



Historical analysis reveals ecological shifts in two omnivorous fish after the invasion of *Limnoperna fortunei* in the Uruguay river

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Abstract Since the Asian golden mussel, *Limnoperna fortunei*, was first reported in the Río de la Plata in the 90's, its invasion has continuously expanded throughout South America, promoting several negative ecosystem consequences. Several fish species consume and assimilate large fractions of *L. fortunei* in their biomass, partially controlling the abundance of this invader, but potential fish dietary and trophic niche modifications caused by the invasion have not been studied in deep. Through gut content, stable isotopes and gut morphometry analysis of field-collected and historical museum samples, the potential dietary, trophic niche and physiological

consequences of the invasion for two predatory fish of the golden mussel were surveyed. The analysis of historical samples of some of the most frequent and abundant *L. fortunei* consumers, such as *Megaleporinus obtusidens* and *Pimelodus maculatus*, revealed changes in trophic niche when fish started to incorporate *L. fortunei*. Specifically, an increase in trophic position and a reduction in diet diversity was observed for both predators. Also, a reduction in dietary generalism occurred for *M. obtusidens*. Furthermore, the digestive tract mass of this species decreased after the invasion, that is, in parallel to a markedly increase in the consumption of animal material. This research raised several questions about the potential effect of *L. fortunei* on growth rates and abundances of *M. obtusidens* and *P. maculatus*, two of the most important species in commercial fisheries in the Uruguay river. Also, it may assist in predicting food web changes to be expected in newly invaded areas.

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Introduction

The Asian golden mussel, *Limnoperna fortunei* (Bivalvia, Mytilidae), is a freshwater mollusc native from South East Asia that was unintentionally

introduced in South America in the 90's decade, with several potential introductions of larvae transported in ballast water from commercial ships (Pastorino et al. 1993; Darrigran and Pastorino 1995). Since then, a fast colonization and invasion started, expanding its distribution towards the Río de la Plata estuary, Uruguay and Paraná river basins (Boltovskoy et al. 2006). By the early 2000', this mussel was already a dominant benthic species in the lower Uruguay River and its large tributaries (Clemente and Brugnoli 2002; Brugnoli et al. 2005; Langone 2005; Muniz et al. 2005; Boltovskoy et al. 2006), and by 2020 it was

widespread along all the higher Uruguay river basin as well (Andrade et al. 2021). Furthermore, the invasion rapidly advances towards coastal Atlantic drainages and towards north in South America (Clemente and Brugnoli 2002; Brugnoli et al. 2005; Muniz et al. 2005; Oliveira et al. 2010; Barbosa et al. 2016). Nowadays the distribution range includes all the large tributaries of Río de la Plata basin and the Brazilian Pantanal up to Río San Francisco basin in North Eastern Brazil (Oliveira et al. 2010; Barbosa et al. 2016) (Fig. 1). The invasion in others basins of South and Central America could occur in a few years, since

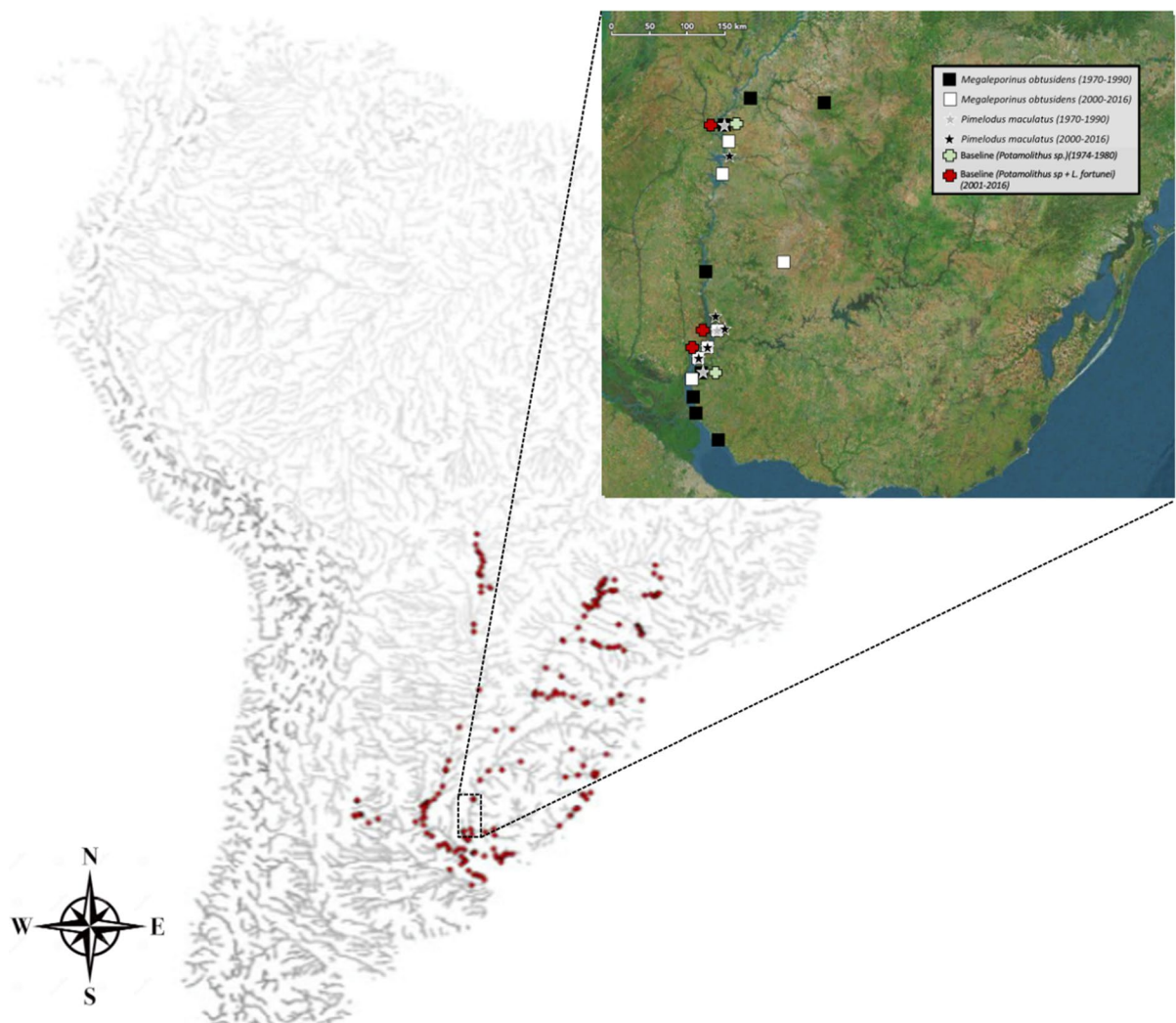


Fig. 1 Map of the study region, showing the current distribution of the invasive mussel *Limnoperna fortunei* in the study area at Uruguay. The sites where specimens of *M. obtusidens*

and *P. maculatus* as well as baseline primary consumers were collected are shown, before (1970–1990) and after (2000–2016)

environmental conditions in other large rivers, as Amazonas and Orinoco, also seem to be suitable for the development of this invasion (Boltovskoy et al. 2006, 2022; Souza Campos et al. 2014).

Several changes in the functioning of freshwater ecosystems have been predicted as *L. fortunei* turns into the dominant benthic invertebrate species (Boltovskoy and Correa 2015). For example, the physical habitat is modified as benthic organic matter accrual increases considerably alongside *L. fortunei* colonies, due to their pseudo-faeces and the increased roughness of surfaces favoring organic matter siltation (Martín and Darrigran 1994; Boltovskoy and Correa 2015). In turn, increased organic matter in sediments seems to modify macroinvertebrate assemblages, favouring anelids and nematods but potentially negatively affecting native gastropods and insects (Martín and Darrigran 1994; Scarabino and Verde 1995; Spaccesi and Rodrigues-Capitulo 2012; Boltovskoy and Correa 2015; Silva et al. 2020, 2021).

It is known that more than 50 fish species from Río de la Plata basin consume *L. fortunei* to some extent, not only in its adult forms but also in its larval stages (Penchaszadeh et al. 2000; García and Protogino 2005; Paolucci et al. 2010; Cataldo 2015; González-Bergonzoni et al. 2010; González-Bergonzoni et al. 2020; López-Rodríguez et al. 2019). More specifically, in the lower and middle Uruguay river at least 28 fish species frequently consumed *L. fortunei*, and estimations using Bayesian stable isotope mixing models showed that more than 10% of the total fish biomass of littoral areas originated from the consumption of *L. fortunei* (González-Bergonzoni et al. 2020). In addition, experimental evidence from in-situ fish exclusion trials showed that fish can reduce up to 80% of mussel abundances in the benthic habitat both in Paraná (Sylvester et al. 2007) and Uruguay rivers (González-Bergonzoni et al. 2020; Silva et al. 2020). Moreover, in rocky shorelines of the lower Uruguay River fish predation not only reduces density, biomass and mussel body size, but also mitigates some of the negative impact of mussel colonization over native macroinvertebrate densities (Silva et al. 2020).

However, despite that we now have an oversight of the predatory role of fish in the present invasion scenario, it remains largely unknown how fish trophic ecology and their digestive physiology may have changed in response to the inclusion in their diet of

this new and abundant resource. Several authors have argued that some of the species that nowadays widely consume *L. fortunei* had previously been recorded to be omnivorous with wide niche breadths, frequently including a significant fraction of vegetal matter in their diets, or perhaps being herbivores in the past (Penchaszadeh et al. 2000; López-Rodríguez et al. 2019). Such a dietary shift, if confirmed, may imply strong changes in different aspects of the trophic niche, such as trophic niche width (e.g. shifting to an omnivore feeding strategy with one major dietary item may imply a decrease in niche breadth) and trophic level (e.g. shifting from herbivory to carnivory may imply an increase in trophic level). These changes may also occur concomitantly with adjustments in digestive tract physiology. The latter may occur because extracting nutritional requirements from low-quality vegetal diet versus high-quality animal diets require contrasting digestive strategies (e.g. Stevens and Hume 1998).

The effect that golden mussel may have over the trophic structure of fish assemblages could be evidenced comparing post-invasion trophic niches with that of museum-preserved specimens collected during pre-invasion periods. Such approaches have been performed in other study cases, such as those analysing historical impacts on food webs due to modifications in the hydrological regime and due to the introduction of invasive fish species around the globe (e.g. Vander Zanden et al. 2003; Turner et al. 2015; Delong and Thoms 2016; Kische-Machumu et al. 2017). Furthermore, comparing aspects of the digestive physiology between pre- and post-invasion periods, such as digestive tract morphology of the fish species currently feeding on *L. fortunei* is also feasible.

Accordingly, in this study changes in the trophic niches and digestive physiology of two fish species, known as predators of *L. fortunei* in the lower and middle Uruguay river, were analysed by comparing gut content, stable isotope ratios and gut morphometry of pre-invasion (preserved) and post-invasion (preserved and fresh) specimens. We predict a reduced trophic niche (number of prey items and isotopic niche), an increased trophic level, and a reduction in gut size after the invasion, i.e., as fish become more specialized in the consumption of *L. fortunei*, a highly abundant resource of potentially elevated nutritive quality. In this way, our research

aims to allow a better understanding of changes in fish trophic niche and digestive physiology caused by the insertion of this exotic resource in the food web.

Material and methods

Study area

The Uruguay river is the second most important tributary to the Río de la Plata estuary (after Paraná river), with an approximate length of 1800 km and a basin area of 365,000 km². The first report of *Limnoperna fortunei* in the Uruguay river dates back to year 2001, when it was reported to cause bio-fouling related problems in waterworks of lower Uruguay River (Langone 2005). Here, we analysed fish collected during the last five decades along the middle and lower Uruguay river sections in Uruguay, which represent one of the oldest focus of invasion in the region (Fig. 1).

Sampling design

We studied trophic niche changes in two species: *Megaleporinus obtusidens*—a species that primary feed on *L. fortunei* at the present—and *Pimelodus maculatus*—a generalist omnivore, which incorporates *L. fortunei* in its diet among several other items (González-Bergonzoni et al. 2020, López-Rodríguez et al. 2019). We focused in these two species based on sample availability in fish collections as well as on availability of data from trophic analyses performed between 2014 and 2016 in lower Uruguay River (Fig. 1). However, we believe that these species may represent good models of a more specialist (*M. obtusidens*) and a more generalist (*P. maculatus*) omnivore strategists, in regard to the feeding response to the invasive mussel.

We used preserved specimens from two collections—Facultad de Ciencias (Universidad de la República) and Museo Nacional de Historia Natural (MNHN), Montevideo, Uruguay— as well as present-day samples collected from sampling campaigns in lower Uruguay River (as detailed below). As pre-invasion samples, we used individuals collected in lower and middle Uruguay river for a period of approximately 20 years, right before the invasion was first reported in the Rio de la Plata basin (1970–1989).

As post invasion period in lower Uruguay river, we used fish collected posterior to the first record of *L. fortunei* in Uruguay River (2001–2016), from both, specimens deposited in collections and from contemporary samplings. Whenever the number of samples and its distribution in time allowed it, the post invasion period was arbitrarily divided in two periods—the early stages of the invasion (ca. first 10 years) and after the invasion was evident and ubiquitous (more than 10 years after the first report)—in an attempt to evaluate a differential effect during the first years of the invasion in comparison to a period when the invasion was truly consolidated. The fish and isotopic baseline samples used in the pre- and post-invasion comparisons were selected to match a similar spatial range within the river, belonging to the same localities in most of the cases or to very proximate ones (see Fig. 1). This way, we try to standardize as much as possible the conditions for the analysis and to avoid a potential spatial bias. Also, it was confirmed that body size of pre- and post-invasion individuals did not differ statistically (ANOVA $p > 0.05$, Table 1), precluding an effect of this factor on the reported results.

Dietary determinations

All guts extracted from conserved or fresh specimens were preserved in ethanol (95°) to be analysed under a 50X magnification Nikon SMZ745 binocular stereomicroscope in the lab. The dietary analysis consists in dissecting guts and determining the proportional contribution of each dietary item (in diet volume, in mm³), following standard procedures (Hyslop 1980). The items were classified to the lowest taxonomic level possible using taxonomic keys for the macroinvertebrates (Domínguez and Fernández 2009).

Samples for stable isotopes

A subsample of both fish species in each site was used for analysis of diet and stable isotopes of carbon (C) and nitrogen (N). For the isotopic analyses, we took samples of a fast turnover tissue (liver, representing a period of days to weeks; Sacramento et al. 2016) and a slower turnover tissue (flank muscle, representing a period of weeks to months; Sacramento et al. 2016) for each individual, in order to evaluate isotopic niche

Table 1 Trophic niche metrics estimations using diet data and stable isotopes data from muscle and liver for *M. obtusidens* and *P. maculatus* (Mean ± SD). Significantly different metrics, and those with higher of 90% of probability to differ in Bayesian analysis, are marked with bold and with different letters in the case of the multiple comparisons. Sample size (n) are provided between brackets

Sample type/trophic niche parameters	Pre-invasion (mean ± SD)	First Years of Invasion (mean ± SD)	Post-Invasion (mean ± SD)	Test parameters (degrees of freedom, F, X ² and p values)	Probability Pre > First years	Probability Pre > Post years
<i>Baseline</i>						
$\delta^{13}\text{C}$ Gastropoda	-26.3 ± 1.8 (A) (n=5)	-20.8 ± 0.4 (B) (n=4)	-22.5 ± 2.8 (B) (n=9)	ANOVA: F = 7.3 df _{res} = 15, p < 0.001		
$\delta^{15}\text{N}$ Gastropoda	11.88 ± 0.9 (n=5)	12.3 ± 1.0 (n=4)	11.83 ± 2.45 (n=9)	ANOVA: F = 0.12 df _{res} = 15, p = 0.85		
$\delta^{13}\text{C}$ L. fortunei		-29.4 ± 2.4 (n=9) (A)	-26.5 ± 1.0 (n=9) (B)	ANOVA: F = 10 df _{res} = 16, p = 0.04		
$\delta^{15}\text{N}$ L. fortunei		9.6 ± 1.4 (n=9) (A)	8.25 ± 0.71 (n=9) (B)	ANOVA: F = 14.5 df _{res} = 16, p = 0.001		
<i>Gut content analysis</i>						
<i>Megaleporinus obtusidens</i>	n = 34	n = 29	N = 44			
Body size (standard length)	25.5 ± 9.5 (ABC)	18.8 ± 9.6 (B)	31.0 ± 12.3 (C)	ANOVA: F = 10.0 df _{res} = 80, p < 0.001		
% Allochthonous diet	58.4 ± 21.0 (A)	7.3 ± 3.6 (B)	8.9 ± 5.0 (B)	K-W, X ² = 14.7 p < 0.001		
% Vegetal diet	71.5 ± 18.4 (A)	10.5 ± 6.3 (B)	11.5 ± 7.2 (B)	K-W, X ² = 17.4 p < 0.001		
Omnivory index	0.37 ± 0.37	0.37 ± 0.14	0.22 ± 0.21	ANOVA: F = 1.73 df _{res} = 78, p = 0.19		
Generalism index	1.8 ± 0.69 (AB)	2.4 ± 1.3 (A)	1.5 ± 0.57 (B)	K-W, X ² = 13.0 p = 0.001		
TP	2.6 ± 0.81 (A)	2.9 ± 0.11 (B)	3.0 ± 0.23 (B)	K-W, X ² = 6.8 p = 0.03		
<i>Pimelodus maculatus</i>	n = 10	n = 16 (all from group 1)				
Body size (standard length)	11.8 ± 4.8	nt	Group 1 = 13.5 ± 5.8; Group 2 = 12.5 ± 7.5	ANOVA: F = 1.0 df _{res} = 25, p = 0.38		

Table 1 (continued)

Sample type/trophic niche parameters	Pre-invasion (mean ± SD)	First Years of Invasion (mean ± SD)	Post-Invasion (mean ± SD)	Test parameters (degrees of freedom, F, X ² and p values)	Probability Pre > First years	Probability Pre > Post years > Post
% <i>Allochthonous diet</i>	0.57 ± 0.11	nt	0.34 ± 0.11	ANOVA: F = 3.1 df _{res} = 21, p = 0.09		
% Vegetal diet	0.28 ± 0.05	nt	0.23 ± 0.09	ANOVA: F = 1.2 df _{res} = 21, p = 0.27		
Omnivory index	0.84 ± 0.03	nt	0.62 ± 0.01	ANOVA: F = 8.7 df _{res} = 18, p = 0.008		
Generalism index	5.1 ± 1.5	nt	5.5 ± 13.8	M-W: W = 61.5, p = 0.94		
TP	3.04 ± 0.14	nt	3.11 ± 0.08	ANOVA: F = 0.09 df _{res} = 18, p = 0.76		
<i>Stable isotope analysis</i>						
<i>M. obtusidens muscle tissue</i>	n = 18	n = 31	N = 76			
δ ¹³ C	-25.5 ± 2.8 (AB)	-24.1 ± 3.1 (A)	-25.6 ± 2.1 (B)	ANOVA: F = 3.6 df _{res} = 121, p = 0.03	0.64	0.84
NR (omnivory)	1.01	0.86	0.41		0.99	0.99
CR (generalism)	2.8	3.9	0.99		0.99	0.94
SEAc (trophic diversity)	15.6 (8.1–23.3)	9.1 (6.1–12.5)	6.7 (5.1–8.1)		0.06	0.35
δ ¹⁵ N based TP	2.2 (2.0–2.5)	2.8 (2.3–3.1)	2.7 (2.4–3.1)			
<i>M. obtusidens liver tissue</i>						
δ ¹³ C	-24.0 ± 2.3 (AB)	-22.1 ± 2.9 (A)	-25.6 ± 1.3 (B)	ANOVA: F = 8.0 df _{res} = 46, p = 0.001	0.44	0.47
NR (omnivory)	0.32	0.46	0.2		0.01	0.99
CR (generalism)	0.44	3.5	0.04		0.76	0.93
SEAc (trophic diversity)	7.5(3.3–12.1)	5.6(3.5–7.8)	3.3(1.4–5.3)			
δ ¹⁵ N based TP	2.44 (2.0–2.9)	2.3 (1.5–2.7)	2.85 (2.2–3.2)		0.73	0.09

Table 1 (continued)

Sample type/trophic niche parameters	Pre-invasion (mean ± SD)	First Years of Invasion (mean ± SD)	Post-Invasion (mean ± SD)	Test parameters (degrees of freedom, F, X ² and p values)	Probability Pre > Post years	Probability First years > Post years
<i>P. maculatus muscle tissue</i>	<i>n</i> = 8		Group 1 <i>n</i> = 31; Group 2 <i>n</i> = 12			
δ ¹³ C	-25.15 ± 1.34 (A)	nt	Group 1 = -26.3 ± 1.84 (A); Group 2 = -28.24 ± 0.98 (B)	ANOVA: F = 9.9 df _{res} = 48; p < 0.001		
NR (omnivory)	2.32	nt	2.3		0.49	
CR (generalism)	1.44	nt	1.9		0.68	
SEAc (trophic diversity)	10.7 (4-18.7)	nt	Group 1 = 3.6 (2.3-5.0); Group 2 = 1.6 (0.8-2.6)		Group 1 = 0.99 Group 2 = 1.0	
δ ¹⁵ N based trophic position	2.1 (1.3-2.8)	nt	Group 1 = 3.02 (2.7-3.2); Group 2 = 2.0 (1.7-2.1)		Group 1 = 0.02 Group 2 = 0.85	

changes at two different temporal scales (e.g. Vander Zanden et al. 2015). However, stable isotopes from liver tissue were only obtained in *M. obtusidens* in sufficient numbers; thus, the study of isotopic niche from *P. maculatus* only includes muscle samples.

To serve as baseline in the estimation of trophic position using stable isotopes of N, it is usually recommended to use primary consumer molluscs abundant in the studied ecosystem, since they integrate larger temporal scales than other resources (Post 2002). Accordingly, here we used gastropods and bivalves for the isotopic baselines. As preserved mollusc samples were not available in collections, we used mollusc found intact inside preserved fish guts for pre-invasion individuals, as some previous works did (e.g. Gkenas et al. 2022). In total we obtained 5 samples from 5 different fish guts (individuals collected between 1974 and 1980) at two locations, one in the lower and one in the middle Uruguay river (Fig. 1). Each sample consisted in 1 to 5 pooled individuals of gastropods from the *Potamolithus* genus. We did not obtain native bivalves in good condition from fish guts, and thus, the isotopic baseline in the pre-invasion scenario consisted only in Gastropods. For the post invasion period, we used molluscs sampled from preserved guts (between 2000 and 2010) and also from fresh individuals collected in the field (between 2014 and 2016). In total, we analysed 26 samples: 13 of gastropods from the *Potamolithus* genus, each containing from 1 to 5 (pooled) individuals; and 13 containing from 1 to 5 (pooled) individuals of *L. fortunei* (Fig. 1). Following this, the modelling for trophic position estimation was made using one benthic baseline in the pre-invasion period and two baselines (one representing benthic chain and other representing the pelagic chain) in the post-invasion period, when fish started to consume *L. fortunei*. This modelling approach resembles the changes occurred in the ecosystems because no pelagic chain seemed to be used previous to the invasion of the golden mussel, as fish rarely consumed native bivalves before *L. fortunei* invasion.

Fresh samples for stable isotope analysis were rapidly frozen (-20 °C) and transported to the lab where they were cleaned and prepared according to standard procedures (Levin and Currin 2012). Samples for stable isotopes taken from formalin-preserved specimens were rinsed in water before being frozen until its analysis. All stable isotope samples were then oven-dried

(for 48 h. at 60 °C), weighed (0.5–1.5 mg), and sent for its analysis in a continuous flow isotope ratio mass spectrometer (IRMS), at UC Davis stable isotope facility, California, USA. The natural abundance of heavy and light C and N stable isotopes ($^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$), relative to the proportions of these isotopes in a standard (Vienna Pee Dee Belemnite rock and atmospheric N, respectively), was determined. As secondary isotopic reference materials the lab used Bovine liver, Nylon 5, Glutamic acid, Enriched Alanine, and Peach leaves. Average analytical precision based on routine analysis of laboratory standards was better than 0.1‰. Proportions are given as delta values ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, in ‰) following:

$$\delta^{13}\text{C} \text{ or } \delta^{15}\text{N} = \left(\frac{R_{\text{sampled}} - R_{\text{standard}}}{R_{\text{standard}}} \right) * 1000$$

As formalin preservation may affect $\delta^{13}\text{C}$, in the few cases where we used fresh samples, we applied a mathematical formula to correct for such effect (see González-Bergonzoni et al. 2015). Preserved consumers and baseline nitrogen values were not corrected for preservation because formalin preservation does not affect isotopic nitrogen ratio values (Edwards 2002; Sarakinos et al. 2002; González-Bergonzoni et al. 2015). Furthermore, stable isotope values of fish and invertebrates were lipid corrected –using the Eq. 3 in Post et al. (2007) for aquatic animals– in all the cases where C:N ratio was higher than 3.5 (see Post et al. 2007).

Gut morphometry metrics

Gut morphometry was only analysed for *M. obtusidens* due to the low sample size of preserved individuals of *P. maculatus* at museum collections. We recorded body length and weight of each individual, dissected its digestive organs, and measured stomach and intestinal lengths to the nearest mm, using a digital caliper. In addition, the stomach, intestine, and liver were oven-dried (48 Hs at 60 °C) and dry weight was recorded in a semi-analytical scale (0.0001 g).

Because the preservation of material may affect gut morphology we only used museum-preserved individuals in this analysis, i.e., we excluded all fresh-collected field specimens. We disregard a potential bias of different preservation time among samples

because all samples were preserved with a solution of 4% formalin for more than 5 years and preservation effects have been shown to stabilize in a few months (Edwards et al. 2002).

Data analysis

Diet based trophic niche metrics

Based on the relative volume of each prey item found in fish guts, we estimated the following trophic niche metrics: (i) trophic position (TP), estimated as $1 + \text{mean trophic position of preys}$ (extracted from Domínguez and Fernández (2009)); (ii) omnivory index, estimated as the standard deviation of the TP of preys; (iii) generalism index, consisting in prey richness (e.g. Lazzaro et al. 2009). Each of these metrics were estimated for each individual, which were then used as replicates in the pre- and post- invasion comparisons. Further, and also using individuals as replicates, we compared the relative volume of animal and vegetal items in the diet, and the relative volume of allochthonous (i.e. terrestrial) and autochthonous (aquatic) items, between pre- and post-invasion periods.

Isotopic metrics

Raw $\delta^{13}\text{C}$ values of baselines and consumers were compared between pre- and post-invasion periods. Then, we estimated, and compared between periods, the following isotopic niche metrics: (i) standard ellipse areas with correction for small sample size (SEAc), i.e., the area occupied in the $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ biplot indicating trophic diversity of species; (ii) the range in $\delta^{15}\text{N}$ values (NR), which is an indicator of omnivory and diversity of $\delta^{15}\text{N}$ in the diet; (iii) the range in $\delta^{13}\text{C}$ values (CR), which is an indicator of the diversity of the origin of food resources assimilated; and (iv) the trophic position, estimated using a single baseline (gastropods) Bayesian model in the pre-invasion scenario and two baselines (gastropods and bivalves) Bayesian model in the post-invasion scenario (Layman et al. 2007, 2012; Jackson et al. 2011, Quezada-Romegalli et al. 2018). For the analysis comparing standard ellipse areas we used the SIBER package in R, building a Bayesian model setting the parameters to 200,000 iterations,

10,000 burnin, 10 thins and 2 chains, and basing the probability test upon 10,000 ellipses (Jackson et al. 2011). For the analysis of trophic position, we used the package tRophicPosition in R, building a Bayesian model setting the parameters to 40,000 iterations, 10 thins and 2 chains, and a sample size per chain of 2,000 (Quezada- Romegalli et al. 2018). The trophic fractionation used was $3.4 \pm 0.98\text{‰}$ for N and $0.39 \pm 1.3\text{‰}$ for C (Post 2002), and the models

were set a lambda value of 2, as we use a baseline of primary consumers.

During preliminary data analysis of standard ellipse areas, we found two seemingly separate groups in the isotopic space of *P. maculatus* for the post-invasion period (see Figs. 2 and 3). Given that in this case the use of a single ellipse might violate modelling assumptions, we decided to divide these data into two groups. The first group (Group 1) was

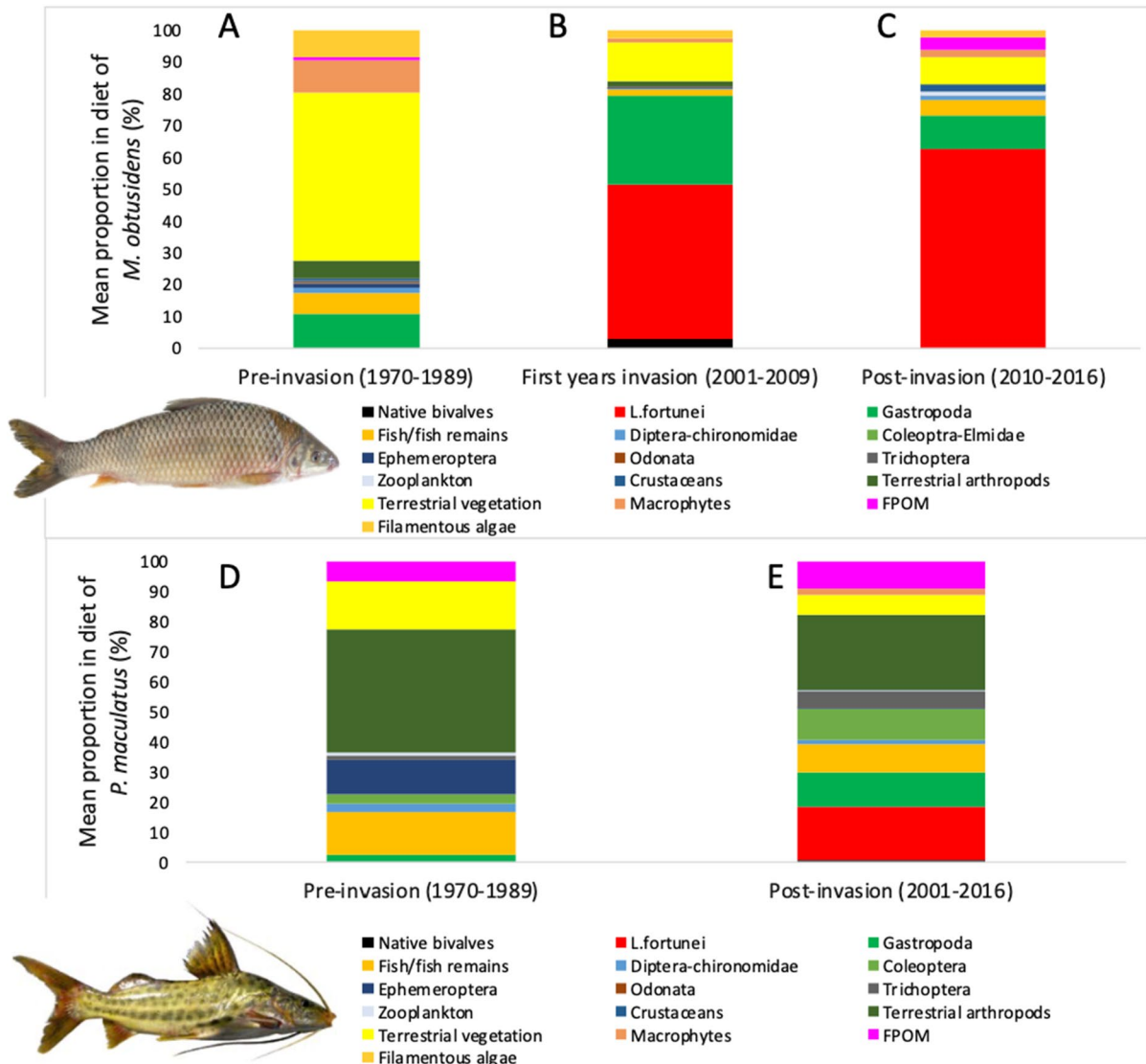


Fig. 2 Relative importance of different dietary items in diet of *M. obtusidens* and *P. maculatus*, before and after the invasion of *L. fortunei* in Uruguay River. A-C) Data from *M. obtusidens* preserved in fish collections from 1970 to 1990 (pre-invasion,

$n = 34$), 2001–2009 (first years of invasion, $n = 29$), and 2011–2016 (post-invasion, $n = 44$); D-E) Data from *P. maculatus* preserved in fish collections from 1970–1990 (pre-invasion, $n = 10$), and 2001–2016 (post-invasion, $n = 16$)

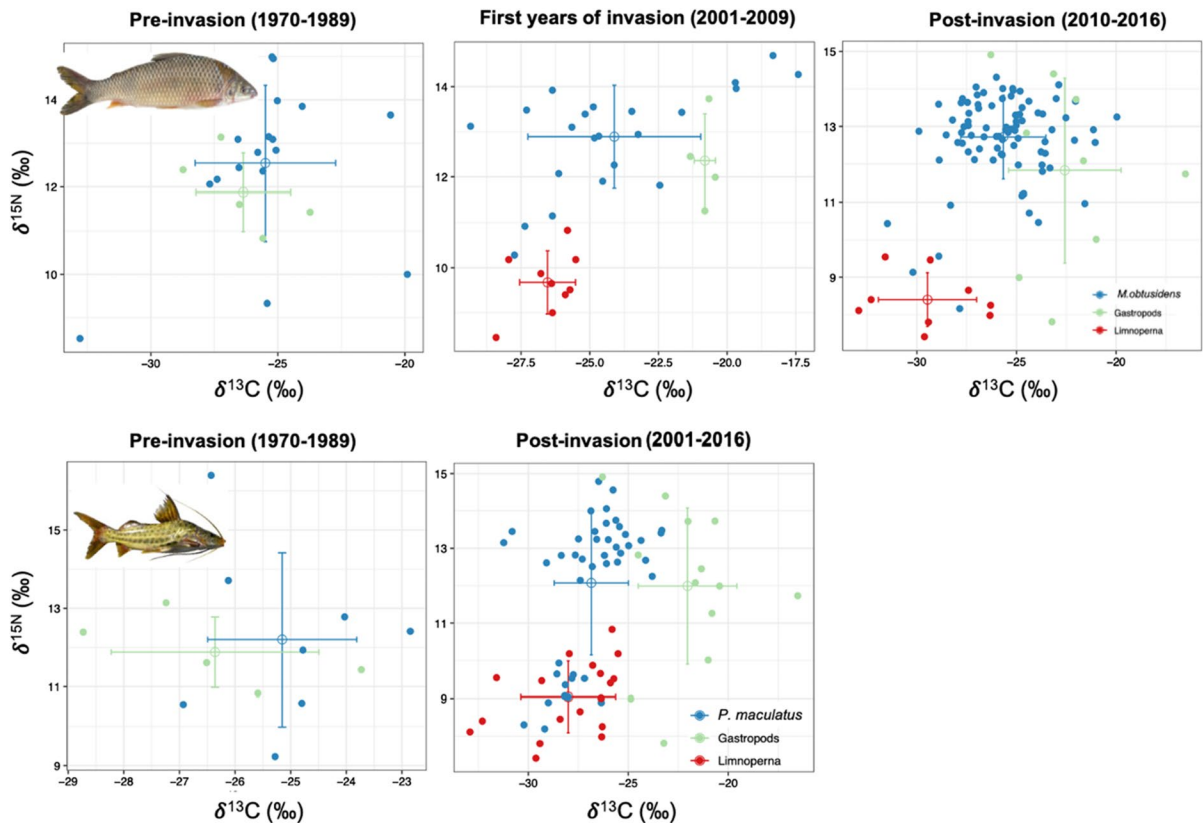


Fig. 3 Biplots of carbon and nitrogen stable isotope ratios, showing consumers (in blue) and baseline data (gastropods, in green) for each period, as well as the means and the standard deviations for each group. Above panels: data for *Megalepori-*

nus obtusidens; below panels: data for *Pimelodus maculatus*. *Limnoperna fortunei* samples in the post invasion periods are shown in red

composed of 31 individuals, collected from 2002 to 2016, with isotopic values for C coarsely between -23‰ and -31‰ and for N between 12‰ and 15‰ . The second group (Group 2) was composed by 12 individuals, collected in 2016, with isotopic values for C between -27‰ and -30‰ and for N between from 8‰ and 10‰ (i.e., clearly lower than those of Group 1). Upon checking at our database for potential determinants of this isotopic difference between the two groups, we found that all individuals were of similar size (Table 1) and, moreover, several individuals belonged to the same locality and sampling time (April 2016). Despite of this, we decided to include both groups separately in the analysis since it may represent individuals or populations with different ecological habits.

Statistical analysis

To disregard any bias caused by differences in body size, we compared body size between periods by using an ANOVA test. In addition, prior the analyses we tested for linear relationships between body size and the gut content metrics estimated, using each individual as a replicate. We thus confirmed that there were no differences in body size between pre-invasion and post invasion periods, neither for *M. obtusidens* nor for *P. maculatus*. Similarly, we did not find any significant relationship between body size and trophic position, omnivory and generalism indexes, and the % of vegetal matter or mussels in diet, for any of the two species in any of the periods analysed (Linear model p always >0.05). Accordingly, we disregard

any confounding effect of body size on the metrics that were considered.

Comparisons in trophic and isotopic niches between periods were evaluated using ANOVA tests ($\alpha=0.05$), followed by Tukey HSD post hoc test. The assumptions of homogeneity of variance and normal distribution were verified by visual inspection of potential patterns in the model residuals (Zuur et al. 2009). Whenever parametric test assumptions were not accomplished, we used non-parametric Kruskal Wallis and Mann–Whitney test (K–W; $\alpha=0.05$). Trophic position, SEAc, NR and CR metrics were estimated in a Bayesian framework and differences between periods were evaluated using packages SIBER (Jackson et al. 2011) and tRophicPosition (Quezada-Romegalli et al. 2018), in R free software (R Core team 2020). In these cases, we also estimated the exact probability of each metric being higher in the pre-invasion period compared to the post-invasion scenario (and whenever possible this last period was split in two, as explained above). For the case of trophic position estimates, we compared a single base model in the pre-invasion scenario with a two base full model in the post-invasion periods. Throughout the text we report all these probability values, but we focus our attention (and interpretation) mainly in those changes where the probability values exceed a 90% threshold (see Mwijage et al. 2021).

Changes in length–weight (or length–length) relationships between periods for the liver, the stomach and the intestine were assessed with generalized least square model tests (GLS, $\alpha=0.05$), using the function VarIdent to account for differences in variances between groups (Zuur et al. 2009). Two-way factorial models were adjusted including the effects of standard length, treatment (pre- and post-invasion) and its interaction. The relationship between standard length and digestive morphometric characteristics was evaluated adjusting generalized additive models (GAM, $\alpha=0.05$), with Varident whenever required (Zuur et al. 2009). All these analyses were carried with the package nlme, in R free software (R Core team 2020).

Results

Before the invasion of *L. fortunei*, the fish *M. obtusidens* had an omnivorous diet, with an important

consumption of vegetal material (63% in volume). Specifically, terrestrial vegetal matter, like tree leaves, fruits and seeds, accounted for 53% of its diet (in volume). Thus, this species seemed to be highly subsidized by allochthonous (i.e. terrestrial) resources. However, even during the first ten years of the invasion, *M. obtusidens* markedly decreased the consumption of vegetal matter (K–W; $X^2=17.4$, $p<0.001$) and allochthonous resources (K–W; $X^2=14.7$, $p<0.001$), while the consumption of *L. fortunei* markedly increased (Table 1, Fig. 2). As for the case of *P. maculatus*, it also included some vegetal material in its diet before the invasion (16% of diet volume), which consisted of terrestrial vegetation. The consumption of both terrestrial vegetation and terrestrial insects determined that this species heavily relied on allochthonous resources before golden mussel invasion (representing a total of 57% of diet volume). However, we did not find significant changes in the importance of vegetal (ANOVA; $F=1.2$ $df_{res}=21$, $p=0.27$) or allochthonous matter (ANOVA; $F=3.1$ $df_{res}=21$, $p=0.09$) in the diet of *P. maculatus* before and after the invasion. Nevertheless, a marginal p-value may suggest a tendency toward a lower relevance of allochthonous items after the invasion by *L. fortunei* (Table 1, Fig. 2).

In line with data from gut content, we found that *M. obtusidens* increased its trophic position immediately after the invasion (K–W, $X^2=6.8$, $p=0.03$, Table 1). In addition, generalism index for this species decreased between the early and the late period of the invasion (K–W, $X^2=13.0$, $p=0.001$), showing a trend for a higher specialization in *L. fortunei* in the more recent years (Table 1). By contrast, the index of omnivory did not change since the 70' decade (ANOVA; $F=1.73$, $df_{res}=78$, $p=0.19$), as this species incorporates prey from contrasting trophic levels (regardless of the aforementioned changes in its relative volume in diet). For *P. maculatus*, neither trophic position estimated from gut content analysis (ANOVA; $F=0.09$, $df_{res}=18$, $p=0.76$) nor generalism level (M–W; $W=61.5$, $p=0.94$) changed after the golden mussel's invasion (Table 1). However, *P. maculatus* decreased its index of omnivory during the post invasion period (ANOVA; $F=8.7$ $df=18$,

$p < 0.01$), as the consumption of prey from contrasting trophic level was reduced (Table 1, Fig. 2).

Carbon isotopic values in the baseline organisms (gastropods) shifted between periods, occurring more depleted values in the pre-invasion period than in the two periods after the invasion of *L. fortunei* (ANOVA; $F = 7.3$ $df_{res} = 15$, $p < 0.001$, Table 1, Fig. 3). However, $\delta^{15}N$ values of gastropods remain unchanged since the pre-invasion period (ANOVA; $F = 0.12$ $df_{res} = 15$, $p = 0.85$). *L. fortunei* $\delta^{13}C$ values became more enriched in the late post-invasion period than in the first years of invasion (ANOVA; $F = 10$ $df_{res} = 16$, $p = 0.04$, Table 1, Fig. 3), while $\delta^{15}N$ values decreased during the late post-invasion period (ANOVA; $F = 14.5$, $df_{res} = 16$, $p = 0.001$ Table 1, Fig. 3). Isotopic $\delta^{13}C$ values in muscle samples of *M. obtusidens* did not change significantly between the pre- and post-invasion periods, but it changed between the early and late scenarios with individuals collected during the last years becoming more depleted than those collected during the first years of the invasion (ANOVA; $F = 3.6$, $df_{res} = 121$, $p = 0.03$).

The same pattern was also observed for liver samples (ANOVA; $F = 8.0$, $df_{res} = 46$, $p = 0.001$, Table 1, Fig. 3). Regarding $\delta^{13}C$ in *P. maculatus* a change between pre- and post-invasion period was only observed for the Group 2 (see above). Individuals in this group had far more depleted values than both, pre-invasion individuals and (post-invasion) individuals in the Group (ANOVA; $F = 8.0$, $df_{res} = 46$, $p = 0.001$, Table 1, Fig. 3).

In *M. obtusidens*, the isotopic metrics based on muscle and liver tissue varied consistently with dietary-based results, as trophic position increased after the invasion. Specifically, the probability of having a higher trophic position in the later period after the invasion was 90% for the muscle and 86% for the liver. However, the strongest evidence regarding post-invasion increase in trophic position comes from Bayesian models for muscle tissue. Direct comparison of posterior distributions reveal a 94% probability of increase, with a trophic position shifting from 2.2 before invasion to 2.8 in the first years of invasion (Table 1, Fig. 4). Trophic

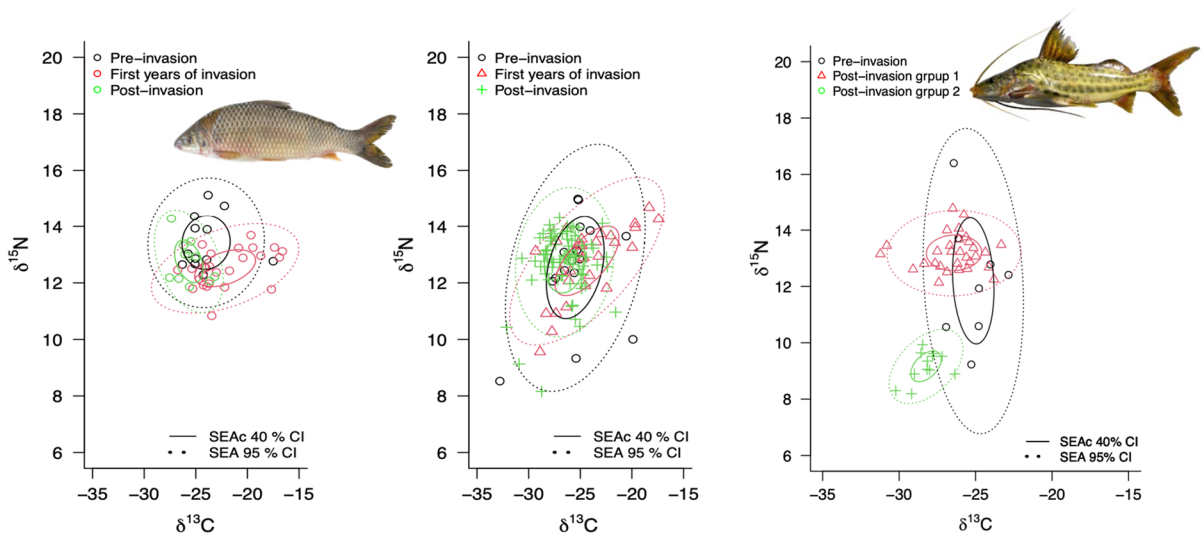


Fig. 4 Changes in isotopic niches of individuals of *M. obtusidens* and *P. maculatus* before and after the invasion of *L. fortunei* in lower Uruguay river, depicted as standard Bayesian ellipse areas (SEAc) occupied in the $\delta^{15}N$ versus $\delta^{13}C$ stable isotope biplot. Left panel): analysis based on liver samples of *M. obtusidens*; Middle panel) analysis based on muscle samples of *M. obtusidens*; Right panel) analysis based on muscle samples of *P. maculatus*. Standard ellipse areas around 40% of data and 95% ellipses are shown in black for the pre-invasion period ($n = 18$ for *M. obtusidens* and $n = 8$ for *P. maculatus*);

in red for the first years of invasion ($n = 31$ for *M. obtusidens*); and in green for the post-invasion period with widespread invasion ($n = 76$ for *M. obtusidens* and $n = 43$ for *P. maculatus*). Note that *P. maculatus* data points are divided in two groups: group 1 ($n = 31$) in red and group 2 ($n = 12$) in green (see details in the main text). A reduction of trophic diversity after the invasion is highly significant for liver and muscle tissues of *M. obtusidens* and for Group 1 of *P. maculatus* (see Table 1 for details)

diversity, estimated as SEAc, decreased immediately after the invasion. Direct comparison of posterior distributions reveal >90% probabilities of observing lower SEAc in both post-invasion periods compared to before invasion (except between pre-invasion and the first years post invasion for SEAc based on liver tissue samples, which showed a probability of decrease of 76%) (Table 1, Fig. 4). Finally, the value of NR, an indicator of omnivory, did not change among the three periods that were compared neither for the muscle nor for the liver (Table 1). Regarding to CR values in liver samples, an immediate increase after the invasion was observed, but a posterior reduction to pre-invasion levels occurred during the last years (Table 1). The consistency of

the results obtained from muscle and liver samples confirm that changes in the trophic behaviour of this species along time, did not depend on the temporal window at which the diet was assessed (i.e., turnover time from days to weeks previous to sampling for the liver and from weeks to months previous to sampling for the muscle).

Values of CR and NR for *P. maculatus* did not differ between pre- and post-invasion periods, indicating that these aspects of the isotopic niche were not affected by the presence of *L. fortunei* (Fig. 5). However, Bayesian estimates for SEAc and trophic position showed substantial changes after the invasion, although this result depends on the group of individuals analysed. While post-invasion SEAc (indicator of

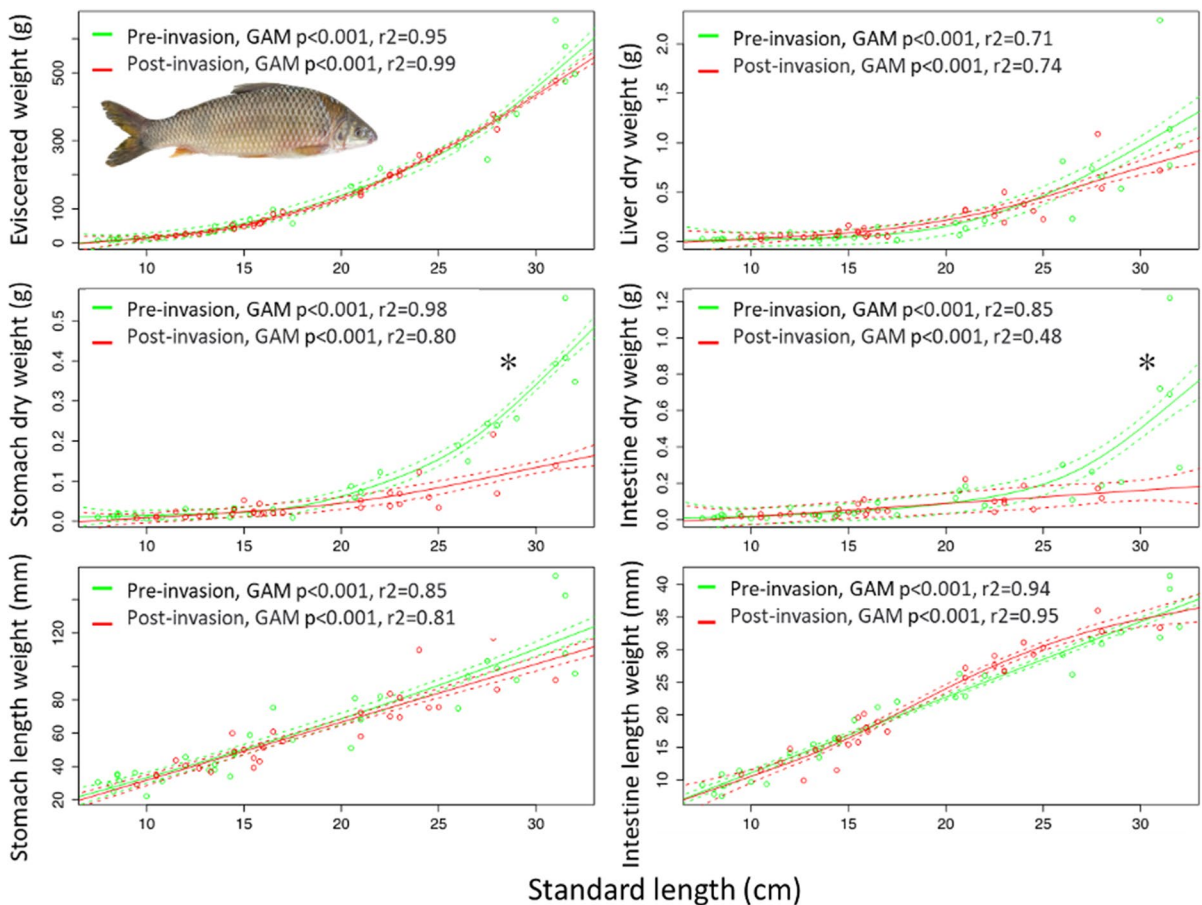


Fig. 5 Changes in fish body mass, liver dry mass, stomach dry mass, intestine dry mass, stomachal length and intestinal length, as a function of standard length, for *M. obtusidens* specimens analysed from fish collections, before (in green), and after (in red) the invasion of *L. fortunei* in Uruguay river.

All the relationships were fitted with GAM, and test statistics are provided inside each panel. An asterisk indicates significant changes in the relationship between pre- and post-invasion periods (two way factorial GLS $p < 0.05$, see Table 2 for statistics)

trophic diversity) decreased with respect to the pre-invasion period for both groups of individuals (the probability of decreased SEAc was more than 90% in all the cases, Table 1), trophic position increased after the invasion for one group (Group 1, with a probability of 98%) but tend to decrease for the other (Group 2, with a probability of 85%).

Regarding digestive morphometry, we observed significant changes for *M. obtusidens* in the dry weight of both, the stomach (GLS; length*treatment interaction: $t=-5.4$; $p<0.0001$) and the intestine (GLS; length*treatment interaction: $t=-3.7$; $p=0.005$). Specifically, we found a decrease in organ mass after the invasion (Table 2, Fig. 4). However, mussel invasion had no effect on the relationships between total length and: (i) total weight (GLS; length*treatment interaction: $t=-0.64$; $p=0.50$), (ii) liver dry weight (GLS; length*treatment interaction: $t=-1.3$; $p=0.17$), (iii) stomach length (GLS; length*treatment interaction: $t=-0.83$; $p=0.40$) and, (iv) intestinal length (GLS; length*treatment interaction: $t=1.8$; $p=0.07$) (Table 2, Fig. 4).

Discussion

The present study reveals differential changes in the diet and trophic niche of two ecological and commercially relevant omnivorous fish species inhabiting the Uruguay river, as a consequence of the introduction of the invasive Asian golden mussel (*L. fortunei*) by the early 2000'. The evidence here shows strong support for our predictions about a reduced trophic niche and increased trophic level

for both fish species as a consequence of the inclusion of *L. fortunei* in their diet. Moreover, our second prediction of a modified digestive physiology as a consequence of the dietary shift following the invasion was also supported for *M. obtusidens* (i.e., the only species that we were able to evaluate from this perspective). Our study reveals modifications in the digestive tract morphology of *M. obtusidens* (i.e. gut size reduction) which are concordant with the increased consumption of *L. fortunei* post invasion and may correspond to a physiological adaptation to maximize the energetic return of increased carnivory (Zaldúa and Naya 2014). In what follows, we will firstly discuss the changes occurred in dietary habits and trophic niche after the mussel invasion, comparing the findings between the two species in the light of similar studies on this topic, and also acknowledging potential sources of error from our methodology. Then, we will focus our discussion on the observed changes in gut morphology of *M. obtusidens*, to finally highlight our main conclusions and perspectives.

The analysis of historical changes in the diet of two *L. fortunei* consumers, *M. obtusidens* and *P. maculatus*, revealed a consistent fall in the direct contribution of terrestrial subsidies in general (although with a marginal p-value for *P. maculatus*), and of vegetal matter in particular for *M. obtusidens*. This shift in the use of direct allochthonous subsidies to autochthonous ones, supports the previous idea that *L. fortunei* may boost autochthonous production by being able to assimilate pelagic carbon directly into benthic food webs (Boltovskoy and Correa 2015). Furthermore, this shift surely modifies the natural flow of carbon,

Table 2 Changes in organs size in *M. obtusidens* after the invasion of *L. fortunei* in Uruguay River. GLS test were applied (using the function VarIdent whenever required) and significant differences are marked in bold. The relationship

between standard length and each morphometric variables was further modelled using GAM (also with the function Varident whenever required)

Parameter	Model type	Test parameters (t; p-value)		
		Pre-post invasion	Standard Length	Interaction
Eviscerated weight	Two-way factorial GLS-VarIdent	-0.43; 0.66	14.7; <0.001	-0.64; 0.50
Liver dry weight	Two-way factorial GLS-VarIdent	0.71; 0.48	6.7; <0.001	-1.3; 0.17
Stomach dry weight	Two-way factorial GLS-VarIdent	3.09; 0.003	10.3; <0.001	-5.4; <0.001
Intestine dry weight	Two-way factorial GLS-VarIdent	2.39; 0.02	5.8; <0.001	-3.7; 0.005
Stomach length	Two-way factorial GLS-VarIdent	0.35; 0.72	13.3; <0.001	-0.83; 0.40
Intestine length	Two-way factorial GLS	-1.45; 0.15	25.2; <0.001	<i>1.8; 0.07</i>

and other materials in food webs, such as diverse contaminants, which might be present in *L. fortunei* tissues (Amaral et al. 2019; Pazos et al. 2020). This might also be consistent with the shifts in carbon isotopic ratios observed in the biplots, where fish isotopic values appeared closer to that of mussel baselines after the invasion (see Fig. 3).

In *M. obtusidens* and *P. maculatus*, strong dietary changes towards an increased trophic position and a reduced trophic diversity, as well as a reduction in prey richness and generalism index for *M. obtusidens*, were detected. In other words, the specialization in mussel consumption caused a fall in the number of trophic links of *M. obtusidens* (index of generalism), probably because consumption of *L. fortunei* alone can meet fish physiological demands. The dietary changes were stronger, and provoked extensive changes in the trophic niche of *M. obtusidens* in comparison to *P. maculatus*, which agree with the fact that *P. maculatus* gut content metrics scarcely differed between periods. Moreover, one group of individuals of *P. maculatus* seemed to be unaffected in its trophic position after the invasion. This might be related to the trophic strategy of *M. obtusidens*, which usually consume large volumes of only one or a few resources (as seen by Penchaszadeh et al. (2000); López-Rodríguez et al. (2019); González Bergonzoni et al. (2020), and confirmed in this study). In contrast, *P. maculatus* strategy seems to include several weak trophic links, consuming lower proportions of many different dietary items (e.g., López Rodríguez et al. (2019); Silva et al. (2019); this study). However, the fact that the isotopic niche of *P. maculatus* in the post-invasion scenario seemed to split in two groups, potentially representing different ecological strategies, could be considered a consequence of the invasion. In this sense, it could be possible that a group of individuals choose the new resource, increasing its trophic position and reducing its trophic diversity (as observed for Group 1, which generally coincides with what was observed for *M. obtusidens*). However, other individuals might have not yet adapted to the consumption of the new resource presented in the system. Alternatively, the second group may also correspond to migratory individuals within the population arriving from a different habitat with contrasting isotopic baseline values, as further discussed below. Although the number of individuals here analysed

is low to demonstrate such pattern, this might be an interesting area for future research.

Behind this contrasting feeding strategies between *P. maculatus* and *M. obtusidens* lie contrasting gape morphological constrains for these two species. *M. obtusidens* belongs to the Anostomidae family of characins, known to bear powerful dentition that allows them to rip mussels apart from hard substrates and to crush them (Teixeira del Mello et al. 2011). By contrast, *P. maculatus* is a pimelodid catfish (Siluriformes, Pimelodidae) with no strong dentition on its mouth, and whose ability to break mussel shells is very limited (most times finding the entire mussel shells on their guts) (Teixeira del Mello et al. 2011). This fact probably allows *M. obtusidens* to specialize on feeding over mussels whenever its present, while individuals of *P. maculatus* only consuming those mussels that occurs in soft substrates or loosely attached to the colonies.

Obviously, the effect of a new food item is expected to be larger when it represents one of the few items preyed upon than if it only constitutes one more of several other items in the new predator diet. This agrees with the postulated stabilizing role of omnivores species with diverse weak interactions (McCann et al. 1998), as the species bearing these characteristics (*P. maculatus* in our study) did not largely shift its trophic niche after the invasion. Consequently, the magnitude of *L. fortunei* effect in food web structure may depend on the trophic niche properties of species composing the original food web. For example, in an assemblage dominated in species richness by more specialist omnivores, like *M. obtusidens*, the control over mussel abundances could be greater, mitigating its impacts over several ecosystem processes, while, in turn, favouring the abundances of these specialized omnivores. However, in an assemblage dominated by generalist omnivores bearing several weak feeding interactions, like *P. maculatus*, the control over mussel abundances would be partial, and mussel impacts over the rest of the ecosystem would be more widespread, although impacting with less strength on specific species and trophic roles. In this sense, the invasion of *L. fortunei* could also be studied as a particular case of anthropogenic modification in resource subsidies to ecosystems which indirectly alters whole food webs by modifying top-down controls as seen for other ecosystems (e.g. Riggi and Bommarco 2019).

A reduced diversity of links associated with a strong preference for a single new resource (as observed in the case of *M. obtusidens* after the invasion) could make food webs more vulnerable to changes, although this is a subject of debate (Schoener 1989; Woodward et al. 2005; Thompson et al. 2012). Furthermore, by preferentially selecting *L. fortunei*, other invertebrate prey may be released from predation pressure to some extent. This agrees with recent findings from an *in-situ* fish exclusion experiment deployed in the lower Uruguay river (Silva et al. 2020). In this experiment, fish access to artificial substrates colonized by native macroinvertebrates, but not extensively by *L. fortunei*, triggered a reduction in native macroinvertebrate densities in comparison to the substrates of fish exclusion modules, likely via direct predation (Silva et al. 2020). However, when the same comparison was made in a scenario of substrates being extensively colonized by *L. fortunei*, fish access to artificial substrates triggered a strong reduction in density of this mussel and an indirect increase in native macroinvertebrate densities in comparison to the substrates of fish exclusion modules (Silva et al. 2020).

The historical analysis using preserved fish, as done in this study, may bring unique and valuable information about trophic and ecological behaviour of species and communities in the past. However, as this framework is under development, several methodological constraints exist, causing that some results should be interpreted with caution. For instance, in our study the lack of preserved primary consumers implied that we had to use preserved molluscs found in fish guts for stable isotopes analyses. Besides the fact that only few samples could be extracted (and not all localities were represented), the use of baseline samples extracted from guts can be risky given to potential effects of digestion. Unfortunately, this was the only option in our case, and to minimize this effect as much as possible, we only used entire gastropods found in the anterior portion of the gut. On the other hand, using invertebrates from guts may have an advantage in terms of the certainty of using basal resources that were truly consumed by consumers. That is, those baseline organisms were effectively consumed by the study subjects for which we assume they represent the base of a trophic chain. In our study, the use of historical samples for stable

isotope analysis is further supported by the fact that both dietary and isotopic results generally match: the increase in the trophic position found by both approaches match the observation of a dietary shift from omnivory-herbivory to omnivory-carnivory. Noticeably, this is not always the case, and several factors can determine that results from stable isotope and dietary analysis strongly differ under some circumstances. For instance, if fish moves between different ecosystems with contrasting resources, or environmental conditions affecting resources isotope ratios, isotopic results might not match those obtained through gut contents analysis (Nielsen et al 2017). In this sense, we also acknowledge that the existence of two groups of isotopic niches in *P. maculatus* might correspond to individuals that have arrived from a different environment (with different resources and a contrasting baseline), as one of many potential alternative explanations. Furthermore, dietary indices are not always directly comparable to isotopic niche results. For instance, a fish diet can be frequently composed by a wide diversity of organisms, and this bear an elevated index of generalism. However, if all individuals behave similarly the isotopic niche can be narrow, suggesting a lower trophic diversity (Flaherty and Ben-David 2010).

As for changes in the gastrointestinal tract of *M. obtusidens*, stomach and intestine dry mass decreased with the inclusion of *L. fortunei* in diet during the post invasion period, which is consistent with the observed dietary increase in animal consumption. While herbivore species have larger digestive tracts to maximize the energy extraction from low nutritive foods, such as vegetal matter, in carnivore species smaller guts are enough to digest and absorb more easily digestible materials (Stevens and Hume 1998; Vidal et al. 2022). The observed reduction in gut mass could be due to micro-evolution and/or could represent a plastic response, and in any of the two cases it is considered a strategy that allow to optimize the energetic trade-off between energetic return from the food consumed and the cost of maintaining gut tissue (Zaldúa and Naya 2014). As a perspective it would be of interest to analyse if this digestive response is plastic (or not) and if changes in less labile traits, such as dentition and the strength of pharyngeal teeth, occur in response to the new food item with strong shells.

Besides its role as predator of *L. fortunei*, *M. obtusidens* represents one of the most important catches in lower Paraná and Uruguay river fisheries (Scarabotti et al. 2021). Thus, it is likely that *L. fortunei* invasion be also affecting growth, survival of juveniles and movements (if feeding migrations occur) in this fisheries stock. In this sense, we highlight that in the last 10-year period the catches of *M. obtusidens* have increased in most artisanal fisheries of the lower Uruguay river (Vidal et al. 2020), similar to the observed by Boltovskoy and Correa (2015) and Scarabotti et al. (2021) for different sections of the also invaded Paraná River. Although many climatic and anthropogenic factors might be causing this trend in fisheries (e.g. Rabuffetti et al. 2021), it is likely that the invasion of *L. fortunei* might also play a role in the observed change.

The information generated here represents a valuable example of the effect of invasive species reshaping food webs and affecting species involved in important ecosystem services, such as the provision of food by fisheries and/or the preservation of biodiversity. Moreover, the evidence shown by our study may assist in the prediction of food web changes occurred after *L. fortunei* invasion.

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Author contributions IGB and DEN participated in the conception and design of the study. All authors carried out the fieldwork. IG, NV and DEN carried out the lab work. IGB analysed the data and drafted the manuscript with the help of all authors. All authors gave final approval for publication.

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Declarations

Conflict of interest The authors declare no conflict of interests.

Availability of data and material The datasets generated during and/or analysed during the current study are available from the corresponding author upon request.

Code availability Does not apply.

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