Invasive crayfishes as a threat to freshwater bivalves: Interspecific differences and conservation implications

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HIGHLIGHTS

- Invasive crayfishes may threaten native freshwater bivalves.
- Predation of freshwater bivalves by invasive crayfishes was distinct among species.
- *Anodonta anatina* was the most preyed species.
- Future studies should assess predation of invasive crayfishes on freshwater bivalves.

GRAPHICAL ABSTRACT
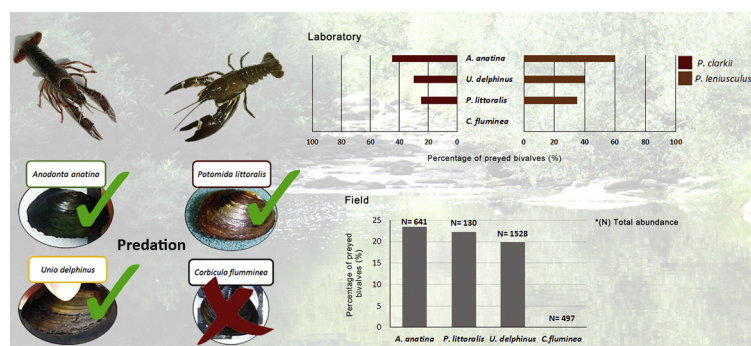
ABSTRACT

Freshwater bivalves have suffered major global declines, being the introduction of invasive alien species (IAS) an important, but not well studied, mechanism of threat. This study assessed the predator-prey relationship between two non-native crayfish species (*Procambarus clarkii* and *Pacifastacus leniusculus*) and three native (*Anodonta anatina*, *Potomida littoralis* and *Unio delphinus*) and one non-native (*Corbicula fluminea*) freshwater bivalve species through experiments in laboratory and validation under natural conditions (Sabor River basin, Portugal). All native bivalve species were preyed both in laboratory and in the field; however, both crayfish species were unable to prey *C. fluminea*. Predation was dependent on crayfish and bivalve species but was not affected neither by crayfish nor bivalve sizes. In the laboratory, the most preyed species by both crayfishes was *A. anatina*. On average, this species was preyed at least 12% more than other species, when crayfishes had a choice. Similar results were found in the field. We also found signs of competition between both crayfishes, being *P. clarkii* more dominant and aggressive as this species, on average, manipulated the bivalves 63.6% more times and 24:33 min longer than *P. leniusculus*, and initiated 55.8% more agnostic bouts. Our results support the idea that *P. clarkii* and *P. leniusculus* can affect native freshwater bivalves, but clear interspecific differences were detected. Both crayfishes may have direct and indirect impacts on bivalve populations by increasing mortality or by reducing their fitness. In addition, since both crayfishes do not prey *C. fluminea*, they offer this IAS

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another advantage over native bivalves. Given the widespread distribution of both *P. clarkii* and *P. leniusculus* and the threatened status of many freshwater bivalves, the dynamics and impacts of this relationship should be taken in account in the implementation of management measures devoted to the conservation of native freshwater bivalves.

1. Introduction

The introduction of invasive alien species (IAS) is among the most important, and sometimes irreversible, human-induced changes on ecosystems, being recognized as one of the biggest threats to biodiversity (Sala et al., 2000; Vitousek et al., 1997). IAS can have numerous impacts in terrestrial and aquatic ecosystems (Olden et al., 2004; Simberloff et al., 2013). For example, 69% of the established aquatic species introduced in six European countries have ecological impacts and these may include changes in biodiversity and ecosystem functioning, trophic interactions and ecosystems' physical properties (García-Berthou et al., 2005; Sousa et al., 2011; Gutiérrez et al., 2014). These changes have possible effects at individual, population, community and ecosystem levels, also leading to high economic losses (Simberloff et al., 2013; Simon and Townsend, 2003; Strayer, 2010; Vilà et al., 2010).

In Europe, numerous crayfish species have been introduced and they can be responsible for several ecological and economic impacts (Gherardi, 2006, Hobbs et al. 1989). Crayfishes can have profound effects in water quality, nutrient dynamics, decomposition and community structure by acting as ecosystem engineers (Carvalho et al., 2016; Creed Jr. and Reed, 2004; Johnson et al., 2010; Matsuzaki et al., 2009; Sousa et al., 2013).

The Signal crayfish *Pacifastacus leniusculus* (Dana, 1852) is a recognized successful invader (Henttonen and Huner, 1999). This species is native to north-western United States of America (USA) and south western Canada (Bondar et al. 2005; Henttonen and Huner, 1999). It was initially introduced in Europe (Sweden in the 1960s) for stocking purposes and as a commercial substitute of the crayfish species *Astacus astacus* (Linnaeus, 1758), which became almost extinct in Europe due to the crayfish plague (Gherardi, 2006). The successful establishment of this species encouraged the later introduction of the Louisiana crayfish, *Procambarus clarkii* (Girard, 1852), in Spain in the 1970s (Gherardi, 2006; Gutiérrez-Yurrita et al., 1999). *Procambarus clarkii* is listed within the top 10 invasive species in Europe with the highest number of impacts on ecosystem services (DAISIE database, 2018; Vilà et al., 2010). This species is native to north-eastern Mexico through south-central USA east to Florida (Gherardi, 2006; Henttonen and Huner, 1999; Hobbs et al., 1989). Both crayfish species spread throughout Europe with considerable success and are now well established. They are also present in Portugal where they occur in sympatry in Sabor River basin (Bernardo et al., 2011; Gutiérrez-Yurrita et al., 1999). *Pacifastacus leniusculus* and *P. clarkii* are known to have a highly plastic diet as they are omnivorous and display generalist and opportunistic feeding habits (Guan and Wiles, 1998; Gutiérrez-Yurrita et al., 1998). *Procambarus clarkii* and *P. leniusculus* may consume large quantities of detritus (e.g. leaf litter) and plants, but they also feed on other animals such as amphibians, fishes, invertebrates, including other crayfishes (Axelsson et al., 1997; Gherardi et al., 2001; Gherardi, 2006; Guan and Wiles, 1998). Their feeding habits are known to have direct and indirect impacts on other species and thus affecting the invaded ecosystems (Axelsson et al., 1997; Gherardi, 2006; Nyström et al., 1996). Even though *P. clarkii* and *P. leniusculus* possible impacts and co-existence in many freshwater ecosystems are recognized, little is known about their role as predators of invertebrate species, such as freshwater bivalves.

Bivalves play a major ecological role in freshwater ecosystems (Howard and Cuffey, 2006; Lopes-Lima et al., 2018; Vaughn and Hakenkamp, 2001; Vaughn et al., 2008); however, in the last decades, freshwater bivalves, especially freshwater mussels from the Unionida order, have suffered a major global decline and are among the most threatened faunal groups in the planet (Lopes-Lima et al., 2014a, 2018; Williams et al., 1993). Since crayfishes can prey on bivalves, the recent introduction of several crayfish species is a possible important threat to the survival of these animals. Nevertheless, very few studies assessed bivalve predation by invasive crayfishes (e.g. Klocker and Strayer, 2004; Machida and Akiyama, 2013) and so the possible consequences of these introductions remain speculative and almost ignored. Given this knowledge gap, the main aim of this study was to describe the potential impacts of *P. clarkii* and *P. leniusculus* on four freshwater bivalve species (the mussels *Anodonta anatina*, *Potomida littoralis* and *Unio delphinus*; and the clam *Corbicula fluminea*) by assessing: i) if predator-prey interactions occur between the invasive crayfishes and bivalves and at what intensity; ii) if some bivalve species are more prone to predation than others; iii) if predation levels depend on predator and prey size; and iv) if *P. clarkii* and *P. leniusculus* compete for the bivalves as a prey. Using laboratory experiments and validations in natural conditions, the null hypothesis of the present study was that the crayfishes have no preference for freshwater bivalve species as prey and both crayfish should have a similar behaviour. However, we predicted that species with thicker shells (*P. littoralis* and *C. fluminea*) will be less prone to predation and *P. clarkii* will present a more aggressive behaviour than *P. leniusculus*.

2. Material and methods

2.1. Study area and animals' collection and maintenance

Sabor River has its source in Zamora province (Spain) and enters in Portugal by crossing the Montesinho mountain (Bragança). This river is a tributary of Douro River and has a wide range of environmental conditions: elevation range between 100 and 1500 m, total annual precipitation between 443 and 1163 mm, mean annual temperature between 6.9 and 15.6 °C. The flow regime in the Sabor River basin is highly seasonal, having some dried streams or disconnected pools during the summer (Filipe et al., 2017). A large dam was built recently in the lower Sabor River and started to operate in 2016. The basin has overall good ecological quality although some problems related with organic pollution and regulation of river flow have arisen over the last years (Sousa et al., 2012).

Throughout this work, three native (*A. anatina*, *P. littoralis* and *U. delphinus*) and one non-native (*C. fluminea*) bivalve species were studied. The four bivalve species have a sympatric distribution in the Sabor River basin also co-existing with the invasive crayfish species *P. leniusculus* and *P. clarkii* (Filipe et al., 2017; Sousa et al., 2012).

Bivalves were collected in the Sabor River basin by two researchers using snorkelling. Specimens were found visually or by searching through the bottom with hands. Both crayfish species were collected in the Sabor River basin by consistently placing several small baited traps ($50 \times 30 \times 20$ cm; 0.5 cm mesh) in the river bottom for 24 h. Only mature males were used in the laboratory experiments in order to minimize possible sex bias in the assessment of the predatory behaviour.

After collection, organisms were immediately transported to the laboratory. Bivalves were maintained in aquariums ($60 \times 30 \times 30$ cm) with

Table 1
Size range of the specimens of each bivalve and crayfish species used in the laboratory experiments.

Bivalve species	Size range (mm)
Corbicula fluminea	15–37
Anodonta anatina	35–126
Potomida littoralis	44–78
Unio delphinus	36–97
Crayfish species	
Pacifastacus leniusculus	74–110
Procambarus clarkii	71–120

20 L of water under aeration and fed on microalgae. The specimens of each crayfish species were maintained in separate aquariums ($60 \times 30 \times 30$ cm) in individual small cages with filtrated water under aeration and fed daily with freshwater fish food sticks. All organisms were always maintained under controlled temperature (15 °C) and photoperiod (12 h in the dark and 12 h with light).

2.2. Laboratory experiments

Permits to conduct laboratory experiments and field surveys were obtained from the Portuguese Conservation Governmental Authority (reference: 203 to 206/2017/CAPT). All ethical requisites were accomplished, being the native animals used in the laboratory studies released in the collection sites after the experiments.

For each experiment animals were placed in plastic containers with 3 cm of fine sand previously washed to promote the interactions between crayfishes and bivalves, and offering crayfish the perfect scenario for predation, but allow bivalves to partly bury in the sediments. The containers were filled with 10 L of water and maintained under controlled temperature (15 °C) and the experiments ran for 72 h. Specimens were only used once and their size was measured (Table 1).

Before the experiments, crayfishes were starved for 3 days to promote their need for nutrition and reduce the effect of previous feeding condition. At the end of each experiment (see below), predation was assessed by checking for the presence of characteristic predation marks on bivalve shells (see Fig. 1A and Machida and Akiyama, 2013) and by visually estimating the percentage of missing shell due to predation comparing to the total shell area.

2.3. Predation experiments

The first experiment (Fig. 1B) aimed to determine if: i) *P. leniusculus* and *P. clarkii* recognized the bivalve species as prey; ii) predation success (whether crayfishes were able to prey on bivalves or not) was dependent on bivalve species and/or the crayfish species; and iii) predation was size dependent.

For this purpose, a single crayfish was placed in a plastic container with a single specimen of one of the four bivalve species (Fig. 1B). Each bivalve-crayfish combination was replicated 20 times ($N = 160$).

2.4. Prey preference

The second experiment (Fig. 1C) aimed to determine if *P. leniusculus* and *P. clarkii* prefer to prey on certain bivalve species. For this, one crayfish of each species was placed on a plastic container with four bivalves (one specimen of each of the four bivalve species; Fig. 1C). Each treatment was replicated 10 times ($N = 20$ for both crayfish species).

2.5. Crayfish competition

The third experiment (Fig. 1D) aimed to determine possible competition between *P. leniusculus* and *P. clarkii* for available prey. For this, one specimen of each crayfish species was placed in a plastic container with one specimen of *A. anatina* (Fig. 1D). The choice of *A. anatina* was based on the results from the two earlier experiments as this species was the most preyed bivalve by both crayfish species (see results). The experiment was replicated 10 times ($N = 10$) and recorded using a webcam (HP® WebCam HD 2300) to assess crayfish behaviour. The time of beginning and ending of all interactions was registered to determine bivalve handling time, the duration of aggressive interactions, and to assess the number of times each of these interactions occurred.

2.6. Sampling and in situ validation

In order to validate the results gathered in the laboratory experiments we performed a field survey in Sabor River basin (North-eastern Portugal) (Fig. 1E).

To assess if predation occurs in the natural environment, a total of 51 sites were sampled during July 2017, 45 in Sabor River and 6 in its major tributary, the Maçãs River (Fig. 2). For each site, and for freshwater bivalve ecological characterization, a river stretch with a minimum of 100 m was surveyed covering the maximum of different habitats as possible (i.e. riffles and pools, banks and center of the channel). These surveys were always performed by a minimum of two researchers using snorkeling and freshwater bivalves were found visually or by hand-searching through the bottom when visibility was low. A total of four replicates lasting 15 min were performed totalizing 60 min of surveyed time per site. In each site, bivalves were collected, including empty shells, and identified to the species level, their size was measured and the percentage of predation by crayfish was estimated using the same methodology as described above for the laboratory experiments.

The abundance of both crayfish species was assessed in 15 of the 51 sites surveyed for bivalves' characterization during August 2017 (Fig. 2). These 15 sites were located along the entire gradient of the Sabor River not subjected to the reservoir influence in an attempt to cover all the river section surveyed for bivalves. Crayfishes were captured by placing 10 funnel traps baited with dead fish, five rectangular ($50 \times 30 \times 20$ cm; 0.5 cm mesh) and five cylindrical (43 cm diameter; 22 cm height; 1.5 cm mesh), per site for 24 h. The crayfishes were identified to the species level, their sex was determined, and their size was measured.
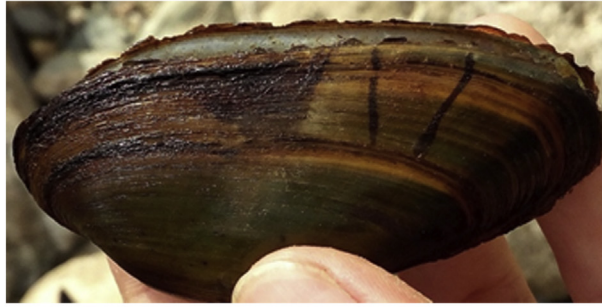
2.7. Statistical analysis

All statistical analyses performed were preceded by Shapiro-Wilk test to determine if data had a Gaussian distribution and by the Bartlett test to verify the homogeneity of variance. When data failed these assumptions, we used non-parametric tests.
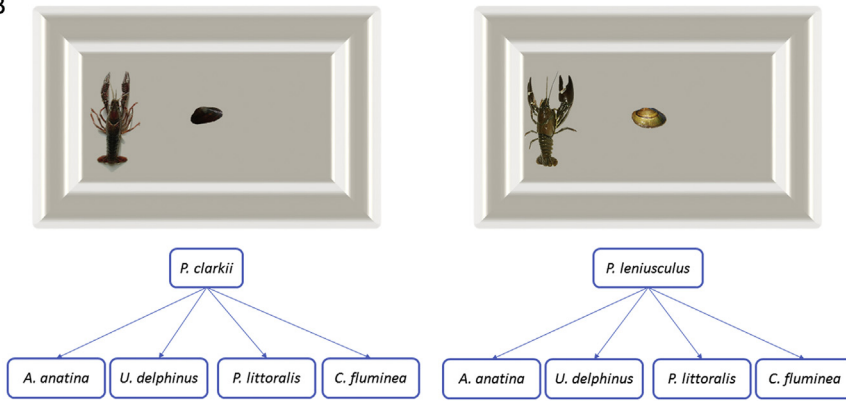
In the first experiment a 2-way permutational univariate analysis of variance (PERMANOVA, Anderson, 2001) (error type-III), with crayfish (2 levels: *P. clarkii* and *P. leniusculus*) and bivalve species (4 levels: *A. anatina*, *U. delphinus*, *P. littoralis* and *C. fluminea*) as fixed factors and percentage of shell loss as response variable, was used to assess the effect of predator and/or prey species on bivalve predation. Pairwise comparisons were used to analyse how predation varied between bivalve species. Pearson's chi-squared and Fisher's exact test of independence were used to test if the percentage of preyed bivalves was significantly different between *P. clarkii* and *P. leniusculus* and between bivalve species. In addition, a Mann Whitney U and Krustal-Wallis tests were

Fig. 1. (A) Crayfish predation marks on the shell of *Unio delphinus* dead specimen. Schematic representation of (B) the predation experiments, in which each bivalve species was tested as prey for each crayfish species (only two specimens were placed in each plastic container - one bivalve and one crayfish); (C) the prey preference experiments, in which five specimens were placed in each plastic container (one bivalve of each of the four studied species and one crayfish); (D) the competition experiments, where each plastic container had three specimens (one specimen of each of the two crayfish species and one specimen of *Anodonta anatina*); (E) the field validation methodology.
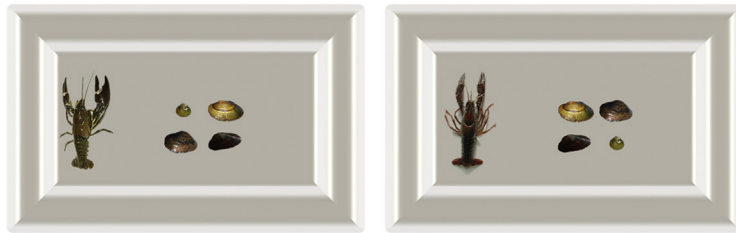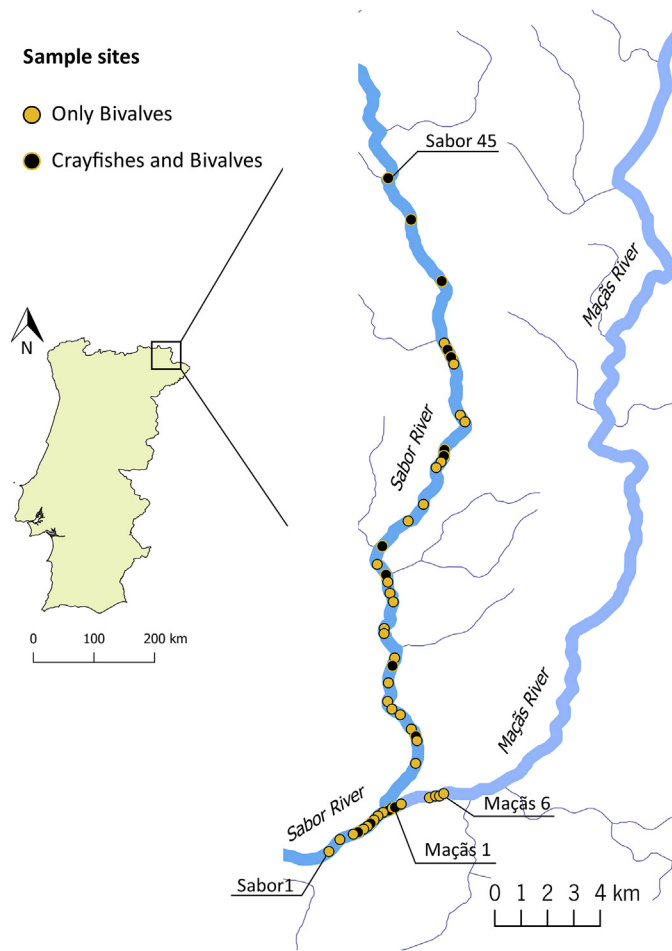
A

B

C

D

E

Fig. 2. Map showing the sampling site locations in Sabor and Maçãs Rivers.

Table 2
Summary of the PERMANOVA results on the effect of crayfish and bivalve species on bivalve shells predation.

Parameter	Effect	df	SS	MS	Pseudo F	P
Shell consumption	Crayfish species	1	44.3	44.3	3.1636	0.0201
	Bivalve species	3	79.76	26.6	1.8982	0.0486
	Crayfish species × bivalve species	3	48.6	16.2	1.1567	0.3392
	Error	152	2128.9	14.0		

and the percentage of preyed bivalves. As *P. littoralis* overall abundance was low and its spatial distribution very restricted, no statistical analyses were done with this species.

All PERMANOVAs were performed with 9999 permutations on the basis of Euclidean distances (Anderson, 2001). PERMANOVAs were done with PRIMER 6 (Primer-E, UK) for Windows. All other analyses were done with R software 3.2.2 for Windows (R Development Core Team, 2015).

3. Results

3.1. Experiment 1: bivalve predation

Procambarus clarkii and *P. leniusculus* preyed on all bivalve species except *C. fluminea*. Predation was dependent on crayfish and bivalve species and there was no interaction between these two factors (PERMANOVA, Table 2, Fig. 3A). Despite this, pairwise comparisons showed significant differences only between the native bivalve species and *C. fluminea*. On average, *P. leniusculus* predated a higher percentage of bivalve shells than *P. clarkii* (Fig. 3A). One specimen of *U. delphinus* was killed and had 44.4% of its shell preyed during the experiment by a signal crayfish. Besides this unusual high value, the maximum shell predation by *P. leniusculus* was 5.0% for *A. anatina*, 7.0% for *U. delphinus* and 6.5% for *P. littoralis*. The maximum shell predation by *P. clarkii* was 10.0% for *A. anatina*, 5.0% for *U. delphinus* and 2.0% for *P. littoralis*.

Considering only preyed bivalves, the average percentage of shell predation was 2.7% in treatments with *P. leniusculus* and 2.6% with *P. clarkii* for *A. anatina*; 9.0% and 1.5% for *U. delphinus*; and 3.0% and 1.0% for *P. littoralis*, respectively (Fig. 3B). On average, *P. leniusculus* predated a higher percentage of shell than *P. clarkii*, but significant differences were only found regarding *U. delphinus* (Mann-Whitney *U* test, U = 8.00, $p = 0.036$). The percentage of bivalve shell predation was not significantly different between bivalve species regardless the crayfish species (Kruskal-Wallis test, $p > 0.05$).

Regarding the percentage of preyed individuals by both crayfish species *A. anatina* presented the highest values, followed by *U. delphinus*, *P. littoralis* and *C. fluminea* (Table 3). Differences between both crayfishes were not significant (Pearson's chi-squared test, $p > 0.05$).

Regarding the predation status (i.e. preyed or not preyed), no significant differences were found considering the sizes of the animals and thus, the success of predation did not depend on crayfish nor bivalve sizes (Welch test, $p > 0.05$). In addition, predation success did not depend on crayfish or bivalve sizes, regardless of crayfish and bivalve species (Spearman's correlation, $p > 0.05$) (Fig. S1). When only considering bivalves that were preyed, results showed that neither crayfish (Fig. S2A) nor bivalve (Fig. S2B) sizes affected the percentage of shell predation (Spearman's correlation, $p > 0.05$).

performed to, respectively, assess if the crayfishes predated different percentage of preyed bivalves' shells and if that percentage was different between bivalve species. Welch's *t*-test was used to evaluate if predation success depends on bivalve and crayfish sizes, while Spearman's correlations were used to assess the relationship between the percentage of predation and size of the animals.

In the second experiment, a Pearson's chi-squared test (Zar, 2010) was performed between the expected and observed percentage of preyed bivalves when crayfishes had a choice.

In the third experiment, the differences between *P. clarkii* and *P. leniusculus* on bivalve handling time, percentage of times the bivalve was handled were examined using Wilcoxon matched-pairs signed ranks tests, and differences on the percentage of initiated agonistics bouts were assessed with aa paired *t*-test.

Data collected in the field was analysed using PERMANOVAs (error type-III), with sampling site (24 levels: Sabor 5, Sabor 12 – Sabor 22, Sabor 25 – Sabor 32, Sabor 36, Sabor 39, Sabor 41, Sabor 44) and bivalve species (2 levels: *A. anatina* and *U. delphinus*) as fixed factors and percentage of shell loss as response variable, to examine the effect of site and bivalve species predation. Additional permutational pairwise comparisons were used to assess which bivalve species was the most preyed within each site and which sites presented higher predation for each bivalve species. Spearman's correlations were used to test the relationship between the percentage of preyed bivalves and crayfish abundance and sex. A Wilcoxon matched-pairs signed ranks test and paired *t*-tests were performed to assess differences in abundance between crayfish species and differences in the abundance of female and male crayfishes. Finally, a Spearman's correlation was used to test the association of sex ratio

Table 3
Percentage of preyed bivalves by *Pacifastacus leniusculus* and *Procambarus clarkii*.

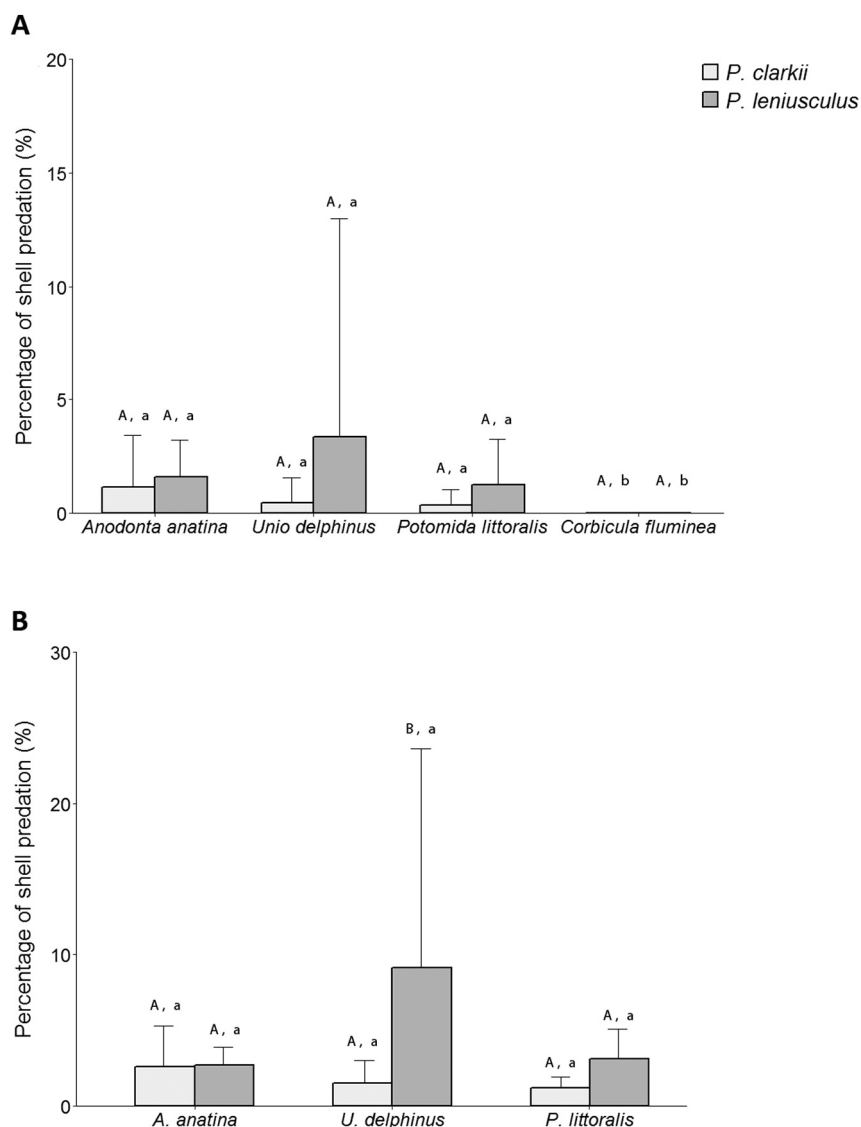|	Percentage of individuals preyed (%)			
	A. anatina	*U. delphinus*	*P. littoralis*	*C. fluminea*
Pacifastacus leniusculus	60	40	35	0
Procambarus clarkii	45	30	25	0

Fig. 3. $\bar{x}$ + SD percentage of shell predation of *Anodonta anatina*, *Unio delphinus*, *Potomida littoralis* and *Corbicula fluminea* by *Procambarus clarkii* and *Pacifastacus leniusculus* considering (A) all bivalves and (B) only preyed bivalves. Different capital letters indicate significant differences in predation between crayfish species and different lowercase letters indicate significant differences in predation between bivalve species.

3.2. Experiment 2: prey preference

When given a choice, *P. clarkii* and *P. leniusculus* preyed on the same bivalve species as in the first experiment. The percentages of preyed bivalves (Table 4) was different than the expected if crayfishes had no choice (Pearson's chi-squared test: *P. leniusculus*: $\chi^2(2) = 38.44$, $p < 0.001$; *P. clarkii*: $\chi^2(2) = 48.17$, $p < 0.001$), being *A. anatina* the most preyed bivalve species by both crayfish species.

3.3. Experiment 3: crayfish competition

When sharing the same space and prey, *P clarkii* was found to handle the bivalve significantly longer than *P. leniusculus* (Wilcoxon matched-pairs signed ranks test, W = 35.00, $p = 0.039$, N = 10)(Fig. 4A). On average, and in 72 h, *P. clarkii* handled bivalves for 27:43 min while *P. leniusculus* did it only for 3:10 min. During the experiments, two Louisiana crayfishes surpassed two hours of handling whereas their opponents handled those bivalves for, approximately, 14 and 6 min. In one replicate none of the crayfishes handled the bivalve and the signal crayfish also did not handle the bivalve in other three replicates.

Procambarus clarkii was found to manipulate bivalves a number of times significantly higher (63.6% on average) than *P. leniusculus* (Wilcoxon matched-pairs signed ranks test, W = 50.00, $p = 0.008$, N = 10) (Fig. 4B).

Procambarus clarkii was also the most aggressive crayfish species, initiating more agonistic bouts than *P. leniusculus* (Paired *t*-test, t(9) = 6.33, $p < 0.001$) (Fig. 4C). On average, the Louisiana crayfish initiated 77.9% of the fights, which translates to 55.8% more initiated agonistic bouts than the signal crayfish. There was one case where *P. leniusculus* initiated 50.0% of the agonistic bouts but on average this species initiated only 22.1% of the aggressive interactions.

3.4. In situ validation

Throughout the 51 sampling sites in Sabor River basin, 2800 (153 dead and preyed, and 2647 alive) bivalve specimens were found being 1531 identified as *U. delphinus*, 642 as *A. anatina*, 130 as *P. littoralis*, and 497 as *C. fluminea* (Fig. 5A). Native bivalves were found in all sampling sites except sites 2, 33 and 34 in Sabor River and sites 4, 5 and 6 in Maças River. Besides being the most abundant species, *U. delphinus* was

Table 4
Average percentage (%) of preyed bivalves (*Anodonta anatina*, *Unio delphinus*, *Potomida littoralis* and *Corbicula fluminea*) by *Pacifastacus leniusculus* and *Procambarus clarkii* when having prey as a choice.

	Mean of preyed individuals' percentage (%)			
	A. anatina	*U. delphinus*	*P. littoralis*	*C. fluminea*
Pacifastacus leniusculus	53.33	13.33	23.33	0
Procambarus clarkii	51.67	39.58	6.67	0

also the most widespread, being present in 45 of the 51 sampling sites. *A. anatina* was found in 36 sites and *P. littoralis* was present in only five sites (Sabor River sites 7, 10 and 12 and Maçãs River sites 1 and 3). The invasive clam *C. fluminea* was found in 14 sites, which include all sites in Maçãs River and sites 4, 6, 7, 8, 9, 10, 11 and 20 in Sabor River.

Regarding the crayfish sampling, in total 1320 crayfishes were collected and 92.1% were identified as *P. leniusculus* and 7.9% as *P. clarkii*, being *P. leniusculus* significantly more abundant than *P. clarkii* (Wilcoxon matched-pairs signed ranks test, $W = 75.00$, $p = 0.016$, $N = 15$). Despite this, the signal crayfish was not found in four of the 15 surveyed sites, while *P. clarkii* was found in all sampling sites except one (Fig. 5B). There were no significant differences between the number of female and

male crayfishes (Paired *t*-test, $p > 0.05$); however, when analysing each crayfish species individually differences were found. Average female signal crayfish abundance was 5.8% higher than its male counterpart (Paired *t*-test, $t(14) = 2.37$, $p = 0.033$), while in the case of the Louisiana crayfish, males were 1.78% more abundant than females (Wilcoxon matched-pairs signed ranks test, $W = 61.00$, $p = 0.049$).

No predation marks were found in any of *C. fluminea* specimens. On the other hand, all native species were found to be preyed by crayfishes (Fig. 5A). In addition, 156 of the native bivalves collected were found dead and presented characteristic marks of crayfish predation (see Fig. 1A and Machida and Akiyama, 2013). The percentage of shell predation of native bivalves varied between 1.0% and 45.0% for *A. anatina* (Fig. 6), 1.0% and 40.0% for *U. delphinus* (Fig. 6), and 1.0% and 10.0% for *P. littoralis* (data not shown). *Potomida littoralis* only showed signs of predation in Maçãs River site 1, which was the site with higher abundance of this species. In this site, 123 *P. littoralis* specimens were collected, 22.7% presented predation marks and the average percentage of shell predation was 3.8%. All the collected bivalves with >15.0% of their shell preyed were found dead except one *U. delphinus* specimen that was found alive with 25.0% of its shell preyed.

When comparing sites where both *U. delphinus* and *A. anatina* were present, results showed that predation was dependent on the river site
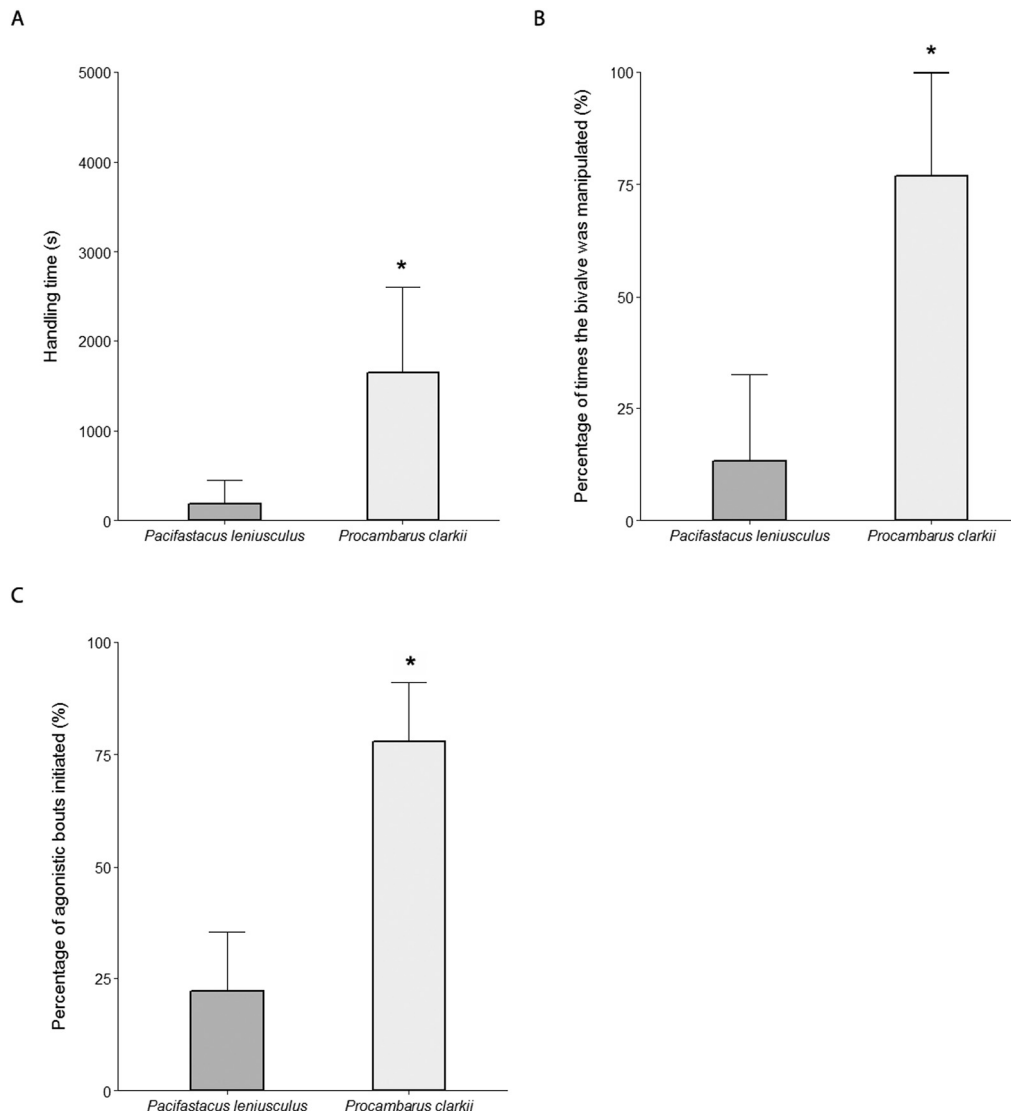

Fig. 4. $\bar{x}$ + SD of (A) bivalve handling time, (B) the percentage of times the bivalve was manipulated and (C) the percentage of times agonistic bouts were initiated by *Pacifastacus leniusculus* and *Procambarus clarkii* when sharing the same space and prey. Asterisk (*) show a significant difference between groups ($p < 005$).
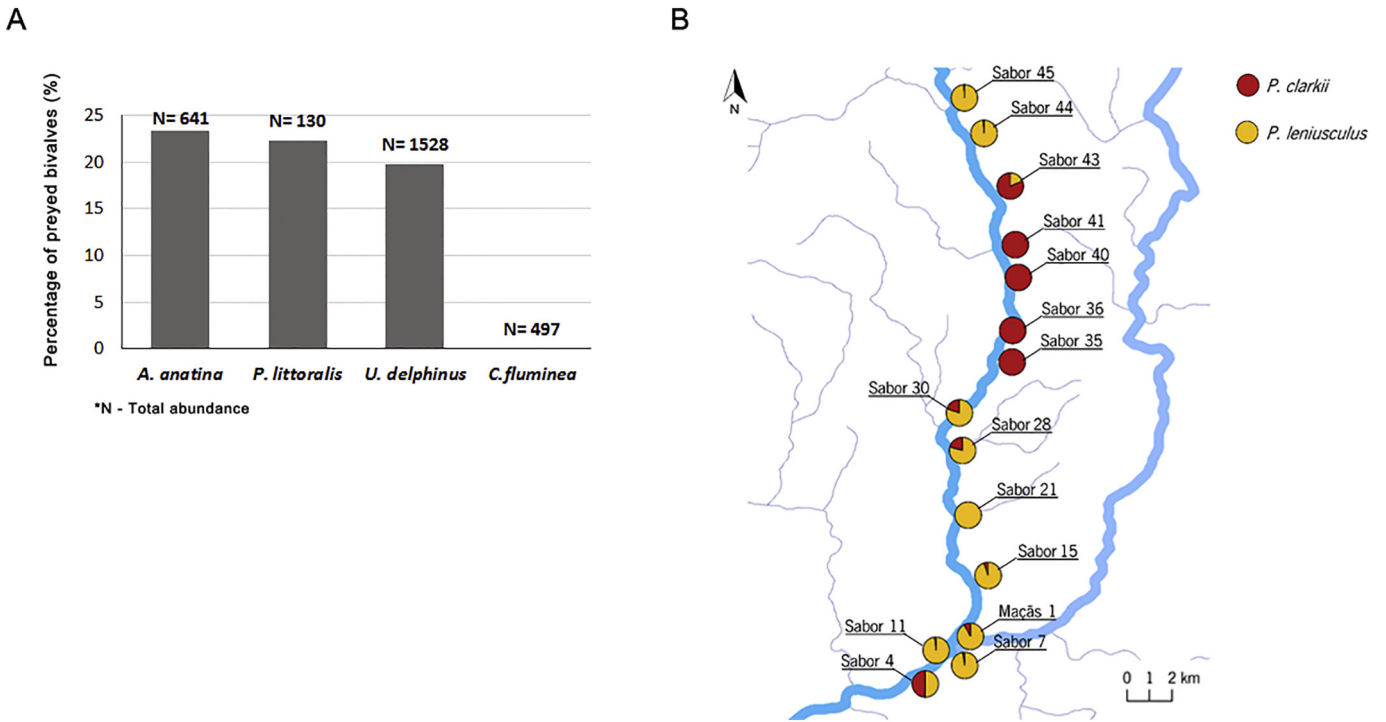
A

B

Fig. 5. (A) Percentage of preyed bivalves in situ and (B) distribution and relative abundance of *Procambarus clarkii* and *Pacifastacus leniusculus* across sampling sites. N represents total abundance.

and bivalve species, with a significant interaction between both factors (PERMANOVA, Table 5). PERMANOVA pair-wise comparisons showed differences on predation between *A. anatina* and *U. delphinus* in five of the analysed sites. On average, *A. anatina* had significantly higher percentage of shell predation in Sabor River sites 29 and 30, while *U. delphinus* had significantly higher percentage in Sabor River sites 18, 26 and 27.

The percentage of shell predation varied between sampling sites for both *A. anatina* and *U. delphinus*. The percentage of preyed *A. anatina*

and *U. delphinus* was not related to crayfish sex (Spearman's correlation, $p > 0.05$) and no correlation was found between the average abundance of crayfish and the percentage of preyed bivalves (Spearman's correlation, $p > 0.05$) (Table S1).

4. Discussion

In this study we showed that the invasive crayfishes *P. clarkii* and *P. leniusculus* can prey on native freshwater bivalves. This comes as no
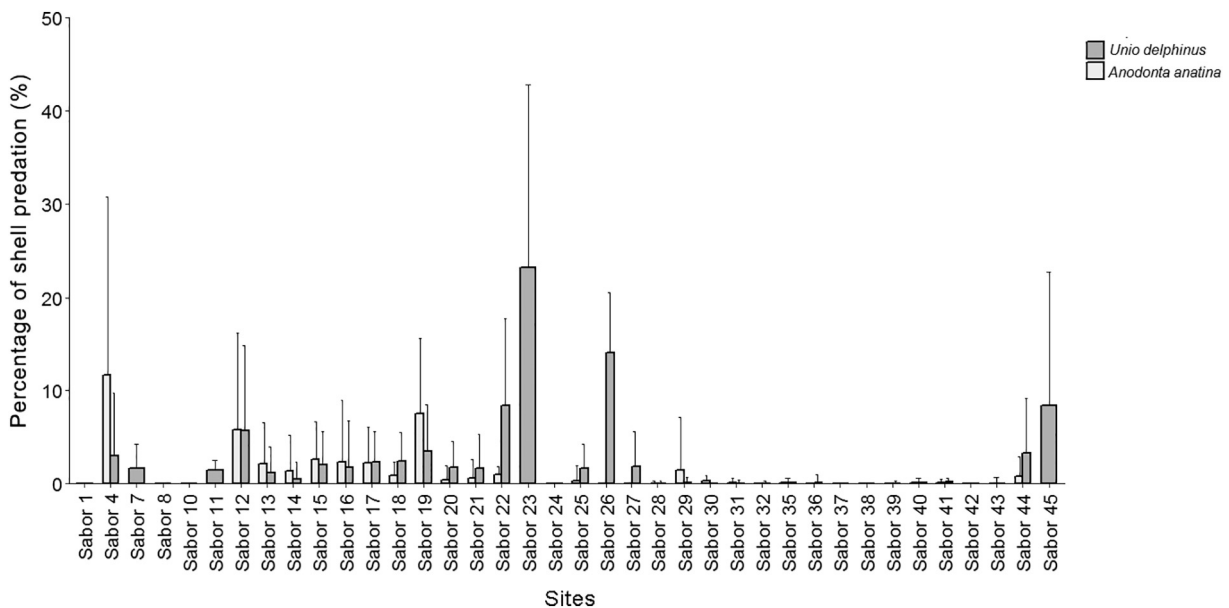

Fig. 6. $\overline{x}$ + SDof the percentage of shell predation of *Anodonta anatina* and *Unio delphinus* by sampling site.

Table 5
Summary of PERMANOVA results on the effect of river site and bivalve species on shell predation by crayfishes.

Parameter	Effect	df	SS	MS	Pseudo F	P
Shell consumption	River site	23	2483.2	107.97	11.555	0.001
	Bivalve species	1	43.86	43.86	4.694	0.034
	River site × bivalve species	23	1413.3	61.45	6.576	0.001
	Error	1796	16,781	9.34		

surprise since other crayfish species are known to prey freshwater bivalves (e.g. Klocker and Strayer, 2004; Perry et al., 1997) and Machida and Akiyama (2013) showed that *P. leniusculus* was capable of preying two endangered *Margaritifera* species.

All native bivalve species used in this study were preyed and results indicated that *P. leniusculus* is a more efficient predator than *P. clarkii*. On average, and in the laboratory experiments, *P. leniusculus* predated a higher percentage of shell of *U. delphinus* than *P. clarkii* but this result was highly influenced by one specimen that was killed, which resulted in 44.4% of its shell eaten. If we exclude this specimen, the average shell predation of *U. delphinus* by the signal crayfish would have been much lower (3.0% instead of 9.0%), which was not significantly different than the average percentage predation by *P. clarkii* (1.5%). Therefore, the possible differences between crayfish species should be interpreted with some caution. Anyway, several studies showed clear differences in the morphology of the mandibles of crayfish species (e.g. Capelli and Capelli, 1980; Kawai, 2012). For example, Harlioğlu (1996) studied *P. leniusculus* and *Astacus leptodactylus* (Eschscholtz, 1823) and suggested that *P. leniusculus* had a feeding advantage over *A. leptodactylus*, which could be explained by differences in their morphology and biology. Thus, the possible differences in bivalve predation between *P. leniusculus* and *P. clarkii* might be also partly explained by differences in morphology; however, future studies must be conducted to address this hypothesis.

When given a choice, both crayfish species preferred *A. anatina* as a prey. Of the four bivalve species used in this study, *A. anatina* has the most fragile and thin shells (Ilarri et al., 2015). Thus, it should be easier for crayfishes to handle and break *A. anatina* shells and energetically more profitable for crayfishes to prey *A. anatina* instead other species. The fact that *P. littoralis* was the less preyed native species in both choice and non-choice laboratory experiments may also be related with the shell thickness since this species presented the most robust and thick shell (Ilarri et al., 2015). Therefore, *P. littoralis* should be more resistant to crayfish predation and less profitable in terms of energy for both crayfishes. *P. littoralis* individuals were also the least preyed native bivalve by both crayfish species when they were allowed to choose their prey.

Potomida littoralis is the most threatened native bivalve species used in this study being listed as endangered by IUCN. Its populations in Portugal (and also in other European and North African countries) are very fragmented and have suffered major declines (Froufe et al., 2016; Lopes-Lima et al., 2014b and 2017) and this situation is also valid for the Sabor River basin (Sousa et al., 2012). Despite this, *P. littoralis* was the least preyed native species in the laboratory experiments. Additionally, only two specimens collected in the field were found dead and the cause did not appear to be directly related to crayfish predation due to the absence of the characteristic marks in the shells. This situation seems to indicate that crayfish predation may not play a major role on the decline of *P. littoralis* in the Sabor River basin (and possibly elsewhere), which may be explained by their thick and hard shells that probably turn the predation by both invasive crayfish more challenging.

Results suggest that *P. clarkii* and *P. leniusculus* predation did not directly cause a high mortality (and consequently a great reduction in abundance) on the native bivalve populations, given the low number of empty shells with predation marks found in the field survey.

However, these results should be interpreted with caution because many empty shells may have disappeared due to shell decay or washed to downstream areas. Anyway, and given the high number of alive bivalves presenting predation marks, it is possible that these species can still suffer sub-lethal effects from these invasive crayfishes. The damage caused to shells should have a physiological cost as energetic resources have to be mobilized for shell regeneration instead of growth and reproduction. In addition, every prey has to deal with the risk of being predated when feeding, thus having to balance the trade-off between predation risk and energy intake (Lima, 1998). Anti-predator behaviour may decrease prey energy intake and thus reduce its fitness and alter resource allocation, which can impact growth, reproduction and/or long-term survival (Johnson & Smee, 2012; Lima, 1998). It has been reported that bivalves can perceive and respond to predation risk (Maire et al., 2010; Naddafi et al., 2007; Wilson et al. 2012). Some studies showed that bivalves filtration rates and the expelling of faeces and pseudofaeces are affected (i.e. reduced) by the presence of predators (Maire et al., 2010; Naddafi et al., 2007). Therefore, it may be reasonable to consider that freshwater bivalves used in the present study may also be affected in their filter feeding activity, due to the presence of *P. clarkii* and *P. leniusculus*, thus having lower fitness and consequently lower growth and reproduction rates. Furthermore, the non-lethal effects of these crayfishes could affect the functional role of bivalves (e.g. nutrient cycling, water filtration and purification, bioturbation of sediments), which may indirectly impact ecosystems.

Most predators prefer small preys when offered a range of different sizes (Juanes, 1992). However, in this study *P. clarkii* and *P. leniusculus* did not showed any preferences regarding the prey size. Machida & Akiyama (2013) observed that *P. liniusculus* particularly injured *Margaritifera* spp. of medium size and suggested that this size class was the most suitable for crayfish manipulation. Nonetheless, and based on Machida and Akiyama (2013) results, these crayfishes may also kill small immature mussels. Besides this, in our study, crayfish size did not affect predation, which shows that smaller crayfishes are as capable of preying bivalves of various sizes as their larger counterparts. In addition, no correlation was found between crayfish sex and bivalve predation in the field suggesting that female and male crayfishes probably have the same level of impact on native freshwater bivalves.

No studies were found about the ability of *P. leniusculus* to prey *C. fluminea*. The lack of information about predator-prey interactions between *P. leniusculus* and *C. fluminea* impair us of making further comparisons. However, a few studies demonstrated that *P. clarkii* was able to prey on this invasive clam (e.g. Covich et al., 1981; Pereira et al., 2016). These earlier results on *P. clarkii* differ from the ones reported here that suggested that neither crayfish species was able to prey *C. fluminea*. The incoherence between the results of this study and those of others means that the absence of predation of the Asian clam is not necessarily due to the lack of ability of these crayfish species to prey it. Indeed, shell morphology and size may also affect the predation of *C. fluminea*. For example, Pereira et al. (2016) showed that *P. clarkii* was only able to prey on Asian clams with <10 mm, as demonstrated earlier by Covich et al. (1981). In our study, the smallest *C. fluminea* individuals used in the laboratory experiments measured 15 mm, which may explain why no crayfish was able to prey this species. Furthermore, *C. fluminea* shell is thicker (Ilarri et al., 2015) and has a spherical shape, probably making it harder for crayfishes to manipulate and open it, unlike the other studied bivalves that have elongated and thinner shells, especially *A. anatina* and *U. delphinus*. Based on the available information (Bernardo et al., 2011; Crespo et al., 2015; Filipe et al., 2017; Sousa et al., 2012), the co-existence of these crayfish species and *C. fluminea* in Sabor River basin is relatively recent and thus, the absence of predation of *C. fluminea* in this study may be explained by the fact that both crayfish species have still not recognized this IAS as prey. Regardless of the reason behind the lack of predation of this IAS, *P. clarkii* and *P. leniusculus* may be able to affect *C. fluminea* recruitment as this species only becomes sexually mature when shell size reaches 6 to 10 mm

(Sousa et al., 2008). However, our results clearly showed that both crayfish species have prey preference when it comes to bivalve species, and as generalist omnivores the probability of them preying *C. fluminea* in natural environments should be very low, as they have numerous and easier to handle alternative food sources, including native bivalves. Thus, *P. clarkii* and *P. leniusculus* would probably not significantly reduce *C. fluminea* abundance and should not be expected to act as control agents of this species in invaded ecosystems. In addition to other possible advantages of *C. fluminea* over the native bivalve species (e.g. rapid growth, early sexual maturity, more plastic feeding; Ferreira-Rodríguez et al., 2018; Novais et al., 2016; Sousa et al., 2008 and 2014), this invasive clam may also benefit by the lower predation pressure in the Sabor River basin and elsewhere (see also Castro et al., 2018). This advantage may increase the pressure of *C. fluminea* over the native bivalve populations.

Our laboratory experiments showed that *P. clarkii* is the dominant and more aggressive crayfish species. This is consistent with the results of Pearl et al. (2013), which showed that *P. clarkii* has the potential to locally displace *P. leniusculus* and that the signal crayfish avoided areas where *P. clarkii* was present. However, in our field validation *P. leniusculus* was more abundant than *P. clarkii*, which suggests that besides aggressiveness, other factors might be important. *Procambarus clarkii* may be more prone to predation by mammals (or other faunal groups) than *P. leniusculus* or the environmental conditions in the Sabor River basin are more favourable to *P. leniusculus*, among other hypotheses. For example, Gherardi et al. (2013) showed that water temperature can affect crayfish behaviour by intensifying or lowering their aggressiveness. Although competition between *P. clarkii* and *P. leniusculus* was confirmed in the laboratory experiments, and their niches overlap, these crayfishes have numerous feeding sources (Axelsson et al., 1997; Gherardi et al., 2001; Gherardi, 2006; Guan & Wiles, 1998), which means that competition for food may be minimized in the field. Although competition for food may be low in the Sabor River basin, these crayfish species may still compete for shelter in order to hide from predators or stressful environmental conditions. Finally, our laboratory results also suggest that *P. clarkii* causes less damage to native bivalves; and thus, native bivalves present in ecosystems where only *P. clarkii* exists will, in theory, suffer less impacts than those where *P. leniusculus* occurs. However, this situation may be highly context dependent and should be further evaluated.

Even though the present study may increase the knowledge about the predatory behaviour of these invasive crayfishes on freshwater bivalves, there are still gaps to explore in order to better understand the dynamics of these relationships and their impacts. To our knowledge this is the first study assembling laboratory and field data demonstrating how the introduction of predators may affect freshwater bivalves, a basic information essential for the conservation of these animals. Finally, the information reported here has also management implications because any measures devoted to the conservation of native freshwater bivalves in ecosystems invaded by *P. clarkii* and/or *P. leniusculus* (or even other crayfish species) should take into account the role of these animals as important predators.

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