

Enhanced fecundity and parasite release in the first amphipod invader on the Iberian Peninsula

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Abstract – *Crangonyx pseudogracilis* is a North American amphipod recently detected in Portugal and it is the first invasive freshwater amphipod detected in the Iberian Peninsula. The aim of this study was to assess the population structure and its invasion range in this area, but also to assess its pathogen profile and its origin through genetic analyses. *Crangonyx pseudogracilis* was only found in the lowlands of the Tagus River basin, a restricted area of the three sampled river basins (Tagus, Guadiana and Sado). Only one mitochondrial DNA (mtDNA) gene cytochrome oxidase subunit 1 (COI) haplotype, identical to the one already detected in Europe and in the Lake Charles, USA, was identified. The obtained data show that the studied populations are well-established, have a high female fecundity and a reproduction period extended throughout the year. No parasites were detected in the 236 dissected specimens. The dissimilarity of parasites prevalence to other European invasive populations opens the possibility of a direct introduction from North America but it also does not exclude the possibility of a secondary introduction from another European country.

Keywords: *Crangonyx pseudogracilis* / amphipod / invasive / parasites / Iberian Peninsula

Résumé – Amélioration de la fécondité et libération de parasites chez le premier amphipode envahisseur de la péninsule ibérique. *Crangonyx pseudogracilis* est un amphipode nord-américain récemment détecté au Portugal et c'est le premier amphipode d'eau douce envahissant détecté dans la péninsule ibérique. L'objectif de cette étude était d'évaluer la structure de la population et son aire de répartition dans cette zone, mais aussi d'évaluer son profil pathogène et son origine par des analyses génétiques. *Crangonyx pseudogracilis* n'a été trouvé que dans les basses plaines du bassin du Tage, une zone restreinte des trois bassins échantillonnés (Tagus, Guadiana et Sado). Un seul haplotype d'ADN mitochondrial (ADNmt) de la sous-unité 1 du gène cytochrome oxydase (COI), identique à celui déjà détecté en Europe et dans le lac Charles, USA, a été identifié. Les données obtenues montrent que les populations étudiées sont bien établies, ont une fécondité femelle élevée et une période de reproduction prolongée tout au long de l'année. Aucun parasite n'a été détecté dans 236 spécimens disséqués. La dissemblance de la prévalence des parasites avec d'autres populations invasives européennes ouvre la possibilité d'une introduction directe depuis l'Amérique du Nord mais n'exclut pas non plus la possibilité d'une introduction secondaire depuis un autre pays européen.

Mots-clés : *Crangonyx pseudogracilis* / amphipode / invasif / parasite / Péninsule ibérique

1 Introduction

Crangonyx pseudogracilis Bousfield, 1958 is a North American amphipod that was unintentionally introduced to Europe in the 1930's, spreading across Great Britain

(Gledhill *et al.*, 1993), northern and central Europe (Berthold and Kaiser, 2004; Pinkster *et al.*, 1980; Silfverberg, 1999; Tittizer *et al.*, 2000). Within the last decade it was spotted in Portugal, being the first invasive amphipod in the Iberian Peninsula (Grabowski *et al.*, 2012). The population colonizing inland waters of Western and Northern Europe originated in the Lake Charles, Louisiana, USA and underwent a strong

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genetic bottleneck during its introduction, which suggests that this was a single introduction event (Slothouber *et al.*, 2010). This was evidenced by the molecular analysis of COI mtDNA in the native and invaded range and it was shown that the whole genetic pool observed in Europe consists only of one haplotype. However, in contrast to previous observations in invasive species parasite release (Torchin *et al.*, 2003), the species maintained its microsporidian parasites: *Fibrillanosema crangonycis*, as well as two other unnamed Microsporidia: *Microsporidium* sp. CRANPA and *Microsporidium* sp. CRANB, which were the only Microsporidia detected in native populations (Slothouber *et al.*, 2010). Moreover, in the invaded range two more parasites were identified: *Microsporidium* sp. CRANPB and *Microsporidium* sp. CRANPC, increasing the parasite species number to five (Slothouber *et al.*, 2010).

Microsporidia are intracellular, obligatory eukaryotic parasites with nonmotile walled spores, belonging to a sister clade to Fungi, Opisthosporidia, which also includes Aphelida and Cryptomycota (Corsaro *et al.*, 2014; Haag *et al.*, 2014; Karpov *et al.*, 2014) and commonly infect amphipod crustaceans (Grabner *et al.*, 2015; Terry *et al.*, 2004). They can be transmitted vertically within the host population (transovarially, from female to offspring) or horizontally (transmission through consumption of infected tissue) causing microsporidiosis (Dunn and Rigaud, 1998; Dunn *et al.*, 2001; Haine *et al.*, 2004) which may be lethal (Haine *et al.*, 2007; Ryan and Kohler, 2010; Stentiford *et al.*, 2013). Nevertheless, some parasite species employ a mixed strategy for transmission (Vizoso and Ebert, 2004). Vertically transmitted Microsporidia are less virulent, enhance the reproduction rate and induce sex ratio distortions that may influence the population structure promoting female individuals and invasive success of the host (Haine *et al.*, 2007; Mautner *et al.*, 2007). Microsporidia that are transmitted horizontally may spread not only to the specific host but to a broader range of species (Bacela-Spychalska *et al.*, 2012). *Crangonyx pseudogracilis* is most commonly infected by *Fibrillanosema crangonycis* (detected all around the Europe) which was suggested to be vertically transmitted (but also present in muscles) and to enhance the success of its invasive host in European waters (Slothouber *et al.*, 2010).

The aim of our study is: (1) to test whether the Iberian population of *C. pseudogracilis* originates from the European source or from the native area as a result of an independent introduction; (2) to reveal the population structure and the extent of the range invaded by the species in the south-western Iberian Peninsula; (3) to test the Iberian populations for the presence of helminths and microsporidian parasites that could promote/impede the invasion of *C. pseudogracilis* in the Iberian Peninsula.

2 Methods

2.1 Samples collection

The field work was conducted between February and December 2014, in 48 sites located in three river basins of central Portugal (Tagus river; Sado river and Guadiana river) (Fig. 1). In order to assess the *C. pseudogracilis* invasion range, a grid of sampling locations containing water

throughout the year (isolated pools) was created around the initial detection locations (Banha and Anastácio, 2015; Grabowski *et al.*, 2012) and extended with new records while conducting this survey. This was important because of the temporary character of streams and rivers in this region. The typical three months without precipitation and high temperatures in the summer of Mediterranean climate regions, promote total or partial drought of watersheds and limits aquatic organisms dispersion, colonization or recolonization (Gasith and Resh, 1999). Sites were sampled downstream and upstream from the locations with presence of *C. pseudogracilis* but also in the other closest watersheds. At each site, the typical microhabitats for amphipods, *i.e.* rich in plants and their roots (Banha and Anastácio, 2015; Grabowski *et al.*, 2012), were inspected with a dip net (1 mm mesh, 60 cm × 40 cm frame) until *C. pseudogracilis* was found, for a maximum of 15 minutes per site. If no *C. pseudogracilis* were found in its favorable microhabitats, the species was considered absent. In the sites with *C. pseudogracilis*, the densities were estimated by sampling one m² of the microhabitat until no more amphipods were captured, following the procedure by Banha and Anastácio (2015). The captured individuals were preserved in 96% alcohol for further studies. The characteristics of each microhabitat, namely water depth, type of sediment substrate (granulometry scale from ASTM (1996), see Tab. 1), vegetation and water flow velocity were registered at each site.

2.2 Sample analyses

In the laboratory all the amphipods were identified to the species level according to the available literature (Holsinger, 1976; Holsinger, 1977; Piscart and Bollache, 2012). The total length of each individual was measured under a stereomicroscope from the anterior margin of a head to the posterior margin of the telson with an accuracy of 0.5 mm using a reflection microscope Nikon SMZ 800 coupled to an image analysis system Precoptic Coolview 2.7.4. The sex of each individual was identified by the presence of calceoli on flagellum of 2nd antennae in males and by the presence of oostegites in females (Zhang and Holsinger, 2003). The number of eggs and embryos in each female was also counted if the brood pouch was undamaged.

2.3 *Crangonyx pseudogracilis* genetic diversity

To estimate the *C. pseudogracilis* genetic diversity and to identify the source of the population colonizing Portuguese inland waters, 186 individuals pooled from all the sampled sites were analyzed (Tab. 2). First, the total DNA was extracted following the procedure used by (Ovcharenko *et al.*, 2010) and the fragment of crustacean mitochondrial DNA (mtDNA) gene cytochrome oxidase subunit 1 (COI) was amplified with a pair of primers LCO1490-JJ (5'-CHAC-WAAYCATAAAGATATYGG-3') and HCO2198-JJ (5'-AWACTTCVGGRTGVCCAAARAATCA-3') following (Astrin and Stüben 2008; Wattier *et al.*, 2007). The amplification program began with 94 °C denaturation for 2 min followed by 34 cycles of 94 °C (20 s), 57 °C (45 s) and 65 °C (45 s). The PCR product was visualized on the 2%

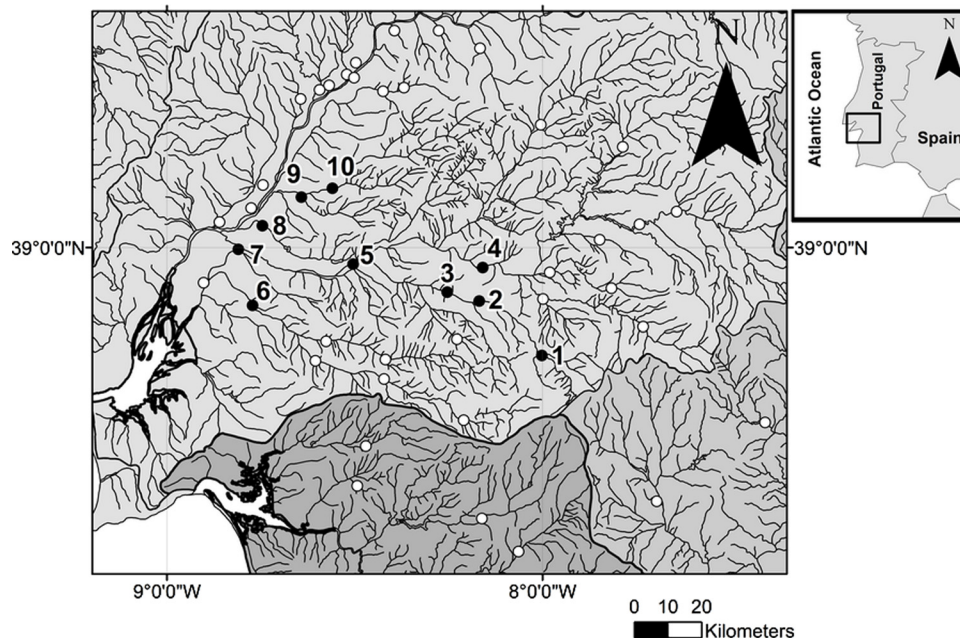


Fig. 1. Study sites in Central Portugal. Sampling stations are represented by dots (Black – *C. pseudogracilis* present; White – *C. pseudogracilis* absent). Different grey shadings correspond to different river basins (dark grey – Sado river basin; medium grey – Guadiana river basin; clear grey – Tagus river basin).

Table 1. Classification of the different types of sediments, adapted from ASTM (1996)

Sediment type	Particle size
Boulders	More than 300 mm
Cobbles	from 75 to 300 mm
Coarse Gravel	from 19 to 75 mm
Fine Gravel	from 4.75 to 19 mm
Sand	from 4.75 mm to 75 μ m
Silt/Clay	Less than 75 μ m

agarose gel to assess the quality of the reaction and then sequences were obtained using Big Dye sequencing protocol (Applied Biosystems 3730xl) by Macrogen Inc., Korea. Amplicon length varied from 614 to 625 base pairs. The sequences were edited and aligned with Geneious R10 (Biomatters Ltd.) as well as identification of haplotypes. The sequences were identified using BLAST (Altschul *et al.*, 1990).

2.4 Parasite detection

Each individual was analyzed to detect parasites: presence of macroparasites as helminths was checked during dissection under light microscope. Presence of the intracellular Microsporidia was tested using molecular methods. In the case of females that had a brood pouch filled with eggs and/or juveniles, the latter were removed before dissection. We amplified a fragment of microsporidian SSU rDNA using a pair of specific primers v1f (Baker *et al.*, 1994) and 530r (Vossbrinck *et al.*, 1993) following Ovcharenko *et al.* (2010). The PCR products being a signature of microsporidian infection, were visualized on the 2% agarose gel. This

assessment provided no microsporidian amplicons and so sequencing was not carried out.

3 Statistical analyses

Differences in proportions of sediment types and in proportions of vegetation types, in the sampling sites with and without amphipod presence, were tested using a Chi-square test on a contingency table. Differences in water flow velocity and water depth between these places were tested using a Mann-Whitney test.

Due to small sample sizes, some data for population structure analysis were aggregated (Tab. 2). The criteria were that the two sites to aggregate were sampled in the same day, were in the same water course and the Euclidean distance between points was less than 10 km. Seven groups were created by this process (1–a; 2+3–b; 4–c; 5–d; 6+7–e; 8–f; 9+10–g) (Fig. 1). Differences in sex ratio among samples were checked using a Chi-square test on a contingency table. A 2-sample t-test was used to test for statistical difference between female and male sizes. After checking the assumptions of normal distribution and homoscedasticity, differences between samples in mean size of each gender were evaluated using an ANOVA followed by a Tukey HSD post hoc test. A female fertility vs. size regression line was adjusted to the data, using discrete size classes of 1 mm total length and number of eggs.

All statistical tests were performed using SPSS version 22, except for the exponential curve adjustment in the fertility vs. size analysis which was performed using Excel.

4 Results

Crangonyx pseudogracilis was found in 10 of the 48 sites sampled in the three river basins. According to our results, the

Table 2. Sampling locations and individuals captured.

Local name	Code (Fig. 1)	Aggregation code for statistical analyses	River name	Coordinates (WGS 84)	Total individuals collected	Male individuals	Genetic sequences obtained	Sample date
N°370 bridge road	1	a	Divor River	38°46'33.98" N 7°59'44.49" W	20	3	19	06/03/2014
N°2 bridge road	2	b	Divor River	38°52'56.18" N 8°10'17.07" W	12	1	12	02/19/2014
Ferrarias	3	b	Divor River	38°54'08.77" N 8°15'39.40" W	25	15	24	02/19/2014
Paço	4	c	Raia River	38°57'21.01" N 8°10'09.08" W	81	35	48	02/19/2014
Coruche	5	d	Sorraia River	38°57'12.06" N 8°31'8.95" W	56	9	46	12/03/2014
Santo Estevão	6	e	Canha Stream	38°51'22.35" N 8°44'34.17" W	10	1	10	05/03/2014
Benavente	7	e	Sorraia River	38°59'30.11" N 8°48'46.62" W	6	4	6	05/03/2014
Paul Magos	8	f	Magos Stream	39°2'9.97" N 8°44'16.95" W	7	3	7	05/03/2014
Granho	9	g	Lamarosa Stream	39°5'48.08" N 8°38'30.05" W	16	5	14	05/03/2014
Raposa	10	g	Muge River	39° 6'44.63" N 8°34'41.38" W	3	2	0	05/03/2014

invasion range of this species in the area is so far restricted to the Tagus river basin, corresponding mainly to the lower stretch of the Sorraia River and some adjacent smaller rivers (Fig. 1). We found a mean relative density of 23.2 individuals m^{-2} , with a minimum of 3 individuals m^{-2} to a maximum of 78 individuals m^{-2} . Within the invasion range, the species was recorded in the river, streams, small oxbow lakes, agricultural canals and ditches, in microhabitats with aquatic plants (*i.e.* *Typha* sp.; *Salix* sp. roots; *Myriophyllum aquaticum*; *Cynodon dactylon*; *Scirpoides holoschoenus*). The average water depth in such localities was 48 cm with a range from 0.30 m to 0.70 m. The sites with the species had low to null water flow (mean = 0.3 ms^{-1} ; range: 0–0.5 ms^{-1}) and mainly fine bottom substrate like fine sand and clay. The exception was concrete agricultural canals with filamentous algae and without plants. The locations with or without amphipods did not differ neither in the water depth (Mann-Whitney: $U=187.0$; $N_1=38$; $N_2=10$; $p>0.05$) nor in water velocity (Mann-Whitney: $U=161.0$; $N_1=38$; $N_2=10$; $p>0.05$). Also, the sites with or without amphipods did not differ, neither regarding the proportion of plant species composing the vegetation ($X^2=9.613$, $df=8$, $p>0.05$), nor the type of substrate (according to ASTM, 1996) (see Tab. 1) ($X^2=6.568$, $df=3$, $p>0.05$).

We detected only one COI haplotype in the analysed material coming from 10 sites. Our sequences were identical to the sequences already detected in Europe (AY529053) and in the Lake Charles, USA (AJ968893). Our best quality sequence (614 bp) was identical to sequences AY529053 and AJ968893 with E value of 0.0 and query coverage 99% and 97% respectively. A Mann Whitney U test indicated that the number of eggs per female was not different between seasons (winter and spring) (Total N = 50, Mann Whitney U = 206, $p>0.05$). The mean fecundity was 33 eggs (32.82 ± 22.29 SD). The maximum number of eggs per female was 91, and the minimum was 1. The total length of females with eggs or juveniles varied from 7.14 to 11.46 mm (Fig. 2). The mean fecundity rose with female body length according to the equation $Y=3.055x+26.005$ ($r^2=0.8417$). Four females carried juveniles/embryos (7, 9 and 9 juveniles/embryos, respectively) and two of these four females carried juveniles/embryos simultaneously with the eggs (9 and 28 juveniles/embryos – 31 and 1 eggs, respectively). The percentage of

females carrying eggs was 30% and 33% during spring and winter, respectively.

The overall sex ratio (M/F) was 0.51. A chi-square test on a contingency table showed that the sex ratio varied significantly among samples ($X^2=21.985$, $df=6$, $p<0.001$).

Females attained larger size than males ($t=-16,702$; $df=230$, $p<0.001$). Mean size and confidence intervals were 7.95 (7.68–8.22) mm and 5.23 (5.05–5.41) mm for females and males, respectively. The largest female was 11.46 mm while the largest male was 9.62 mm, which was an unusually large size (in fact, an outlier).

Samples from different sites presented significantly different mean female sizes (ANOVA, $F=3.599$, $p<0.05$), but male size was not significantly different depending on the sampling sites (ANOVA, $F=2.092$, $p>0.05$). Size was not different between spring and winter for females ($t=-0.499$; $df=43$; $p>0.05$) and males ($t=-0.474$; $df=17$; $p>0.05$).

Altogether, 236 individuals were checked for the presence of the parasites. No macro-parasites from the body cavities nor Microsporidia (no positive PCR reaction) were detected in any of the analyzed individuals.

5 Discussion

The present study reveals the invasion range and distinctive biological characteristics of the first amphipod invader detected in the Iberian Peninsula. We found out that recently installed populations are genetically similar to other European populations but with population structure similarities to North American native populations (Embodly, 1911).

Genetic analyses show that the Portuguese populations of *C. pseudogracilis* share the same genetic background as other European populations. It is highly probable that the population in Portugal may be of European origin, as a further invasion step on the European continent. Bearing in mind the ability of *C. pseudogracilis* to cling to waterfowl (Rachalewski *et al.*, 2013), the role of zoochory in the spread of this amphipod within Europe cannot be underestimated. However, it may not be excluded that it was introduced again from the same native area in the Louisiana, USA, that was the source for the previous introduction to Europe. Nevertheless, low parasite prevalence when compared with invasive populations in Europe, may support the hypothesis of independent

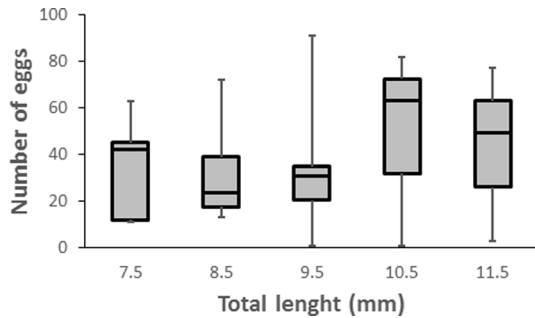


Fig. 2. Box and whiskers plots for number of eggs for each size class of *Crangonyx pseudogracilis* female body length.

introduction from the native range which in a consequence led to parasite release in Portugal. The introduction from a population without parasites from a native area seems more likely than from other European populations, which are predominantly characterized by very high parasite prevalence (Slothouber *et al.*, 2010).

On the other hand, the lack of microsporidian parasites in Portuguese populations of *C. pseudogracilis* seems to be evidence for parasite release in this recent invasion process, if it was a case of a secondary introduction from other European country. Loss of natural enemies is one of the major hypothesis for the success of biological invasions (Torchin *et al.*, 2003) and therefore we assume that the lack of virulent Microsporidia is favoring the ongoing invasion of the Iberian Peninsula. Slothouber *et al.* (2010) demonstrated that *C. pseudogracilis* underwent a post-invasion genetic bottleneck. However, they did not observe parasite loss in populations in France, UK, Belgium and Netherlands. The most abundant microsporidium species in those populations is the vertically transmitted *Fibrillanosema crangonycis* which can reach a prevalence up to 100% (Slothouber *et al.*, 2010). Interestingly, within the north-American native range, *F. crangonycis* does not exceed a 10% prevalence, possibly because this microsporidium shows high level of pathogenesis (Slothouber *et al.*, 2004). No studies were conducted to test the impact of this parasite on its host. By its mode of transmission it was suggested to enhance the spread of the host, but this parasite was also found in muscles (Slothouber *et al.*, 2004), thus it may serve as pathogen affecting the host fitness. Other Microsporidia were rare, as they were recorded in single individuals in single sites and their presence did not impact the host populations (Slothouber *et al.*, 2004). So, in Portugal, the likely scenario for the absence of parasites may be a recent introduction of a small population of uninfected hosts directly from North America.

Similarly, histopathology (Bojko *et al.*, 2013) and PCR-based (Arundell *et al.*, 2015) studies of UK populations of the invasive amphipod *Dikerogammarus villosus* showed a distinct lack of pathogens and parasites when compared in invasive range across central (Etxabe *et al.*, 2015) populations of *D. villosus* is suggestive of single-point introductions, rather than continual incursion events as previously observed throughout its continental invasive (Bojko *et al.*, 2013). In contrast, *Dikerogammarus haemobaphes*, presents UK populations with high prevalence of parasites, such as the Microsporidia *Dictyocoela berillonum* and *Cucumispora ornate* (Bojko *et al.*, 2015) or the Nematoda *Hysterothylacium deardorffover-*

streetorum (Etxabe *et al.*, 2015). In this, case it is possible that the initial invasive population consisted of a small number of infected individuals and that the current infection prevalence represents a parasitic founder-effect (Etxabe *et al.*, 2015). Despite, the potentially lower invasive performance of *D. haemobaphes* in comparison with *D. villosus*, the transmission of parasites to native amphipods by the latter species, via cross-taxa transmission, may pose a significant ecological impact, as observed in other European regions (Bacela-Spychalska *et al.*, 2012).

The negative impacts known for introduced amphipods are related with replacing native amphipod species and community disturbance (Conlan, 1994; MacNeil *et al.*, 1997). However, the impacts in Portuguese waters are still poorly studied. No native amphipod species were found in the locations during this study, nor in the literature regarding the corresponding sampling areas. Nevertheless, other crustaceans such as the invasive red swamp crayfish *Procambarus clarkii* (Girard, 1852) and the native river shrimp *Atyaephyra desmarestii* (Millet, 1831), were detected (Banha and Anastácio, 2011). Indeed, the lack of amphipod competitors in this region and the wide range of potential dispersal vectors could promote a rapid expansion in the Iberian Peninsula, since natural vectors, *e.g.* waterfowl (Rachalewski *et al.*, 2013) and those related with human activities, such as sport fishing, are very frequent in the area (Banha and Anastácio, 2015). Range expansion may also be enhanced due to the high suitability of environmental conditions found in most river networks, especially in the south of the Iberian Peninsula and in lowlands. *Crangonyx pseudogracilis* seems to thrive best in marginal habitats, particularly shallow eutrophic waters (Dick *et al.*, 1999), rich in macrophytes and/or roots (Grabowski *et al.*, 2012). Generally, this species is associated to low water quality (polluted and low oxygen content) but it is also able to colonize areas of high water quality (MacNeil *et al.*, 2000). Thus, it is possible that *C. pseudogracilis* will be able to invade nearby water bodies and poor-quality rivers which are very common in the study area. Lotic ecosystems with permanent and fast flow are frequent in highlands and in the north of the Iberian Peninsula, some of them being the habitat of native amphipods (Gama *et al.*, 2017) and these could also be at risk of invasion. *Echinogammarus meridionalis* (Pinkster, 1973) is one example of an endemic Iberian amphipod potentially threatened by the arrival of *C. pseudogracilis* and it has populations in an adjacent river basin (Mondego). However, a short-term laboratory experiment with both species, studying food intake, molting and survival, suggests that direct effects of *C. pseudogracilis* on *E. meridionalis* may be scarce (Gama *et al.*, 2017). Nevertheless, some community disturbance is expected in the invaded range, namely due to a variety of complex interspecific prey-predator interactions with native and invasive fish species (MacNeil *et al.*, 1999). Altogether, no evidence for negative ecological or economic impact is currently known, but this species has high invasive potential, as assessed according to the Great Britain non-native organism risk assessment scheme (Dunn, 2013). The absence of parasites in Portuguese populations should minimize the risk of parasite transmission to native amphipods, however, this also increases the fitness of the host. Our preliminary results from *C. pseudogracilis* biology seems to reveal that warmer climate conditions in Portugal are beneficial for *C. pseudogracilis* reproduction and invasion. In the north of Europe, cold temperatures almost prevent reproduction during winter

(Pinkster and Platvoet, 1983) and in the laboratory it was shown that the time needed to reach sexual maturity is also affected by temperature (80–106 days at 15 °C and 60–85 days at 25 °C) (Sutcliffe and Carrick, 1981). *Crangonyx pseudogracilis* is characterized by high fecundity while compared to local species (Zhang and Holsinger, 2003) and our results seem to be congruent with this statement. Our data suggest that the studied populations are well established and high temperatures of the Iberian Peninsula may be beneficial for female fecundity (mean number of eggs was 32.82 eggs) which is higher than in north-European populations (mean number of eggs 25.94) and the reproduction period does not have an intermission during the winter (percentage of females with eggs is 30 % in Portugal and less than 10 % in north-Europe) (Pinkster and Platvoet, 1983). An exception was observed in an UK population that also presented high fecundity (mean number of 34.3 eggs) and breeding period during the entire year (Hynes, 1955). These UK and Portuguese population features are shared with the native populations that appear to breed at all seasons and have also high fecundity (mean number eggs 45) (Embody, 1911). As previously found by Goedmakers (1981) for other freshwater amphipods, our results showed that fecundity increases with female size.

Concluding, we found a *C. pseudogracilis* restricted invasion range, limited to a few south margin Tagus river tributaries in central Portugal. We cannot exclude that this may be the result of a recent invasion directly from the native area in the USA, since we found highly fertile populations, without parasites and the surrounding wetlands have both excellent environmental conditions for these species and abundance of potential dispersal vectors. Finally, we highlight the importance of studies for invasive amphipods in the Iberian Peninsula due to a virtual lack of studies of invasive amphipods presence, distribution and ecology (García-Berthou *et al.*, 2007). This is a matter of high conservation concern in view of the potential for new endemic species discovery in the inland waters of the Iberian Peninsula.

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References

- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. *J Mol Biol* 215: 403–410.
- Arundell K, Dunn A, Alexander J, Shearman R, Archer N, Ironside JE. 2015. Enemy release and genetic founder effects in invasive killer shrimp populations of Great Britain. *Biol Invasions* 17: 1439–1451.
- ASTM E. 1996. 1737-96: Standard test method for J-integral characterization of fracture toughness. Annual Book of ASTM Standards 3: 994–1017.
- Astrin JJ, Stüben PE. 2008. Phylogeny in cryptic weevils: molecules, morphology and new genera of western Palaearctic Cryptorhynchinae (Coleoptera: Curculionidae). *Invertebr Syst* 22: 503–522.
- Bacela-Spychalska K, Wattier RA, Genton C, Rigaud T. 2012. Microsporidian disease of the invasive amphipod *Dikerogammarus villosus* and the potential for its transfer to local invertebrate fauna. *Biol Invasions* 14: 1831–1842.
- Baker MD, Vossbrinck CR, Maddox JV, Undeen AH. 1994. Phylogenetic relationships among *Vairimorpha* and *Nosema* species (Microspora) based on ribosomal RNA sequence data. *J Invertebr Pathol* 30: 509–518.
- Banha F, Anastácio PM. 2011. Interactions between invasive crayfish and native river shrimp. *Knowl Manag Aquat Ecosyst* 401: 17.
- Banha F, Anastácio PM. 2015. Live bait capture and crayfish trapping as potential vectors for freshwater invasive fauna. *Limnol-Eco Manag Inland Waters* 51: 63–69.
- Berthold E, Kaiser I. 2004. Weitere Funde von *Crangonyx pseudogracilis* und *Chelicorophium robustum* (Amphipoda) im Main. *Lauterbornia* 50: 15–17.
- Bojko J, Stebbing P, Bateman K, Meatyard J, Bacela-Spychalska K, Dunn A, Stentiford G. 2013. Baseline histopathological survey of a recently invading island population of 'killer shrimp', *Dikerogammarus villosus*. *Dis Aquat Org* 106: 241–253.
- Bojko J, Dunn AM, Stebbing PD, Ross SH, Kerr RC, Stentiford GD. 2015. *Cucumispora ornata* n. sp. (Fungi: Microsporidia) infecting invasive 'demon shrimp' (*Dikerogammarus haemobaphes*) in the United Kingdom. *J Invertebr Pathol* 128: 22–30.
- Conlan K. 1994. Amphipod crustaceans and environmental disturbance: a review. *J Nat Hist* 28: 519–554.
- Corsaro D, Walochnik J, Venditti D, Steinmann J, Müller K-D., Michel R. 2014. Microsporidia-like parasites of amoebae belong to the early fungal lineage Rozellomycota. *Parasitol Res* 113: 1909–1918.
- Dick JT, MacNeil C, Anderson R. 1999. The distribution of *Crangonyx pseudogracilis* Bousfield, 1958 (Crustacea: Amphipoda) in Northern Ireland, with notes on its ecology and behaviour. *Irish Nat J* 26: 236–240.
- Dunn A. 2013. GB Non-native Organism Risk Assessment for *Crangonyx pseudogracilis*. www.nonnativespecies.org Access at 21 December 2017
- Dunn A, Rigaud T. 1998. Horizontal transfer of parasitic sex ratio distorters between crustacean hosts. *Parasitology* 117: 15–19.
- Dunn AM, Terry RS, Smith JE. 2001. Transovarial transmission in the microsporidia. *Adv Parasitol* 48: 57–100.
- Embody GC. 1911. A preliminary study of the distribution, food and reproductive capacity of some fresh-water amphipods. *Int Revue der gesamten Hydrobiol Hydrogr Suppl* 3: 1–35.
- Etxabe AG, Short S, Flood T, Johns T, Ford AT. 2015. Pronounced and prevalent intersexuality does not impede the 'Demon Shrimp' invasion. *Peer J* 3: e757.
- Gama M, Marquéz L, Banha F, Anastácio P. 2017. Coexistence patterns between the invasive amphipod *Crangonyx pseudogracilis* and native *Echinogammarus meridionalis*: a laboratory approach. *Fund Appl Limnol/Archiv für Hydrobiol* 190: 133–140.
- García-Berthou E, Boix D, Clavero M. 2007. Non-indigenous animal species naturalized in Iberian inland waters. *Biological invaders in inland waters: profiles, distribution, and threats*: 123–140.
- Gasith A, Resh VH. 1999. Streams in Mediterranean climate regions: abiotic influences and biotic responses to predictable seasonal events *Annu Rev Ecol Syst* 30: 51–81.
- Gledhill T, Sutcliffe DW, Williams WD. 1993. British Freshwater Crustacea Malacostraca: a Key with ecological notes, Scientific Publication. Freshwater Biological Association, Ambleside.
- Goedmakers A. 1981. Population dynamics of three gammarid species (Crustacea, Amphipoda) in a french chalk stream. II. *Standing crop. Bijdr. Dierk.* 51: 31–69.

- Grabner DS, Weigand AM, Leese F, Winking C, Hering D, Tollrian R, Sures B. 2015. Invaders, natives and their enemies: distribution patterns of amphipods and their microsporidian parasites in the Ruhr Metropolis, Germany. *Parasites & vectors* 8: 419.
- Grabowski M, Rachalewski M, Banha F, Anastacio P. 2012. *Crangonyx pseudogracilis* Bousfield, 1958-the first alien amphipod crustacean in freshwaters of Iberian Peninsula (Portugal). *Knowl Manag Aquat Ecosyst* 404: 11.
- Haag KL, James TY, Pombert J-F, Larsson R, Schaer TM, Refardt D, Ebert D. 2014. Evolution of a morphological novelty occurred before genome compaction in a lineage of extreme parasites. *Proc Natl Acad Sci USA* 111: 15480–15485.
- Haine ER, Brondani E, Hume KD, Perrot-Minnot M-J, Gaillard M, Rigaud T. 2004. Coexistence of three microsporidia parasites in populations of the freshwater amphipod *Gammarus roeseli*: evidence for vertical transmission and positive effect on reproduction. *Int J Parasitol* 34: 1137–1146.
- Haine ER, Motreuil S, Rigaud T. 2007. Infection by a vertically-transmitted microsporidian parasite is associated with a female-biased sex ratio and survival advantage in the amphipod *Gammarus roeseli*. *Parasitology* 134: 1363–1367.
- Holsinger JR. 1976. The freshwater amphipod crustaceans (Gammaridae) of North America. US Environmental Protection Agency, Office of Research and Development, Environmental Monitoring and Support Laboratory, Biological Methods Branch, Aquatic Biology Section. Cincinnati, Ohio, 89 p.
- Holsinger JR. 1977. A review of the systematics of the holarctic amphipod family Crangonyctidae. *Crustaceana Suppl.* 244–281.
- Hynes H. 1955. The reproductive cycle of some British freshwater Gammaridae. *J Animal Ecol.* 352–387.
- Karpov SA, Mamkaeva MA, Aleoshin VV, Nasonova E, Lilje O, Gleason FH. 2014. Morphology, phylogeny, and ecology of the aphelids (Aphelidea, Opisthokonta) and proposal for the new superphylum Opisthosporidia. *Front microbiol.* 5.
- MacNeil C, Dick JT, Elwood RW. 1997. The trophic ecology of freshwater Gammarus spp. (Crustacea: Amphipoda): problems and perspectives concerning the functional feeding group concept. *Bio Rev* 72: 349–364.
- MacNeil C, Dick JT, Elwood RW. 2000. Differential physico-chemical tolerances of amphipod species revealed by field transplantations. *Oecologia* 124: 1–7.
- MacNeil C, Elwood RW, Dick JT. 1999. Predator-prey interactions between brown trout *Salmo trutta* and native and introduced amphipods; their implications for fish diets. *Ecography* 22: 686–696.
- Mautner SI, Cook KA, Forbes MR, McCurdy DG, Dunn AM. 2007. Evidence for sex ratio distortion by a new microsporidian parasite of a Corophiid amphipod. *Parasitology* 134: 1567–1573.
- Ovcharenko MO, Bacela K, Wilkinson T, Ironside JE, Rigaud T, Wattier RA. 2010. Cucumispora dikerogammari n. gen. (Fungi: Microsporidia) infecting the invasive amphipod *Dikerogammarus villosus*: a potential emerging disease in European rivers. *Parasitology* 137: 191–204.
- Pinkster S. 1973. The *Echinogammarus berilloni*-group, a number of predominantly iberian amphipod species (Crustacea). *Bijdr tot de Dierkd* 43: 1–36.
- Pinkster S, Dieleman J, Platvoet D. 1980. The Present Position of *Gammarus tigrinus* Sexton, 1939, in the Netherlands, with the Description of a Newly Discovered Amphipod Species, *Crangonyx pseudogracilis* Bousfield, 1958 (Crustacea, Amphipoda), 7. Bulletin. Zoologisch Museum, Universiteit van Amsterdam.
- Pinkster S, Platvoet D. 1983. Further observations on the distribution and biology of two alien amphipods, *Gammarus tigrinus* Sexton, 1939, and *Crangonyx pseudogracilis* Bousfield, 1958, in the Netherlands (Crustacea, Amphipoda). *Bull Zoologisch Mus* 9: 153–162.
- Piscart C, Bollache L. 2012. Crustacés amphipodes de surface: gammares d'eau douce. Association française de limnologie.
- Rachalewski M, Banha F, Grabowski M, Anastácio PM. 2013. Ectozoochory as a possible vector enhancing the spread of an alien amphipod *Crangonyx pseudogracilis*. *Hydrobiologia* 717: 109–117.
- Ryan JA, Kohler SL. 2010. Virulence is context-dependent in a vertically transmitted aquatic host-microparasite system. *Int J Parasitol* 40: 1665–1673.
- Silfverberg H. 1999. A provisional list of Finnish Crustacea. *Memo Soc Fauna Flora Fenn* 75: 15–37.
- Slothouber Galbreath J, Smith J, Terry R, Becnel J, Dunn A. 2004. Invasion success of *Fibrillanosema crangonyctis*, n.sp., n.g.: a novel vertically transmitted microsporidian parasite from the invasive amphipod host *Crangonyx pseudogracilis*. *Int J Parasitol* 34: 235–244.
- Slothouber Galbreath JG, Smith JE, Becnel JJ, Butlin RK, Dunn AM. 2010. Reduction in post-invasion genetic diversity in *Crangonyx pseudogracilis* (Amphipoda: Crustacea): a genetic bottleneck or the work of hitchhiking vertically transmitted microparasites? *Biol Invasions* 12: 191–209.
- Stentiford GD, Feist SW, Stone DM, Bateman KS, Dunn AM. 2013. Microsporidia: diverse, dynamic, and emergent pathogens in aquatic systems. *Trends Parasitol* 29: 567–578.
- Sutcliffe D, Carrick T. 1981. Effect of temperature on the duration of egg development, and moulting and growth in juveniles of *Crangonyx pseudogracilis* (Crustacea: Amphipoda) in the laboratory. *Freshwater Biol* 11: 511–522.
- Terry RS, Smith JE, Sharpe RG, Rigaud T, Littlewood DTJ, Ironside JE, Rollinson D, Bouchon D, MacNeil C, Dick JT. 2004. Widespread vertical transmission and associated host sex-ratio distortion within the eukaryotic phylum Microspora. *Proc R Soc Lond B* 271: 1783–1789.
- Tittizer T, Schöll F, Banning M, Haybach A, Schleuter M. 2000. Aquatische Neozoen im Makrozoobenthos der Binnenwasserstraßen Deutschlands. *Lauterbornia* 39: 1–72.
- Torchin ME, Lafferty KD, Dobson AP, McKenzie VJ, Kuris AM. 2003. Introduced species and their missing parasites. *Nature* 421: 628–630.
- Vizoso DB, Ebert D. 2004. Within-host dynamics of a microsporidium with horizontal and vertical transmission: *Octospora bayeri* in *Daphnia magna*. *Parasitology* 128: 31–38.
- Vossbrinck CR, Baker MD, Didier ES, Debrunner-Vossbrinck BA, Shaddock JA. 1993. Ribosomal DNA sequences of *Encephalitozoon hellem* and *Encephalitozoon cuniculi*: species identification and phylogenetic construction. *Eukaryot Microbiol* 40: 354–362.
- Wattier R, Haine E, Beguet J, Martin G, Bollache L, Musko I, Platvoet D, Rigaud T. 2007. No genetic bottleneck or associated microparasite loss in invasive populations of a freshwater amphipod. *Oikos* 116: 1941–1953.
- Zhang J, Holsinger JR. 2003. Systematics of the Freshwater Amphipod Genus *Crangonyx* (Crangonyctidae) in North America: Virginia Museum of Natural History.