Universidade de Évora - Escola de Ciências e Tecnologia

Mestrado em Biologia da Conservação

Dissertação

Distribuição e dinâmica populacional de *Crangonyx pseudogracilis*, um anfípode invasor.

Daniela Filipa Pereira Correia

Orientadores(es) | Pedro Manuel Silva Gentil Anastácio
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Pedro Manuel Anastácio (Universidade de Évora) (Orientador)

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“Distribuição e dinâmica populacional de *Crangonyx pseudogracilis*, um anfípode invasor”

Resumo

Um dos principais fatores de perda de biodiversidade é a introdução de espécies exóticas invasoras. Em 2011, foi detetada em Portugal uma população abundante de *Crangonyx pseudogracilis*, um anfípode de água doce nativo da América do Norte. Este estudo permitiu analisar a sua dinâmica populacional em Ferrarias e Coruche durante o ano de 2016/2017 e acompanhar a sua expansão, detetando eventuais sobreposições com espécies nativas de anfípodes. Os resultados mostraram que a espécie se reproduz em Ferrarias durante a maior parte do ano, mas em Coruche apenas de março a julho. A densidade de anfípodes diminui de maio a outubro e aumenta de novembro a abril. Tanto a proporção como o comprimento das fêmeas é maior que o dos machos em ambos os locais. Finalmente, notou-se um grande aumento na área de distribuição de *C. pseudogracilis* em relação ao observado em 2014. No entanto, nenhuma sobreposição foi detetada entre espécies de anfípodes nativas e a exótica.

Palavras-chave: Invasões biológicas, Dispersão, Expansão, Período de recrutamento, Estrutura populacional

“Distribution and population dynamics of *Crangonyx pseudogracilis*, an invasive amphipod”

Abstract

One of the main drivers of biodiversity loss is the introduction of exotic invasive species. In 2011, an abundant population of *Crangonyx pseugogracilis*, a freshwater amphipod native to North America, was detected in Portugal. This study allowed us to analyse its population dynamics in Ferrarias and Coruche during the year 2016/2017 and to monitor its expansion, detecting possible overlaps with native species of amphipods. The results showed that this species reproduces in Ferrarias during most of the year, but in Coruche only from March to July. Amphipod density decreases from May to October and increases from November to April. Both the proportion and the length of females are greater than males at both sampling locations. Finally, we noticed a great increase in *C. pseudogracilis* area of distribution compared to that observed in 2014. However, no overlap was yet detected between native amphipod species occurrence and this exotic species.

Keywords: Biological Invasions, Dispersion, Expansion, Recruitment period, Population structure

Introdução Geral

Os ecossistemas de água doce são *hotspots* de biodiversidade e possuem diversas comunidades, sendo algumas delas bastante sensíveis a perturbações ambientais e antropogénicas (Aylward *et al*., 2005). Estes ecossistemas, bem como as suas comunidades, são essenciais para a sobrevivência humana, por exemplo como recurso alimentar, uma vez que permitem a reciclagem de nutrientes e ao mesmo tempo mantêm a qualidade da água (Aylward *et al*., 2005). No entanto fatores como a destruição de habitats, a poluição, a subexploração dos recursos, as mudanças climáticas e a introdução de espécies exóticas (Moyle & Leidy, 1992) afetam constantemente os ecossistemas de água doce. Estes fatores têm frequentemente como consequência extinções de espécies e consequente perda de biodiversidade.

Os anfípodes são uma das várias comunidades que fazem parte dos ecossistemas de água doce. Estes pertencem ao filo Arthropoda, sub-filo Crustacea, classe Malacostraca, super ordem Peracarida e ordem Amphipoda (Rocha, 2012). O seu corpo é desprovido de carapaça, geralmente comprimido lateralmente e subdividido em cabeça (com dois pares de antenas), pereon (especializado em várias funções como a reprodução, a locomoção e a alimentação), pleossoma, urossoma e telson terminal (Rocha, 2012).

Estes macroinvertebrados podem ser encontrados em diferentes habitats, tais como na coluna de água, sendo considerados organismos pelágicos, também podem ser encontrados enterrados no sedimento, considerados assim organismos bentónicos ou podem simplesmente estar escondidos entre os detritos (Ruppert *et al*., 2004). Tendo em conta a diversidade de habitats que os anfípodes habitam, também é de esperar que estes possuam diferentes regimes alimentares, podendo ser detritívoros, herbívoros, filtradores e ocasionalmente carnívoros (Schram, 1986).

Quando os anfípodes se encontram na época de reprodução é comum as fêmeas desenvolverem lamelas com numerosas sedas formando no seu conjunto uma bolsa, que também pode ser chamada de marsúpio. É nessa bolsa que ocorre a deposição dos ovos e o desenvolvimento embrionário, até á eclosão dos juvenis (Rocha, 2012).

Assim sendo, os anfípodes apresentam um desenvolvimento direto, sem fase larvar (ovo – embrião – juvenil - adulto), com diversas mudas, sem metamorfose, onde se acentua após cada uma delas os caracteres sexuais (Forest *et al*., 1999).

Nos ecossistemas de água doce os anfípodes apresentam um papel fundamental no seu bom funcionamento. Estes são muito importantes nas redes alimentares aquáticas, uma vez que atuam como condutores de nutrientes e de energia para níveis tróficos mais elevados (Väinölä *et al*., 2007). Temos como exemplo o facto de os anfípodes serem uma das espécies importantes na dieta dos peixes, servindo também com frequência como hospedeiros intermediários dos seus parasitas (Väinölä *et al*., 2007). Em Portugal são conhecidas três espécies epígeas nativas de anfípodes de água doce, sendo elas *Echinogammarus meridionalis* Pinkster 1973*, Echinogammarus simoni* Chevreux, 1894 e *Echinogammarus lusitanus* Schellenberg 1943 (Pinkster, 1973, 1993; Dobson, 2012; Anastácio, 2019).

Apesar de os anfípodes estarem entre as comunidades bem mais bem-sucedidas, também são as espécies invasoras de invertebrados aquáticos mais comuns (Altermatt *et al*., 2014). Quando uma espécie exótica invasora se estabelece num ecossistema de água doce, é quase impossível eliminá-la (Cuhel & Aguilar, 2013). Para além disso, também é um dos principais fatores de perda de biodiversidade. A presença de espécies invasoras pode promover a predação de espécies nativas, a competição entre espécies exóticas e nativas, hibridação, alteração das propriedades físicas e químicas do habitat, mudanças na cadeia trófica, bioacumulação de substâncias tóxicas e transmissão de doenças e parasitas (Guerra & Gaudêncio, 2016). No caso dos anfípodes, poderá ainda afetar diretamente a reciclagem de nutrientes e a transferência de energia para níveis tróficos mais altos (Piscart *et al*., 2011; Jourdan *et al*., 2016), o que em casos extremos pode causar a eliminação total de populações nativas.

Segundo Katsanevakis *et al*. (2013), para implementar medidas eficazes de prevenção, controlo e monotorização de espécies exóticas invasoras, é fundamental conhecer as características biológicas e ecológicas dessas espécies, bem como monitorizar a sua ocorrência, os seus vetores de introdução e a sua expansão geográfica. Em 2011, uma população abundante de *Crangonyx pseudogracilis* Bousfield 1958 foi detetada no distrito de Santarém, em Portugal (Grabowski *et al.*, 2012). Este anfípode de água doce da família *Crangonyctidae* é nativo da América do Norte, originário de Lake Charles, Louisiana. Em 1939, invadiu a Europa Ocidental a partir da Inglaterra (Crawford, 1937; Tattersal, 1937; Gledhill *et al.*, 1993), Holanda (Pinkster *et al.*, 1980; Zhang & Holsinger, 2003), País de Gales (Gledhill *et al.*, 1993) Escócia (Gledhill *et al.*, 1993), Bélgica (Pinkster *et al.*, 1980), Alemanha (Silfverberg, 1999), Finlândia (Silfverberg, 1999), Irlanda (Holmes, 1975; Dick *et al.*, 1999) e finalmente em 2011, foi detetado em Portugal (Grabowski *et al.*, 2012). Este também foi o primeiro anfípode de água doce exótico detetado na Península Ibérica (Grabowski *et al.*, 2012).

Crangonyx pseudogracilis pode ser encontrado principalmente em ribeiros, rios e lagos com fluxo lento (Holland, 1976; Zhang & Holsinger, 2003; Josens *et al.*, 2005). Apesar de ser uma espécie de água doce, consegue tolerar alguma salinidade (Cloreto: 250–350 mg/l) (Galbreath *et al.*, 2009). Esses anfípodes, quando comparados a outros Gamarídeos, apresentam um comportamento locomotor diferente, pois nadam verticalmente e geralmente ocupam as áreas mais poluídas dos ribeiros, enquanto as espécies nativas preferem ocupar áreas mais bem oxigenadas (Gama *et al.*, 2017). Segundo Grabowski *et al.* (2012), esta espécie exótica reproduz-se mais rapidamente que as espécies epígeas nativas presentes em Portugal, género *Echinogammarus*, pois as fêmeas atingem a maturidade sexual em dois a três meses, e têm uma longevidade que pode chegar até aos dois anos e durante esse período são capazes de produzir cerca de oito ninhadas.

A introdução de *Crangonyx pseudogracilis* na Europa pode ter resultado do transporte de peixes vivos e/ou plantas aquáticas, através de embarcações (Zhang & Holsinger, 2003), ou também devido ao comércio de madeira (Maitland & Adams, 2001). A dispersão desta espécie exótica, após a sua introdução, está também associada aos vetores antropogénicos anteriormente mencionados, mas existem outras possibilidades. Alguns exemplos são, através da pesca (Banha & Anastácio, 2015), na plumagem e pernas das aves, onde os anfípodes se agarram (Rachalewski *et al.*, 2013) ou então através de corredores de migração aquática. No entanto, na

Península Ibérica, este último processo é limitado pelo número reduzido (comparativamente com a Europa central) de canais artificiais que interconectam as bacias hidrográficas (Rachalewski *et al.*, 2013), sendo as outras duas hipóteses mais relevantes para a sua dispersão.

Em Portugal a espécie *Crangonyx pseudogracilis* foi encontrada na bacia do rio Tejo, nomeadamente em alguns afluentes do Sul (Banha *et al.*, 2018). Estes locais possuem um baixo fluxo de água e são ricos em vegetação e substrato de fundo fino, como areia fina e argila (Banha *et al.*, 2018). Levando em conta as definições apresentadas pela Convenção sobre Diversidade Biológica (2020), espécies exóticas são aquelas que foram introduzidas fora da sua área original de distribuição, enquanto espécies exóticas invasoras são aquelas que, para além de terem sido introduzidas fora da sua área de distribuição, possuem um crescimento e/ou reprodução muito superior ao das espécies nativas, competindo por alimento e/ou habitat e apresentando assim riscos negativos para as nativas. *Crangonyx pseudogracilis* não é ainda considerada legalmente uma espécie invasora, no entanto, existem espécies nativas de anfípodes que podem ser ameaçadas pela chegada desta espécie exótica. De facto, é conhecida a presença de populações importantes do género *Echinogammarus*, endémicas da Península Ibérica, em bacias hidrográficas adjacentes, como por exemplo no Mondego e Ribeiras do Oeste.

Os objetivos deste estudo são, monitorizar a expansão de um anfípode exótico de água doce (*Crangonyx pseudogracilis*), identificando distribuições sobrepostas com anfípodes nativos, e analisar a dinâmica populacional dessa espécie em dois locais (Ferrarias e Coruche), durante um ano. A análise da atual área de distribuição deste anfípode, em Portugal, e como ela mudou desde a última avaliação (2014), permitirá estimar o seu coeficiente de difusão e a sua taxa de progressão. A análise da estrutura populacional e da dinâmica populacional fornecerá informações importantes para estimar o potencial invasor da espécie.

Os resultados do nosso estudo serão expostos sob a forma de artigo científico (já submetido), mas mais alongado, no capítulo seguinte desta dissertação.

Research paper

Distribution and population dynamics of *Crangonyx pseudogracilis*, an invasive amphipod

Abstract

One of the main drivers of biodiversity loss is the introduction of exotic invasive species. In 2011, an abundant population of *Crangonyx pseudogracilis*, a freshwater amphipod native to North America, was detected in Portugal. This study allowed us to better understand its biology, analysing its population dynamics in a temporary river pool and a small lake for one year, and to follow its expansion. Our results showed that this species reproduces in the temporary river pool during most of the year, but in the lake only from March to July. Amphipod density decreases from May to October and increases from November to April. As usual, females were larger than males, but the proportion of females was higher than males at both sampling locations. Finally, we noticed a great increase in *C. pseudogracilis* distribution area in relation to what was observed in 2014, with a diffusion coefficient of 2495.27 Km^2/year and a spread rate of 26 Km/year. However, no overlap was yet detected between native and exotic amphipod species. Since the distance to known native amphipods locations is very small, there is great potential for *C. pseudogracilis* to have a negative impact on native species in a very near future.

Keywords: Biological Invasions, Dispersion, Expansion, Recruitment period, Population structure

1. Introduction

Freshwater ecosystems are biodiversity hotspots with diverse communities, some of which are very sensitive to environmental and anthropogenic changes (Aylward *et al.*, 2005). These ecosystems, as well as their communities, are essential for human survival, for example as a food resource, and to allow nutrient recycling and maintaining the water quality at the same time (Aylward *et al.*, 2005).

Amphipods are one of the several communities that are part of freshwater ecosystems. These belong to the phylum Arthropoda, subphylum Crustacea, class Malacostraca, superorder Peracarida and order Amphipoda (Rocha, 2012). Its body has a carapace, usually laterally compressed and subdivided into the head (with two pairs of antennae) pereon, pleosome, urosome and telson (Figure 1) (Rocha, 2012).

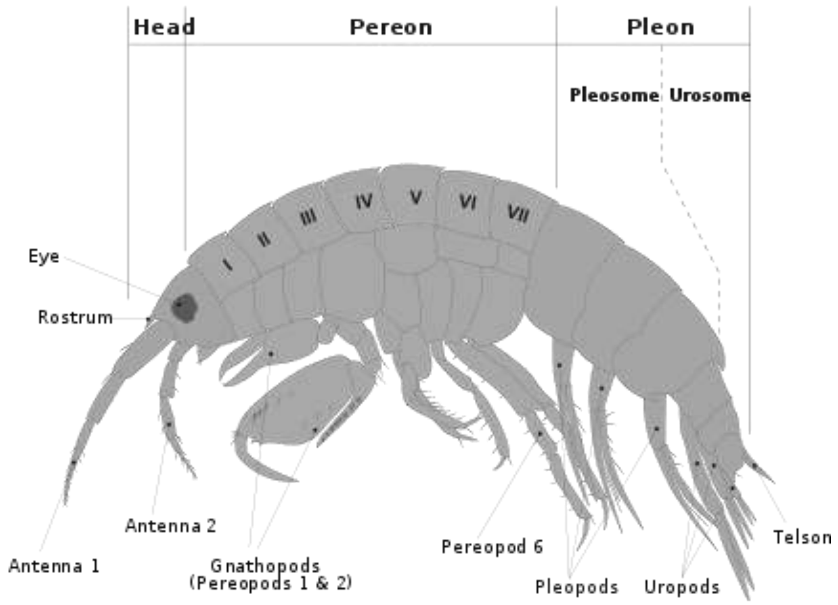


Figure 1. Anatomy of an amphipod. Source: Love (2017).

These macroinvertebrates can be found in different habitats such as the water column, being considered pelagic, or in the sediment, buried or hidden among the detritus, being considered benthic organisms (Ruppert *et al.*, 2004). When amphipods are in the reproduction season, it is common for females to develop lamellae with numerous silks, forming a pouch (Figure 2). Eggs are deposited and develop in this pouch until they hatch into embryos (Rocha, 2012), which are also kept in the pouch.

Figure 2. Female with eggs in a pouch.

In freshwater ecosystems, the amphipods have a fundamental role in their proper functioning. They are very important in aquatic food networks since they act as nutrient and energy conductors for higher trophic levels (Väinölä *et al.*, 2007). In Portugal, we can find three native epigean species of freshwater amphipods. These are *Echinogammarus meridionalis* Pinkster 1973, *Echinogammarus simoni* Chevreux, 1894 and *Echinogammarus lusitanus* Schellenberg, 1943 (Pinkster, 1973, 1993; Dobson, 2012; Anastácio, 2019).

One of the main factors of biodiversity loss is the introduction of exotic invasive species in freshwater ecosystems. This promotes the predation of native species; competition between non-native and native species; hybridization; alteration of the physical and chemical proprieties of the habitat; changes in the food web; bioaccumulation of toxic substances and transmission of diseases and parasites (Guerra & Gaudêncio, 2016). In the case of amphipods, it will affect the recycling of nutrients and the transfer of energy to higher trophic levels (Piscart *et al.*, 2011; Jourdan *et al.*, 2016), sometimes causing the total elimination of native populations. According to Katsanevakis *et al.* (2013), to implement effective measures for the prevention, control, and management of invasive exotic species, it is fundamental to know the biological and ecological characteristics of these species, as well as to monitor their occurrence, vectors of introduction and geographical expansion.

In 2011, an abundant population of *Crangonyx pseugogracilis* Bousfield 1958 was detected in the Santarém district of Portugal (Grabowski *et al*., 2012) (Figure 3). This freshwater amphipod from the *Crangonyctidae* family is native to North America and can be found for example in Lake Charles, Louisiana (Slothouber *et al*., 2010). In 1939 it invaded Western Europe starting in England (Crawford, 1937; Tattersal, 1937; Gledhill *et al*., 1993), Netherlands (Pinkster *et al*., 1980; Zhang & Holsinger, 2003), Wales (Gledhill *et al*., 1993) Scotland (Gledhill *et al*., 1993), Belgium (Pinkster *et al*., 1980), Germany (Silfverberg, 1999), Finland (Silfverberg, 1999), Ireland (Holmes, 1975; Dick *et al*., 1999) and finally in 2011 it was detected in Portugal (Grabowski *et al*., 2012). This is the first exotic freshwater amphipod detected in the Iberian Peninsula (Grabowski *et al*., 2012).

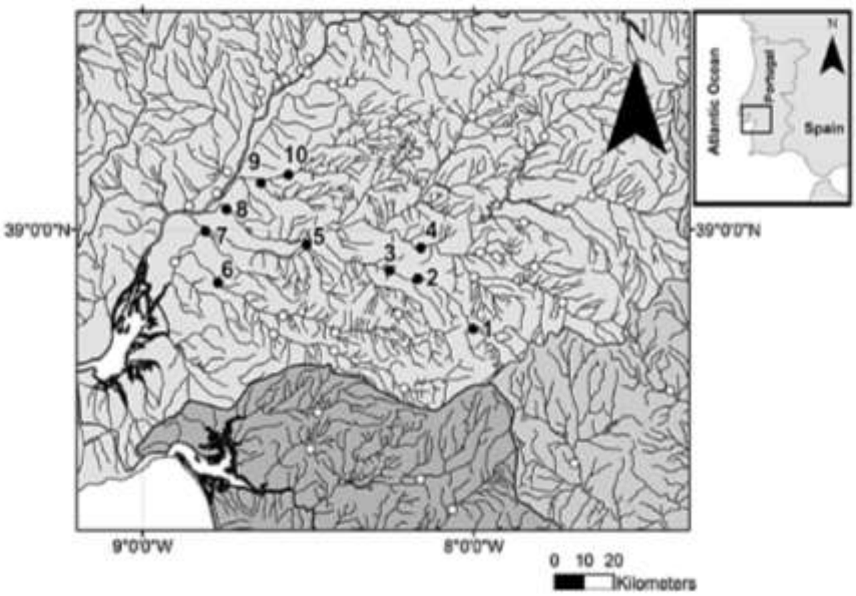


Figure 3. The distribution area of *Crangonyx pseudogracilis* in the Tagus basin in 2011. Black points indicate the presence of this species while white points indicate absence. Source: Banha *et al*. (2018).

Crangonyx pseudogracilis can be found mainly in streams, rivers and lakes with slow flow (Holland, 1976; Zhang & Holsinger, 2003; Josens *et al*., 2005). Despite being a freshwater species, it can tolerate some salinity (Chloride: 250–350 mg/l) (Galbreath *et al.*, 2009). These amphipods, when compared to other *Gammarids*, present a different locomotor behaviour, as they swim vertically and usually occupy the most polluted areas of the streams (Gama *et al.*, 2017). According to Hynes (1955), Sutcliffe & Carrick (1981), Pinkster & Platvoet (1983) and Dick *et al*. (1998, 1999), this exotic species reproduces faster than the native epigean species present in Portugal, genus *Echinogammarus*, as females reach sexual maturity in two to three months, having longevity that can reach up to two years, and during this period are capable of producing up to eight broods.

The introduction of this species in Europe may have resulted from the transport of live fishes and/or aquatic plants, on boats (Zhang & Holsinger, 2003), or from the timber trade (Maitland & Adams, 2001). *Crangonyx pseugogracilis* dispersion after the introduction is associated with the previously mentioned human vectors or other activities like fishing (Banha & Anastácio, 2015) but there are other possibilities, such as on the plumage and legs of birds (Rachalewski *et al.*, 2013) or through aquatic migration corridors. However, in the Iberian Peninsula, the latter is limited by the reduced number of artificial channels that interconnect the river basins (Rachalewski *et al.*, 2013).

In Portugal, *Crangonyx pseudogracilis* was found in the Tagus river basin namely in some south tributaries (Banha *et al.*, 2018). These sites have a low water flow, are rich in vegetation and present fine substrate such as fine sand and clay (Banha *et al.*, 2018). The definitions presented by the Convention on Biological Diversity (2020) mention that an exotic species is a species introduced outside its original area of distribution, and an exotic invasive species is an exotic species with negative risks for native species (from that distribution area). *Crangonyx pseudogracilis* is not yet legally considered an invasive species, however, there are native species of amphipods that might be threatened by the arrival of this exotic species. In Portugal, there are some populations of the genus *Echinogammarus*, endemic to the Iberian Peninsula, in several river basins, such as for example the Mondego and Ribeiras do Oeste which are adjacent to the currently invaded Tagus basin.

The objectives of this study are to monitor the expansion of an exotic freshwater amphipod (*Crangonyx pseudogracilis)* in Portugal, identifying overlapping distributions with native amphipods and to analyse the population dynamics of this species at two sites (Ferrarias and Coruche). The analysis of the current distribution area of this amphipod in Portugal and how it has changed since the last fieldwork assessment (2014), will allow us to estimate its diffusion coefficient. Population structure and population dynamics analysis will provide important information to estimate the invasive potential of the species.

2. Methodology

2.1. Field sampling for population dynamics

In order to study the population dynamics of *Crangonyx pseudogracilis*, samples were taken in two locations (Ferrarias, 38º54'08.77''N, 8º15'39.40''W; and Coruche, 38°57'12.06"N, 8°31'8.95"W), that are 23.09 km away from each other), where the presence of this species had already been detected in 2011 (Figure 3). These samples were collected between May 2016 and June 2017, with a 1 mm mesh dip net and stirring the substrate. The total sampled area was always constant, 1.5 m^2 for each location, i.e. 3 replicates of 0.5 m^2, allowing for density comparisons. To assure the collection of all individuals in the sampled area, the same effort of 10 minutes per replicate was always used. This was previously tested and considered enough to collect all individuals in that sampling area size. Samples were kept in bottles containing 96% ethylic alcohol, which also allows for DNA preservation, in case further genetic studies are needed. For each location during the sampling period (14 months), physicochemical variables, such as pH, dissolved oxygen, conductivity, temperature was monitored using a portable WTW field probe model MultiLine P4 and a Hanna model HI 93703-11 was used for turbidity measurements. One Tinytag Aquatic 2 Datalogger model TG-4100 was left at each location, but the one in Ferrarias disappeared. Microhabitat characteristics, such as water depth, type of vegetation, type of substrate and speed of water flow were also recorded (Grabowski *et al*., 2012).

2.2. Field sampling for *Crangonyx pseudogracilis* distribution

To know the current distribution of *Crangonyx pseudogracilis*, in Portugal, samples were taken during 2019 (June, July and October). These included locations where the species was not found in 2011 (Tagus river Basin) (Figure 3) and the sampling was expanded to locations provided by the Administration of the Tagus and West Hydrographic Region (ARHTO) where amphipods were previously found but not identified. A total of 57 locations were sampled in the Tagus river basin and Western Portugal (small river basins). The physicochemical variables were measured, and microhabitat characteristics of each site were recorded, as previously described. All sampling sites contained water throughout the year (Banha *et al*., 2018). The sampled area varied from place to place, since in this case, the purpose was only to assess the presence of amphipods in each location during a maximum period of one hour. In the locations where amphipods were found, they were collected using a 1 mm mesh dip net and stored in bottles containing 96% ethanol, for further identification in the laboratory as native or exotic amphipods.

2.3. Population dynamics sample analysis

An *OLYMPUS SZ40* Stereo Zoom Microscope was used to identify the sex of each individual, count the number of eggs and/or embryos present in fertilized females and measure the length of each amphipod. Mature reproductive females (Figure 4A) have a pouch with eggs (Figure 4B) and/or embryos in the abdomen (Figure 4C). Males have *calceoli,* which are clavate structures, in the second pair of antennas (Figure 5). The number of eggs and/or embryos was counted whenever detected (Banha *et al*., 2018). Amphipods were measured head to telson (Banha *et al*., 2018) using a *Leica EC3* video camera for image capture, which were analysed and measured using *IC Measure* software.

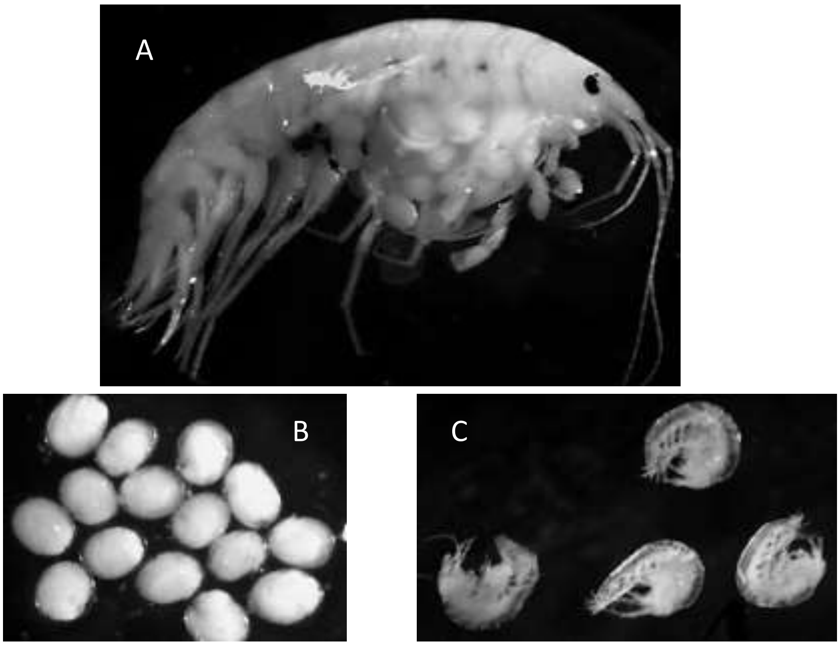


Figure 4. A- Female with eggs and embryos; B- *Crangonyx pseudogracilis* eggs; C- *Crangonyx pseudogracilis* embryos.

Figure 5. Clavate structures (*calceoli*) present in *Crangonyx pseudogracilis* males.

Figure 6. *Crangonyx pseudogracilis* measurement was made from the head to the telson.

2.4. *Crangonyx pseudogracilis* distribution sample analysis

The samples collected in 2019 to assess the distribution of *Crangonyx pseudogracilis*, were analysed in the laboratory, to identify whether the amphipods collected were native (e.g. *Echinogammarus meridionalis*), or non-native (e.g. *Crangonyx pseudogracilis*). This identification was made with an *OLYMPUS SZ40* Stereo Zoom Microscope and was based on the identification keys "*Identifying Invasive Freshwater Shrimps and Isopods*" and "*Key to the epigean freshwater amphipods in continental Portugal*" by Dobson (2013) and Anastácio (2019), respectively. For *Crangonyx pseudogracilis*, the sex of each individual and the number of eggs and/or embryos were registered. All the specimens were photographed with a *Leica EC3* video camera, attached to an *OLYMPUS SZ40* Stereo Zoom Microscope.

2.5. Population dynamics statistical analysis

One mm size classes were created, starting at 1 mm and ending at 16 mm, and size-frequency histograms were produced for males and for females, at each location and sampling date. *FiSAT II Software* (version 1.2.2, FAO-ICLARM) was used for modal progression analysis of size-frequency distribution data (Anastácio *et al*., 2018). The initial analysis was made using the *Bhattacharya* method (Sparre *et al*., 1992) and then the *Normsep* method with the *Simplex* algorithm was applied (Anastácio *et al*., 2018). For each of the cohorts, the standard deviation and average size of males and females were calculated. With these measures, scatter plots were created, where it was possible to identify the cohorts and to calculate the growth rate of some cohorts.

In order to find out if the males and females of each of the sites were of the same size, the Independent samples t-test (Zar, 1999) was used, comparing separately the size of males and females in each location. To compare the proportion of females and males in each month for each location separately during the sampling period, the *Chi-Square* test was used. The *Wilcoxon* test was used to compare the proportion of females between the sampled locations (Ferrarias and Coruche). The *Wilcoxon* test was also used to compare each of the physical-chemical variables (dissolved oxygen, pH, conductivity, turbidity, temperature, water depth) between sampling sites. *SPSS* *software* (version 24.0) was used for these statistical tests. Regarding the physical-chemical variables, boxplots were created for each of the sampled locations, using *GraphPad Prism software* (version 5.01).

2.6. Species distribution mapping

With the data obtained and using the *ArcGIS Software v. 10.6*, *Crangonyx pseudogracilis* distribution maps were created, thus updating the existing information. These maps contain a total of 75 points mapped from north to south of Portugal (Figure 7), with more emphasis on the Tagus river basin and West zone, which was where our sampling was focused. Of these 75 points, 10 of them correspond to points where the exotic species had already been detected in 2014 and 18 points correspond to samples that were provided to us by other research teams, where amphipods were present. In order to know the current area of the species and what it occupied in 2014, the minimum convex polygon area was calculated in *ArcGIS* software. From the difference in the area occupied by the species in 2014 and now in 2019 it was possible to calculate the speed of the invasion of *Crangonyx pseudogracilis*, namely its diffusion coefficient (D), expressed as Km^2 per year (see for ex. Lockwood *et al*. 2007 for further details). Measuring the distance from new points (2019) to the nearest old points (2014), it was possible to estimate the mean Spread rate (*c*) in Km per year. Finally, using the equation $c = 2\sqrt{rD}$ it was possible to estimate the intrinsic growth rate (r) of the population (see Lockwood *et al*. 2007 for more details).

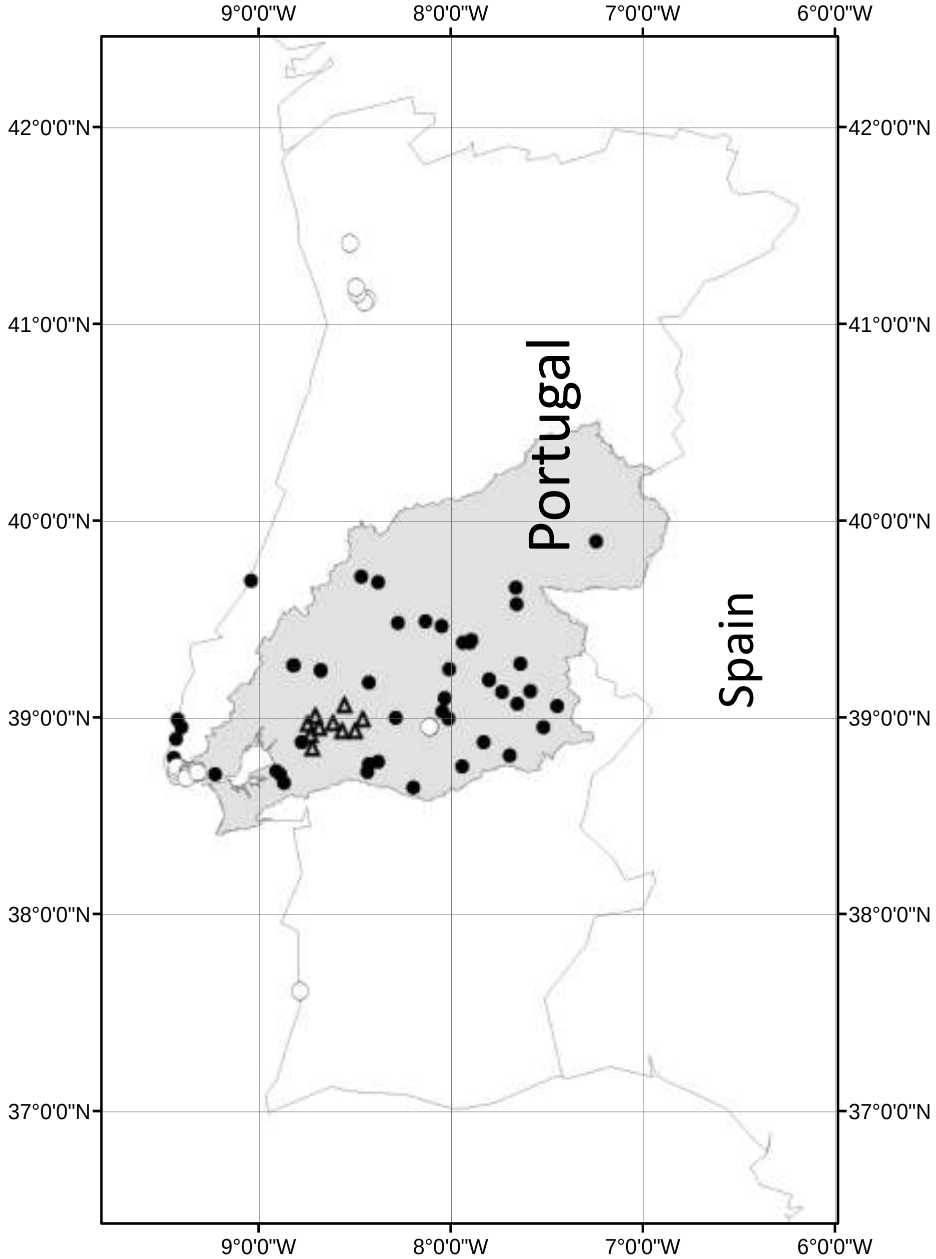


Figure 7. Map with the 75 points sampled. The black circles represent our points (2019). The black triangles represent the 2014 points. White circles represent other research teams' points. Grey indicates the Tagus river basin in Portugal.

3. Results

3.1. Population dynamics in Ferrarias and Coruche

In Ferrarias the total number of individuals captured during the sampling period was 1440, with a mean density from 14 to 680 amphipods m^{-2} (Figure 8). Regarding Coruche the total number of individuals captured between May 2016 and June 2017 was 1099, with a mean density from 22 to 342 amphipods m^{-2} (Figure 8). Densities are clearly higher in Coruche during winter and spring. A somewhat similar pattern of abundance occurs in Ferrarias, but with a distinct peak in August.

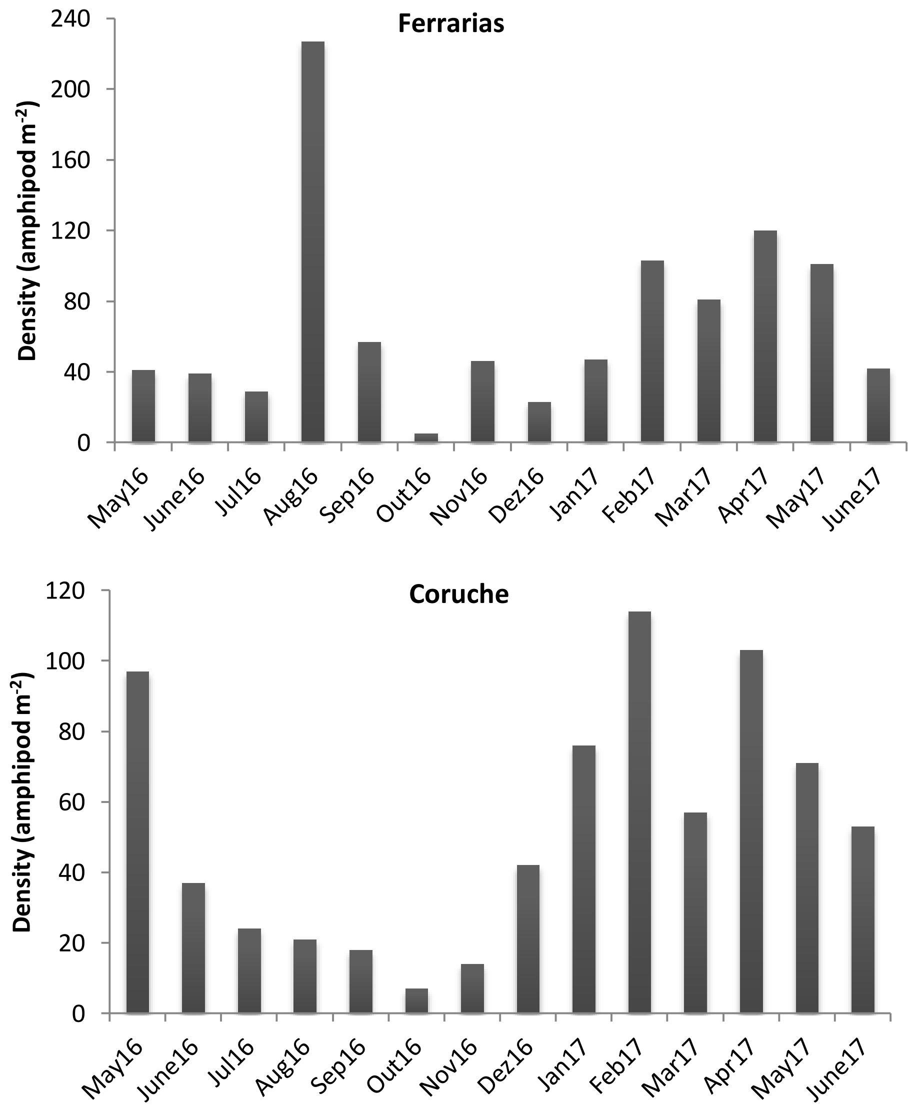


Figure 8. Density of amphipods in Coruche and Ferrarias

In general, and for almost every month, the proportion of females was higher than males for both locations (Figure 9). In Ferrarias we obtained an overall proportion of 0.76 females to 0.24 males and in Coruche the total proportion was 0.63 females to 0.37 males (Figure 9). Regarding the total proportion of females with eggs, this was 0.11 in Coruche and 0.05 in Ferrarias. The total proportion of females with embryos was 0.01 for both Coruche and Ferrarias (Figure 9).

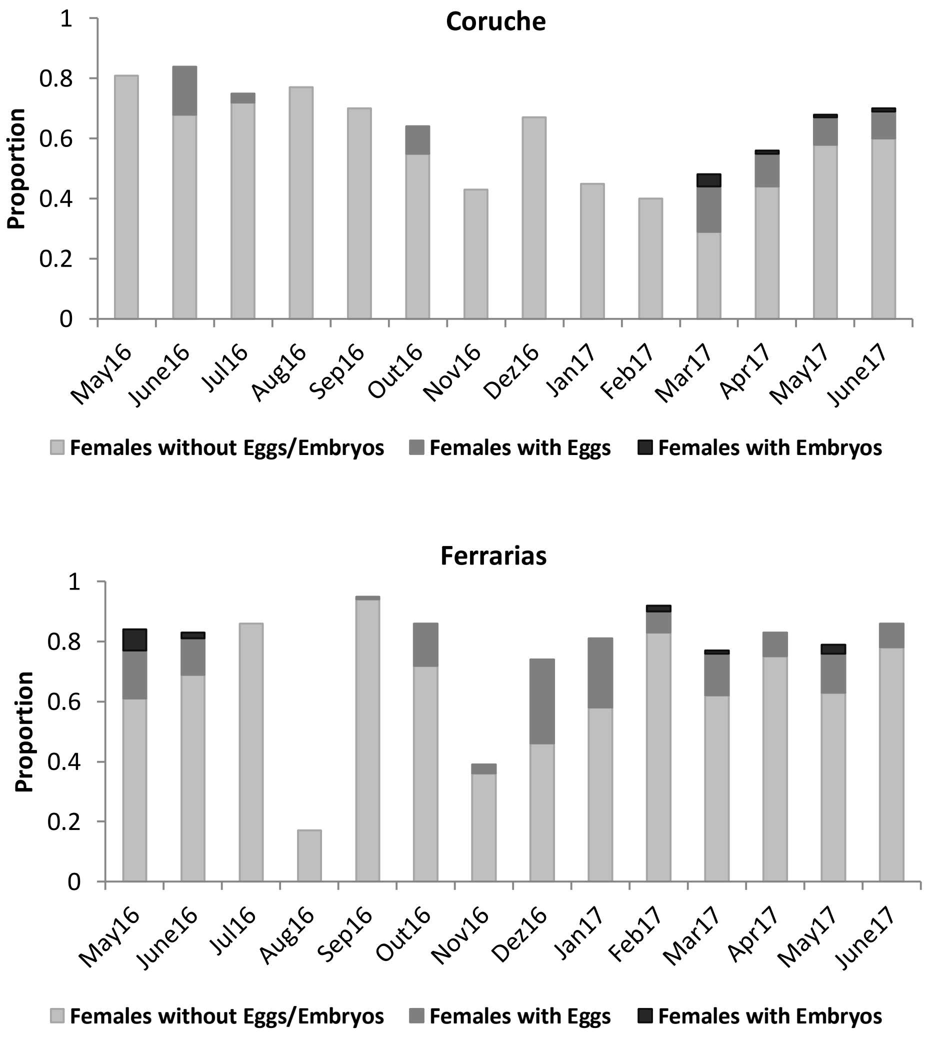


Figure 9. Proportion of females in Coruche and Ferrarias.

Figure 10 represents the reproduction season of *Crangonyx pseudogracilis*, in Coruche and Ferrarias. In Ferrarias females with eggs occur from January to June and September to December, with higher incidence from January to June and in December.

Females with embryos occur in February, March and June with a greater incidence in May. In Coruche, we found females with eggs from March to July and in October, with a higher incidence from March to June. Females with embryos occur in the months of March to June, especially in March.

In Ferrarias reproduction occurs during most of the year, except for July and August, with a peak from December to June. In the case of Coruche, the reproduction season of *Crangonyx pseudogracilis* occurs during the months of March to July.

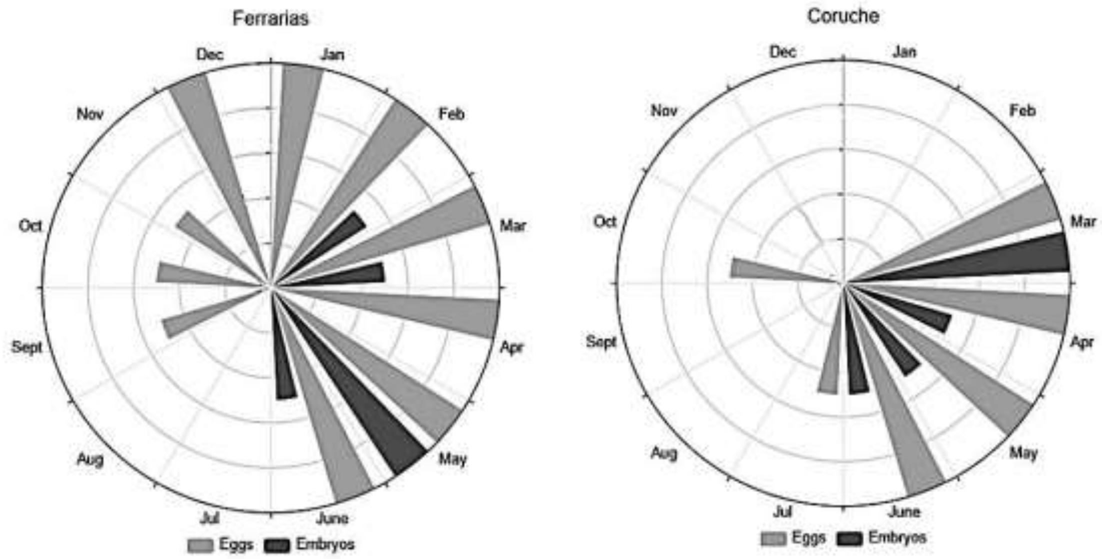


Figure 10. Reproduction intensity in Ferrarias and Coruche.

For Coruche, a total of 559 eggs were counted with an average of 10.96 per female, in which the minimum size of females with eggs was 2.99 mm. In Ferrarias there was a total of 2464 eggs with an average of 25.94 eggs per female, and the minimum size of females with eggs was 3.24 mm. The total number of embryos observed in females for Coruche was 59, with an average of 9.38 embryos per female, and the minimum size of females with embryos was 5.08 mm. For Ferrarias, the total number of embryos counted was 56, with an average of 5.09 per female, and the minimum size of females with embryos was 6.72 mm.

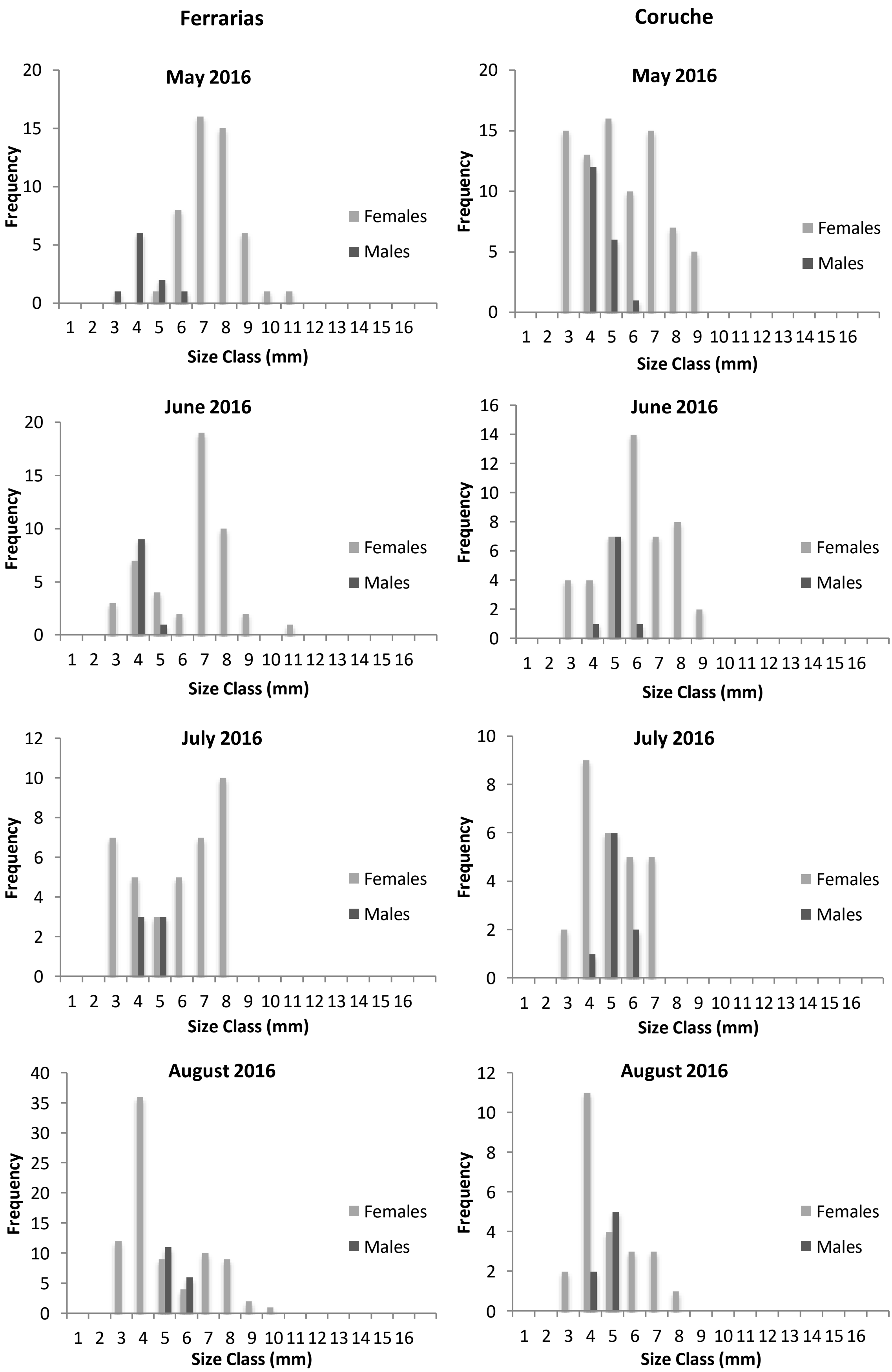


Figure 11. Histograms of males and females for each month in each of the sampled location.

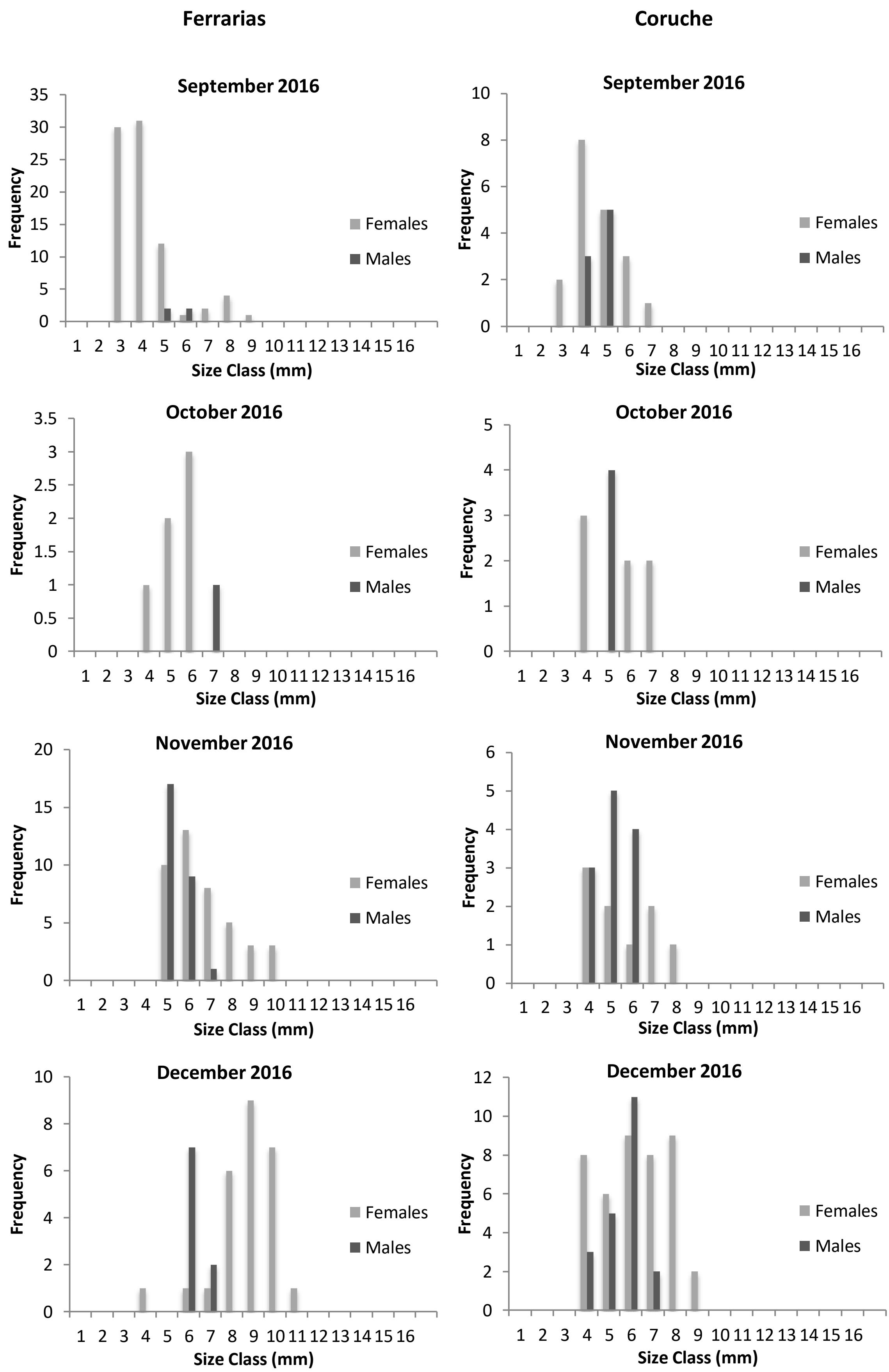


Figure 11. Histograms of males and females for each month in each of the sampled location (continued).

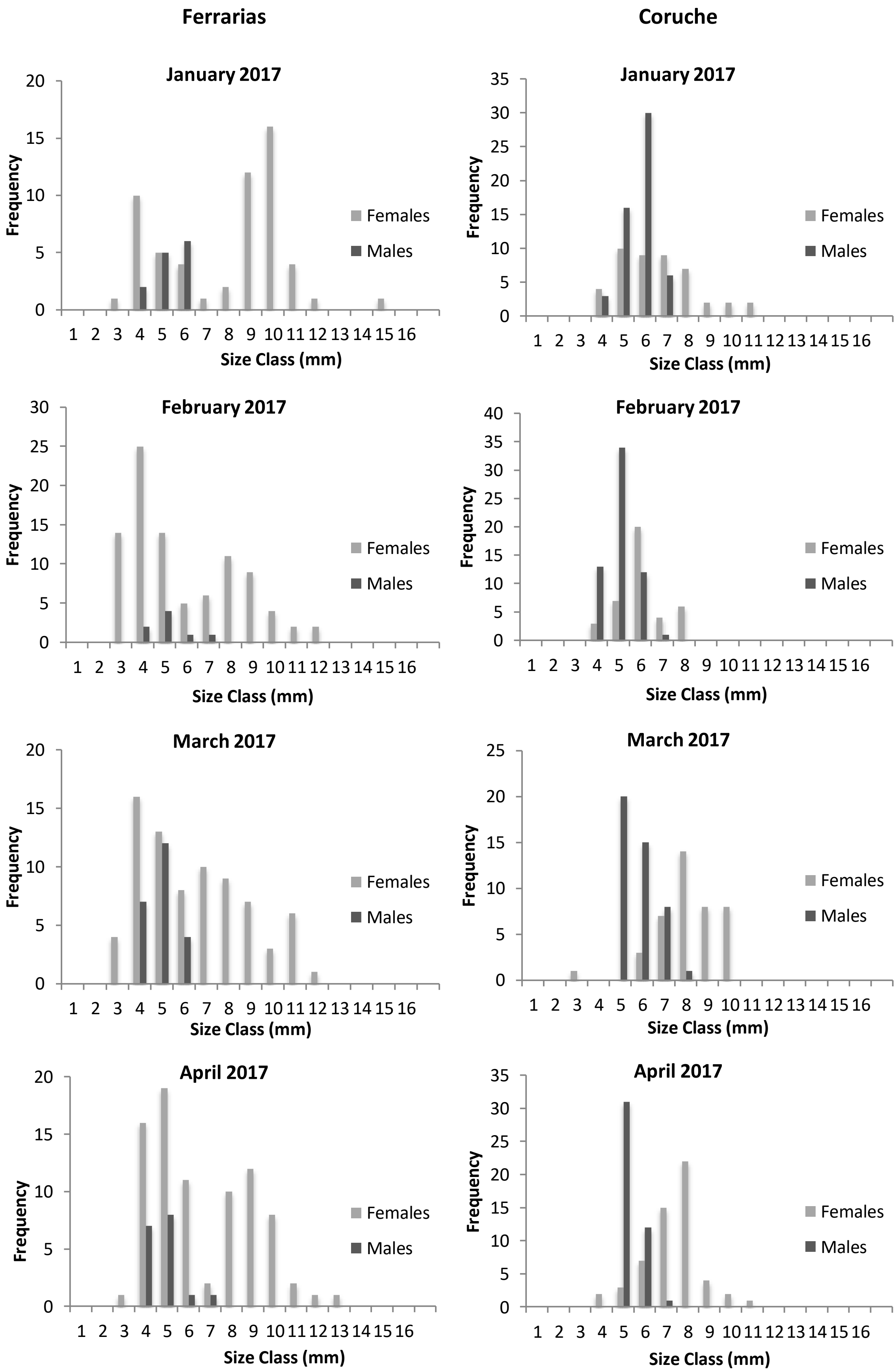


Figure 11. Histograms of males and females for each month in each of the sampled location (continued).

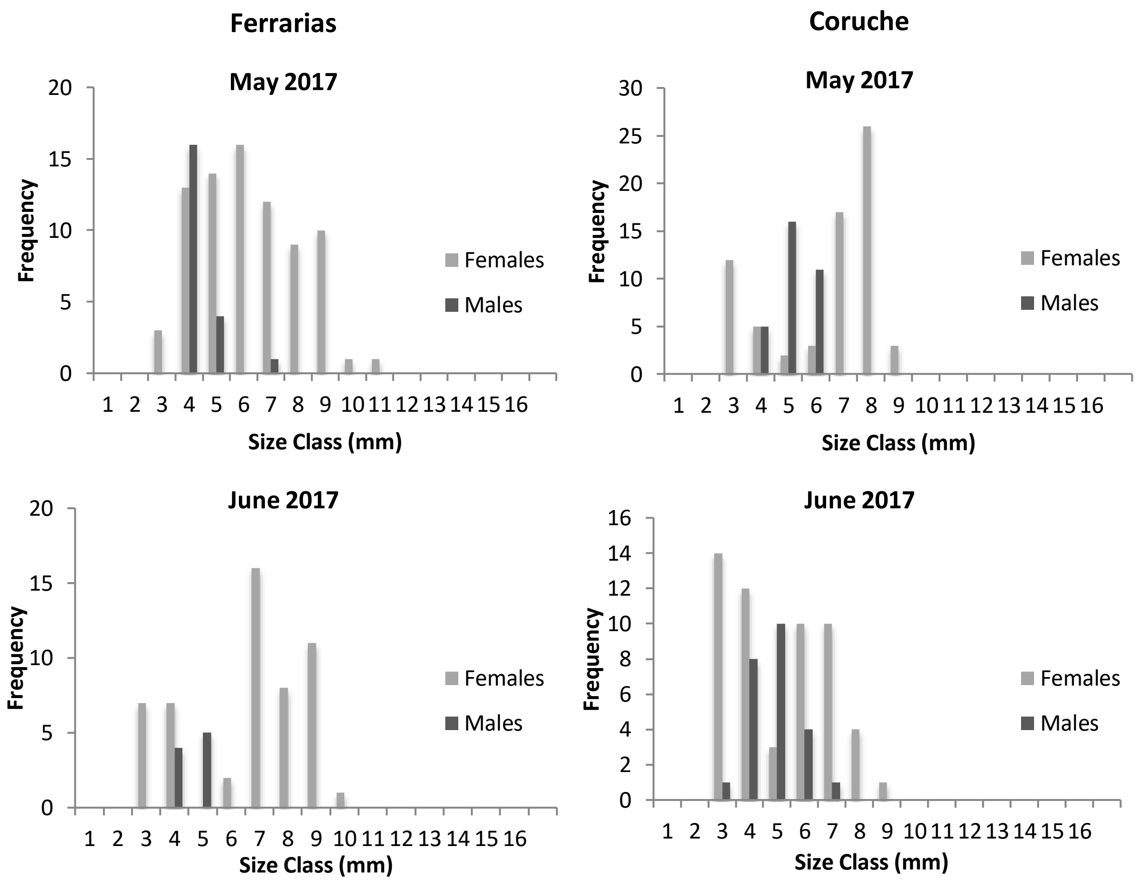
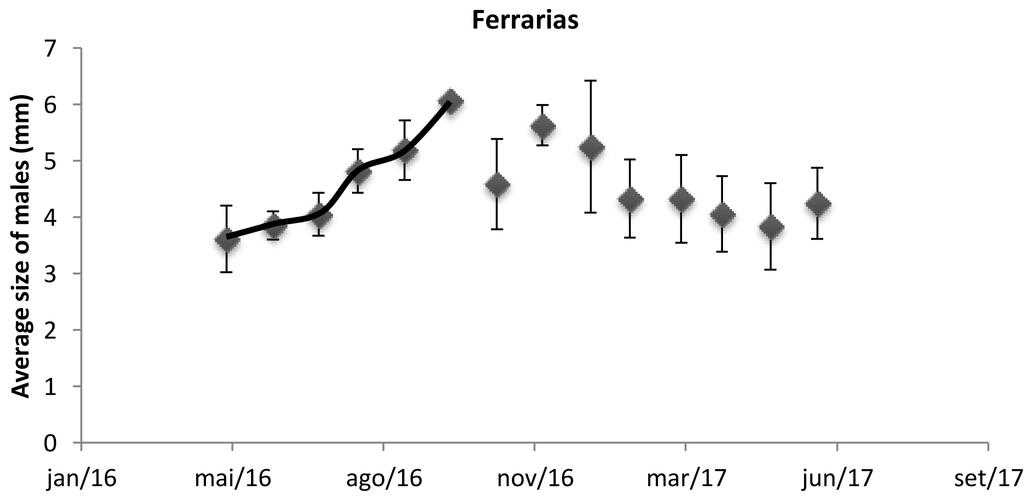


Figure 11. Histograms of males and females for each month in each of the sampled location (continued).

In Figure 11, male and female histograms are shown for each month in each of the sampled locations. Females have a greater range of size classes than males in all months and for both sampled locations. We found that females are larger than males (approximately 1 mm), both for Coruche (independent samples t-test: $p \leq 0.001$) and for Ferrarias (independent samples t-test: $p \leq 0.001$). In Coruche we obtained a mean length of 5.56 mm (1.99 - 10.46 mm range) for females and 4.74 mm (2.99 - 7.99 mm range) for males. In the case of Ferrarias, the mean was 6.11 mm (1.82 - 14.54 mm range) for females and 4.51 mm (2.93 - 6.75 mm range) for males. Females with eggs had a mean length of 7.32 mm (2.99 - 9.91 mm range) in Coruche and 8.34 mm (3.24 - 12.58 mm range) in Ferrarias. In the case of the females with embryos, the mean length was 7.3 mm (5.08 - 8.48 mm range) in Coruche and 7.84 mm (6.72 - 9.6 mm range) in Ferrarias.

In Ferrarias, females have a greater range of size classes in January, (2 - 15 mm). Males have a greater range of size classes in May 2016, (2 - 6 mm), and in February, April and May 2017 (3 - 7 mm). In Coruche, females have a larger range of size classes in January (3 - 11 mm), March (2 - 10 mm) and April (3 - 11 mm). Males have a greater range of size classes in January (3 - 7 mm). For both males and females, from Coruche and Ferrarias, October presents the smallest range of size classes.

Figure 12 presents the cohorts for females and males from Ferrarias. For females we tracked a cohort from February to June 2017, with an average growth rate of 0.80 mm/month. A male cohort was tracked from May to October 2016, with an average growth rate of 0.49 mm/month. Male and female cohorts for Coruche are represented in Figure 13. However, it is not possible, neither for males nor females, to track the growth rate for any of the cohorts.



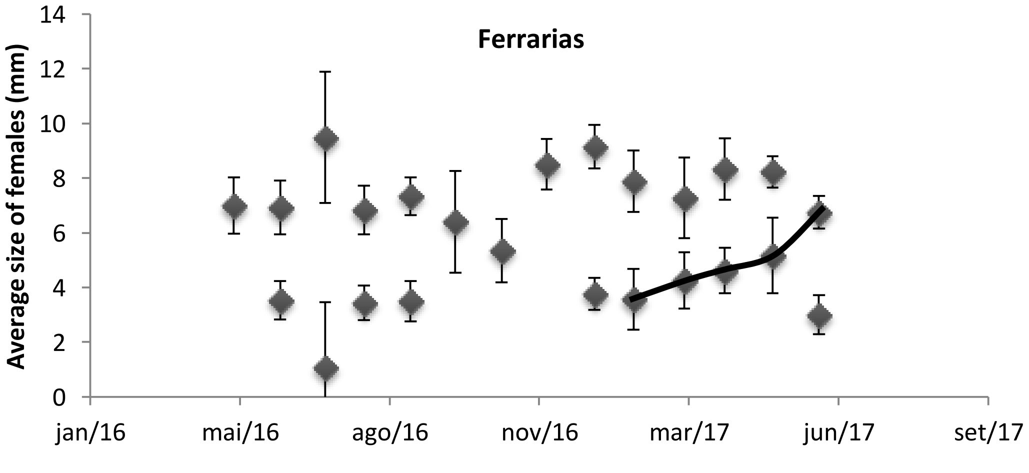


Figure 12. Males and female cohorts detected in Ferrarias. A tracked cohort is represented in each figure.

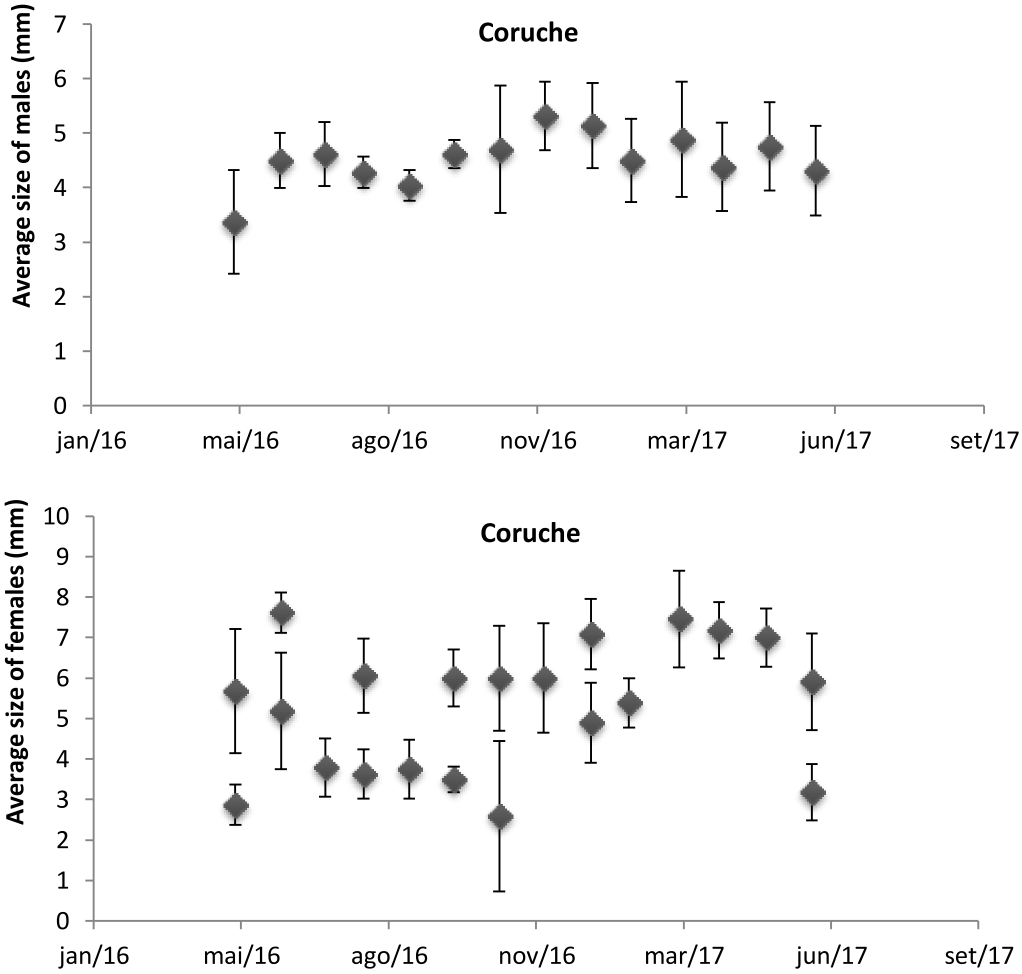


Figure 13. Males and female cohorts detected in Coruche.

The proportion of males and females differs in Coruche (Chi-square test: x^2=104.013, d.f.=13; p<0.001) and in Ferrarias (Chi-square test: x^2=272.447, d.f.=13; p<0.001). The proportion of females was significantly different in Ferrarias and Coruche (Wilcoxon test: Z=-2.355; p<0.05), which was higher for Ferrarias than for Coruche. All physical-chemical variables were significantly different in Ferrarias and Coruche according to a Wilcoxon test (O_2: Z=-3.040, p=0.002; depth: Z=-2.806, p=0.005; turbidity: Z=-2.803; p=0.005; conductivity: Z=-3.181; p=0.001; temperature: Z=-2.552; p=0.011; pH: Z=-2.621; p=0.009) (Figure 14).

In Figure 14 we can see that, in Coruche, depth presents a greater amplitude of variation, attaining larger values than in Ferrarias. In the case of dissolved O_2, it is in Ferrarias that there is a higher variation. As for turbidity, it is in Coruche that there is a greater variation. Ferrarias presents a greater variation of the conductivity, however, values are higher for Coruche. In the case of the temperature, the variation for Ferrarias and Coruche is practically the same, being slightly higher in Ferrarias. Finally, pH presents an identical range of variation in both locations.

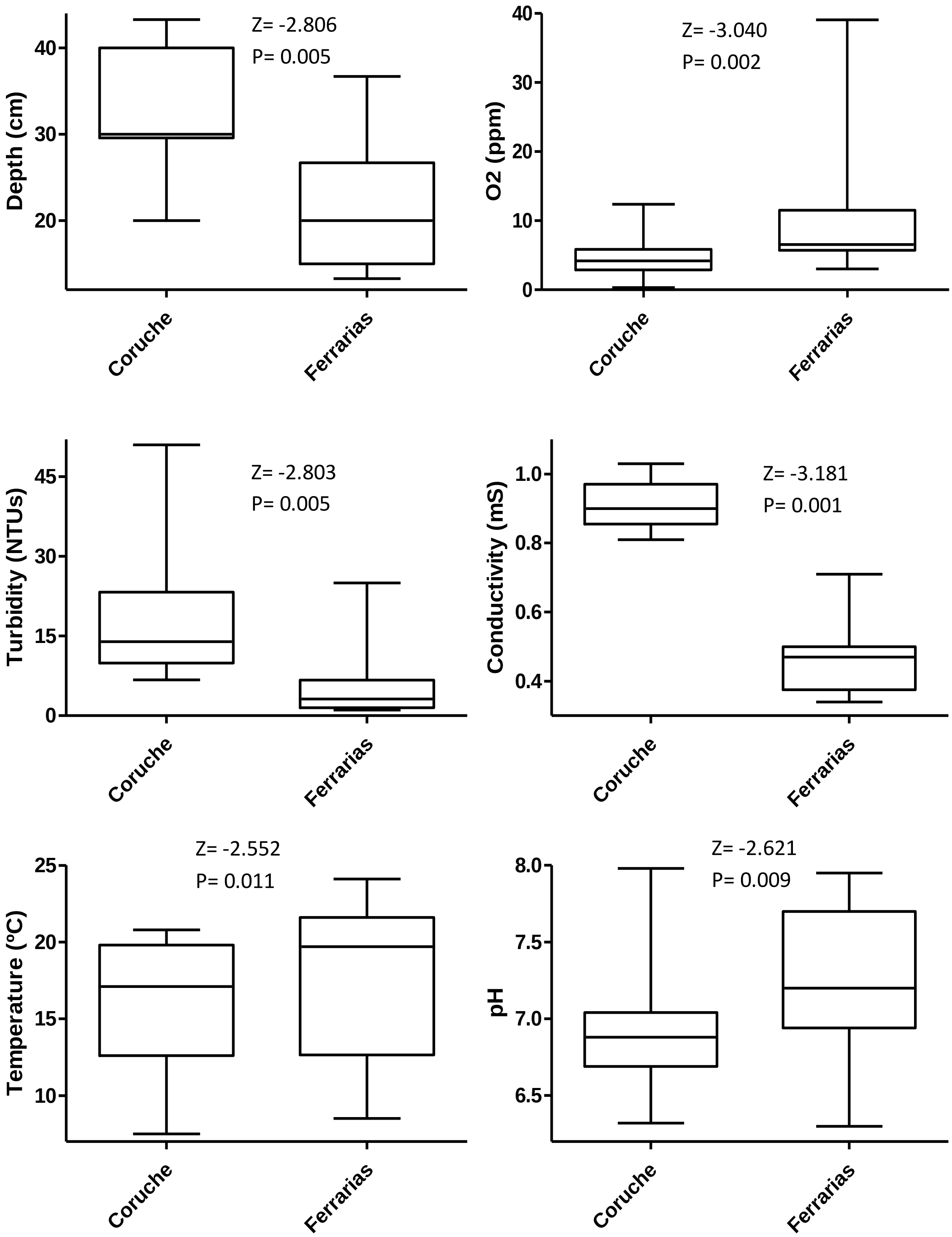


Figure 14. Boxplot of the physical-chemical variables (Depth, O_2, Turbidity, Conductivity, Temperature and pH) in Coruche and Ferrarias. Results of each Wilcoxon test are also presented.

Water temperature in Coruche is displayed in Figure 15. In this location, the maximum temperature varied between 7 °C and 38 °C, while the average temperature varied among 6 °C and 26 °C and finally the minimum temperature varied between 6 °C and 25 °C.

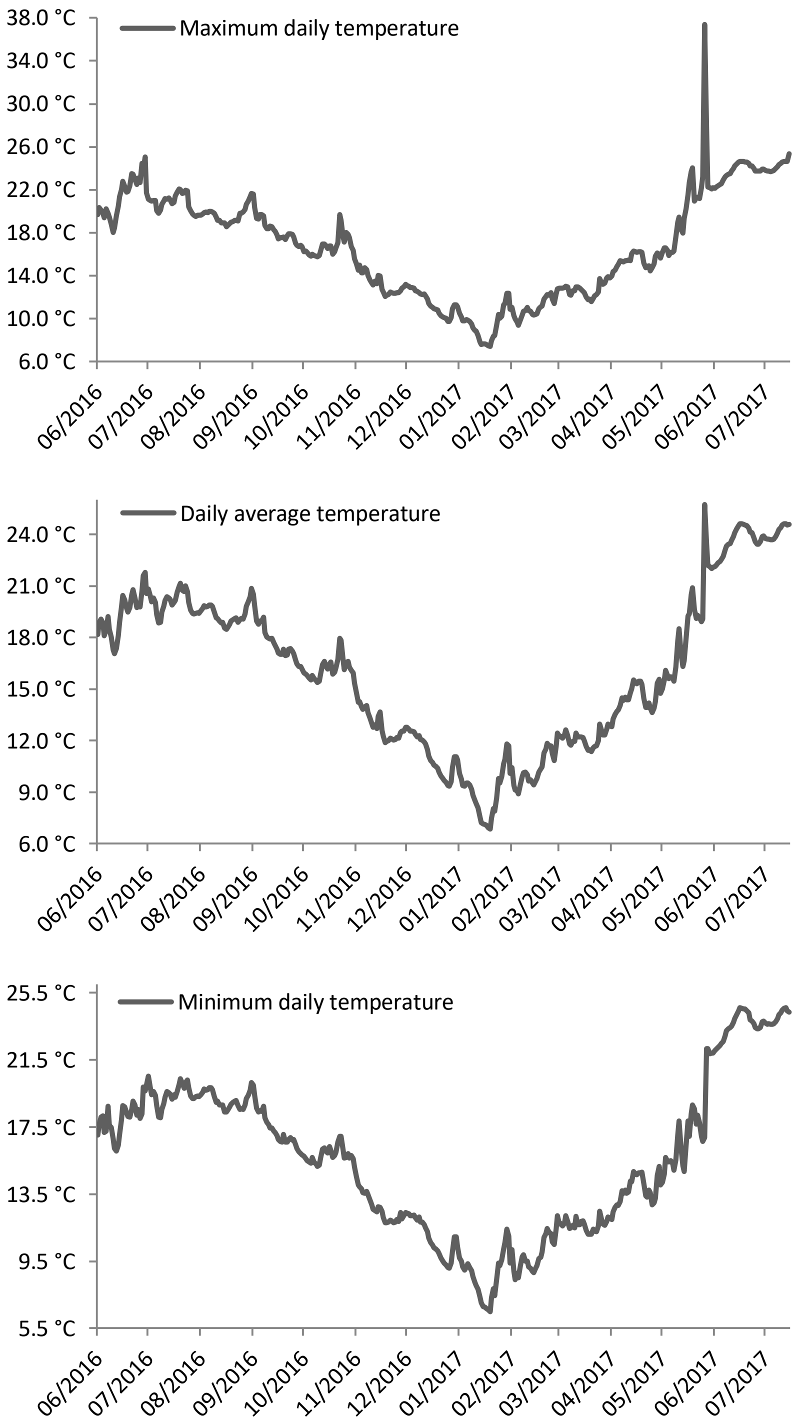


Figure 15. Water temperature in Coruche.

3.2. Species distribution analysis

In 2014 only 10 points were identified in the area of the Tagus basis with the presence of *Crangonyx pseudogracilis* (Banha *et al*., 2018), occupying an area of 579.13 Km^2. Currently, the distribution area has increased in Portugal, no longer being restricted to a small part of the Tagus basin and now it includes a large part of the Tagus river basin, with an area of 13055.46 Km^2. Furthermore, we are aware, through the analysis of amphipod samples provided by other research teams, that this species reached the north (Douro river basin and Ave river basin) and south (small basin in south-west) of the country. Knowing that the area of distribution of the exotic species in the Tagus and west basins increased by 12476.33 Km^2 in 5 years, it has an approximate diffusion coefficient (D) of 2495.27 Km^2/year. By calculating the distance from the furthest new point (2019) to the old point (2014) closest to that, we obtained an approximate spread rate (*c*) of 26.06 Km/year. Knowing the diffusion coefficient (D) and the spread rate (*c*), the intrinsic growth rate (r) was estimated at 0.07 /year.

Of the 57 sites sampled by our team in the Tagus and west area, the presence of *Crangonyx pseudogracilis* was found in 28 locations and the presence of the native species, genus *Echinogammarus*, was detected in 8 points. Regarding the 18 points corresponding to the samples provided by other researcher teams, the presence of *Crangonyx pseudogracilis* was detected in 8 of these points and in 2 of them native amphipods (genus *Echinogammarus*) were found. However, there was no overlap between the exotic species and the native species for any of the mapped locations (Figure 16).

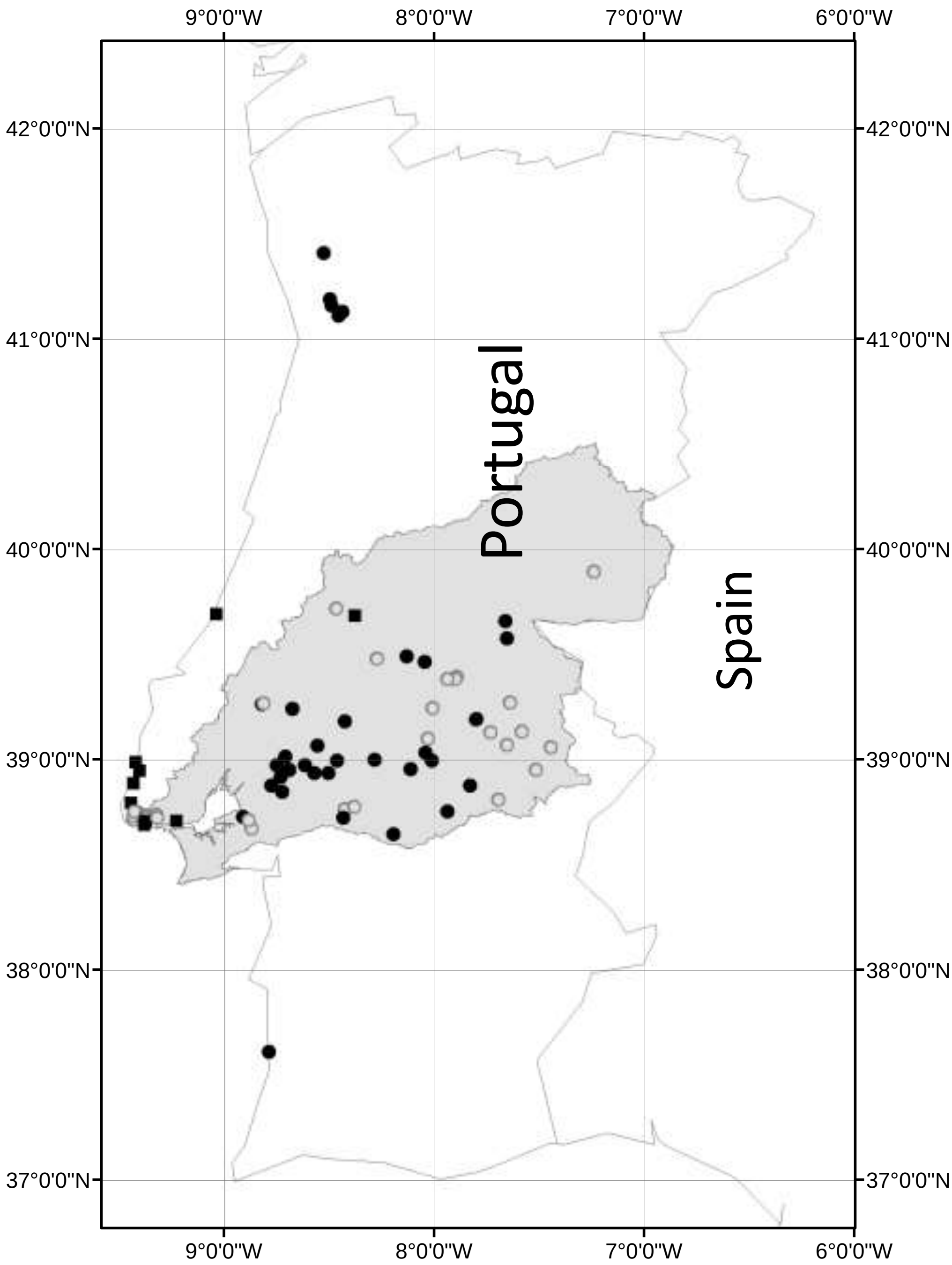


Figure 16. Map of the current distribution of *Crangonyx pseudogracilis*, in Portugal. Black circles represent the points with *Crangonyx pseudogracilis*. White circles represent points without amphipods. Black rectangles represent points with native species (genus *Echinogammarus)*. Grey indicates the Tagus river basin in Portugal.

4. Discussion

This study allowed us to better understand the biology of *Crangonyx pseudogracilis* and to follow the expansion of this exotic freshwater amphipod in Portugal, showing a great increase in its area of distribution in relation to what was observed in 2014. We found that *Crangonyx pseudogracilis* females are larger than males, approximately 1.2 mm larger in Ferrarias and 0.85 mm in Coruche. This is in line with Henry & Tarter (1997), but in their case, the difference was 3 mm. This species reproduces in Ferrarias during most of the year (December to June), but in Coruche only from March to July. We also found that the density of amphipods decreases from May to October and increases from November to April. Our results, regarding the reproduction season in Ferrarias and the fact that the density of amphipods increases and decreases at certain times of the year, are in accordance with the studies in New York, by DiSalvo *et al*. (2006 and 2015).

The dispersion of *Crangonyx pseudogracilis* occurs mainly due to human activities such as the transport of live fish and/or aquatic plants, in boats (Zhang & Holsinger, 2003), the timber trade (Maitland & Adams, 2001) or fishing with dip nets (Banha & Anastácio, 2015). Other dispersion mechanisms are through aquatic migration corridors and the plumage and legs of water birds (Rachalewski *et al*., 2013). The main pathway of dispersion is through the inland waterways, however, in their absence, human activities and animal movements are an efficient substitute (Bilton *et al*., 2001) and this was demonstrated by Banha & Anastácio (2015) and by Rachalewski *et al*. (2013). According to the study by Banha & Anastácio (2015), when fishermen capture live bait using dip nets, they also capture invasive species. One of these species is *Crangonyx pseudogracilis*, which can thus be transported accidentally for 50 km, considering its ability to survive desiccation. Based on the study by Rachalewski *et al*. (2013), *Crangonyx pseudogracilis* may cling to birds, in this case, mallards, and remain there during flight. Anseriformes fly at speeds of 61.2 to 86.4 km/h (Bruderer & Boldt, 2001; Clausen *et al*., 2002), mostly over a distance of 1 to 2 km (Legagneux *et al*., 2009) and during that period about half of the amphipods that cling to the ducks may survive.

We found that in the months with more amphipods, the temperatures were lower. More precisely, the months with temperatures above 12 °C showed a lower density of captured amphipods than the months with temperatures below 12 °C. The most likely hypothesis that can explain this is that the optimal temperature is important for the survival and reproduction of amphipods. This species seems to prefer lower temperatures than higher temperatures, so this will be reflected in a greater or lesser population density, respectively. Another hypothesis is that when amphipods are subjected to higher temperatures this results in a faster growth rate, which also leads to a faster sexual maturation and also resulting in shorter longevity (Biorede, 2020), which in a way may be related to the hypothesis previously presented. In contrast, amphipods subjected to lower temperatures have longer longevity since they have a slower growth rate, which results in a slower sexual maturity (Biorede, 2020). According to Brown *et al*. (2015), the time between moults depends mainly on the temperature. Longer intermoult periods correspond to slower growth rate and this occurs at low temperatures. In contrast, shorter intermoult periods correspond to higher growth rates, and this occurs at high temperatures. However, amphipods are not only subjected to temperature variations, but also many other variables such as predation, food availability, pH, water depth, oxygen dissolved, conductivity and turbidity, and changes in environmental conditions can affect the densities of *Crangonyx pseudogracilis* (Brown *et al*., 2015).

According to the "Atlas das Aves Invernantes e Migradoras de Portugal" (2018), most water birds, present in our sampling sites, spend the winter there and then migrate to their nesting grounds. It should be noted that in our study the density of amphipods increases from November to April and decreases from May to October. When we compare the density of amphipods with the density of water birds, we realize that waterbird abundance and density of amphipods seem to be temporally matched. Taking into account the experiences of Rachalewski *et al*. (2013), in which water birds are considered an important means of dispersion of amphipods, this reinforces the potential risk of birds as a dispersion vector for this invasive species (in Ferrarias and Coruche). Another important dispersion vector are dip nets used by fishermen to capture live bait. Based on the studies by Banha *et al*. (2016), large amphipod densities and fishing activity coincide from May to July. Moreover, the distances reported in that study suggest that this vector could disperse *C. pseudogracilis* to much greater distances than birds.

It was noticed that the proportion of females was higher than that of males. Some hypotheses may explain this difference, for example, a greater susceptibility of one of the genders to predation, which is directly related to the difference in sizes and behaviour. Although females of *Crangonyx pseudogracilis* are larger than males, males must find females to mate, thus being more exposed to predators (Jacobucci & Leite, 2006; Castiglioni *et al*., 2016). In addition to males being more exposed to predators, there may also be a dispute between them for a female during the reproduction season, which can lead to mortality and/or abandonment of the colonies (Lewbel, 1978). Studies by Lewbel (1978) report that there is a higher probability of juvenile male's mortality after hatching from the pouch. The fact that the sexes behave differently may lead them to use different habitats, which may be subjected to different temperature or vegetation resulting in different growth rates, maturity and longevity. Likewise, the availability of food, presence of parasites and habitat fragmentation can influence the sex ratio in amphipods (Jacobucci & Leite, 2006; Castiglioni *et al*., 2016). Finally, a study by Watts *et al*. (2002) revealed that the exposure of amphipods to certain chemicals present in the residual water, such as 17α - ethinylestradiol or also known as environmental estrogen (EE), can favour females more than males, resulting in a marked difference in proportions between the genders.

For both Coruche and Ferrarias, females reached a greater length than the males and this is not uncommon for amphipods. In fact, Kinne (1959) and Sheader (1983) suggested that males tend to select larger females since the size of the females is positively related to the number of eggs they can carry in their pouches. Females may pass through a larger number of moults, thus reaching a larger size than males and certain environmental conditions may benefit the growth of females more than that of males (Biorede, 2020). We observed that females also have a higher growth rate than males and this is in line with Devin *et al*. (2004), in France, with *Dikerogammarus villosus*, in which the growth rate is also higher for females than for males. We also found that the average growth rate estimated in our study (0.80 mm/month for females and 0.49 mm/month for males) is low but in line with studies such as the one by Brown *et al*. (2015) in Antarctica, with *Paramoera walkeri* (0.84 mm/month).

The reproduction season of *Crangonyx pseudogracilis* differed between locations. In Coruche, the reproduction season occurs only from March to July and in Ferrarias the reproduction season occurs during most of the year, but with a greater incidence in the months of December to June. These locations have different habitat characteristics (temporary stream vs. natural lake), which results in different micro-habitat characteristics at each site (water depth, type of substrate, type of vegetation and speed of water flow) and also different values of the physical-chemical variables (pH, depth, oxygen level, conductivity, temperature and turbidity). These differences may be reflected in unequal behaviour, growth rate and reproduction seasons for amphipod populations present in each of the sites. In Coruche, the habitat is always the same, a natural lake, which makes it more stable. However, in Ferrarias the habitat is a temporary river, which flows during the winter and is reduced to pools in the summer, making it less stable. When young females are faced with favourable environmental conditions, they can reach sexual maturity in just one month (Biorede, 2020), which explains why we find 2.99 mm long females with eggs only in Coruche and 3.24 mm in Ferrarias.

Finally, in comparison with the distribution of *Crangonyx pseudogracilis* reported for 2014, there was a very large increase in the Tagus and West hydrographic zone. Additionally, it is also present in the north and south of the country, apparently involving long-distance dispersal mechanisms. However, the total area that it occupies now it is not yet known, and additional sampling effort is needed in the north and south of Portugal. Despite the increment of the distribution area, no overlap was yet detected between native amphipod species occurrence and this exotic species. It was found that the area occupied by *Crangonyx pseudogracilis* is increasingly approaching the area where there are native species of the genus *Echinogammarus*. This is especially notorious for the Mondego river basin and also for the western Portuguese river basins. Since *C. pseudogracilis* can reproduce throughout the year when conditions are favourable and reproduces faster than native species (Grabowski *et al.*, 2012), this indicates a possible future risk for native species. Despite predictable future native/exotic species distribution overlap, *C. pseudogracilis* may not have a negative impact on native species, both coexisting in the same habitat. Laboratory experiments have shown that during short term interactions with *Echinogammarus meridionalis* there was no effect on the survival rate of the native species when it is in the presence of the exotic. Additionally, the species is apparently not carrying transmissible microsporidian parasites (Banha *et al*. 2018) and there was no short-term competition for food, nor predation, in the absence of food, of the native by the exotic (Gama *et al*., 2017). *Crangonyx pseudogracilis* inhabits a wide range of freshwater habitats, however, it is more commonly found in shallow ponds and more polluted areas (Grabowski *et al*., 2012). *Echinogammarus meridionalis*, on the other hand, prefers well-oxygenated, less polluted areas (MacNeil *et al*. 2001). These differences may allow the coexistence of both species in the same place but in different micro-habitats (MacNeil & Dick, 2014). Despite the experiments carried out, there is also the possibility of having a negative impact on native species through long-term competition either for food or for habitat, which can lead to the exclusion of native species from their current area of distribution and, in a more serious case, their extinction. If the latter is detected, then it will be necessary to apply measures both to control the exotic species and to conserve native species.

5. Acknowledgements

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Considerações Finais

Este estudo permitiu entender melhor a biologia de *Crangonyx pseudogracilis*, analisando a sua dinâmica populacional em dois locais (Ferrarias e Coruche) durante um ano, e permitiu também avaliar a sua expansão. A análise de sua área atual de distribuição e como ela se modificou desde a última avaliação por trabalho de campo (2014), permitiu estimar o coeficiente de difusão e a taxa de propagação. A análise da estrutura populacional e da dinâmica populacional forneceu informações importantes para avaliar o potencial invasivo da espécie.

A principal via de dispersão, desta espécie exótica, é através de canais artificiais entre bacias hidrográficas, porém, na sua ausência, as atividades humanas e os movimentos dos animais são um substituto eficiente (Bilton *et al.*, 2001) e isso foi demonstrado por Banha & Anastácio (2015) e por Rachalewski *et al*. (2013). De acordo com o estudo de Banha & Anastácio (2015), quando os pescadores capturam isco vivo usando camaroeiros, também capturam espécies invasoras. Uma dessas espécies é *Crangonyx pseudogracilis*, que pode ser transportada acidentalmente por 50 km, considerando a sua capacidade de sobreviver à dessecação. Com base no estudo de Rachalewski *et al*. (2013), *Crangonyx pseudogracilis* pode-se agarrar às pernas e patas das aves, e permanecer lá durante o voo. Sabe-se que muitos Anseriformes voam maioritariamente distâncias de 1 a 2 km (Legagneux *et al*., 2009) e durante esse período cerca de metade dos anfípodes transportados pelas aves conseguem sobreviver. Devido à capacidade de sobrevivência desta espécie, conclui-se que o vetor pesca pode dispersar *C. pseudogracilis* a distâncias muito maiores que o vetor aves.

Os locais amostrados apresentam diferentes características de habitat. Ferrarias é um rio temporário que flui durante o inverno, reduzindo-se a pegos durante o verão e Coruche é um lago natural durante todo o ano, sendo por isso mais estável. Estas diferenças vão resultar também em diferentes características de micro-habitat em cada local (profundidade da água, tipo de substrato, tipo de vegetação e velocidade do fluxo de água) e também valores diferentes das variáveis físico-químicas (pH, profundidade, nível de oxigénio, condutividade, temperatura e turbidez). Isto vai-se refletir em diferenças na taxa de crescimento e nas épocas de reprodução das populações de anfípodes presentes em cada um dos locais.

Verificamos então que a espécie exótica se reproduz em Ferrarias durante a maior parte do ano (dezembro a junho), mas em Coruche apenas de março a julho. Também se verificou que a densidade de anfípodes diminui de maio a outubro e aumenta de novembro a abril, coincidindo os meses com mais anfípodes, com as temperaturas mais baixas. Isto pode ser indicativo que esta espécie prefere temperaturas mais baixas. A temperatura ideal é importante para a sobrevivência e a reprodução dos anfípodes, mas não o único fator, pois existem outras variáveis como predação, disponibilidade de alimentos, pH, profundidade da água, oxigénio dissolvido, condutividade e turbidez, e mudanças nas condições ambientais que podem afetar as densidades de *Crangonyx pseudogracilis* (Brown *et al*., 2015).

Verificou-se como habitualmente, que as fêmeas eram maiores que os machos, aproximadamente 1.2 mm em Ferrarias e 0.85 mm em Coruche. Mas também se encontrou uma proporção de fêmeas maior que a de machos nos dois locais de amostragem. Isto pode dever-se aos machos terem que se deslocar para encontrar as fêmeas para acasalar, ficando assim mais expostos aos predadores (Jacobucci & Leite, 2006; Castiglioni *et al*., 2016). Além disso, também pode existir disputa entre eles por uma fêmea durante a época reprodutiva, o que pode levar à mortalidade e/ou ao abandono das colónias. O facto de os sexos se comportarem de maneira diferente pode levá-los a usar micro-habitats diferentes, que podem estar sujeitos a diferentes temperaturas ou vegetação, resultando em diferentes taxas de crescimento, maturidade e longevidade. Da mesma forma, a disponibilidade de alimentos, a presença de parasitas e a fragmentação do habitat podem influenciar a proporção entre os sexos nos anfípodes.

Finalmente, notou-se um grande aumento na área de distribuição de *C. pseudogracilis,* em relação ao observado em 2014, na zona hidrográfica do Tejo e Oeste, com um coeficiente de difusão de 2495,27 Km^2/ano e uma taxa de propagação de 26 Km/ano. Além disso, a espécie, também está presente a norte e sul do país, aparentemente envolvendo mecanismos de dispersão a longa distância. No entanto, a área total que ocupa agora não é ainda conhecida com exatidão e é necessário um esforço de amostragem adicional a norte e sul de Portugal. Apesar disto, ainda não foi detetada sobreposição entre a ocorrência de espécies de anfípodes nativas e esta espécie exótica. Existe, no entanto, a possibilidade desta espécie exótica ter um impacto negativo sobre as espécies nativas num futuro muito próximo. Na verdade, a distância para locais conhecidos com anfípodes nativos é muito pequena e *C. pseudogracilis* pode-se reproduzir ao longo de todo o ano quando as condições são favoráveis, e cresce mais rapidamente que as espécies nativas (Grabowski *et al*., 2012). Se se verificar um impacte negativo, pode ser necessário aplicar medidas para controlar a espécie exótica ou preservar as espécies nativas. No entanto e apesar da provável sobreposição futura de áreas de distribuição de espécies nativas e exótica, pode suceder que *C. pseudogracilis* não tenha um impacto negativo nas espécies nativas, coexistindo com estas. *Crangonyx pseudogracilis* habita uma ampla gama de habitats de água doce, sendo mais comum em lagoas rasas e áreas mais poluídas (Grabowski *et al*., 2012). As espécies nativas, género *Echinogammarus,* preferem pelo contrário áreas bem oxigenadas e menos poluídas (MacNeil *et al*., 2001). Essas diferenças poderão eventualmente permitir a coexistência de ambas as espécies no mesmo local, mas em diferentes micro-habitats (MacNeil & Dick, 2014).

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Anexo I

Sampled Locations			
Number	Name	Location	Identification
1	Abrantes	39.4751205288, -8.24095063825	Absence
2	Alcochete	38.9159495548, -8.72787929536	Crangonyx
3	Algés	38.7133642295, -9.22423327524	Native
4	Almoster	39.264493384, -8.81500275108	Crangonyx
5	Alvisquer	39.2409227751, -8.67241872017	Crangonyx
6	Arcês	39.4885302857, -8.12813889298	Crangonyx
7	Benavente	38.9721498829, -8.74554541113	Crangonyx
8	Cabanas	39.2391548332, -8.6727572419	Crangonyx
9	Cabeço de Vide	39.1335760053, -7.58097861931	Absence
10	Cabido	38.753894931, -7.93624701105	Crangonyx
11	Camões	38.9952810746, -8.00843235833	Crangonyx
12	Carregal	39.464434723, -8.04292524943	Crangonyx
13	Chão das Eiras	39.6857961183, -8.37608355964	Native
14	Colares	38.47535, -9.26142	Native
15	Coruche	38.9351396059, -8.56658098104	Crangonyx
16	Crato	39.2716142161, -7.63649897013	Absence
17	Cuco	38.9905827953, -9.41558858162	Native
18	Fajarda	38.9734745941,	Crangonyx

		-8.61298324489	
19	Freixo	39.8931786054, -7.24017866049	Absence
20	Lamarosa	39.0648565027, -8.55345335406	Crangonyx
21	Marinhais	39.0127883513, -8.70590692065	Crangonyx
22	Margem	39.3942289013, -7.89079290699	Absence
23	Margem	39.3807411473, -7.89591844667	Absence
24	Monforte	39.0576709591, -7.44376140129	Absence
25	Monte Novo	39.0980062234, -8.02730179321	Absence
26	Mora	39.0322089576, -8.03841730586	Crangonyx
27	Paredes de Vitória	39.7020599471, -9.04862048675	Native
28	Pé de Erra	38.9960048671, -8.45764715151	Crangonyx
29	Porto Alto	38.9721498829, -8.74554541113	Crangonyx
30	Porto Carvoeira	38.9494014487, -9.3957928169	Native
31	Porto do Tejo	39.6575020624, -7.65851139888	Crangonyx
32	Pt. Freiria	39.2665916255, -8.81033631389	Absence
33	Ramalheira	39.7256896839, -8.45425831952	Absence
34	Ribeira de Benavila	39.1294436189, -7.72819801629	Absence
35	Ribeira de Canha	38.7236574929, -8.4289682927	Crangonyx
36	Ribeira Grande	39.070483355, -7.65108678662	Absence
37	Ribeira de Lavre	38.7639488391, -8.42126534367	Absence
38	Ribeira de Muge	39.1797003853,	Crangonyx

		-8.42237122817	
39	Ribeira de Nisa	39.5753956771, -7.65320529883	Crangonyx
40	Ribeira de Santo Estevão	38.8461937677, -8.720730943	Crangonyx
41	Ribeira de Seda	39.1891089316, -7.79910680657	Crangonyx
42	Ribeira de Sor	39.2460339138, -8.00562951326	Absence
43	Ribeira de Tera	38.8759766259, -7.826767387	Crangonyx
44	Rio Almansor	38.6446284164, -8.19201440395	Crangonyx
45	Salgueirinha	38.6700643574, -8.86409566852	Absence
46	Samarra	38.8921636196, -9.42603399559	Native
47	Santo Estevão	38.8763722726, -8.77200456645	Crangonyx
48	Seda	39.1891089316, -7.79910680657	Absence
49	Simarros	38.7236574929, -8.4289682927	Absence
50	São Bartolomeu	39.3820341417, -7.93358800637	Absence
51	S. Pedro do Estoril	38.7128961154, -9.36791668593	Native
52	Tejo	38.7280657851, -8.90439851797	Crangonyx
53	Trejoito	38.9159495548, -8.72787929536	Crangonyx
54	Vala da Mare	38.7107971215, -8.88141167214	Absence
55	Vale de Poços	38.9352624072, -8.49926960495	Crangonyx
56	Veiros	38.9511212266, -7.51361047332	Absence
57	Vimieiro	38.8094745906, -7.69128889436	Absence

Donated Samples			
Number	Name	Location	Identification
1	Alcabideche	38.733246956, -9.36720329028	Absence
2	Alcabideche	38.731886326, -9.42912192616	Absence
3	Alcabideche	38.7526034419, -9.42414916058	Absence
4	Alcabideche	38.7486286762, -9.41021988197	Absence
5	Cavaleiro	37.611005158, -8.7832321853	Crangonyx
6	Cascais	38.7103569419, -9.42660488309	Absence
7	Covelo	41.110810481, -8.45099497168	Crangonyx
8	Estoril	38.7133941082, -9.36722497857	Native
9	Marginal	38.6951376667, -9.37546213998	Native
10	Mora-Gameiro	38.9557373391, -8.11012484823	Crangonyx
11	Mora-Gameiro	38.9535122351, -8.10834579953	Crangonyx
12	Paredes	41.1286836099, -8.43335934603	Crangonyx
13	Pateiras do Ave	41.4080124985, -8.52385768068	Crangonyx
14	Ribeiro das Parreiras	38.7372365512, -9.31920052078	Absence
15	São Domingos de Rana	38.723295964, -9.31602540334	Absence
16	São Domingos de Rana	38.7218994815, -9.36702737551	Absence
17	Valongo	41.1868074792, -8.49431357584	Crangonyx
18	Valongo	41.1570092449, -8.48411194205	Crangonyx