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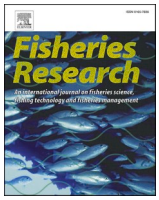

Macro and microscopic maturation stage key of green crab (*Carcinus maenas*, Linnaeus 1758): Reproductive cycle and differences among estuarine systems

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ABSTRACT

Gonadal histology is a helpful tool to validate species' macroscopic maturity stages in fisheries biology. Regardless of the many studies on *Carcinus maenas*, there are still few concerning gonads tissue histology and description of maturity stages. In Portugal, information regarding this species' biology to help support the regulation of crab fisheries, including the minimum landing size, has not yet been defined. In this work, the macro and microscopic characteristics of the reproductive cycle of *C. maenas*, oogenesis, and spermatogenesis development stages along the Portuguese coast are described, and a new macroscopic scale based on the histological analyses is suggested. During 2019 and 2020, adult *C. maenas* (both males and females) were collected from the Ria de Aveiro estuary, Rio Sado estuary, and Ria Formosa lagoon, respectively, North, Center, and South Portugal. No-significant differences ($P > 0.05$) were observed in carapace width and individual weight between all systems. Significant differences ($P < 0.05$) were observed in gonad weight, gonadosomatic index, and Fulton's condition index between the Rio Sado estuary and Ria Formosa lagoon. Furthermore, significant differences ($P < 0.05$) in the oocyte diameter between the northern and southernmost locations suggest a geographic variation related to the local environmental conditions of each system. Principal component analysis (PCA) revealed maturity stage and carapace width an association, and a similar morphometry between Ria de Aveiro and Ria Formosa. Temperature was correlated to both maturity stage and carapace width in Ria de Aveiro and Ria Formosa. From the histological analysis and based on microscopic criteria, it was observed that females previously classified within the late macroscopic development stage 2 should be considered mature, so a classification change in the current ovary's developmental stage is proposed. These findings can allow fisheries researchers to reclassify the estimations of maturity ogives and help support the regulation of this species' fishery. The proposed macroscopic scale was validated by histological analyses and can be used elsewhere.

1. Introduction

The European green crab, *Carcinus maenas* (Linnaeus, 1758), is one of the most widely distributed benthic predators in marine and estuarine intertidal areas (Glamuzina et al., 2017; Klassen and Locke, 2007; Leignel et al., 2014; Waser et al., 2018). It is a small decapod, native to the coast and estuaries of the Northeast Atlantic, from Mauritania to Iceland, occurring also in the British Isles and Norway (Darling et al., 2008; Roman and Palumbi, 2004). In the past two centuries, this species has been identified as an invasive species in southern Africa, Australia, Pacific region, and North and South America Atlantic coast (Cohen et al., 1995). *C. maenas* is an important and valuable species within its area of occurrence, but it is at the same classified as one of the world's worst invasive species by the International Union for Conservation of Nature

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(IUCN) (Leignel et al., 2014) due to its phenotypic plasticity, wide adaptability to different habitat types and as an opportunistic feeding behavior (Young and Elliott, 2020).

In Portugal, *C. maenas* is still considered as a common species, registering high population densities throughout its distribution along the continental coast from the Ria Formosa to the Rio Minho estuary (Amaral et al., 2009; Elumalai et al., 2007; Monteiro et al., 2022; Queiroga, 1996). It's a species with high ecological and commercial value, mainly in Europe, being fished along the Portuguese coast. During the 1990's, in the local estuaries it was harvested in large quantities (hundreds of tons per year) to supply the Iberian food industry (Pascoal et al., 2009). Currently, the *C. maenas* is commercialized as bait for octopus trap fishing, as bait for recreational fishing (mainly for gilthead seabream, *Sparus aurata*) or sold fresh for human consumption (Leitão et al., 2021).

In crustacean species, with a high commercial value or ecological potential, the study of reproductive biology has been widely studied (Castiglioni and Negreiros-Fransozo, 2006). The relationship between size and reproduction events, such as mating and physiological sexual maturity, is crucial to understand stock dynamics (Bianchini and Ragonese, 2008) and managing adaptations for the preservation and/or commercial exploitation of populations (Hartnoll,1974). Within its area of distribution, the *C. maenas* may endorse different reproductive periods per year depending on the water temperature (Audet et al., 2008). In temperate waters, the species has only one reproductive period per year, whereas in warmer waters there are two (Lovell et al., 2007; Young and Elliott, 2020). Along the Portuguese coast, the reproductive period occurs between October to June, when the water temperature remains below 23ºC, with a peak during the cooler winter months (January and February) (Sprung, 2001; Young and Elliott, 2020). Temperature also affects the duration of the reproductive cycle of *C. maenas*, which has been observed to vary across systems (Hines et al., 2004). Therefore, regional patterns in the number of breeding periods (Young and Elliott, 2020), size and number of eggs (Collin et al., 2018; Rodríguez-Félix et al., 2018), and time of the larval period (Nagaraj, 1992; Spitzner et al., 2019) mostly depend on the environmental differences among areas.

Fisheries assessment models use the information on the maturation stages of the species to know the spawning season and the proportion of mature and immature individuals to estimate the maturity ogives (Sparre et al., 1989; Rothschild et al., 1987). The maturation stages are assigned based on the macroscopic development of the gonads of the species under study. In each phase of the reproductive cycle, the gonads present specific characteristics which result from the maturation of the oocytes in the case of female gonads and of the spermatozoa in male gonads. Maturation stages are generally assigned by observation of the external morphology of the gonad. The use of macroscopic maturity stage keys makes it possible to analyze many individuals with relatively little effort. However, the construction of macroscopic keys should be based on microscopic criteria that allow an objective identification of the various stages of maturation and the proper discrimination between them. Such accurate information is required for fisheries researchers for instance to validation of maturity ogives (Costa & Borges, 1996). The reproductive cycle of a crustacean includes a series of morphological and physiological events. In specimens completing the juvenile stages, these events include the proliferation of gonadal cells (activation of gametogenesis), differentiation and growth of gametes to maturation (gamete production), reproductive behavior associated with mating, the release of gametes; ovulation, spawning, and incubation of embryos until hatching to release larvae or juveniles (Sastry, 1983).

Despite many scientific studies on *C. maenas* ecology (Baeta et al., 2005; Bessa et al., 2010; Young et al., 2017) and larval development (Behrens Yamada et al., 2021; Spitzner et al., 2018; Sprung, 2001; Young and Elliott, 2020), only a limited number of studies have addressed the histological analysis of both male and female reproductive cycle with those being restricted to studies made on nonindigenous populations from the northeast coast of North America (Audet et al., 2008; Best et al., 2017), in the southwest of Ireland (Lyons et al., 2012), in Patagonia, Argentina (H. Vinuesa, 2005) and the Mediterranean of the congeneric species *Carcinus aestuarii* (Baklouti et al., 2013; Özbek et al., 2012). There few studies describing morphological and histological aspects of the gonads of *C. maenas* in NE Atlantic, with exception of Ireland Sea and the east and southeast coast of Newfoundland. Despite the economic importance of the species there is a lack of information for morphological and histological aspects of the gonads of *C. maenas* also along Portuguese coast. In fact, few studies describe the reproductive cycle using histological analysis of both species sexes e.g. *C. maenas* (Audet et al., 2008; Best et al., 2017; Lyons et al., 2012) congeneric species *C. aestuari* (Baklouti et al., 2013); other crab species (Castiglioni et al., 2007; Santos et al., 2009) and in fishes (Blazer, 2002).

C. maenas is caught in artisanal estuarine/lagoon fishery, and little/none attention has been given regarding Portuguese crab fishery biology, the main motive for the lack of crab fishing regulations. The main objective of this study was: i) to provide information to fully describe the male and female gametogenesis of the European green crab *C. maenas*, ii) to establish a relationship between the macroscopic and microscopic stages previously identified along the Portuguese coast, iii) to provide fundamental information to help estimate the species maturity ogives and the crab length at first maturity to be used in the species fishery regulation, and, iv) to understand the role of local environmental conditions in gametogenic development.

2. Material and methods

2.1. Study site

Carcinus maenas were collected in three selected locations along the Portuguese coast, Ria de Aveiro (North Portugal), Rio Sado (Center Portugal), and Ria Formosa (South Portugal) (Fig. 1), were according to DGRM (Direcção Geral de Recursos Naturais, Segurança e Serviços Marítimos) the official *C. maenas* landing statistics are particularly relevant. Ria de Aveiro is a mesotrophic shallow coastal lagoon (Almeida et al., 2005), located on the northwest coast of Portugal formed by a very complex system of channels and wide intertidal areas, mudflats, and salt marshes (Dias and Lopes, 2006). This mesotidal, temperate, and well-mixed system characterizes by the lowest water

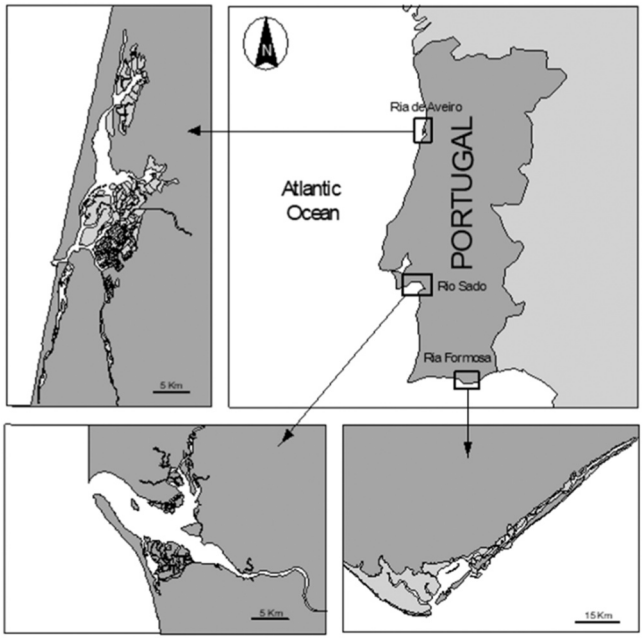


Fig. 1. Geographical location of the different study areas: Ria de Aveiro, Rio Sado, and Ria Formosa, along the Portuguese coast.

temperature (Table A.1) (Matias et al., 2013) and is considered one of the largest continuous salt marshes in Europe (Sousa et al., 2017). It is a system with a mesotidal regime, with an average amplitude at the inlet of 2 m, ranging between 0.6 m from neap tides to 3.2 m in spring tides (Dias et al., 1999).

Rio Sado is located on the western coast, being the second largest Portuguese estuary with an area of approximately 180 km^2 and is one of the few European rivers flowing from South to North (Ferreira et al., 2003). It is a mesotidal and, a well-mixed estuary with a tidal that range varies from 0.6 m on neap tides to 1.6 m on spring tides, dominated by semidiurnal tides (Ferreira et al., 2003). According to OSPAR (2002), the intertidal area includes large areas of salt marshes and intertidal flats and corresponds to approximately 30 % of the total estuary area. The salinity of estuarine waters is influenced by the runoff derived from the exploitation of the salt pans in the surrounding area (Gonçalves et al., 2015).

Ria Formosa is a highly productive and multi-inlet barrier island system extending along 55 km, located in southern Portugal (Falcao and Vale, 1990). It is a shallow mesotidal coastal lagoon with an average depth, relative to mean sea level, of approximately 2 m. The hydrodynamics of the Ria Formosa is forced by tides, which are semidiurnal and range from about 3.6 m and 0.6 m for spring and neap tides, respectively. The tidal effect is responsible for 50–75 % of the daily lagoon water exchanges (Newton and Mudge, 2003). This lagoon system has an important ecological role, as a nursery and growth area for several commercially valuable species (Brito et al., 2012; Cravo et al., 2012).

2.2. Sampling strategy

The *C. maenas* sampling period was carried out between October 2019 to January 2020, within the peak spawning period of this species in the three selected locations. The crabs were captured by professional fishermen using baited traps. The traps were baited with various fish species (chub mackerel, sardines, and golden grey mullet) and deployed between 12 and 24 h at depths between 1 and 5 m, regardless of the geographic location.

2.3. Laboratory procedures

After capture, crabs were placed on ice to reduce their activity. The crabs were then transported to the laboratory and anesthetized by a hypothermic shock in the freezer (−20ºC) for five minutes. Before dissection, carapace width (CW, mm), total body mass (BM, g), and gonad weight (GW, g) were measured/weighted. Then, the carapace was removed, and the gonads of 30 females and 20 males, macroscopically identified, were excised, weighed (g) and fixed in a 4 % formaldehyde solution buffered hydrated borate of sodium ($Na_2H_{20}B_4O_{17}$), for at least 48 h before histological tissue processing.

The environmental data from each river/lagoon system, namely, annual mean temperature (°C) and seawater salinity (PSU), were obtained from the Eumetsat database (©EUMETSAT2022) from Copernicus Programme (https://www.copernicus.eu/pt).

2.4. Histological techniques

The sample histology protocol was carried out following Campinho et al. (2007). After fixation, the gonad tissue was placed in histological cassettes (n = 5 by each maturity stage) and transferred to 70 % ethanol for at least two hours until further processing. For paraffin embedding the tissues were dehydrated through a graded ethanol series from 70 % to 100 % followed by xylene (100 %) using an automated tissue processor (Leica, TP1020), and finally embedded in paraffin wax (Histosec, Merk, Germany). The blocks were stored at − 20ºC until use. Transversal serial sections of embedded tissue (5 μm) were cut using a rotary microtome (MICROM, HM340E), mounted on glass slides coated with 3-Aminopropyltriethoxysilane (APES; Sigma-Aldrich), and dried overnight in an oven at 37 °C. Standard hematoxylin-eosin staining was performed on dewaxed and rehydrated sections. Briefly, tissues were immersed in Harris hematoxylin solution for 2 min and washed in tap water with three drops of ammonia and distilled water. Afterward, immersed in eosin solution for 30 s, washed in distilled water with a few drops of acetic acid, and the excess stain was removed in distilled water. Stained sections were dehydrated, cleared, and mounted in DPX (Sigma–Aldrich), and allowed to dry overnight at room temperature. Photomicrographs of histological sections of *C. maenas* gonad tissue were taken using a ZEISS Axiolab drb KT optical microscope coupled to an OPTICA 3 digital camera. Image analysis was carried out using open-source software, Image-J (https://imagej.nih.gov/ij/download.html).

2.5. Data analysis

Data was used to calculate the Fulton's condition factor (K) (Gonçalves et al., 2017), where W is the total crab weight (g) including gonads and L is the crab width (cm). K was calculated by sex (F and M) and river/lagoonary system.

$$K = \frac{W}{L^3} * 100$$

Gonadosomatic index (GSI) (Zaleski and Tamone, 2014), which describes the percentage of the wet gonad weight relative to the crab's total weight.

$$GSI = \frac{Wet\quad Gonad\quad weight(g)}{Total\quad weight(g)} * 100$$

One Way Analyses of Variance (ANOVA) was used to test average difference in Carapace width (CW) and weight (W) between systems. An analysis of covariance (ANCOVA) was applied to assess the relationship and differences between the dependent variables (gonadosomatic index (GSI), Fulton's condition index, oocyte diameter, and oocyte type) and sampled sites (Ria de Aveiro, Rio Sado, and Ria Formosa) as independent factors. The correlation between CW and W were tested for collinearity significantly showing correlation (Spearman correlation) among these two measurements in all systems (Ria Aveiro: $\rho = 0.904$; $p > 0.001$; Rio Sado: $\rho = 0.969$; $p > 0.001$; Ria Formosa: $\rho = 0.953$; $p > 0.001$). The carapace width (CW) as then selected as the covariate to further ANCOVA analyses. The inclusion of the carapace width (CW) as a covariable in the ANCOVA model allowed us to assess differences between variables, after removing the CW for each individual used in the analyses. The level of significance was set at $p < 0.5$.

The PCA aimed to correlate the relative difference among sampling sites (Ria de Aveiro, Rio Sado, and Ria Formosa) in relation to their overall gonadal developmental stage. For each individual analyzed, the mean oocyte diameter by each oocyte development stage (named as oocyte type) was determined. The PCA was carried out using the following variables: oocyte type, mean oocyte diameter, CW, microscopic maturity stage, salinity, temperature, and sampling site. The PRINCOMP procedure of R was applied after data standardization and PCA was based on the correlation matrix.

Data statistical analyses were carried out with IBM SPSS software (Statistical Package for the Social Sciences) and R 4.1.0 (R Core Team, 2021).

3. Results

The observed average Carapace width (CW) and individual weight (W) did not varied (Table A.2) statistically between systems (CW-ANOVA: F=0.66; P = 0.52; W-ANOVA: F=0.80; P = 0.45).

3.1. Carapace width – gonad weight relationships

Gonad weight (GW) and CW were related in all coastal systems, with

all GW-CW regressions producing statistically significant slopes (ANCOVA p-value fitting: 0.01; Fig. A.1). The GW-CW correlation regression value was higher at Ria Formosa and lower at the Ria de Aveiro and Rio Sado. There was a statistical effect of CW on the GW ($F_{(1,94)} = 7.936$; $p < 0.05$) and GW varied statistically among systems ($F_{(2,94)} = 3.020$; $p < 0.05$). Sidak's Post-hoc comparisons showed statistical differences between Rio Sado and Ria Formosa ($p < 0.05$). The higher increment rate GW was observed in Rio Sado relative to Ria Formosa, with GW reaching higher values for the same CW (see also Table A.2).

3.2. Carapace width – Gonadosomatic index relationships

The Gonadosomatic index (GSI) and CW ANCOVA model fitting were statistically significant (ANCOVA p-value model fitting: <0.01; Fig. A.2). The analyses showed no statistical effect of CW on the GSI ($F_{(1103)} = 0.835$; $p > 0.05$) but there was a statistical difference in GSI among systems ($F_{(2103)} = 6.760$; $p < 0.05$). Sidak's Post-hoc revealed differences between Rio Sado and Ria Formosa ($p < 0.05$). The higher GSI increment rate was in Rio Sado, then in Ria de Aveiro, and finally in Ria Formosa (Table A.2).

3.3. Carapace width – Fulton's condition index relationships

The Fulton's condition index (FC) and Carapace width (CW) ANCOVA model was statistically significant (ANCOVA p-value fitting: <0.04; Fig. A.3). There was an effect of the covariate carapace width on Fulton's condition index ($F_{(1100)} = 2.776$; $p < 0.05$) but there was a statistical difference in Fulton's condition index among systems ($F_{(2100)} = 3.726$; $p < 0.05$). Sidak's Post-hoc revealed differences between Ria Formosa and Rio Sado ($p < 0.05$). The higher increment rate Fulton's condition index was in Rio Sado, then in Ria de Aveiro, and finally in Ria Formosa.

3.4. Reproductive cycle

Macroscopic analysis of gonad maturation (adapted from Lyons et al. (2012) allowed us to relate the observed gonadal development, with the gametogenic development based on histological analyses. Gonads from all development stages of maturation were obtained, regardless of the sex (Table A.3).

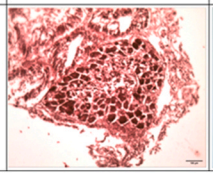
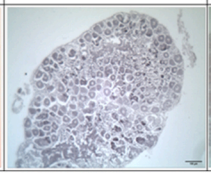
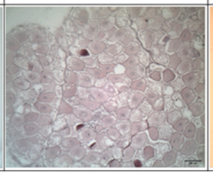
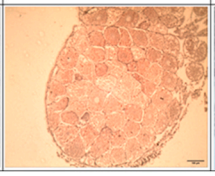
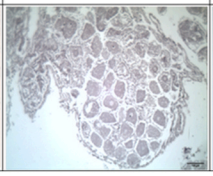
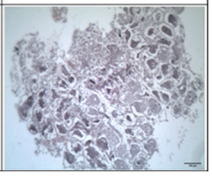
3.4.1. Female gametogenic development

Based on histological analysis, the female's reproductive cycle was divided into six developmental stages. Those stages were assigned according to the different gametogenic cells. When at an intermediate stage, many gametogenic cells were observed and represent different development phases, the stage decision criteria are based on the one that occupied the largest part of the gonad.

Microscopically, it was possible to observe the presence of primary oocytes in all the gonadal development stages (I to V), with oocytes varying in number within each stage of maturation. Females were considered immature at stages 0 and 1, and mature at stages 2, 3, 4, and 5 (Table 1). The difference between stage 1 and 2 is that in stage 1 only non-vitellogenic oocytes (primary oocytes) are present. The oocyte diameter between stage 1 and 2 is similar (Table A.2). Some oocytes in stage 2 were at beginning of vitellogenesis (partial yolked) and some yolked, although with a lower frequency, which is associated with the increase of gonad weight (Table 1). Stage 3 (Mature) presents those different types of oocytes: non-yolked, at the beginning of vitellogenesis (partially yolked), vitellogenic stage (which include also the oocytes at migratory nucleus stage), the latter are larger in number present in the gonad and also on diameter, being in an advanced maturation stage. Stage 4 (spawning stage) is the stage that presents the hydrated oocytes (Table A.2, Fig. 2). If the hydrated oocytes are present in the ovary, even in small number, but together with the atretic oocytes and post-ovulatory follicles (POFs) this is an indication of recent spawning or that spawning has just ended. On stage 5 (Spent) vitellogenic oocytes in different stages could be present, but the most evident characteristic is the present of POFs, indicating the release of the hydrated oocytes and the end of spawning (Table A.2). Atretic oocytes could also be present and in a significant number by the end of the spawning period, which indicates the degeneration of oocytes in which the vitellogenic process by any reason stopped taking place the reabsorption of non-viable oocytes.

Oocyte diameter significantly increased with oocyte maturation (Fig. 3). The diameter measurements of the hydrated oocytes are merely indicative, and it correspond to the higher diameter observed, since

Table 1
Reproductive stage key applied to classify the different gonadal maturity stages of *Carcinus maenas*, including illustrative photomicrographs and a brief description of each female macro and microscopic maturity stage. Scale bars correspond to 100 μm.

Stage	0 - Immature	1 – Early Development	2 – Late Development	3 - Mature	4 - Spawning	5 – Spent/ Resorbing
Macroscopic maturity stage	No gonad tissue could be visually identified	Thread-like ovary, embedded in/difficult to distinguish from, the hepatopancreas. Translucent in colour. Oocytes are not visible.	Ovary has increased in size. More discernable from the hepatopancreas. Creamy in colour. Oocytes are not visible.	Ovary had dramatically increased in size. Occupies the majority of the body cavity. Orange to deep red in colour. Visible oocytes.	Ovary reduced in size. Varying colour from yellow to red. Residual oocytes are still visible.	Ovary reduced in size. Varying colour from yellow to red. Residual oocytes are still visible
Gonad external image						
Microscopic maturity stage	Only oogonia (OG) and primary growth (PG) oocytes are present. There is no evidence of oil droplets in PG oocytes. No atresia (AT) or muscle bundles. Thin ovarian wall and little space between oocytes.	Cortical alveoli (CA) and/or early vitellogenic (VT1) oocytes are present. In case, this is a developing from a previous spawning (regenerating stage), the presence of atretic oocytes (AT) could be observed.	PG, CA, and/or VT1 and/or mid vitellogenic (VT2) oocytes are present. Some AT can be present.	Late vitellogenic (VT3) oocytes, migratory nucleus (MIG) and/or hydrated (HYD) are mainly present. PG together with CA can also be observed.	Post-ovulatory follicles (POF's) present in dominance, some PG, CA can be present. Thick ovarian wall. Remaining hydrated oocytes (HYD), or HYD at atretic state may also be present.	PG oocytes dominate. Massive AT and POF's present some CA and/or, VT1, VT2 can be present.
Gonad internal image						

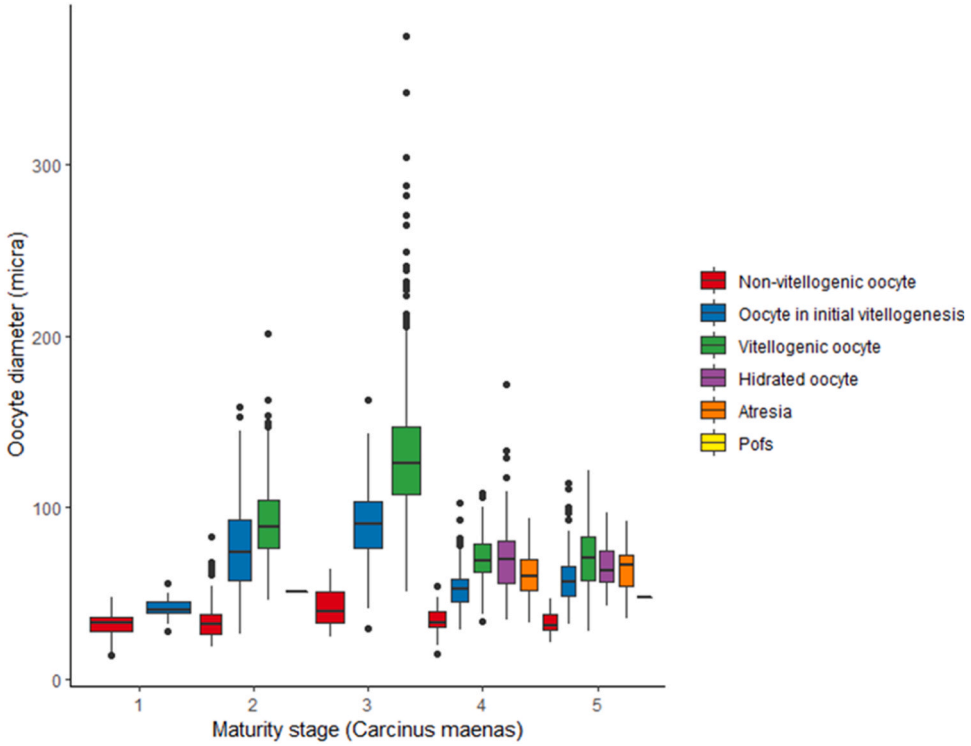


Fig. 2. Oocyte diameter (μm), observed in each type of oocyte, at the various stages of ovarian development of *C. maenas* along the Portuguese coast.

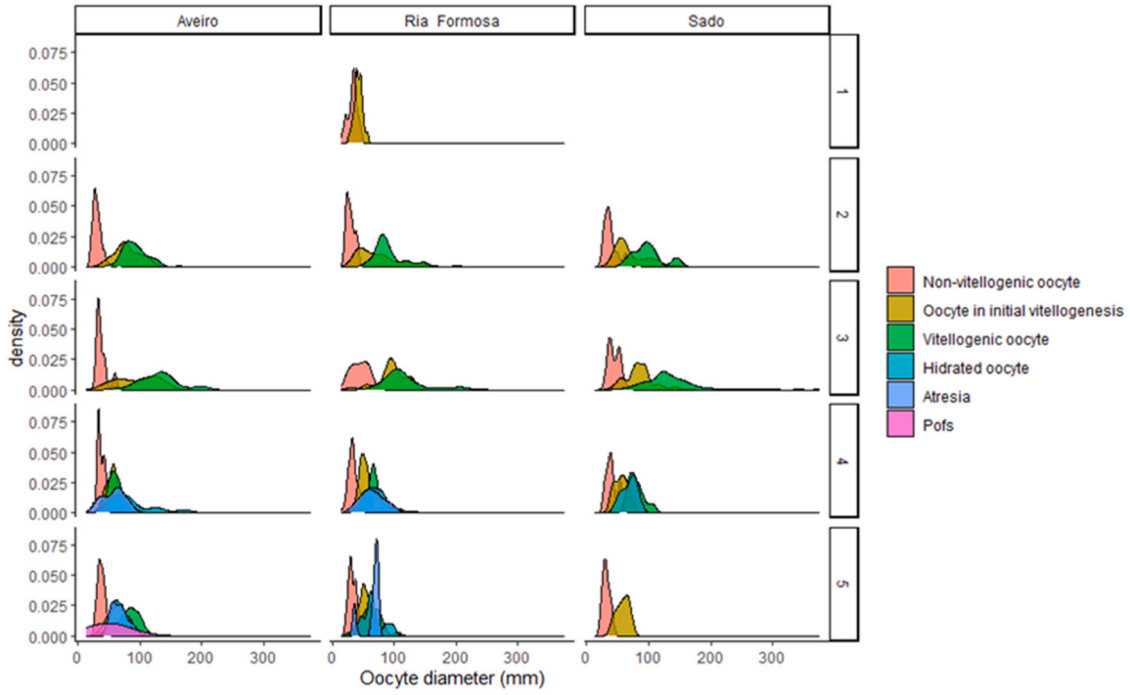


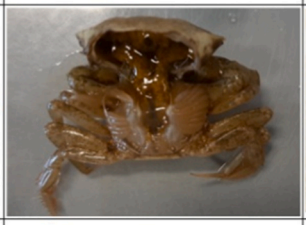
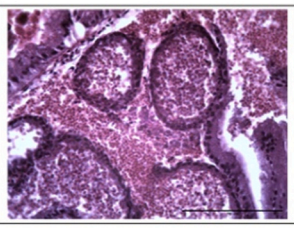
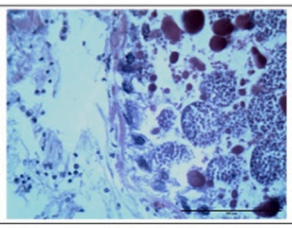
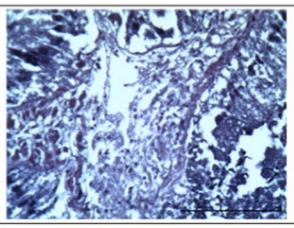
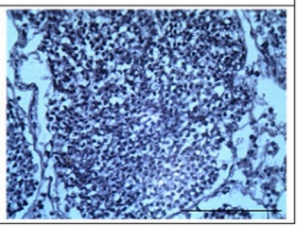
Fig. 3. Density of oocytes, observed in each type of oocyte, at the various stages of ovarian development (1−5), grouped according to oocyte diameter (mm) of *C. maenas* from three lagoon and estuarine systems from Portugal (Ria Aveiro, Ria Formosa and Rio Sado).

during the histological gonad processing the dehydration of the tissues makes, they lose their spherical shape and became convoluted, which doesn't enable the correct diameter measurement. On POF's due to the reabsorption process the membrane and to the loss of the circular shape, the measurement was taken on the bigger axes of the structure on the gonad. There were significant differences between all study sites regarding oocyte diameter increase with maturation (ANCOVA: $F_{(2,4404)} = 76.303$; $p < 0.05$).

3.4.2. *Male gametogenic development*

Male reproductive maturity was categorized into four developmental stages (Table 2). From a microscopic point of view, gonads are identified as belonging to stages 0 and 1 have only spermatogonia A and B (Sg A and Sg B). Stage 2 represents mature testis, in which spermatozoa cells

Table 2
Reproductive stage key applied to classify the different gonadal maturity stages of *Carcinus maenas*, including illustrative photomicrographs and a brief description of each male macro and microscopic maturity stage. Scale bars correspond to 100 μm.

Stage	0 - Immature	1 – Development	2 – Spawning	3 - Spent
Macroscopic maturity stage	No gonad tissue could be visually identified	Thread-like testis, embedded in/difficult to distinguish from, the hepatopancreas. Translucent in colour	Testis has greatly increased in diameter. More discernable from the hepatopancreas. Creamy/ off white in colour	Testis reduced in size. Creamy/ off white in colour
Gonad external image				
Microscopic maturity stage	Only spermatogonia A (SgA) are present. The spermatogonia A are single germ cells.	When spermatogonia B (SgB) are present germ lineage has started. In this stage also spermatocytes (Sc), spermatids (St).	Spermatozoa (Sz) are present throughout the testis, either in the sperm duct, cysts or lobules. Spermatogonia (Sg) and spermatids are present.	Residual spermatozoa (Res Sz) present in the sperm duct. Degeneration and reabsorption of the remaining sperm cells, empty spaces are visible in the ducts.
Gonad internal image				

(Sz) are present, and is the most well-represented stage in the samples (Table A.3). On stage 3 shows already residual spermatozoa (Res Sz) and resorption of cells.

3.5. Principal component analysis (PCA)

PCA was applied to females to understand which variables better explain the overall variability in ovarian development in the three study areas (Ria de Aveiro, Rio Sado, and Ria Formosa), and how these variables are related to each other or with the study sites. The selected variables were oocyte diameter and type, maturity stage, and CW. In this analysis, the two main components explain 59 % of the variance of the results. PC1 accounts for 37 % of the variance, and PC2 accounts for 22 %. Fig. 4 shows the PCA results, illustrating the associations of the variables, with the projection of the respective study area. PC1 shows an association between the Ria de Aveiro and Ria Formosa study area, related to maturity stage and carapace width (CW). The projection of the sites shows that Ria de Aveiro and Ria Formosa have the most similar morphometry. The second factor (PC2) is influenced by oocyte diameter. The projection of the sites shows that the largest oocyte diameters are in

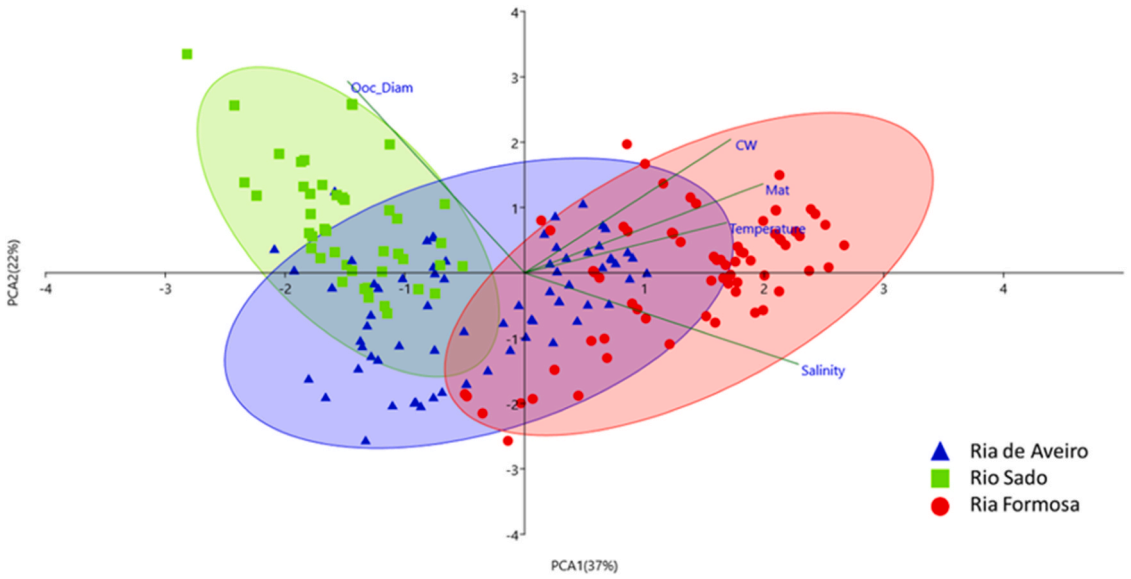


Fig. 4. Principal component analysis (PCA). PC1 vs. PC2 was applied to the data (Carapace width (CW); maturity stage (Mat); oocyte diameter (Ooc_Diam), Temperature and Salinity) of the three study sites: Ria de Aveiro; Rio Sado; Ria Formosa.

the Rio Sado while the Ria de Aveiro and Ria Formosa present the smallest oocyte size. The results also showed the oocyte diameter are associated to lower salinity. The temperature were correlated to both maturity stage and CW in Ria de Aveiro and Ria Formosa.

4. Discussion

Considered the most common intertidal decapod crustacean in Europe and with the largest distribution worldwide (Gomes, 1991), *C. maenas* can change many aspects of its biology to adapt to particular environments. Studies on this species mostly focus on laboratory experiments and observations of physiological and biological processes (Young and Elliott, 2020) whereas, little is known on the reproduction issues at the microscopic scale (Lyons et al., 2012; Best et al., 2017).

In the present study, it was observed that specimens with higher GW presented more developed gonads at higher maturity stages. This relationship is explained by carapace width: the longer the CW the greater the GW (greater area of gonad occupied in the abdominal cavity). Our results are in line with other studies (Audet et al., 2008; Rodríguez-Félix et al., 2018) on marine species where increased female body size is also associated with increased fecundity. Such were the cases for other nonindigenous populations of *C. maenas* (Audet et al., 2008; Griffen, 2014), *C. aestuarii* (Baklouti et al., 2013), and other crab species (*Callinectes bellicosus*, Rodríguez-Félix et al., 2018) and fish (Quinn et al., 2011). The gonadosomatic index (GSI) values were used to measure the sexual maturation index of *C. maenas*, related with the development of the ovary and testis. During our study, it was found that during the spawning season GSI condition index increase with CW. This relationship is explained by the fact that GSI follows gonadal maturation, reaching higher values in the maturation phase, and then decreasing after spawning, especially in females (Table 1). There was also an effect of CW in Fulton's condition index (FC), which is an indicator of the reproductive period of the species by an increase or decrease in weight due to reproductive activity.

GSI and FC vary according to the reproductive strategies of the species and are often used to compare the effect of biotic and abiotic factors on the physiological condition of a population (Le cren, 1951). Several authors (Leignel et al., 2014; Lyons et al., 2012) report that reproduction in crustaceans is strongly influenced by environmental factors (especially water temperature, salinity, light cycle, and feeding conditions) and differences are found to be related to region/systems. As sampling was made in the same period across systems, we would expect the relationships between GSI and FC to be similar across areas. Based on GSI and FC indexes our results confirmed the existence of regional differences in the reproductive condition of *C. maenas*. Results reveal that these indexes varied between the center and southern systems which are Rio Sado and Ria Formosa lagoon. Usually, the temperature is found to influence reproduction as recorded in non-indigenous populations in the Northeast coast of North America (Audet et al., 2008; Griffen, 2014) and other crustacean species along the North-Western coast of Iberia (*Afropinnotheres monodi*, Perez-Miguel et al., 2020). As no significant differences ($p < 0.05$) in the condition indexes were recorded for between the North (Ria Aveiro) and South (Ria Formosa) for *C. maenas* samples, differences justified by the temperature gradient also lack to fully explain the observed results. Therefore, other existing variables may help explain the difference among center and south systems, namely the condition index and the morphometry and size of the oocytes as denoted by the PCA. The differences between Rio Sado and Ria Formosa lagoon can be related to the lower salinity of Rio Sado (Table A.1) relatively to the other two sites (Monteiro et al., 2022). The breeding period in the Sado occurs between October and May (Sprung, 2001), with the sampling months coinciding with this time and with the raining peak season. In the Rio Sado, this period coincides with an increase in runoff, leading to a decrease in salinity. PCA reveal that diameter and type of oocytes were associated to system sampled and mostly with salinity, namely in the Rio Sado while in Ria Formosa (the system with low oocyte sizes) the size of oocytes was negative correlated with both temperature and salinity. In opposition in Ria Formosa, where temperature is higher than in the other systems and the discharge of freshwater is negligible (Falcao and Vale, 1990), maturity was associated to higher temperature and salinity. This is also mention in some similar works that recorded that oceanographic aspects of the study systems can affect the physiological conditions of the species (Young and Elliott, 2020), as seen in the diameter and type of oocytes and maturity by the PCA. Thus, the above results are in line with one of the principles of marine ecology in which marine invertebrates adapt their reproductive and local environmental strategies to optimize their biological reproduction condition (Lyons et al., 2012).

In our study, continuous development of oocytes was observed from the primary oocyte stage to its resorption and start of a new cycle. These reproductive periods have also been verified for other brachyura species such as *Callinectes ornatos* (Mantelatto and Fransozo, 1999), *C. sapidus* (Cilenti et al., 2015), *C. aestuarii* (Baklouti et al., 2013) and other crab species (Rodríguez-Félix et al., 2018). Even though the methods used in this study may be regarded as labor-intensive and expensive, we believe our work provides a valuable methodology not often found in other studies (both quantitative and qualitative gonadal development analysis) namely when comparative analyses are made across systems. For fisheries management overall the macroscopic scales validated by histological analyses reveal that from North to South the same macroscopic protocol can be used by researchers to evaluate spawning aspects of the species (e.g. for determine seasonal periods or estimation of size of first maturity).

The histological analysis of the gonads of *C. maenas* comprises six stages of female gonadal development and four stages of male gonadal development (Tables 1 and 2, respectively). Previous studies (Best et al., 2017; Lyons et al., 2012) considered that the microscopic stage II of ovarian maturation status was immature. However, our results on the microscopic analysis denoted that regardless of the place of origin, stage 2 (late development) of female gametogenic development was already mature due to the presence of mature partially vitelogenic oocytes.

One other finding that requires some attention regarding macroscopic stage 2 is the presence of oocyte types that are characteristic of stages 4 and 5 (hydrated oocytes and post-ovulatory follicles). This is indicative of a post-spawning phase or a recovery to the beginning of a new spawning event. The fact that this species presents a biannual ovarian development (Lyons et al., 2012) may be the contributing factor to the presence of these types of oocytes at this stage, and to this misclassification on the macroscopic maturity assigned. Some of these results can affect the assignment of individuals macroscopically as mature or immature. Nevertheless, the macroscopic key already take into account our histological findings/scale, in which we consider females in stage 2 as mature. Such changes in the macroscopic maturity scale as previously suggested by Lyons et al. (2012) and Best et al. (2017), assigning stage 2 as mature at the macroscopic level rather than immature, are particularly relevant in fisheries biology studies, as it may alter the size of the first maturity estimated by the L_{50} from the maturity ogives.

The microscopic analysis of the males agrees with that reported by Lyons et al. (2012). Few studies are using histological analysis of the male gonads, but several authors report that the production of male gametes occur all over the year, as in the case of the species *Telmessus cheiragonus* by Nagao and Munehara (2003), *C. maenas* by Baeta et al. (2005) and the mangrove land crab, *U. cordatus* by Castilho et al. (2008). However not all Brachyura species have continuous gamete production. Some species, such as the red frog crab, *Ranina ranina* by Minagawa et al. (1994), and the blue crab, *C. sapidus* by Johnson (1980), have reported slight seasonal changes in the testicular lobes during different developmental stages.

This study contributed to a better characterization of male and female reproductive biology during the gonad development and maturation of the green crab, *C. maenas* across different systems with different

environmental features. The present macroscopic and microscopic maturity stage scale keys here developed can be used on fisheries researcher for studies that include the determination of maturity ogives and in the regulation of minimum landing size for crabs in different areas of the Portuguese coast. The present macroscopic scale was validated by histological analyses and can be used elsewhere.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

Acknowledgements

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Appendix

Table A.1
Physico-chemical parameters of three different river/lagoon systems of the Portuguese coast. Annual mean sea surface temperature (°C) and mean salinity (PSU) between 2019 and 2020.

System	Annual Mean SST (°C)	Annual Mean Salinity
Ria de Aveiro	15.58 ± 0.07	35.36 ± 0.02
Rio Sado	16.60 ± 0.20	35.62 ± 0.02
Ria Formosa	18.05 ± 0.13	35.77 ± 0.03

Table A.2
Biometric data, gonadosomatic, and Fulton's condition index of *C. maenas* in the three systems (Ria de Aveiro, Rio Sado and Ria Formosa). N, the number of individuals; M, males; F, females; T, the total number of individuals (males + females); CW, carapace width (cm); SD, standard deviation; W, weight (g); GW, gonad weight (g); GSI, gonadosomatic index; FC, Fulton's condition index.

System	Ria de Aveiro			Rio Sado			Ria Formosa		
Sex / Total (N)	M (12)	F (23)	T (35)	M (8)	F (23)	T (31)	M (15)	F (22)	T (37)
Max CW	5.84	5.64	5.84	5.31	5.27	5.31	5.90	5.87	5.90
Min CW	2.79	2.23	2.23	2.70	2.99	2.70	2.62	1.94	1.94
Mean CW (cm)	4.16 ± 1.13	4.00 ± 0.80	4.06 ± 0.93	3.86 ± 0.86	4.04 ± 0.62	3.99 ± 0.70	4.15 ± 1.05	4.30 ± 1.00	4.23 ± 1.03
Max W	45.69	47.00	47.00	37.79	34.96	37.79	54.87	46.58	54.87
Min W	5.64	2.70	2.70	4.32	4.82	4.32	4.13	4.41	4.13
Mean W (g)	19.52 ± 14.18	18.55 ± 11.03	18.88 ± 12.21	16.58 ± 10.80	17.12 ± 8.42	16.98 ± 9.10	18.92 ± 14.30	22.08 ± 12.46	20.62 ± 13.44
Max GW	0.26	0.87	0.87	0.11	1.69	1.69	0.60	0.48	0.60
Min GW	0.001	0.001	0.001	0.01	0.00	0.00	0.00	0.00	0.00
Mean GW (g)	0.08 ± 0.08	0.20 ± 0.24	0.15 ± 0.21	0.05 ± 0.04	0.28 ± 0.43	0.22 ± 0.38	0.08 ± 0.14	0.09 ± 0.12	0.09 ± 0.13
Max GSI	0.87	4.94	4.94	0.41	6.37	6.37	1.09	1.27	1.27
Min GSI	0.01	0.00	0.00	0.14	0.02	2.70	0.00	0.01	0.00
Mean GSI	0.30 ± 0.24	1.05 ± 1.34	0.79 ± 1.15	0.27 ± 0.11	1.73 ± 2.08	1.35 ± 1.90	0.24 ± 0.28	0.32 ± 0.37	0.28 ± 0.34
Max FC	27.14	84.78	87.78	26.95	27.68	27.68	26.70	254.78	254.78
Min FC	19.51	16.02	16.02	20.82	18.09	18.09	17.18	19.62	17.18
Mean FC	23.02 ± 1.93	27.20 ± 14.75	25.77 ± 12.17	24.34 ± 1.85	23.92 ± 2.72	24.03 ± 2.53	22.03 ± 2.26	33.43 ± 48.37	28.15 ± 35.92

Table A.3
Carcinus maenas gonadal development data in the three study sites (Ria de Aveiro, Rio Sado and Ria Formosa). *MS, microscopic maturity stage; OT, oocytes type; N, number of oocytes; M±SD (μm), mean and standard deviation; OD Min-Max (μm), minimum and maximum oocyte diameter.* Remark: (*) Due to dehydration, the oocyte is difficult to measure, the measurements were only made for the largest diameter.

System	MS	OT	N	M±SD (μm)	OD Min-Max (μm)
Aveiro	1	non-vitellated	20	32.69 ± 14.72	20.01–65.63
		partially vitellated	60	61.95 ± 8.15	31.01–88.13
	2	non-vitellated	118	30.25 ± 27.49	19.38–44.43
		partially vitellated	520	81.21 ± 37.46	34.00–154.13

(*continued on next page*)

Table A.3 (*continued*)

System	MS	OT	N	M±SD (μm)	OD Min-Max (μm)
		vitellated	298	107.23 ± 28.29	57.20–170.47
	3	non-vitellated	16	38.01 ± 39.59	29.89–59.38
		partially vitellated	22	95.00 ± 42.72	47.84–134.59
		vitellated	274	127.77 ± 47.87	57.04–212.49
	4	non-vitellated	57	35.91 ± 20.71	23.95–44.77
		partially vitellated	246	57.89 ± 27.48	29.31–114.70
		vitellated	49	75.53 ± 19.82	38.31–121.64
		Hydrated	34	82.34 ± 20.04*	46.32–171.73*
		Atretic	30	65.40 ± 22.20	32.85–91.93
		Pof's	2	48.19 ± 21.88	48.19–48.19
	5	non-vitellated	2	45.12 ± 13.93	45.12–45.12
		partially vitellated	64	61.39 ± 20.65	38.42–86.22
		vitellated	12	71.25 ± 15.24	56.61–86.39
		Hydrated	8	60.45 ± 15.64*	45.67–75.19*
		Atretic	8	57.22 ± 16.19	50.83–66.80
Sado	1	-	-	-	-
	2	non-vitellated	149	39.46 ± 27.49	19.67–83.19
		partially vitellated	136	70.84 ± 37.46	38.18–141.64
		vitellated	165	92.61 ± 28.29	51.39–148.65
	3	non-vitellated	16	54.01 ± 39.59	38.13–75.86
		partially vitellated	176	125.10 ± 42.72	50.35–223.10
		vitellated	590	131.97 ± 47.87	61.73–341.84
	4	-	-	-	-
	5	non-vitellated	8	31.38 ± 13.93	23.17–41.26
		partially vitellated	12	57.97 ± 20.65	41.84–70.28
		Hydrated	6	68.41 ± 32.12*	51.20–81.15*
Ria Formosa	1	non-vitellated	220	32.27 ± 14.72	14.20–47.94
		partially vitellated	80	42.86 ± 11.05	27.93–59.71
	2	non-vitellated	17	26.01 ± 27.49	19.99–38.74
		partially vitellated	133	77.03 ± 37.46	26.97–158.22
		vitellated	129	91.90 ± 28.29	46.34–201.19
		Atretic	1	51.46 ± 0.00	51.46–51.46
	3	non-vitellated	11	42.05 ± 39.59	24.88–60.50
		partially vitellated	70	90.81 ± 42.72	29.88–129.20
		vitellated	79	117.89 ± 47.87	58.53–231.95
	4	non-vitellated	4	23.96 ± 20.71	15.31–34.23
		partially vitellated	11	49.07 ± 27.48	36.62–129.20
		vitellated	32	86.34 ± 19.82	34.40–231.95
		Hydrated	12	73.98 ± 20.04*	49.28–104.61*
		Atretic	1	58.42 ± 22.20	58.42–58.42
	5	non-vitellated	97	33.17 ± 13.93	21.74–47.79
		partially vitellated	194	51.97 ± 20.65	32.91–80.70
		vitellated	67	65.11 ± 15.24	28.46–105.20
		Hydrated	33	64.37 ± 15.64*	33.59–129.20*
		Atretic	22	63.93 ± 16.19	35.26–93.74

Table A.4
Numbers, sizes, and weights of male and female C. *maenas* at different maturation stages in the three study sites (Ria de Aveiro, Rio Sado and Ria Formosa). MS, microscopic maturity stage; N, number of specimens per sex; CW, carapace width (cm); W, weight (g); GW, gonad weight (g); GSI, gonadosomatic index; FC, Fulton's condition index.

System	MS	Sex	N	CW (cm)	W (g)	GW (g)	GSI	FC
Ria de Aveiro	1	M	3	4.05 ± 1.13	17.08 ± 12.67	0.04 ± 0.05	0.16 ± 0.14	22.11 ± 1.83
		F	0	-	-	-	-	-
	2	M	7	4.37 ± 1.15	22.70 ± 15.34	0.09 ± 0.09	0.29 ± 0.17	23.07 ± 1.80
		F	9	3.74 ± 0.80	11.96 ± 6.92	0.14 ± 0.18	0.80 ± 0.84	20.42 ± 2.26
	3	M	2	3.57 ± 0.78	12.05 ± 6.41	0.09 ± 0.08	0.52 ± 0.34	24.25 ± 1.81
		F	4	3.81 ± 0.43	13.20 ± 4.71	0.43 ± 0.32	2.97 ± 1.74	22.60 ± 1.31
	4	M	N/A	N/A	N/A	N/A	N/A	N/A
		F	4	4.53 ± 0.47	24.56 ± 8.05	0.04 ± 0.02	0.15 ± 0.10	25.43 ± 0.90
	5	M	NA	N/A	N/A	N/A	N/A	N/A
		F	4	4.61 ± 0.88	28.96 ± 14.26	0.08 ± 0.06	0.22 ± 0.12	26.15 ± 2.12
Rio Sado	1	M	2	2.72 ± 0.02	4.40 ± 0.07	0.01 ± 0.00	0.16 ± 0.03	27.59 ± 0.93
		F	1	3.73 ± 0.00	14.26 ± 0.00	0.03 ± 0.00	0.20 ± 0.00	27.59 ± 0.00
	2	M	6	4.24 ± 0.64	20.64 ± 9.46	0.06 ± 0.03	0.31 ± 0.10	24.28 ± 1.13
		F	8	4.30 ± 0.66	20.27 ± 8.29	0.29 ± 0.54	1.59 ± 2.21	23.96 ± 1.98
	3	M	0	-	-	-	-	-
		F	11	3.95 ± 0.56	15.93 ± 8.53	0.25 ± 0.21	1.83 ± 1.70	23.79 ± 2.73
	4	M	N/A	N/A	N/A	N/A	N/A	N/A
		F	2	4.17 ± 0.17	18.72 ± 3.27	0.72 ± 0.68	3.34 ± 3.03	25.49 ± 1.46
	5	M	N/A	N/A	N/A	N/A	N/A	N/A
		F	1	2.99 ± 0.00	4.82 ± 0.00	0.00 ± 0.00	0.02 ± 0.00	18.09 ± 0.00
Ria Formosa	0	M	N/A	N/A	N/A	N/A	N/A	N/A

(*continued on next page*)

Table A.4 (*continued*)

System	MS	Sex	N	CW (cm)	W (g)	GW (g)	GSI	FC
		F	5	3.18 ± 0.21	7.02 ± 1.44	0.00 ± 0.00	0.02 ± 0.00	21.46 ± 0.64
	1	M	2	3.44 ± 0.20	8.94 ± 0.44	0.00 ± 0.00	0.04 ± 0.03	22.20 ± 2.72
		F	1	3.88 ± 0.00	12.26 ± 0.00	0.02 ± 0.00	0.15 ± 0.00	20.99 ± 0.00
	2	M	13	4.64 ± 0.90	24.51 ± 14.09	0.12 ± 0.15	0.34 ± 0.29	21.90 ± 2.49
		F	5	4.86 ± 0.51	26.05 ± 8.53	0.11 ± 0.07	0.47 ± 0.37	22.12 ± 2.87
	3	M	0	-	-	-	-	-
		F	3	5.33 ± 0.46	34.70 ± 8.15	0.32 ± 0.11	0.97 ± 0.33	22.43 ± 0.36
	4	M	N/A	N/A	N/A	N/A	N/A	N/A
		F	0	-	-	-	-	-
	5	M	N/A	N/A	N/A	N/A	N/A	N/A
		F	3	4.46 ± 0.76	25.50 ± 14.95	0.06 ± 0.05	0.18 ± 0.09	25.59 ± 1.65

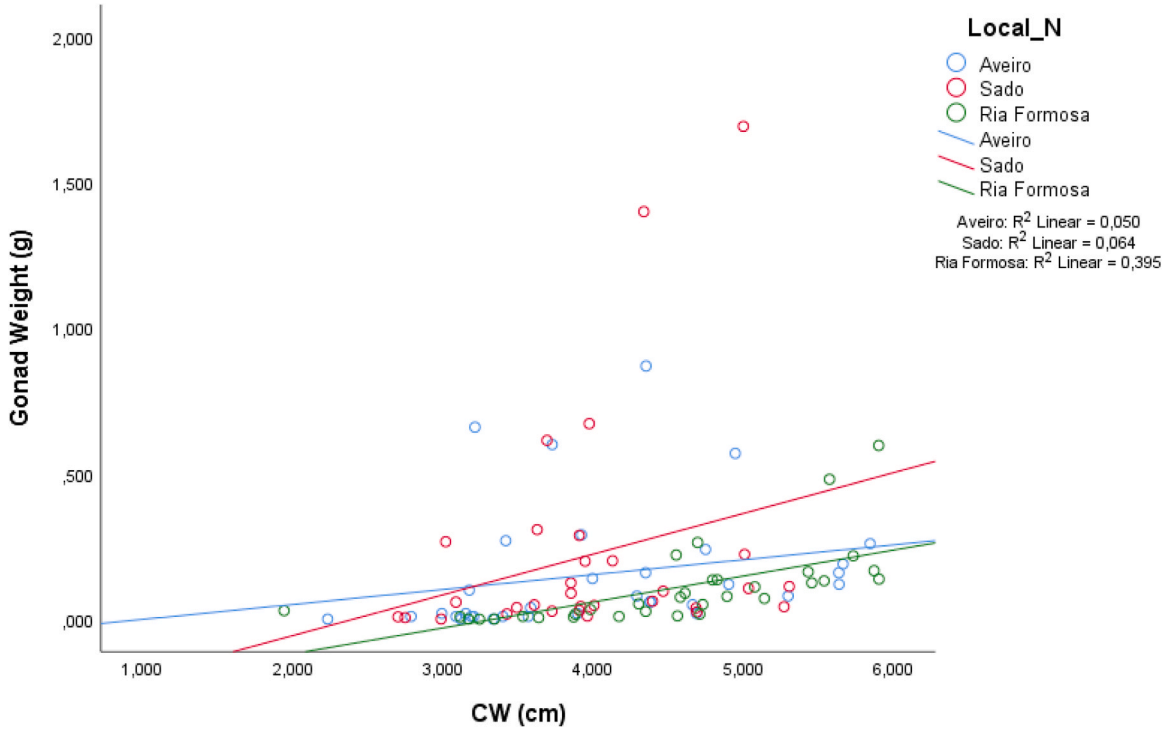

Figure A.1. *Carcinus maenas* GW regressed against CW and compared among three lagoon and estuarine systems from Portugal.

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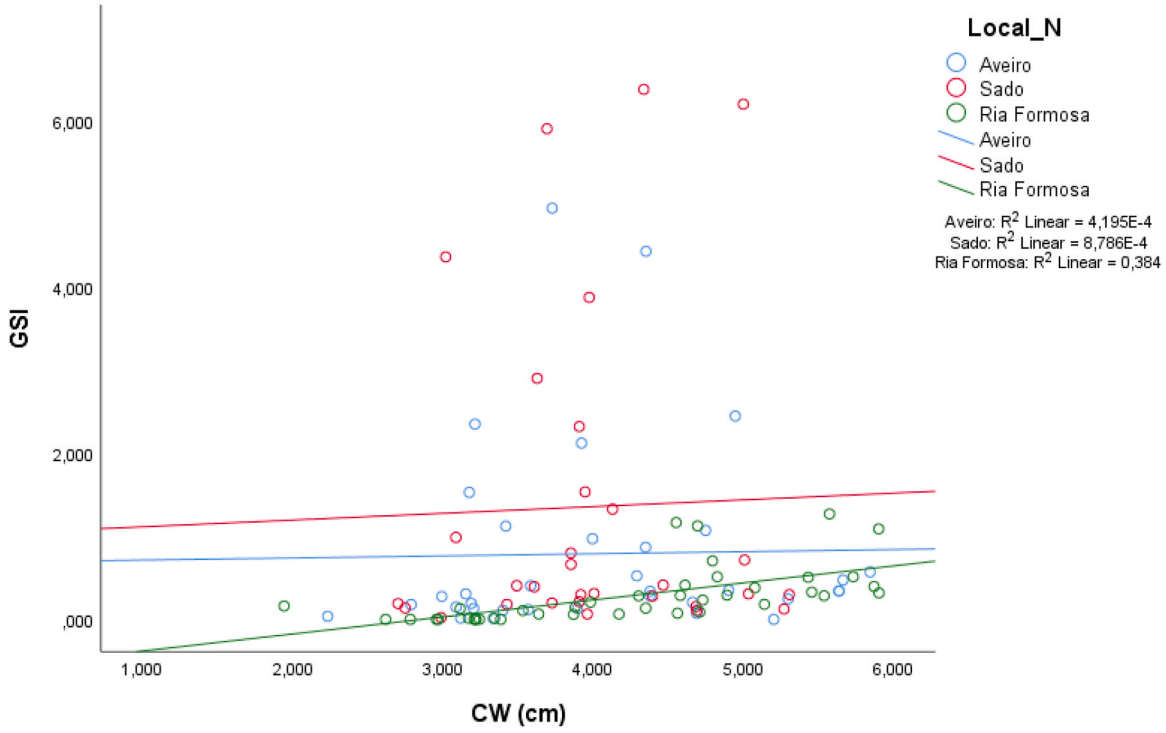

Figure A.2. *Carcinus maenas* GSI regressed against CW and compared among three lagoon and estuarine systems from Portugal.

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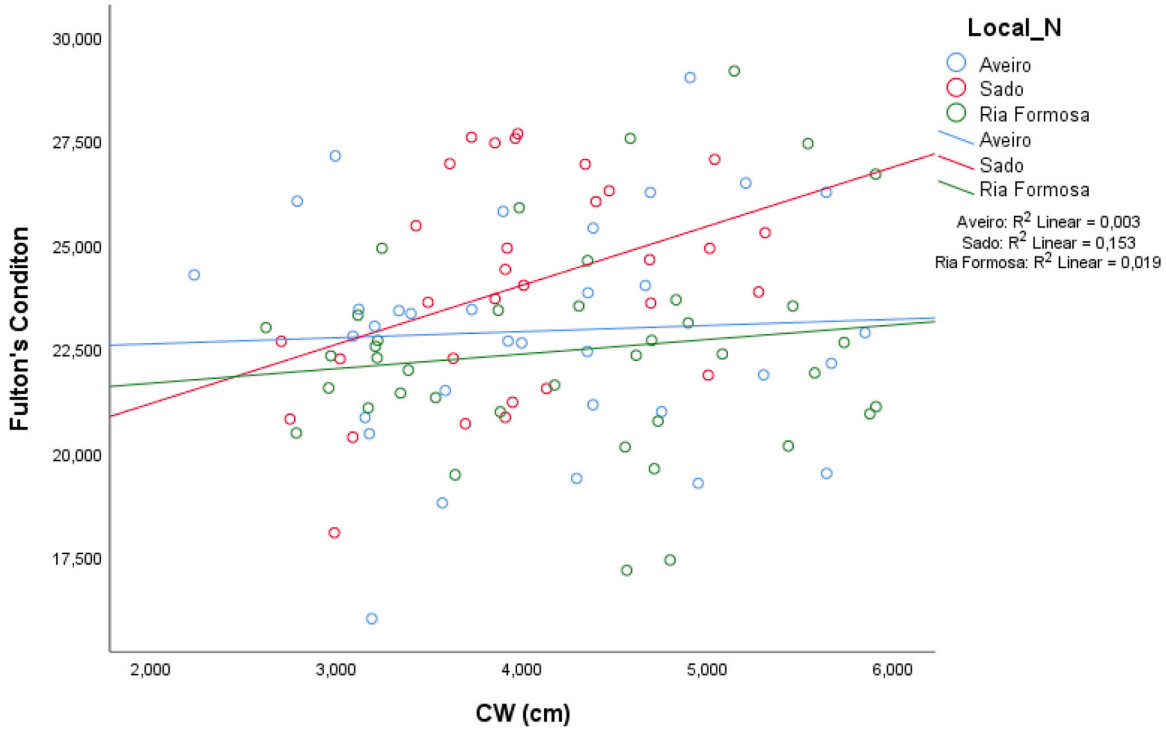

Figure A.3. *Carcinus maenas* FC regressed against CW and compared among three lagoon and estuarine systems from Portugal.

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