DETAILED DESCRIPTION OF TASKS

INPACTAR PTDC/MAR/111537/2009

Task 1- Data collection and processing

1.1.Field campaigns

Between July 2011 and August 2012, the 3 estuaries (Minho, Ave and Mondego) were monthly sampled except when weather conditions did not allow field work to take place (Minho-October 2011 and all 3 systems-December 2011). Three sampling stations were chosen within each estuary according to suitability for *E. marinus* populations, distance to shore and representing different habitats for the amphipod.

In Minho estuary, "Embocadura" is the sampling station with the closest location to the estuary mouth, characterized by the presence of abundant *Fucus* sp. on intertidal mudflats; "Intermedia" is located upstream from "Embocadura" on a more disturbed area, characterised by the presence of a small fishermen harbour and close to a domestic effluent, where *Fucus* sp. was present along with significant allochtonous organic material; "Sapal" is the most upstream station in the Minho, located within Coura saltmarsh area, where *Fucus* sp. was present on mudflats with significant organic matter content.

In Ave estuary," Praia" is the most downstream station with a high influence of seawater where *Fucus sp.* was present on sandy sediments. "Canal" field station is located in the main channel of the Ave estuary, characterized by high hydrodynamics; *Fucus* sp. were mostly attached to rocks and very few were present on the sediment; "Estaleiro" is a very disturbed station, located close to Vila do Conde boatyard facilities; *Fucus* sp. populations were sparse and located near a rocky belt: "

In the Mondego estuary, "Doca" station was the most downstream station, being located near an abandon boatyard, where *Fucus* sp. lays on mud-sand sediments; "Armazens" is located further upstream with abundant mats of *Fucus sp.* on mud-sand sediments; "Pranto" is the most upstream station with abundant *Fucus* sp. on quite muddy sediments.

During sampling campaigns, which took place during low tide, at each station, 3 replicates of a representative *Fucus* sp. spot containing *E. marinus* were sampled plus the initial 5 cm of sediment below the focus spot; the area of the spot was photographed for posterior area

calculation with appropriate software. Water temperature, salinity, dissolved oxygen, pH, ROP were measured in nearby water retention pools; water samples were collected for posterior analyses of nitrate, nitrite, ammonia, phosphate and silica. Sediment samples were collected for posterior organic matter content analysis. Seasonal samples of sediments were also collected for analyses of the following contaminants: Cd, Cu, TBT and PAH.

Biological samples from the field were divided in two fractions: *Fucus* and sediment. The sediment fraction, which was previously sieved in the field with a 0.5 mm mesh, was stored in plastic bags and preserved with 4% buffered formalin. The *Fucus* fraction, containing most *Fucus spp.* and amphipods was carefully washed with tap water in order to remove all organisms which were then stored in flasks containing 80% ethanol until sorting. Subsequently, *Fucus spp.* was weighted to determine its fresh weight, dried in a stove at 60ºC for at least 72h in order to determine its dry weight for further biomass estimation. Some samples of *Fucus* were also burned in a muffle at 450ºC for 8h to ensure a correct determination of the ash-free dry weight (AFDW).

Organic matter samples were placed in paper boxes (10x10x5cm) and left to dry in a stove at 60ºC for at least 72h after which they were macerated into ceramic crucibles and burned in a muffle for 450ºC for 8h to determine the AFDW and subsequently account for the site's organic matter content.

Water samples were filtered upon arrival to the laboratory using a vacuum pump and Whatman GF/F glass-fibre filters and the filtered samples were stored in 40 ml vials at -18ºC until they were analysed for their nutrient content.

Nutrient analysis was performed using the Skalar® San++System Autoanalyzer, a segmented continuous flow multiparameter analyser, which determined the contents of phosphate, nitrate, nitrite and ammonia in our water samples.

To determine the phosphate content, the sample was mixed with potassium peroxodisulfate. The organic phosphates were destructed by means of UV radiation and sulfuric acid was added to the sample stream and the solution heated to 107ºC. Complex inorganic phosphates were digested to ortho-phosphate and sodium hydroxide was added to neutralize the solution. Ammonium heptamolybdate catalysed by potassium antimony(III) oxide tartrate reacts in an acidic medium with diluted solutions of phosphate to form a phospho-molybdic acid complex. This complex was then reduced in an intensely blue coloured complex by

L(+)ascorbic acid and measured at 880 nm. For nitrates and nitrites, the water sample was mixed with a potassium peroxodisulfate/sodium hydroxide solution. The solution was then mixed and brought into an UV digester and heated to 107°C. After dialysis the nitrate was determined by the Griess reaction after reduction of nitrate to nitrite by a cadmium copper reductor. The colour was measured at 540 nm.

Ammonia was determined through the Berthelot reaction where it was buffered and chlorinated to monochloramine which reacts with phenol. After oxidation and oxidative coupling a green coloured complex is formed. The reaction was catalysed by nitroprusside and sodium hypochlorite was used for chlorine donation. The formed complex was measured at 630 nm.

1.2. Identification procedures

After sorting, the amphipods were identified using a stereomicroscope but only the *Echinogammarus marinus* individuals were counted and the cephalic length was measured to the nearest 0.02 mm, between the extremity of the rostrum and the base of the head, using a calibrated stereomicroscope. Measuring the cephalic length is more convenient than the total length because the comma-shaped bodies of *E. marinus* make it harder to determine age and growth (Marques and Nogueira, 1991).

The sex of each individual was determined based on the presence or absence of female characters, oostegites and/or broods and male characters, genital papillae. Females were also classified as mature and immature, thus considering an immature female as having oostegites without setae. Individuals without these features were considered to be juveniles and individuals with both features were classified as intersex males, if they had genital papillae and rudimentary brood plates, or intersex females if the less developed character were the genital papillae (Ford et al. 2003).

In the presence of brooding females, the eggs were removed and counted to estimate fecundity and examined in order to determine the development stage. Five stages were considered, based on the criteria described by Skadsheim (1982) and adapted by Marques and Nogueira (1991), respectively:

A – Newly laid eggs, spherical grouped and resembling a gelatinous mass;

B – Eggs well separated, oblong internally homogeneous;

C – Embryo comma-shape with vestigial pereopods already visible;

D – Constriction of the comma clearly visible, appendages segmented and looking slender, eyes visible, cephalotorax orange-red;

E – Hatched and free juveniles (these may stay some days in the brood pouch).

Due to some difficulties in distinguishing stages A and B, data regarding these two stages were pooled in an AB stage.

Immediately, after field work, in the lab *Fucus sp*. replicates were washed from sediments and *E. marinus* individuals using appropriate sieves (0,5 mm); all *Fucus sp.* replicates were weighted (fresh weight) and *E. marinus* individuals were preserved on 70% ethanol for posterior identification; the below *Fucus* sediment samples were preserved on 4% formaldehyd solution for posterior sorting. Water samples were filter and frozen until nutrient analysis. Sediment was weighted, dried and burnt for organic matter content determination. Sediment contaminant samples were frozen until further analysis.

1.3. Consumption and feeding preference experiments

During November 2012, *U. intestinalis* and *F. vesiculosus* fronds with *E. marinus* individuals (visually confirmed) were collected from the intertidal area of the south arm of Mondego estuary (Western coast of Portugal: 40°08' N, 8°50' W) and taken to the lab. Seawater and sand were also collected and taken to the lab for preparation of acclimatization procedures. During acclimation, individuals of *E. marinus* corresponding to 3 different areas were assigned to 3 different aquaria to ensure independency of data (Underwood and Clarke, 2005). Each aquarium, containing 6L of filtered seawater continuously aerated and a sand-bottom layer, was kept at constant temperature and light conditions (20°C and 12h:12h light: dark cycle) for 4 days. During this period, amphipods were fed *ad libitum* with fresh fronds of *F. vesiculosus* and *U. intestinalis*. One day prior to the experiment, 10 amphipods were removed from each of the 3 acclimation aquaria (Total=30 individuals). Amphipods were individually allocated to 14cm x 14cm aquaria containing 400 mL of filtered seawater, continuously aerated, and kept in starvation for 1 day to equalize the hunger state among individuals (Underwood et al., 2004). In the same day, fresh *F. vesiculosus* and *U. intestinalis* were collected from the previously referred site in the Mondego estuary.

On the day of the experiment, algal discs were cut from the newly collected *F. vesiculosus* and *U. intestinalis* using a metallic cylinder (Ø = 1.8 cm). *Fucus* discs were never cut on the apical part of the alga to avoid bias derived from potentially different inter-individual growth rates. At the beginning of the experiment, which lasted for 3 days, algal discs were distinctly added to the 30 aquaria containing starved *E. marinus* individuals: 10 aquaria were provided only with *Ulva* discs, 10 aquaria were provided only with *Fucus* discs and 10 aquaria were provided both with *Ulva* and *Fucus* discs. 10 controls were also run for each treatment (Total=30 controls) (Fig. 1).

This experimental design allows running stage 1, when animals are separately fed with different food items and proportions eaten are registered (p1 and p2 as 2 different items are being tested) and stage 2, when animals are simultaneously fed with different food items and proportions eaten are also registered (q1 and q2) (Underwood and Clarke, 2005). The null hypothesis of no preference is:

H_0: $q_1=\theta p_1$; $q_2=\theta p_2$

θ is a constant, unknown parameter.

Proportions of algae eaten were estimated as consumption rates, where consumption of algae by *E. marinus* (g g fresh wt-1 d-1) was calculated as in Canhoto and Graça (1995):

$$\text{Consumption}=(Ai-(Af\ .k))/(Wf.d) \qquad \text{(Eq. 1.1)}$$

Ai and Af are algal initial and final fresh weight, respectively. Wf is the animal's final fresh weight, d is the duration of the experiment in days;

$$k=Ci/Cf \qquad \text{(Eq. 1.2)}$$

Ci and Cf are initial and final fresh weight of algae in controls, respectively.

T-tests were performed with STATISTICA software package (StatSoft).

1.4. *Echinogammarus marinus* PCR-based parasite screening

In order to assess parasite prevalence 95 *Echinogammarus marinus* adults were collected from 3 different estuaries, 18 males and 18 females from the Mondego estuary, 17 males and 18 females from de Ave estuary and 12 males and 12 females from de Minho estuary. The original design accounted for 54 adults of each sex (108 total), however logistical constraints

prevented the fulfilment of our plan. All samples were preserved in 70% ethanol and stored in 1,5ml eppendorfs at -80ºC until further processing.

The parasite screening (Microsporidia and Paramixea) was performed by polymerase chain reaction (PCR). DNA extraction from the samples was performed using the DNeasy® Blood and Tissue Kit (QIAGEN®) following the manufacturer's protocol. Gonads and all muscle tissue of each sample was dissected and placed in 180 µl of buffer ATL. These tissues were then homogenised using a disposable pestle or the QIAGEN TissueLyser with disruption beads (40s at 30 Hz). 20 µl of proteinase K were added to the solution, vortexed and incubated in a thermomixer at 56ºC under 650 rpm for a minimum of 2h. In order to obtain RNA-free DNA 20µl of RNase A (10mg/ml) were added to the solution which was left at rest for a minimum of 2 minutes and then vortexed for 15s. 200 µl of buffer AL and 200 µl of 96% ethanol were added and, after vortexing thoroughly and spinning, the mixture was pipetted into a DNeasy Mini spin column and centrifuged for 1 minute at 8000 rpm. After discarding the flow-through, 500 µl of buffer AW1 were added to the spin column and centrifuged for 1 minute at 8000 rpm. After discarding the flow-through, 500 µl of buffer AW2 were added and centrifuged at 14000 rpm for 3 minutes. An extra centrifugation of 1 minute at 14000 rpm was done to ensure that no residual ethanol was carried over to the following elution. The DNeasy Mini spin column was placed in a 1.5 ml microcentrifuge tube and, to elute the DNA, 100 µl of buffer AE were pipetted directly onto the spin column's membrane. After incubating for 1 minute at room temperature the spin column was centrifuged at 8000 rpm for 1 minute and the eluted DNA collected in the 1.5 ml tube.

The quantity of extracted DNA was assessed using the NanoDrop spectrophotometer (Thermo Scientific®) using buffer AE as the blank solution for calibration and the quality of DNA samples was checked through PCR amplification of the host (*E. marinus*) cytochrome c oxidase (COI) gene. The microsporidian and paramixean parasites screening was performed by PCR amplification of the microsporidian 16S rRNA gene and the 18S rRNA gene, respectively. All PCR reactions were performed in 25 µl solutions containing 2.5 µl of Buffer DreamTaq 10x (Thermo Scientific), 2.5 µl of dNTPs 2mM, 1.25 µl of each primmer 10 µM (Table 1), 0.2 µl of DreamTaq 5U/µl (Thermo Scientific) and ±20 ng of sample DNA. A negative control was also done for every batch of PCRs using water instead of sample DNA and also a positive control (DNA from a known infected sample) was used when screening for parasite infection. All reactions were carried out in a thermal cycler (Bio-Rad) using primers and thermal cycling conditions described in Table 1. The PCR products were

separated by 1% agarose gel electrophoresis, stained with GreenSafe Premium (NZYtech) and visualized under a UV transilluminator.

A selection of the amplified microsporidian 16S rRNA gene product were cut from the agarose gel, purified using the QIAquick® Gel Extraction Kit (Qiagen®) according to the manufacturer's instructions and sequenced in an Automatic Sequencer 3730xl under BigDye^{TM} terminator cycling conditions at Macrogen® (Korea) using the same primers as in PCR. Sequences analyses were carried out using BioEdit (Hall, 1999) and homologous sequences in the GenBank database were searched using BLAST (Altschul et al., 1997).

Table 1. Primers and PCR conditions used for *Echinogammarus marinus* parasite screening

Gene	Primer	Primer Source	Sequence 5' end to 3'	Thermal Cycle	Approximate size of amplified fragment (bp)
E.marinus COI gene	LCO1490	Folmer et al. (1994)	GGTCAACAAATCATAAAGATATTGG	94ºC (5 min), 40 cycles of 94ºC (30 s), 45ºC (30 s) and 72ºC (1 min), a final incubation of 72ºC (5 min) and idle at 10ºC	700
	HCO2198		TAAACTTCAGGGTGACCAAAAAATCA		
Microsporidia 16S rRNA gene	VIF	Weiss et al. (1994)	CACCAGGTTGATTCTGCCTGAC	94ºC (5 min), 42 cycles of 94ºC (45 s), 62ºC (45 s) and 72ºC (1 min 45s), a final incubation of 72ºC (5 min) and idle at 10ºC	1200
	1342AC	Yang et al. (2011)	ACGGGCGGTGTGTACAAGGTACAG		
Paramyxean 18S rRNA gene	PARA18SF5	Short et al. (2012)	CCAAACCAAACGATCGAAGT	94ºC (5 min), 42 cycles of 94ºC (45 s), 63ºC (45 s) and 72ºC (1 min), a final incubation of 72ºC (5 min) and idle at 10ºC	1000
	PARA18SR3		GGGCGGTGTGTACAAAGG		

1.4.1. PCR results

DNA was extracted from 95 individuals but for only 60 DNA samples the COI gene was successfully amplified (Annex1). This fact could be justified by the presence of some PCR inhibitors in some of the DNA samples. Several dilutions of the obtained DNA solution were

tested in the COI PCR to try to overtake this problem. Out of these 60 samples, 9 were found positive for microsporidia yet no trace of paramyxean infection was detected in any tested sample. In the Mondego estuary 10 out of 36 samples had a successful amplification of the COI gene but only 1 showed the presence of microsporidia infection (10%) (Fig.1). Regarding the Ave estuary, out of 35 samples the COI gene was successfully amplified in 28 and 6 of these were found infected with microsporidia (21.4%). As for the Minho estuary, 22 out of 24 samples showed successful amplification of the COI gene and only 2 out these 22 were infected with microsporidian parasites (9.1%).

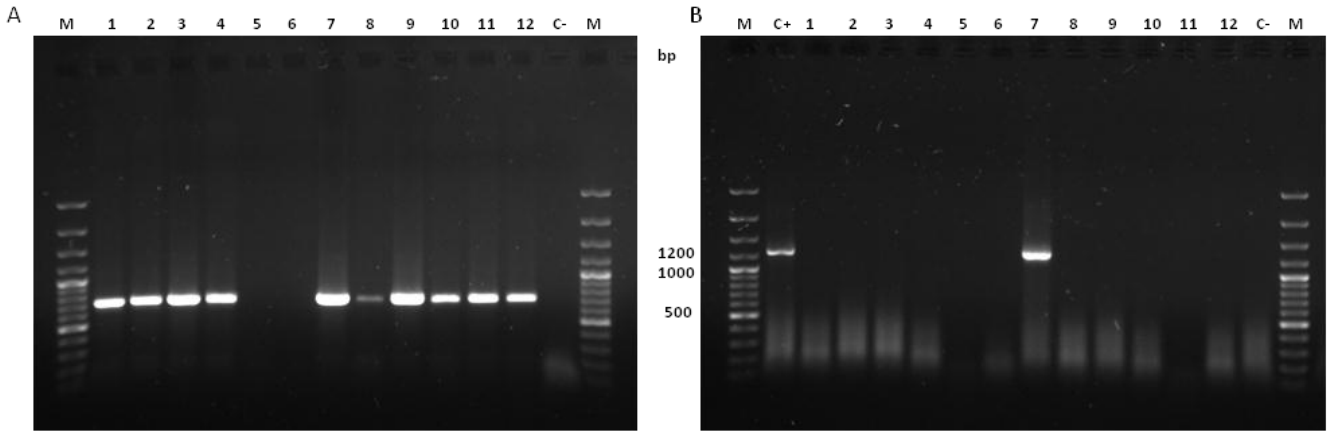

Figure 1. Typical examples of inference of DNA quality with PCR amplification of *Echinogammarus marinus* COI gene (700 bp product) (A) and microsporidia parasite infection with PCR amplification of the 16S rRNA gene (1200 bp product). Agarose gel electrophoreses of PCR products from DNA samples from Mondego estuary (1-12) and from a known microsporidia infected sample (C+); a non-template control (C-) and GeneRuler 100 bp plus DNA Ladder (Thermo Scientific) (M).

These results are consistent with the previous sugestion that amphipods from highly impacted sites are more susceptible to infection by parasites (Ford et al, 2006), which in turn has been associated with intersexuality incidence in amphipods (Short et al, 2012). In the severely impacted Ave estuary the percentage of infected individuals with microsporidia was the highest among the three estuaries, 21.4%. Mondego samples displayed 10% infection while the Minho estuary presented 9.1% of infected samples. Although samples from the Minho estuary presented the lowest values of infection, they were very close to the ones from Mondego (10 and 9.1%, respectively). This was somewhat unexpected because the Minho estuary was regarded as a reference site with very little pollution and therefore should present very low levels of infection when compared with the Mondego estuary, a medium-impacted one (Leite et al, 2014). Although our samples were infected with microsporidian parasites

none of them displayed any sign of intersexuality, suggesting that feminising parasites such as microsporidia might not be the cause of intersexuality occurrence.

From the sequencing results obtained for the 8 microsporidia rDNA amplified fragments, only one of the samples (from the Minho estuary) gave a single sequence with 99% of sequence identity to the *Dictyocoela duebenum* 16S ribosomal RNA gene, partial sequence (accession number: JQ673483). All the other sequenced products gave mix sequencing signals revealing the presence of more than one Microsporidia species. Further studies must be performed in order to identify the microsporidia species present in these samples. Coinfection of different microsporidia parasites in the same host individual was reported to be very rare (Haine et al., 2004; Terry et al., 2004) however, coinfection of *E. marinus* with two microsporidia species (*Dictyocoela duebenum* and *Dictyocoela berillonum*) has been previously reported (Yang et al., 2011).

A paper entitled "Parasite screening in *Echinogammarus marinus* populations from three estuaries with different environmental impact levels" is under preparation.

Annex 1. List referring to the provenience, sex, id, nucleic acid concentration of each sample and the respective PCR result for the COI gene and microsporidia screening – positive (+) or negative (-).

Estuary	Station	Sex	Sample ID	Nucleic Acid Conc.	COI	MIC
Minho	Embocadura	Male	ME1M	260,7	+	-
Minho	Embocadura	Male	ME2M	219,6	+	-
Minho	Embocadura	Male	ME3M	203,4	+	-
Minho	Embocadura	Male	ME4M	273,4	+	-
Minho	Embocadura	Male	ME5M	255,9	+	-
Minho	Embocadura	Male	ME6M	306,9	+	-
Minho	Embocadura	Female	ME1F	8,9	+	-
Minho	Embocadura	Female	ME2F	8,4	+	-
Minho	Embocadura	Female	ME3F	40	+	+
Minho	Embocadura	Female	ME4F	54,4	+	+
Minho	Embocadura	Female	ME5F	3,5	+	-
Minho	Embocadura	Female	ME7F	7,5	+	-
Minho	Intermédia	Male	MI1M	7,9	+	-
Minho	Intermédia	Male	MI2M	4,4	+	-
Minho	Intermédia	Male	MI3M	9,2	+	-
Minho	Intermédia	Male	MI4M	6,2	+	-
Minho	Intermédia	Male	MI5M	16,4	-	-
Minho	Intermédia	Male	MI6M	9,4	+	-

Minho	Intermédia	Female	MI1F	6,3	-	-
Minho	Intermédia	Female	MI2F	10,6	+	-
Minho	Intermédia	Female	MI3F	5,8	+	-
Minho	Intermédia	Female	MI4F	8,6	+	-
Minho	Intermédia	Female	MI5F	4,9	+	-
Minho	Intermédia	Female	MI6F	11,7	+	-
Ave	Praia	Male	AP1M	53	+	+
Ave	Praia	Male	AP2M	223,4	+	-
Ave	Praia	Male	AP3M	32,4	+	-
Ave	Praia	Male	AP4M	23,2	+	-
Ave	Praia	Male	AP5M	16,5	+	-
Ave	Praia	Male	AP6M	17,9	+	-
Ave	Praia	Female	AP1F	26,6	+	-
Ave	Praia	Female	AP2F	62,4	+	-
Ave	Praia	Female	AP3F	65,4	+	-
Ave	Praia	Female	AP4F	130,5	-	-
Ave	Praia	Female	AP5F	449,5	-	-
Ave	Praia	Female	AP6F	15,6	+	+
Ave	Estaleiro	Male	AE1M	56,5	+	-
Ave	Estaleiro	Male	AE2M	104	+	-
Ave	Estaleiro	Male	AE3M	21,9	+	-
Ave	Estaleiro	Male	AE4M	30,9	+	-
Ave	Estaleiro	Male	AE5M	70,2	+	-
Ave	Estaleiro	Male	AE6M	50,8	+	-
Ave	Estaleiro	Female	AE1F	50,9	+	-
Ave	Estaleiro	Female	AE2F	22,4	+	-
Ave	Estaleiro	Female	AE3F	41,6	+	-
Ave	Estaleiro	Female	AE4F	28,2	+	-
Ave	Estaleiro	Female	AE5F	14	-	-
Ave	Estaleiro	Female	AE6F	14,2	+	+
Ave	Canal	Male	AC1M	30,5	-	-
Ave	Canal	Male	AC2M	26	+	+
Ave	Canal	Male	AC3M	40,4	-	-
Ave	Canal	Male	AC4M	29	-	-
Ave	Canal	Male	AC5M	119,2	+	-
Ave	Canal	Female	AC1F	62,6	-	-
Ave	Canal	Female	AC2F	53,4	+	-
Ave	Canal	Female	AC3F	59,4	+	-
Ave	Canal	Female	AC4F	63,5	+	-
Ave	Canal	Female	AC5F	68	+	+
Ave	Canal	Female	AC6F	25,3	+	+
Mondego	Armazéns	Male	MoA1M	70,7	+	-
Mondego	Armazéns	Male	MoA2M	63,6	+	-
Mondego	Armazéns	Male	MoA3M	74	-	-
Mondego	Armazéns	Male	MoA4M	81,3	+	-

Mondego	Armazéns	Male	MoA5M	75,2	-	-
Mondego	Armazéns	Male	MoA6M	70,3	-	-
Mondego	Armazéns	Female	MoA1F	50,2	-	-
Mondego	Armazéns	Female	MoA2F	59,9	+	-
Mondego	Armazéns	Female	MoA3F	87,4	-	-
Mondego	Armazéns	Female	MoA4F	190,6	-	-
Mondego	Armazéns	Female	MoA5F	95,7	-	-
Mondego	Armazéns	Female	MoA6F	79,5	-	-
Mondego	Pranto	Male	MoP1M	81,1	-	-
Mondego	Pranto	Male	MoP2M	69,3	-	-
Mondego	Pranto	Male	MoP3M	73	-	-
Mondego	Pranto	Male	MoP4M	97,7	-	-
Mondego	Pranto	Male	MoP5M	94,7	-	-
Mondego	Pranto	Male	MoP6M	72,2	-	-
Mondego	Pranto	Female	MoP1F	58,1	-	-
Mondego	Pranto	Female	MoP2F	68,4	-	-
Mondego	Pranto	Female	MoP3F	63,8	-	-
Mondego	Pranto	Female	MoP4F	56,4	-	-
Mondego	Pranto	Female	MoP5F	79,3	-	-
Mondego	Pranto	Female	MoP6F	71,7	+	+
Mondego	Doca	Male	MoD1M	108,1	-	-
Mondego	Doca	Male	MoD2M	73,3	-	-
Mondego	Doca	Male	MoD3M	94,8	+	-
Mondego	Doca	Male	MoD4M	52,8	+	-
Mondego	Doca	Male	MoD5M	70,6	-	-
Mondego	Doca	Male	MoD6M	99,8	-	-
Mondego	Doca	Female	MoD1F	49,8	-	-
Mondego	Doca	Female	MoD2F	88,1	+	-
Mondego	Doca	Female	MoD3F	95,7	-	-
Mondego	Doca	Female	MoD4F	78,5	+	-
Mondego	Doca	Female	MoD5F	68,5	-	-
Mondego	Doca	Female	MoD6F	116,4	+	-

Task 2- Population production and dynamics

2.1. Annual production estimation

Secondary production was calculated using the Size–Frequency method (Hynes & Coleman, 1968; Hamilton, 1969; Benke, 1979).

Raw data for size-frequency production estimates include observations of the density (Y) of *E. marinus* in the algae cover (number of individuals.m^{-2}) at intervals over 13 months. The density was related with both sampled area and algal density through the formula:

$$Y = A \times B \qquad \text{(Eg. 2.1)}$$

where A is the number of amphipods per gram of algae in each sample and B is the average weight of algae (g) per square meter on a sample date (Marques and Nogueira, 1991).

The size distributions calculated for each sample date were used to build a mean-size-frequency distribution that helped the final calculation of *E. marinus* production. The population was divided into 109 (Mondego), 99 (Minho) and 89 (Ave) arbitrary size length classes.

In the following equations, sampling dates are indexed by *i* and proceed from 1 through *n*, where *n* represents the total number of sampling dates (*i* = 1, 2, . . . , *n*).

Size classes are indexed by *j* (*j* = 1, 2, . . . , *a*) from the smallest to the largest. Samples within date *i* are ordered by *k* (*k* = 1, 2, . . . , b_i) where b_i represents the total number of samples taken on date *i*.

The mean number of individuals by size class *j* observed on the *i*th date is:

$$\bar{Y}_{ij.} = \frac{\sum_{k=1}^{b_i} Y_{ij.}}{b_i} \qquad \text{(Eq. 2.2)}$$

where Y_{ijk} is the number of individuals observed on the *i*th date in the *j*th size class from the *k*th sample drawn.

The estimate of the number of individuals on the average during the sampling year for each size class ($\bar{Y}_{.j}$) is weighted by sampling interval length. This calculation of $\bar{Y}_{.j.}$ can easily be thought of as the prevalence of the *j*th size class in the population. $\bar{Y}_{.j.}$ is expressed as:

$$\bar{Y}_{.j.} = \frac{\sum_{i=1}^{n-1}(D_{i+1} - D_i)\ \frac{\bar{Y}_{ij.} + \bar{Y}_{i+1j.}}{2}}{D_n - D_1} \qquad \text{(Eq. 2.3)}$$

where D_i is the number of days between the first sampling day and the *i*th sampling date.

The size values were converted to biomass values by interpolating the length-dry weight equations described by Marques and Nogueira (1991):

$$W = 1.592924 \times C_L^{3.94344} \qquad \text{(Eq. 2.4)}$$

where W refers to ash-free dry weight (mg AFDW) and $\mathbf{C_L}$ is the cephalic length (mm).

In the production calculation given here, the geometric mean $(W_j W_{j+1})^{0.5}$ of the mean weights per individual of size classes *j* and *j + 1* (see Sokal and Rohlf 1969) is used to estimate the weights of individuals lost.

This modification of the size-frequency production calculation can be written as:

$$P = a\left[\sum_{j=1}^{a-1}\left(\bar{Y}_{.j.} + \bar{Y}_{.j+1.}\right)\left(W_j W_{j+1}\right)^{0.5}\right] \qquad \text{(Eq. 2.5)}$$

where P is the annual-production and W_j is the mean weight per individual in size class *j*.

The calculations described above can be used only for univoltine species where pupal, adult, or egg stages do not comprise a significant portion of the total generation time. In these study, because *E. marinus* is a multivoltine specie, the production estimate (P) must be multiplied by 395/CPI (Benke 1979),

$$P = a\left[\sum_{j=1}^{a-1}\left(\bar{Y}_{.j.} + \bar{Y}_{.j+1.}\right)\left(W_j W_{j+1}\right)^{0.5}\right] 395/\mathrm{CPI} \qquad \text{(Eq. 2.6)}$$

where CPI (i.e., cohort production interval) is the mean development time in days from hatching to final size. We estimated a mean CPI of 360 (Mondego), 390 (Minho) and 360 (Ave) days, based upon interpretation of size-frequency histograms.

2.2. Population dynamics and life history traits

For the population dynamics and life history traits study several parameters were determined, including:

Abundance/density**:**

The density was calculated through the equation 2.1.

The size values were converted to biomass values by interpolating the length-dry weight equations described by Marques and Nogueira (1991) as in equation 2.4.

Population structure:

Size-frequency distributions along the time were analysed to interpret the population structure. The determination of each sex group was based on the presence or absence of oostegites and/or broods (females), and of genital papillae (males). Animals without these

features were considered to be juveniles. Maturity of non-brooding females was determined by the presence of setae on the oostegites.

Reproduction:

The percentage of ovigerous females over the total female population, through time was analyzed to determine the period of reproduction.

Fecundity:

The number of eggs by females of different size classes (because, a significant correlation was found between number of eggs within the brood pouches and size of females), were counted to estimate the fecundity (number of juveniles released), measured and examined to determine the development stage.

For this purpose we did not take into consideration embryos in stage E, that can freely get in and out of brood.

Sex-ratio variation

Sex-ratio (males/females) variation through time

A paper entitled "Does production and life history traits of the amphipod *Echinogammarus marinus* reflect the ecological status of estuaries and coastal systems?" is under preparation.

Task 4 – Ecological models

Based on data obtained in task 1 and results obtained in task 2, *E. marinus* populations from the 3 sampling sites within the 3 estuaries were divided into the following four groups: juveniles, immature (non-reproductive) females, adult, (reproductive) females and males. Growth, maturation (the rate of transfer from immature females to adult females), mortality and recruitment (the reproduction rate of adult females) regulate the density variation in each group throughout time. In addition to density estimation, the model also calculates the weight variation of each group throughout time, according to a temperature-dependent modified von Bertalanffy model; which has been considered suitable to describe *E. marinus* growth (see Marques, 1989; Marques and Nogueira, 1991). According to experimental evidence, the number of newly hatched juveniles is dependent on female's size, temperature and salinity

(Maranhão and Marques, 2003). The growth and reproduction rate of *E. marinus* populations are temperature- and salinity-dependent; these two environmental parameters act as the basic forcing functions of the model. The main equations of the model are shown in table 1.

The structure of the model is essentially the same for all the sampling sites because *E. marinus* population composition was found similar in all sites exhibiting structured populations. However, two sampling sites in Ave estuary exhibited disrupted populations with a sparse number of individuals over the sampling period; consequently, it was not possible to run the model for these two sites. Model initial values and forcing functions are site-dependent and assume real values obtained during data collection.

Currently, the model is being calibrated for the different sampling sites. Preliminary results indicate that *E. marinus* populations from Mondego estuary show long-term stability unless annual water temperature increases more than 2°C; Minho populations seem to be negatively affected by the significant salinity decrease observed in the system during autumn/winter but during spring and summer, *E. marinus* populations from the Embocadura field station (Minho) show high abundance of individuals particularly juveniles and very high productivity values. Thus, long-term simulations suggest that the productivity and stability of *E. marinus* in Minho may depend on the frequency of rainfall and on the amount of rain; extreme rainy seasons may affect population stability and resilience.

Only one field station from Ave estuary (Praia) exhibited a structured and stable population of *E. marinus*. Long-term simulations indicate that this population can be severely affected by sea level rise or intense rainfall.

Two papers are under preparation within this task: “Long-term variations of the productivity and life history traits of amphipod populations from different habitats and under different environmental stressors” and “How will different populations of the same species react to climate change?

Table 2 - Main equations of the model. *J* – Juveniles (Individuals m^{-2}); *IF* –Immature Females (Individuals m^{-2}); *AF* –Adult Females (Individuals m^{-2}) and *M* – Males (Individuals m^{-2}). *AvW* – average dry weight (mg), W_{i-1} – dry weight at the beginning of the population group *i* (mg), W_i – dry weight at the end of the population group *i* (mg), $days_{i-1,i}$ – average duration of a certain population group (days), *h* – anabolism parameter, *k* – catabolism parameter, *DW*- ash free dry weight (mg AFDW), *CL* – cephalic length (mm), *T* – temperature (ºC), T_{op} – optimum temperature for grow, T_{max} – maximum temperature at which growth ceases, *S* – salinity (psu) (for a better explanation of the biomass calculation module see Martins et al., 2002).

$\frac{\partial J}{\partial t} = recruitment - mort_J - transf1 - transf2$	Variation of juvenile density per unit of time
$\frac{\partial IF}{\partial t} = growth_{IF} - mort_{IF} - maturation$	Variation of young female density per unit of time
$\frac{\partial M}{\partial t} = growth_M - mort_M$	Variation of male density per unit of time
$\frac{\partial AF}{\partial t} = maturation - mort_{AF}$	Variation of adult female density per unit of time
$mort_{J,IF,AF,M} = mort_{J,IF,AF,M} \times Density_{J,IF,AF,M}$	Mortality rate of population groups as a function of density
$recruitment = NBJ \times f(S) \times f(T) \times 0.0291 \times AF$	Number of newly hatched juveniles (NBJ) from adult females per unit of time
$AvW = \frac{W_{i-1} + W_i}{2}$	Average weight of population groups
$W_i = W_{i-1} + f(T) \times days_{i-1,i} \times \left(h + W_{i-1}^{2/3} - k \times W_{i-1}\right)$	Weight of *E. marinus* at the end of population group i
$DW = 1{,}592924 \times CL^{3{,}94344}$	Regression between dry weight and cephalic length
$NBJ = 0$ for salinity ≤ 4 $NBJ = -70.3 + 62.8 \times CL_{AF}$ for temperature < 15 $NBJ = -50.4 + 44.8 \times CL_{AF}$ for temperature ≥ 15 and < 23 $NBJ = -11.7 + 12.2 \times CL_{AF}$ for temperature ≥ 23	Significant regression ($p<0.05$) between the number of newly hatched juveniles and females cephalic length and dependency of NBJ on salinity and temperature
$f(T) = \left(\frac{T}{T_{opt}}\right)^{2.5} \times e^{\left(1-\left(\frac{T}{T_{opt}}\right)^{2.5}\right)}$ for $T < T_{opt}$	Effect of temperature on recruitment

$f(T) = 1 - \left(\frac{T - T_{opt}}{T_{max} - T_{opt}}\right)^2$ for $T \geq T_{opt}$	
$f(S) = 0$ else $f(S) = 1$ for salinity $= 4$ or salinity < 4	Effect of salinity on recruitment

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