ‰ for $\delta^{13}\text{C}$ and 1.20 ± 1.26 for $\delta^{15}\text{N}$ for animals, and 1.57 ± 0.98 ‰ for $\delta^{13}\text{C}$ and 2.94 ± 1.12 for $\delta^{15}\text{N}$ for plants and detritus, were used for crayfish. For fish, the TDFs for muscle tissue from McCutchan et al. (2003), namely

1.3 ± 1.27 ‰ for $\delta^{13}\text{C}$ and 2.9 ± 1.24 for $\delta^{15}\text{N}$, were used for both vegetal and animal preys.

All the statistical analyses were performed in the R environment (v. 4.3; R Core Team, 2023), and data visualizations were conducted using the R package ggplot2 (Wickham, 2016).

Results

In total, 178 adult crayfish (90 *P. virginalis*, 80 *F. limosus* and 8 *P. clarkii*) and 108 adult fish (47 *G. holbrooki* and 61 *G. aculeatus*) were sampled (Table S1). All crayfish species tended to occupy a trophic position comprised between 2.0 and 3.0 in all sections and seasons, except for *P. virginalis* in spring in the lower section (3.09) and in autumn in the upper section (3.30), and both *P. virginalis* and *F. limosus* (3.47 and 3.37, respectively) in summer in the lower section (Table S1). The trophic position of *F. limosus* in both sections increased from winter to summer and then decreased in autumn. A similar pattern was observed for the trophic position of *P. virginalis* in the lower section, while in the upper section the trophic position continuously increased from spring to autumn (Table S1). Both fish species occupied in all sections and seasons a trophic position comprised between 3.0 and 4.0, except for *G. holbrooki* in the upper section, which had slightly lower TP values (2.8–2.9). The TP of both species was relatively constant across seasons, with *G. holbrooki* having a lower TP than *G. aculeatus* in each season and site (Table S1).

Both the crayfishes' and fishes' isotopic niches did not show any clear pattern across species, seasons or sections (Table S2). The isotopic niches of crayfish as well as of fish were quite conservative, occupying a similar niche space across seasons and sections (Figs. 2 and 3). This is confirmed by the results of the general PERMANOVAs, which found that the combined effect of species, season and section had no significant effect on the isotopic niche position for both crayfish and fish (Table 1). However, for crayfish there were significant interactions between season and section (pseudoF_{3,162} = 16.28, $P < 0.001$) and between species and season (pseudoF_{3,162} = 5.88, $P < 0.001$) (Table 1), with *F. limosus* that tended to have higher $\delta^{13}\text{C}_{\text{cor}}$ values than *P. virginalis*, especially in summer (Fig. 2). Similarly, fishes showed significant differences in their niche according to the interaction

between season and section (pseudoF_{2,95} = 3.83, $P < 0.05$; Table 1), with *G. aculeatus* having higher $\delta^{15}\text{N}_{\text{cor}}$ (as mentioned earlier for the TP) and, in the upper section, lower $\delta^{13}\text{C}_{\text{cor}}$ values than *G. holbrooki* (Fig. 3).

Looking at the niche overlap in each sampling event, we found interspecific differences among crayfish niches only in summer. In the lower section (Fig. 2), we found no significant differences during autumn among the niches of *P. virginalis*, *F. limosus* and *P. clarkii* (pseudoF_{2,23} = 0.35, $P = 0.77$) and in spring between the niches of *P. virginalis* and *F. limosus* (pseudoF_{1,27} = 2.75, $P = 0.10$). However, we found a marginal significance in winter (pseudoF_{1,17} = 4.04, $P = 0.05$) and a significant difference in summer (pseudoF_{1,25} = 8.81, $P < 0.01$), with *F. limosus* having higher $\delta^{13}\text{C}_{\text{cor}}$ (pseudoF_{1,25} = 21.58, $P < 0.001$) and lower $\delta^{15}\text{N}_{\text{cor}}$ (pseudoF_{1,25} = 6.27, $P < 0.05$) than *P. virginalis*. In the upper section (Fig. 2), we found no significant differences between the niches of *P. virginalis* and *F. limosus* during spring (pseudoF_{1,25} = 1.70, $P = 0.19$) and autumn (pseudoF_{1,20} = 0.21, $P = 0.66$), but significant differences between the two species during summer (pseudoF_{1,23} = 5.99, $P < 0.05$). Similarly to the lower section, in summer *F. limosus* had higher $\delta^{13}\text{C}_{\text{cor}}$ (pseudoF_{1,23} = 26.25, $P < 0.001$) and lower $\delta^{15}\text{N}_{\text{cor}}$ (pseudoF_{1,23} = 5.48, $P < 0.05$) than *P. virginalis*. In winter, only three specimens of *F. limosus* were found.

The fish isotopic niches (Fig. 3) were significantly differentiated in all sampling seasons and in both sections where they co-occurred. In the lower section, the niches of the two species were partitioned in spring (pseudoF_{1,14} = 31.77, $P < 0.001$), summer (pseudoF_{1,19} = 15.66, $P < 0.001$) and autumn (pseudoF_{1,11} = 26.00, $P < 0.01$), in all cases for their $\delta^{15}\text{N}_{\text{cor}}$ values, higher in *G. aculeatus* than *G. holbrooki* (spring: pseudoF_{1,14} = 40.24, $P < 0.001$; summer: pseudoF_{1,19} = 16.72, $P < 0.01$; autumn: pseudoF_{1,11} = 30.21, $P < 0.001$), but not their $\delta^{13}\text{C}_{\text{cor}}$ values (spring: pseudoF_{1,14} = 2.08, $P = 0.18$; summer: pseudoF_{1,19} = 0.68, $P = 0.42$; autumn: pseudoF_{1,11} = 2.88, $P = 0.12$). In the upper section, the fish niches were differentiated in winter (pseudoF_{1,17} = 34.39, $P < 0.001$), spring (pseudoF_{1,14} = 23.08, $P < 0.001$) and summer (pseudoF_{1,16} = 47.80, $P < 0.001$), in all cases with *G. aculeatus* having higher $\delta^{15}\text{N}_{\text{cor}}$ (winter: pseudoF_{1,17} = 36.99, $P < 0.001$; spring:

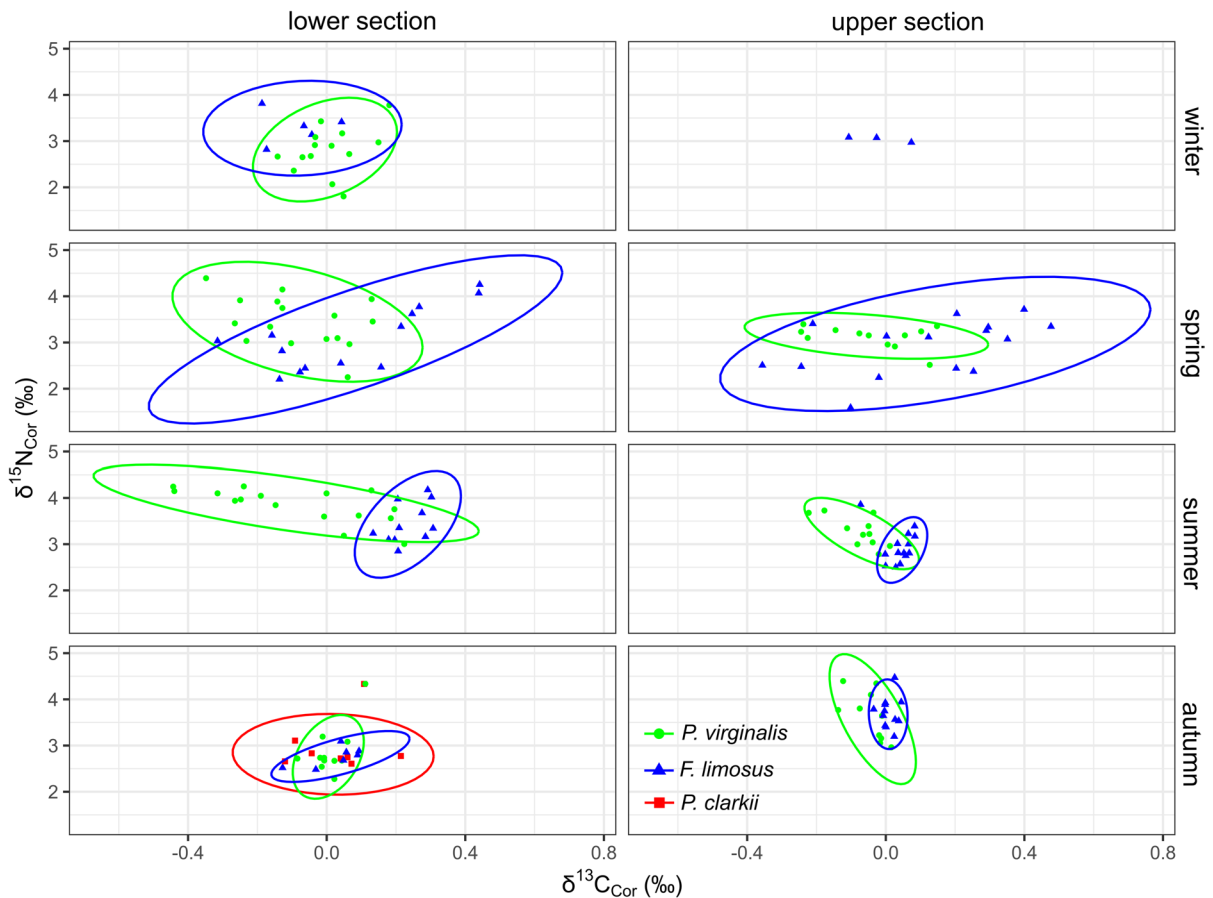


Fig. 2 Isotopic niches (represented as the 95% standard ellipse areas) occupied by the three crayfish species (*P. clarkii*=*Procambarus clarkii*, *P. virginalis*=*Procambarus virginalis* and *F. limosus*=*Faxonius limosus*) in the four

seasons in the two sampling sections. Note that in the upper section in winter, only three *F. limosus* specimens were found, so that ellipses could not be drawn

pseudo $F_{1,14}$ = 23.96, $P < 0.001$; summer: pseudo $F_{1,16}$ = 48.74, $P < 0.001$) and more negative $\delta^{13}C_{cor}$ (winter: pseudo $F_{1,17}$ = 21.91, $P < 0.001$; spring: pseudo $F_{1,14}$ = 17.35, $P < 0.001$; summer: pseudo $F_{1,16}$ = 22.30, $P < 0.001$) than *G. holbrooki*.

As for the intraspecific isotopic niche, we found interseasonal differences for all crayfish species in both sections (Fig. 4). The niche of *P. virginalis* was significantly different among seasons in both sections (lower: pseudo $F_{3,53}$ = 13.45, $P < 0.001$; upper: pseudo $F_{3,30}$ = 5.18, $P < 0.01$), similarly to the niche of *F. limosus* (lower: pseudo $F_{3,32}$ = 3.11, $P < 0.05$; upper: pseudo $F_{3,40}$ = 7.93, $P < 0.001$). *Procambarus clarkii* was found only in one season, so this analysis could not be carried on for this species. In particular, in

the lower section, the niche of *P. virginalis* in winter was significantly different from spring ($P < 0.01$) and summer ($P < 0.001$), and significant differences were found also between spring and summer ($P < 0.05$), spring and autumn ($P < 0.05$), and summer and autumn ($P < 0.001$). In the upper section, the niche of *P. virginalis* was significantly differentiated only between spring and autumn ($P < 0.05$). The niche of *F. limosus* in the lower section was significantly different between summer and autumn ($P < 0.01$), and autumn and winter ($P < 0.05$), while in the upper section significant differences between winter and autumn ($P < 0.01$), spring and autumn ($P < 0.001$), and summer and autumn ($P < 0.001$) were identified by the multiple comparisons (Table S3).

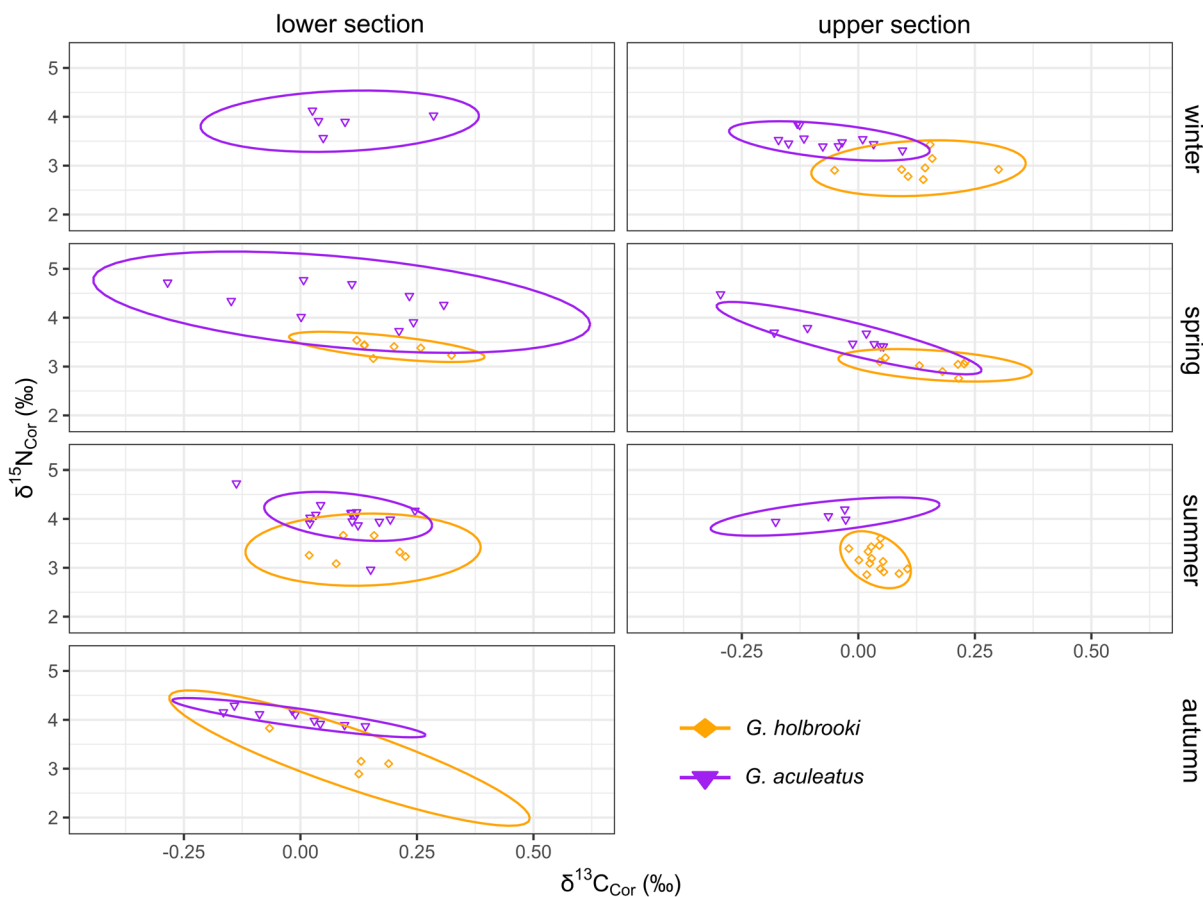


Fig. 3 Isotopic niches (represented as the 95% standard ellipse areas) occupied by the two fish species (*G. holbrooki*=*Gambusia holbrooki* and *G. aculeatus*=*Gasterosteus aculeatus*)

in the four seasons in the two sampling sections. Note that no adult fish was found in autumn in the upper section

In fish (Fig. 5), we found significant differences in the niche among seasons only in the upper section for both *G. holbrooki* (pseudo $F_{2,27}=3.33$, $P<0.05$) and *G. aculeatus* (pseudo $F_{2,20}=5.52$, $P<0.01$). In this latter species, the niche differed between summer and winter ($P<0.01$), while in *G. holbrooki* this significance became marginal during the pairwise multiple comparisons (Table S4). On the contrary, no significant seasonal differences were found in the lower section for both *G. holbrooki* (pseudo $F_{2,14}=0.53$, $P=0.64$) and *G. aculeatus* (pseudo $F_{3,34}=2.33$, $P=0.07$).

The mixing models for crayfish (Table 2) showed some general patterns between *P. virginialis* and *F. limosus*. For both species, in the lower section, the consumption of zoobenthos was very high in winter; then, it decreased till summer and again increased

in autumn. Similarly, plant consumption was high in winter, decreased in spring/summer and then increased again in autumn. Algae were an important food source for both species in spring; then, their proportion in the diet of both crayfish species decreased toward autumn. In the upper section, instead, zoobenthos was very highly consumed by both species in spring; then, it declined till autumn. A similar pattern was also observed for the proportion of *G. holbrooki* juveniles, although with a lower importance. An increase in the consumption of detritus, plants, algae and crayfish juveniles from spring to summer was also observed in both species. In both sections and species, the proportion of detritus and zoobenthos showed opposite trends across the seasons.

Some patterns could also be found in the seasonal dietary trends of both species across sections. In

Table 1 Results of the permutational multivariate analyses of variance (PERMANOVA, 9999 permutations, Euclidean distance) on the isotopic niche of crayfish (*Procambarus clarkii*, *Procambarus virginalis* and *Faxonius limosus*) and fish (*Gambusia holbrooki* and *Gasterosteus aculeatus*), using species, season and section (and all their interactions) as predictors. Asterisks highlight the statistical significance levels: $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***)

group	Predictor	df	Sum of Squares	R ²	pseudoF	P
Crayfish	Species	2	2.07	0.03	4.29	<0.05*
	Season	3	4.01	0.06	5.54	<0.001***
	Section	1	0.12	0.002	0.48	0.53
	Species:Season	3	4.25	0.07	5.88	<0.001***
	Species:Section	1	0.07	0.001	0.29	0.64
	Season:Section	3	11.80	0.19	16.28	<0.001***
	Species:Season:Section	2	0.13	0.002	0.27	0.81
	Residual	162	39.14	0.64		
	Total	177	61.62	1.00000		
	Fish	Species	1	15.45	0.54	193.16
Season		3	2.12	0.07	8.82	<0.001***
Section		1	2.14	0.07	26.72	<0.001***
Species:Season		3	0.32	0.01	1.34	0.26
Species:Section		1	0.08	0.03	1.00	0.32
Season:Section		2	0.61	0.02	3.83	<0.05*
Species:Season:Section		1	0.25	0.01	3.09	0.08
Residual		95	7.60	0.27		
Total		107	28.56	1.00000		

P. virginalis, there was an increase in detritus and plant consumption toward autumn, while zoobenthos and, to a lesser extent, *G. holbrooki* juveniles were important food sources in spring/summer. Moreover, an important predation on crayfish juveniles was also estimated in summer and autumn. In *F. limosus*, a considerable plant consumption was estimated for winter and autumn, while there was an overall important proportion of zoobenthos, detritus, *G. holbrooki* juveniles and crayfish juveniles across the seasons. On the only occasion where it was caught, *P. clarkii* mostly fed on crayfish juveniles and zoobenthos.

The mixing models for fish (Table 3) showed some similar patterns in the lower section, where for both species the proportion of zoobenthos increased till summer, but not in the upper section. In the lower section, *G. holbrooki* consumed mostly zoobenthos in all seasons, followed by algae and then detritus. Algae were particularly consumed in spring (when they had a similar proportion in the diet as zoobenthos), after which they showed an opposite trend compared to zoobenthos. In the lower section, *P. virginalis* was the main prey of *G. aculeatus* across seasons, followed by juveniles of *G. holbrooki* and then zoobenthos, this latter being as high as *P. virginalis* in summer. Adults of *G. holbrooki* were also important throughout the

year, as well as *F. limosus*, which showed an opposite trend than *P. virginalis* (i.e., the contribution of either increased when the other's one decreased). In the upper section, *G. holbrooki* consumed mainly zoobenthos in all seasons, with a peak in spring (when it was the only prey found). In winter, detritus was also an important food source for this species. In the upper section, zoobenthos was the main food source for *G. aculeatus*, with a decreasing trend from winter to summer. In spring and summer, *P. virginalis* was also an important prey for this species, while *F. limosus* was an important prey in winter (when *P. virginalis* was not present) and, to a lesser degree, in spring and summer. Both adults and juveniles of *G. holbrooki* were important food sources in the diet of *G. aculeatus* throughout the seasons, especially juveniles during summer, when they represented the main prey together with *P. virginalis*.

Comparing the seasonal trends between the two sections for either species, few patterns were evident. In *G. holbrooki*, zoobenthos was the main prey in all cases. In both sections, the diet of *G. aculeatus* showed an overall importance of *G. holbrooki* juveniles, especially in autumn when it was the main food source for this consumer. Crayfish were also important preys, with *P. virginalis* being particularly prominent in spring and summer, and *F. limosus* being consumed less than *P.*

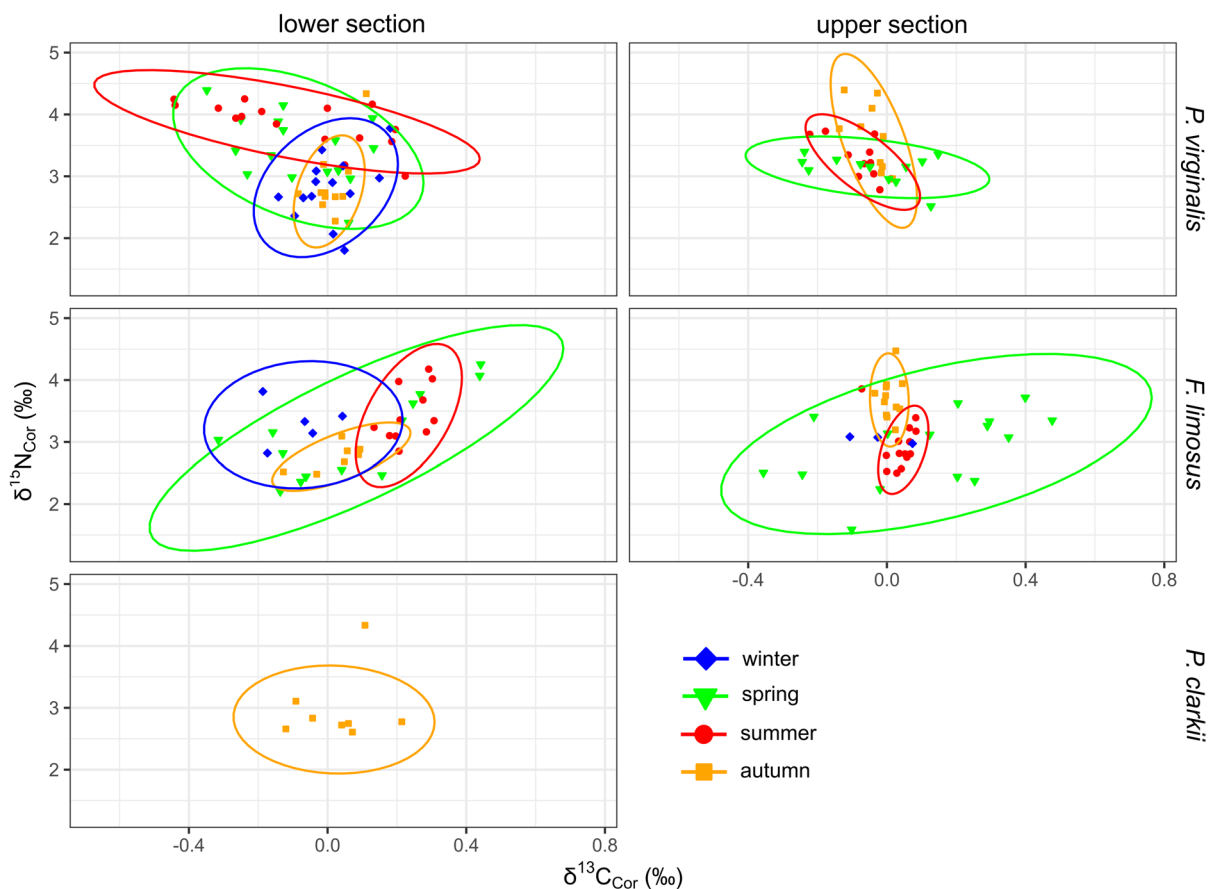


Fig. 4 Isotopic niches (represented as the 95% standard ellipse areas) occupied by the three crayfish species (*P. clarkii*=*Procambarus clarkii*, *P. virginialis*=*Procambarus virginialis* and *F. limosus*=*Faxonius limosus*) in the four

virginialis, although a clear opposite trend occurred in both sections.

Discussion

To assess and predict the impacts of non-native species in new environments, it is crucial to investigate their novel arisen trophic interactions, for example in terms of feeding competition and predation (Britton, 2018; Balzani & Haubrock, 2022; Tarkan et al., 2023). For example, the consumption of the same food source by multiple non-native species can result in increased predation pressure on a certain prey (Jackson et al., 2017), while competition between non-native species can, on the other hand, contain their impacts on the other

seasons in the lower (left column) and upper (right column) section. Note that *P. clarkii* was found only in the lower section in autumn

species, with both non-native populations limiting each other (Aksu et al., 2025). Here, we assessed the intra- and interspecific differences in the diet and trophic niche of the populations of three non-native crayfish and two non-native fish along a gradient of annual temperature variation across the four seasons. Contrary to our expectations, we did not find greater conservatism (i.e., stability) throughout the year in the trophic niche, diet or potential interspecific competition in the studied populations in the section with a more constant water temperature compared to the section with higher water temperature variability.

Crayfish are omnivorous macroinvertebrates and play important roles in food webs (Dorn & Wojdak, 2004; Reynolds et al., 2013; Lipták et al., 2019). Indeed, they often reach a substantial biomass and are prey for multiple predators (Neveu, 2009; Reynolds,

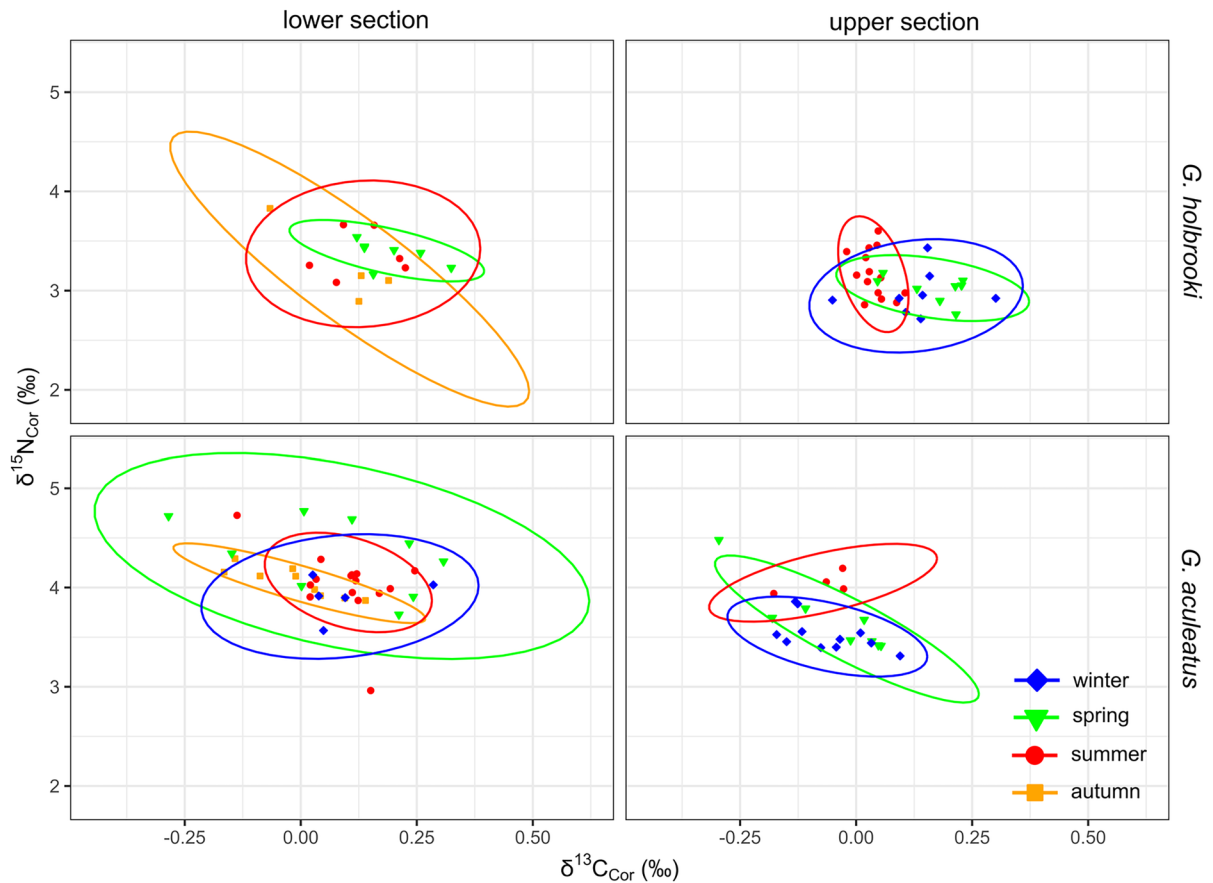


Fig. 5 Isotopic niches (represented as the 95% standard ellipse areas) occupied by the two fish species (*G. holbrooki*=*Gambusia holbrooki* and *G. aculeatus*=*Gasterosteus aculeatus*) in the four seasons in the lower (left column) and upper (right column) section

2011), mediating energy flows within and among ecosystems (Correia & Anastacio, 2008; Cruz et al., 2008; Grey & Jackson, 2012). Our results are in line with this expectation, with all populations occupying an omnivore trophic position in almost all seasons and sections. On a few occasions, however, we found both *P. virginalis* and *F. limosus* showing a more predatory trophic position. The occasional predatory role of these species was already observed in previous studies (Linzmaier et al., 2020). This might be explained by crayfish opportunistic feeding (Jackson et al., 2017), while varying prey availability throughout the year could explain the interseasonal variation in the trophic position and isotopic niche we observed for both species. Moreover, the crayfish niche position in the isotopic space did not show major shifts, although some were significant among seasons between sections and species. In particular, *F. limosus*

had higher $\delta^{13}\text{C}_{\text{cor}}$ values than *P. virginalis*, especially in summer, suggesting a greater consumption of food sources of terrestrial origin compared to *P. virginalis*. This is in line, at least for the lower section, with the high amount of detritus (potentially of allochthonous origin) estimated by mixing models in the diet of *F. limosus*, in accordance with the results of Veselý et al. (2021) from the same study site.

Both *G. holbrooki* and *G. aculeatus* are small predatory fish, mainly feeding on aquatic insects and zooplankton (Pyke, 2005; Bretzel et al., 2021; Saç, 2023). However, *G. holbrooki* can also feed on plants, algae and detritus, if other resources become scarce (Yoğurtçuoğlu & Ekmekçi, 2017; Andolina et al., 2022). Our results, especially the lower trophic position of *G. holbrooki* compared to *G. aculeatus* in all seasons and sections confirm these previous results. In accordance with the results about crayfish,

Table 2 Results of the stable isotopes mixing models on *Procambarus virginialis*, *Faxonius limosus* and *Procambarus clarkii* in the four seasons in the two sampling sections

Consumer	Prey	Lower section (A)				Upper section (B)			
		Winter	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn
<i>Procambarus virginialis</i>	Zoobenthos	0.29 (0.04–0.59)	0.19 (0.02–0.46)	0.10 (0.01–0.28)	0.20 (0.02–0.48)	NA	0.83 (0.65–0.97)	0.19 (0.02–0.44)	0.18 (0.02–0.46)
	Plants	0.28 (0.04–0.58)	0.07 (0.01–0.25)	0.10 (0.01–0.31)	0.13 (0.01–0.40)	NA	–	0.08 (0–0.29)	0.14 (0.01–0.44)
	Aquatic moss	0.20 (0.02–0.55)	0.36 (0.12–0.60)	0.13 (0.01–0.34)	0.10 (0.01–0.27)	NA	–	0.09 (0–0.33)	–
	Algae	–	0.12 (0.01–0.37)	0.11 (0.01–0.35)	0.10 (0.01–0.34)	NA	–	0.10 (0.01–0.35)	0.12 (0.01–0.39)
	Detritus	–	0.08 (0.01–0.26)	0.08 (0.01–0.25)	0.11 (0.01–0.35)	NA	–	0.10 (0.01–0.32)	0.15 (0.01–0.47)
	Juvenile crayfish	0.08 (0.01–0.29)	–	0.22 (0.07–0.38)	0.14 (0.01–0.45)	NA	–	0.14 (0.01–0.41)	0.18 (0.02–0.51)
	Juvenile <i>G. holbrooki</i>	0.06 (0–0.20)	0.09 (0.01–0.20)	0.13 (0.01–0.34)	0.05 (0.01–0.21)	NA	0.17 (0.03–0.35)	0.14 (0.02–0.34)	0.07 (0.01–0.24)
<i>Faxonius limosus</i>	Zoobenthos	0.22 (0.02–0.54)	0.19 (0.03–0.42)	0.06 (0.01–0.21)	0.22 (0.03–0.51)	0.30 (0.05–0.59)	0.76 (0.45–0.95)	0.33 (0.10–0.53)	0.12 (0.01–0.36)
	Plants	0.14 (0.01–0.40)	0.10 (0.01–0.32)	0.05 (0–0.20)	0.13 (0.01–0.39)	0.21 (0.02–0.56)	–	0.05 (0–0.17)	0.12 (0.01–0.39)
	Aquatic moss	0.14 (0.02–0.43)	0.13 (0.01–0.37)	0.04 (0–0.18)	0.10 (0.01–0.31)	–	–	0.10 (0.01–0.34)	–
	Algae	–	0.18 (0.02–0.48)	0.14 (0.01–0.42)	0.12 (0.01–0.36)	–	–	0.08 (0.01–0.26)	0.11 (0.01–0.38)
	Detritus	–	0.23 (0.03–0.53)	0.30 (0.04–0.59)	0.15 (0.02–0.42)	0.17 (0.02–0.50)	–	0.12 (0.01–0.35)	0.20 (0.02–0.53)
	Juvenile crayfish	0.22 (0.02–0.56)	–	0.04 (0–0.13)	0.08 (0.01–0.38)	–	–	0.12 (0.01–0.36)	0.23 (0.02–0.56)
	Juvenile <i>G. holbrooki</i>	0.15 (0.01–0.43)	0.06 (0.01–0.19)	0.26 (0.04–0.54)	0.03 (0–0.16)	0.23 (0.05–0.51)	0.24 (0.05–0.55)	0.08 (0.01–0.24)	0.07 (0.01–0.24)
<i>Procambarus clarkii</i>	Zoobenthos	NA	NA	NA	0.17 (0.02–0.42)	NA	NA	NA	NA
	Plants	NA	NA	NA	0.13 (0.01–0.37)	NA	NA	NA	NA
	Aquatic moss	NA	NA	NA	0.09 (0.01–0.28)	NA	NA	NA	NA
	Algae	NA	NA	NA	0.11 (0.01–0.34)	NA	NA	NA	NA
	Detritus	NA	NA	NA	0.11 (0.01–0.34)	NA	NA	NA	NA
	Juvenile crayfish	NA	NA	NA	0.17 (0.02–0.47)	NA	NA	NA	NA
	Juvenile <i>G. holbrooki</i>	NA	NA	NA	0.07 (0.01–0.26)	NA	NA	NA	NA

The proportion of each prey in the diet of these consumers is indicated as mean and its lower and upper 95% credible intervals (CI) in brackets. 0 indicates proportions < 0.01, while ‘–’ indicates that a certain prey or consumer was not present in a certain section and season. ‘NA’ indicates that a certain consumer was not present in a certain section and season. Juvenile crayfish = *P. virginialis*

Table 3 Results of the stable isotopes mixing models on *Gambusia holbrooki* and *Gasterosteus aculeatus* in the four seasons in the two sampling sections

Consumer	Prey	Lower section (A)				Upper section (B)			
		Winter	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn
<i>G. holbrooki</i>	Zoobenthos	NA	0.38 (0.14–0.69)	0.47 (0.23–0.75)	0.46 (0.16–0.78)	0.61 (0.28–0.92)	1.00	0.65 (0.44–0.83)	NA
	Algae	NA	0.40 (0.06–0.73)	0.29 (0.03–0.63)	0.27 (0.03–0.64)	-	-	0.22 (0.04–0.44)	NA
	Detritus	NA	0.19 (0.02–0.49)	0.20 (0.02–0.47)	0.21 (0.02–0.55)	0.39 (0.08–0.71)	-	0.11 (0.01–0.33)	NA
<i>G. aculeatus</i>	Zoobenthos	0.13 (0.02–0.36)	0.16 (0.02–0.41)	0.32 (0.12–0.49)	0.10 (0.01–0.29)	0.54 (0.32–0.77)	0.32 (0.06–0.65)	0.10 (0.01–0.31)	NA
	<i>Faxonius limosus</i>	0.20 (0.02–0.49)	0.09 (0.01–0.29)	0.07 (0.01–0.22)	0.11 (0.01–0.30)	0.26 (0.03–0.52)	0.12 (0.01–0.38)	0.11 (0.01–0.35)	NA
	<i>Procambarus virginalis</i>	0.16 (0.02–0.43)	0.27 (0.04–0.61)	0.32 (0.10–0.52)	0.11 (0.01–0.31)	-	0.17 (0.01–0.46)	0.19 (0.02–0.49)	NA
	<i>G. holbrooki</i>	-	0.16 (0.02–0.45)	0.10 (0.01–0.31)	0.13 (0.01–0.38)	0.07 (0.01–0.24)	0.09 (0.01–0.33)	0.14 (0.01–0.42)	NA
	Juvenile <i>G. holbrooki</i>	0.18 (0.02–0.46)	0.21 (0.02–0.50)	0.13 (0.01–0.42)	0.32 (0.06–0.52)	0.08 (0–0.28)	0.18 (0.01–0.47)	0.18 (0.01–0.52)	NA
	Juvenile crayfish	0.22 (0.02–0.55)	-	-	0.15 (0.02–0.40)	-	-	0.12 (0.01–0.37)	NA

The proportion of each prey in the diet of these consumers is indicated as mean and its lower and upper 95% credible intervals (CI) in brackets. 0 indicates proportions < 0.01, while '-' indicates that a certain prey or consumer was not present in a certain section and season. 'NA' indicates that a certain consumer was not present in a certain section and season. Juvenile crayfish = *P. virginalis*

the fish niches also varied across seasons in the upper section, supporting our interpretation of temporal variability in prey availability. Indeed, the diets of both studied fish are flexible and opportunistic, showing seasonal variations in response to prey availability (Remon et al., 2016; Bretzel et al., 2021; Pirroni et al., 2021), also independently of prey availability (Dirnberger & Love, 2016). Interestingly and contrary to our expectations, no seasonal variations were found in the fish niche in the lower section, suggesting that in this section some food source remained stable throughout the year, while prey availability varied between the two sections.

We did not find any clear pattern regarding the niche width or the interindividual dispersion in the isotopic niches of crayfishes and fishes. This indicates an absence, for all species, of increased generalism or specialization (and the subsequent change in the intraspecific potential competition) over the seasons and between sections, again suggesting that no higher variability in prey availability occurred in the lower section as a consequence of the higher water temperature fluctuations. The combined results of

niche features and niche position of fishes over the seasons suggest that fish in the upper section kept their feeding specialization degree even shifting their niche according to the available sources. Examining each sampling event separately, we found interspecific niche differences among crayfish only in summer, with *F. limosus* having higher $\delta^{13}\text{C}_{\text{cor}}$ and lower $\delta^{15}\text{N}_{\text{cor}}$ than *P. virginalis* in both sections. This suggests a higher consumption of terrestrial food sources for *F. limosus* than *P. virginalis*. A previous study found a low isotopic niche overlap between these two species in spring–summer (Linzmaier et al., 2020), although another study conducted in autumn in our same study area described a considerable niche overlap between these species (Vesely et al., 2021). However, while Vesely et al. (2021) found a segregated niche with higher trophic position for *P. clarkii*, in the present study we found that in the only case where *P. clarkii* was also present (in the lower section in autumn), the crayfish niches were all overlapped. Moreover, in line with Vesely et al. (2021), the niche width of both *F. limosus* and *P. virginalis* was shrunk in the presence of *P. clarkii*

compared to the other seasons, suggesting increased interspecific competition. A similar result was found in Czechia for the native noble crayfish *Astacus astacus* (Linnaeus, 1758) and the non-native signal crayfish *Pacifastacus leniusculus* (Dana, 1852), whose isotopic niche areas were narrower when in sympatry than in allopatry (Ercoli et al., 2025).

The isotopic niches of fish were partitioned in all cases where the two species co-occurred, with *G. aculeatus* having always higher $\delta^{15}\text{N}_{\text{cor}}$ values than *G. holbrooki*. Moreover, we found *G. holbrooki* having more positive $\delta^{13}\text{C}_{\text{cor}}$ values than *G. aculeatus* in the upper section, suggesting a higher consumption of terrestrial sources, as also previously documented for this species (Specziár, 2004). Previous studies found a dietary overlap between non-native *G. holbrooki* and the native toothcarp *Valencia letourneuxi* (Sauvage, 1880) (Kalogianni et al., 2014), and a niche partitioning between non-native *G. holbrooki* and the native *Atherina boyeri* Risso, 1810 (Andolina et al., 2022). To our knowledge, the present study is the first one that investigated the potential competition between *G. holbrooki* and *G. aculeatus*, and our results suggest a low potential feeding competition between these two species.

The results of the mixing models seem to confirm the idea of varying prey availability across seasons in the two sections. Noteworthy is the importance of *G. holbrooki* and crayfish juveniles in the diet of both *P. virginalis* and *F. limosus*, as well as crayfish juveniles in *P. clarkii*. Since all crayfish juveniles belonged to *P. virginalis*, this result implies both intra- and interspecific predation. Cannibalism was described in all the here-studied species (Kouba et al., 2021) and represents a common crayfish habit (Kouba et al., 2011; Veselý et al., 2020; Ercoli et al., 2021). Reproduction occurs more often in *P. virginalis* than the other crayfishes (Hossain et al., 2018), so that the probability of finding some juveniles is higher for this species. Despite *G. holbrooki* having been found in the diet of *P. clarkii* (Gutiérrez-Yurrita et al., 1998; Souty-Grosset et al., 2016), there was no evidence in the literature of such predation by *F. limosus* and *P. virginalis*. Another result worth of attention is the predation of *G. aculeatus* on *F. limosus* and *P. virginalis*, which has recently been demonstrated under controlled conditions (Musil et al., 2024). Moreover, we found a considerable proportion of both juvenile and adult

G. holbrooki in the diet of this species, in accordance with the known predation of *G. aculeatus* on smaller fish (Lucas et al., 2021; Gugele et al., 2023).

This studied man-made thermal tributary is currently dominated by non-native species. In the main Barát brook, to which our study stream is a tributary and which is directly connected to the Danube River, 15 native and 8 non-native fish species have been recorded during our long-term surveys of the fish community (Gál et al., 2018). These species can use the lower and estuary section of the Barát brook if the water level of the Danube River is sufficiently high. However, over the last decade, the lower 1.5-km stretch of the Barát brook has completely dried up. Similarly, the native noble crayfish (*A. astacus*) was occurring in the main Barát brook (Puky et al., 2005). However, this species has now almost completely disappeared from the lower and middle sections of all tributaries of the Danube River, due to urbanization and water pollution, leaving ecological niches to the spread of non-native crayfish species, which started colonizing not only the main arm of the Danube River but also all connected water bodies (Ludányi et al., 2016; Mozsár et al., 2021). Interestingly, non-native crayfish invasions also varied through time in our study site, with *P. clarkii* arriving only in the last sampling season from the Danube River (Oficialdegui et al., 2025). Since then, it has considerably spread and displaced the other non-native crayfishes in both sections (unpublished data).

Therefore, our results cannot measure the impacts of non-native species on native fish or crayfish species. The only native species occurring in the tributary stream are amphibians, especially the marsh frog *Pelophylax ridibundus* (Pallas, 1771), of which not enough individuals could be caught during our sampling campaigns. Thus, the impacts of non-native species on native amphibians (especially on their eggs and larval stages) in our study site deserve *ad hoc* investigations. Overall, our results provide information on the interspecific interactions among non-native species in a peculiar ecosystem, characterized by different levels of temperature variability, that may represent environmental conditions found in other sites or in the future due to climate change. However, some caveats due to a lower sample number for some species on some sampling occasions (see Table S1), due to the natural fluctuations of the sampled populations, should also

be acknowledged and the associated results taken with caution.

In conclusion, we found that non-native crayfish generally had overlapped trophic niches, except in summer, while non-native fish always had segregated isotopic niches. We also found seasonal changes in the trophic position and diet in crayfish and fish, while the isotopic niche was shifted across seasons in all sections for crayfish and only in the upper section for fish. Our study suggests that the studied non-native species had a considerable trophic plasticity, thus being able to tackle with different food source availability. At the same time, our results showed that the temperature variability gradient characterizing our study site did not affect directly the trophic ecology of our focal species. Our study provides valuable information on how non-native populations may respond to environmental changes such as climate change, which may be useful to improve their management and impact mitigation.

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Data availability All data will be made available upon reasonable request.

Declarations

Conflict of interest statement The authors declare no conflict of interest.

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