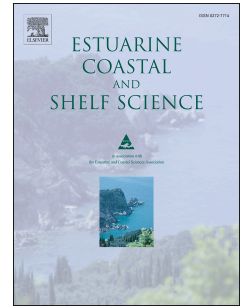# Accepted Manuscript

RNA:DNA ratios as a proxy of egg production rates of *Acartia*

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**RNA:DNA ratios as a proxy of Egg Production Rates of *Acartia***

Joana Cruz[1,4*], M. Alexandra Teodósio[1], Radhouane Ben-Hamadou[3], Luís Chícharo[5], Susana Garrido[2,4], Pedro Ré[4] and A. Miguel P. Santos[2]

[1] Centro de Ciências do Mar do Algarve, Universidade do Algarve. Campus de Gambelas, 8005-139 Faro, Portugal.

[2] Instituto Português do Mar e da Atmosfera - IPMA, Av. Brasília s/n, 1449-006 Lisboa, Portugal.

[3] Department of Biological and Environmental Sciences, College of Arts and Sciences, Qatar University, PO Box 2713, Doha, Qatar.

[4] Marine and Environmental Sciences Centre, Faculdade de Ciências, Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal

[5] Centre for Marine and Environmental Research (CIMA), University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

* Corresponding author: Tel: Tel.: (+351)289800800; e-mail: joanacruz23@hotmail.com

**Abstract**

Estimates of copepod secondary production are of great importance to infer the global organic matter fluxes in aquatic ecosystems and species-specific responses of zooplankton to hydrologic variability. However, there is still no routine method to determine copepods secondary production in order to eliminate time consuming experimental analyses. Therefore, we determined whether there is a correlation between Egg Production Rates (EPR) and RNA:DNA ratios of *Acartia*, by measuring their seasonal and spatial variability and the influence of environmental factors for *Acartia* sp. collected in the the Guadiana river estuary. EPR of *A. tonsa* was positively related with chlorophyll *a* concentration, freshwater inflow and biomass of dinoflagellates, while *A. clausi* was only related to dinoflagellates. Dinoflagellates seem to be the optimal food item influencing the reproduction of both *Acartia* species in the studied area. The biochemical index RNA:DNA was positively related to EPR, indicating that it is a good proxy of copepod production and a promising method to use in the future to estimate secondary production.

*Keywords*: Guadiana river estuary, zooplankton, *Acartia clausi*, *Acartia tonsa*, hatching success, secondary production

**1. Introduction**

Planktonic copepods are usually considered relevant herbivores (Nybakken, 2001) playing a key role in the biogeochemical cycles of carbon and other elements in estuarine and nearshore

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ecosystems (Hernández-León and Ikeda, 2005; Buitenhuis et al., 2006). Copepods and their developing progeny form the main food supply for planktivorous predators, such as pelagic fish and medusa (Purcell, 1997; Garrido and van der Lingen, 2014). Estimates of copepod secondary production are of great importance to infer the global organic matter fluxes in aquatic ecosystems, and species-specific responses of zooplankton to hydrologic variability. Furthermore, these estimates integrate recent feeding history and physiological adaptations to environmental variability (Hay, 1995, Calliari et al., 2006; Peck et al., 2014), which is particularly useful in dynamic estuarine systems suffering important anthropogenic impacts.

The Guadiana river basin has been highly modified over the last decades with numerous dams constructed that have significantly reduced the river freshwater flow to the estuary. In February 2002, the Alqueva dam, the largest and most recent dam built in the Guadiana basin (Chícharo et al., 2006a), was completed. The present study is the first to analyze the secondary production of this estuary after the Alqueva dam construction event. As in most temperate estuaries, *Acartia* is the most represented genus in the Guadiana estuary (Chícharo et al., 2006b). It is considered one of the most abundant mesozooplanktonic genus, as well as the most widespread (Day et al., 1989); its distribution ranges from nearly fresh to hypersaline waters, from 0 to 40 °C temperature, clear to turbid, shallow to deep, and polar to tropical estuarine and coastal ecosystems (Sautour and Castel, 1995). Typically, the most common species occurring in temperate estuaries and adjacent coastal areas are *Acartia tonsa* and *Acartia clausi*, and due to their characteristics and life history adaptations *A. tonsa* is more commonly found inside the estuaries while *A. clausi* is more abundant outside (Chinnery and Williams, 2004; Azeiteiro et al., 2005; Calliari et al., 2006). The adults of these species do not build up large energy reserves and invest most of the energy into reproduction (Kiørboe et al., 1985), thus reflecting the environmental conditions present *in situ*. Few studies have examined the effects of freshwater flow and salinity on the EPR of calanoid copepods, which is surprising given the high abundance of these species within estuarine areas (Paffenhöfer and Stearns, 1988; Calliari et al, 2006; Peck et al., 2014).

Although EPR is still one of the most commonly used methods for inferring copepods production *in situ*, in recent decades there has been an effort in research to develop biochemical methods to estimate growth and production, with the aim to eliminate time-consuming experimental analyses and possible artifacts. The RNA:DNA ratio analysis is based on the fact that DNA content per somatic cell is assumed to be constant in mature adults, therefore this index translates the protein synthetic capacity, once that RNA is needed for the protein synthesis reflecting the growth condition (Bulow, 1987). This technique measures the total amount of nucleic acids of the entire organism or it can be applied to specific tissues of the organism (Olivar et al., 2009). Nucleic acid

derived indices of growth and condition have been used in recent decades in several marine organisms such as fish, bivalves, cephalopods and crustaceans (e.g. Gorokhova and Kyle, 2002; Sykes et al., 2004; Chícharo and Chícharo, 2008; Amaral et al., 2009). More specifically, it has been developed to assess copepod physiological conditions (Chícharo and Chícharo, 2008), as an indicator of nutritional condition (Wagner et al., 1998; Vehmaa et al., 2012), growth (Elser et al., 2000; Wagner et al., 2001), dormant condition (Kobari et al., 2013) and egg viability (Hogfors et al., 2011). Previous studies on individual copepods such as *Paracalanus* sp. (Nakata et al., 1994), *Acartia grani* (Saiz et al. 1998), *Acartia bifilosa* (Gorokhova, 2003) and *Calanus sinicus* (Ning et al., 2013) have successfully established the relationships between RNA:DNA ratios and protein synthetic activities determined as egg production rates, that is proved to be influenced by temperature and food quantity and quality (Saiz et al., 1998; Gusmão et al., 2009). Nonetheless, until now this correlation has been proved for few species and more evidence must be achieved considering the high diversity of Copepoda (Mauchline, 1998). Furthermore, most of the studies have been made under laboratory conditions, lacking field studies proving the relationship between the RNA:DNA ratio and egg production rates throughout the seasons.

Considering these features, this study aims to: i) determine the seasonal and spatial variability of *Acartia tonsa* and *Acartia clausi* egg production rates and hatching success in the Guadiana estuary relating to the environmental factors potentially influencing reproduction, and consequently estimate seasonal secondary production of females and recruitment; ii) measure RNA:DNA ratios in order to determine if they can be considered a good proxy for secondary production.

## 2. Materials and methods

### 2.1. Study area and sampling

This study took place in the lower part of Guadiana estuary and in the adjacent coastal area, located in the south-western Iberian Peninsula, Portugal (Fig. 1). This estuary has the fourth largest catchment basin of the Iberian Peninsula (~67,500 km$^2$) and extends along approximately 70 km, with the lower 50 km constituting the southern border of Portugal and Spain (Iberian Peninsula, Europe). It is considered a mesotidal estuarine system, with tidal amplitudes that range from 1.3 to 3.5 m and an average depth of 6.5 m. It is influenced by a temperate Mediterranean climate, exhibiting moderate humid winters and hot dry summers. The average annual rainfall fluctuates between 561 and 600 mm in the Portuguese basin, with considerable variation between years. Before the Alqueva dam construction, freshwater inputs to the estuarine zone used to vary sharply between dry and humid months (1995–2000: 333 ± 1,096 m$^3$s$^{-1}$: retrieved from http://www.snirh.pt), while since 2002 a more regular freshwater flow throughout the year has been

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occurring. The total volume of water retained in the river not reaching the estuary is estimated to be 13,000 hm$^3$ year$^{-1}$ (Dias et al., 2004). Guadiana estuary has become dominated by freshwater during winter and flood periods, while in summer and spring season it is a salt-wedge estuary (Rocha et al., 2002). It is also influenced by weak coastal upwelling events (Fiúza, 1983). Numerous fish species, use the estuary, as an important nursery area, such as the pelagic species with high economic importance in the area, the anchovy *Engraulis encrausicolus* and the sardine *Sardina pilchardus,* as well as Sparidae (Faria et al., 2006).

From December 2008 until June 2010, two stations located in the lower part of the Guadiana estuary (~ 4 m depth, 37º13'05.11" N - 7º24'46.60" W) and in the adjacent coastal area (~ 10 m depth, 37º07'37.42" N - 7º24'39.30" W) (Fig. 1) were sampled monthly during flood tide (fortnightly during December 2008, January 2009 and March to May 2009 and no sampling during August, December 2009 and February, March 2010).
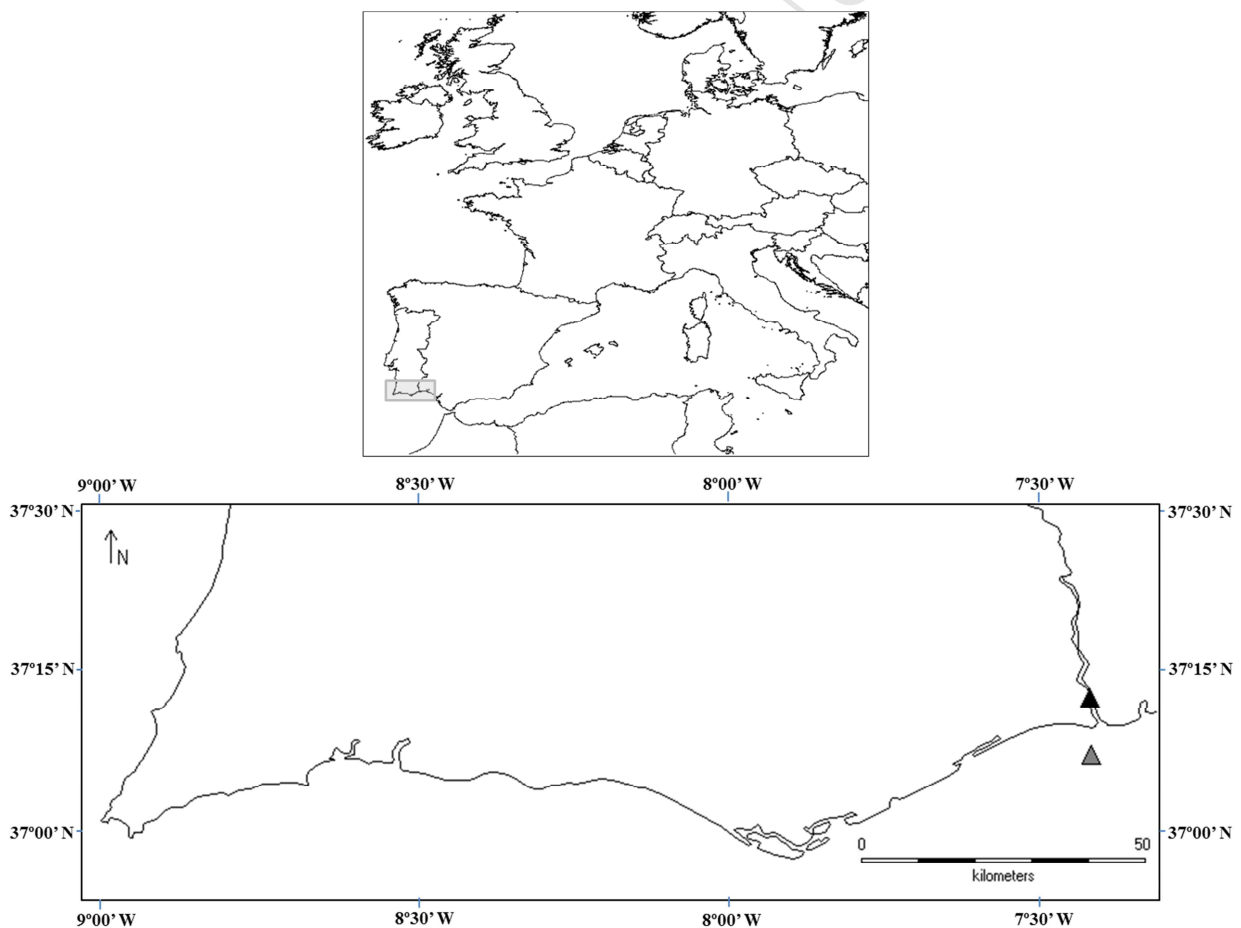


Figure 1 – Map of the studied area with the two sampling stations, black triangle corresponds to inside Guadiana river estuary and grey triangle to adjacent coastal area.

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Sampling was always carried out during daylight hours (9 – 15h). Zooplankton was sampled with a conical net (0.13 m$^2$ mouth opening and 200 µm mesh size) hauled horizontally just below the sea surface for 5 min at approximately 2 knots, equipped with a HydroBios flow meter. Two vertical hauls were taken at each station: one catch was fixed in 4 % formalin buffered for determination of abundance of the target species (samples sorted for the selected species identified with reference under a stereomicroscope), and on the other catch the cod end content was gently transferred to clean insulated containers and diluted with surface seawater for transport back to the laboratory within one hour. Temperature and salinity were measured with a hand-held meter (YSI 85). In situ chlorophyll *a* was determined using a fluorometer (10 AU Turner). Water samples were also collected to determine major microplankton groups in the laboratory. To assess microplankton composition and abundance, samples were preserved with acid Lugol's solution and subsamples of 50 ml were concentrated by gravimetric sedimentation by the Utermöhl technique (Hasle, 1978). Samples were identified to the highest taxonomic separation using a Zeiss IM35 inverted microscope with phase contrast and bright field illumination. The carbon content of the main microplankton groups were calculated based on the equations given in Smayda (1978), using the cells measurements performed by Garrido et al. (2008). The freshwater inflow data measured at the Pulo do Lobo hydrometric station were obtained from the Sistema Nacional Informação Recursos Hídricos (http://snirh.pt/)

## 2.2. Egg production and hatching success experiments

Egg production rates and hatching success were determined for the free-spawning copepods *Acartia clausi* and *Acartia tonsa*. Water from each station was passed through a 50 µm mesh to remove any copepod eggs and metazoan zooplankton prior to the experiments. *Acartia* females were gently sorted from the samples using a glass pipette, and 5 to 8 undamaged and actively swimming individuals were placed in 500 ml bottles. Three replicate bottles per station were incubated at the same water temperature as that of the sampling station. The incubation time was 24-26 hours. After this period, all females were removed from the bottle by sieving the water through a 200 µm mesh and immediately placed in liquid nitrogen for posterior RNA:DNA ratio analysis. All the eggs produced by the females during the experimental period were incubated for an extra 48 hours at the same temperature in order to estimate the hatching success. At that period, all nauplii were counted.

## 2.3. Biomass and secondary production determination

Carbon-specific egg production rates (SEP) for the copepod species were calculated according to the equation:

$$SEP = EP \times \frac{We}{Wf}$$

where, *EP* is the number of eggs female$^{-1}$ day$^{-1}$, *We* is the egg carbon content and *Wf* is the female carbon biomass. Egg carbon content was assumed to be 0.045 and 0.04 µg C egg$^{-1}$, for *Acartia tonsa* and *Acartia clausi*, respectively (Kiørboe and Sabatini, 1995). Female carbon weights were estimated from prosome lengths using the equations, for *Acartia clausi*:

$$\log(Wf) = 3.005 \times \log(PL)^{-8.444} \text{ (Ayukai, 1987)}$$

and for *Acartia tonsa*:

$$\log(Wf) = 2.476 \times \log(PL)^{-0.698} \text{ (Thompson et al., 1994).}$$

The total biomass was calculated multiplying the average female carbon weight by the abundance of females. Copepod female production was then calculated by multiplying the female biomass with *SEP*, and recruitment was estimated by multiplying the production with hatching success as described in Poulet et al. (1995).

### 2.4. RNA:DNA ratio analysis

Nucleic acids were analyzed for adult females of *Acartia clausi* and *Acartia tonsa* collected from March 2009 to June 2010 for both sampling sites. Nucleic acids were obtained using a method based in the microplate fluorescent assay (MFA) by Ikeda et al. (2007), which is a modification of the sequential fluorometric method of Bentle et al. (1981). This method is based on the use of an ethidium bromide fluorometric technique, where the nucleic acids are sequentially degraded by nucleases (RNase and DNase). Wagner et al. (1998) modified the sequential fluorometric method to the MFA with 96-well microtiter plates by adopting a sarcosyl extraction technique, eliminating the DNase step, and allowing the measurement of nucleic acids of several samples at the same time.

Prior to the assay, the wet weight (WW) of a batch of 5 to 30 specimens was measured to obtain more than 0.5 mg WW. Copepods were homogenized by sonication (3 pulses 50 A during 1 min) with a volume of 100 µl (0.5%) cold sarcosyl extraction buffer. Then all the samples were shaken for 30 minutes at room temperature using a vortex mixer equipped with a multiple-vial head. Afterwards, the samples were centrifuged (12000 r.p.m, at 0-4ºC) for 15 min to sediment any copepod remain particles. The samples were diluted 1:10 with Tris buffer to reduce the sarcosyl concentration to 0.05%. In each run, duplicate 50 µl aliquots of supernatants of the samples and duplicates of 0, 0.6, 1.1, 1.7 and 2.3 µg ml$^{-1}$ DNA standard solutions (λ-phagus 0.25 mg ml$^{-1}$ from Roche), and 0, 3.6, 7.3, 10.9 and 14.6 µg ml$^{-1}$ RNA standard solutions (16s-23s *E. coli* 4 µg µl$^{-1}$ from Roche) were transferred to Nunclon 96-well, black, round-bottom microplates. The

concentrations of DNA and RNA standard were chosen because the fluorescence has been shown to be linear within these ranges, and values of the samples fit within these values. The mean ratio of the slopes of the standard curves (slope of DNA standard curve/slope of RNA standard curve) was $2.8 \pm 0.05$, which can be used to compare RNA:DNA ratio results determined by other protocols (Caldarone et al., 2006). Gel red solution (30 μl) was added to each well, and the plates were shaken gently at room temperature. The fluorescence was then scanned after addition of the fluorescent dye on a microplate reader (Biotek synergy HT model SIAFRTD) with 360 nm (excitation) and 590 nm (emission) (first scan-total fluorescence RNA and DNA). Following the first scan, RNase solution (15 μl, 0.12 µg ml-1) was added to each well and incubated at 37 ºC for 30 minutes. The concentration of DNA was calculated directly using the standard curve. The concentration of RNA was determined indirectly by subtracting the DNA fluorescence (second scan) from the total fluorescence (first scan).

## 2.5. Data analysis

The Wilcoxon test was used to verify if there were significant differences between both sampling sites of the main environmental variables (temperature, salinity and chlorophyll *a*). Generalized linear models (GLM; Venables and Ripley, 2002) were used to analyze the spatial and temporal variability of egg production rates, hatching success and RNA:DNA ratio of the *Acartia* species under study. For the egg production rates, a negative binomial GLM with a logit link was used, while for the hatching success a quasibinomial GLM with a logit link was chosen. The following independent variables were included: water temperature, chlorophyll *a*, freshwater inflow, diatom-, dinoflagellate-, ciliate biomass. Salinity was not used in the model due to its obvious negative correlation with the freshwater inflow. In order to analyze the RNA:DNA ratio variability and possible relation to egg production rates, a Gaussian GLM was used with an identity link. As there were no significant differences in the seasonal variability of RNA:DNA between both species, further analyses were made including all the data in the same model (370 individuals in 20 sampling dates). The independent variables used were: temperature, chlorophyll *a*, diatoms, dinoflagellates, ciliates and egg production rate. All the GLM were chosen accordingly the distribution of the analyzed variables. Model predictors were selected using the Akaike Information Criterion (AIC; Sakamoto et al., 1986). Predictors were removed by backward elimination based on AIC which balances the degree of fit of a model with the number of variables, in order to find the most parsimonious model. Only those predictors which contributed significantly to the model were kept. The predictors freshwater inflow, diatoms, dinoflagellates, ciliates and RNA:DNA ratios were log-transformed, in order to normalize the data. Statistical analysis was performed using the open source software R 2.15.3 (R Development Core Team 2013).

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# 3. Results

## 3.1. Hydrography and food environment

Water temperature exhibited a typical seasonal cycle in both stations, with maximum values obtained in June and minimum values in December, varying from 12.1 to 25.1 °C. Generally, lower temperatures during colder months and higher temperatures during warmer months were found inside the estuary when compared to the coastal area (Fig. 2), although there were no significant differences between both stations (Wilcoxon test $Z=103.5$, $p > 0.05$). Salinity was lower inside the estuary, reaching minimum values during February 2009, January and June 2010, while in the coastal area salinity was more constant throughout the time, with minimum values in January and June 2010, showing significantly differences between both stations (Wilcoxon test $Z=209$, $p < 0.05$). The salinity ranged inside the estuary from 2 to 33.5 psu and in the coastal area from 25.1 to 38 psu (Fig. 2). Although chlorophyll $a$ presented similar values in both stations, the Wilcoxon test revealed that they were significantly different ($Z = 41$, $p < 0.05$). Inside the estuary the maximum values were recorded in May and June 2010, and ranged from 0.24 to 15.42 µg $L^{-1}$, while in the coastal area maximum values were registered in April of 2009 and 2010, ranging from 0.22 to 9.36 µg $L^{-1}$ (Fig. 2). The freshwater inflow varied strongly between years, with maximum values in March 2010 (Fig. 3), as a consequence of the discharge of Alqueva dam after the occurrence of high precipitation during winter (December 2009 to February 2010).

Food availability for the copepods varied significantly during the studied period (Figure 4). At the station located inside the estuary, diatoms showed a higher relative abundance during spring months (April 2009 and May 2010, carbon content: ~ $99.8 \pm 81.8$ µg C $L^{-1}$) and summer (July 2009, carbon content: 118.1 µg C $L^{-1}$), while dinoflagellate concentration was higher in May and June 2009 and April 2010 (carbon content: ~ $33.7 \pm 10.9$ µg C $L^{-1}$). Ciliate concentration was lower than the other two main groups, with maximum values in March, October and November 2009 (carbon content: ~ $9.9 \pm 2.3$ µg C $L^{-1}$). At the coastal area, diatoms also presented high percentages during spring 2009 (April and May, carbon content: ~ $132.7 \pm 30.2$ µg C $L^{-1}$) and 2010 (May and June, carbon content: ~ $104 \pm 73.7$ µg C $L^{-1}$). Dinoflagellates were less abundant in this area when compared to the station inside the estuary, with maxima in July and September 2009 (carbon content: ~ $11.1 \pm 0.9$ µg C $L^{-1}$). Contrary, ciliates were more abundant in the coastal area, peaking in March and May 2009 and April 2010 (carbon content: ~ $13.1 \pm 6.1$ µg C $L^{-1}$).
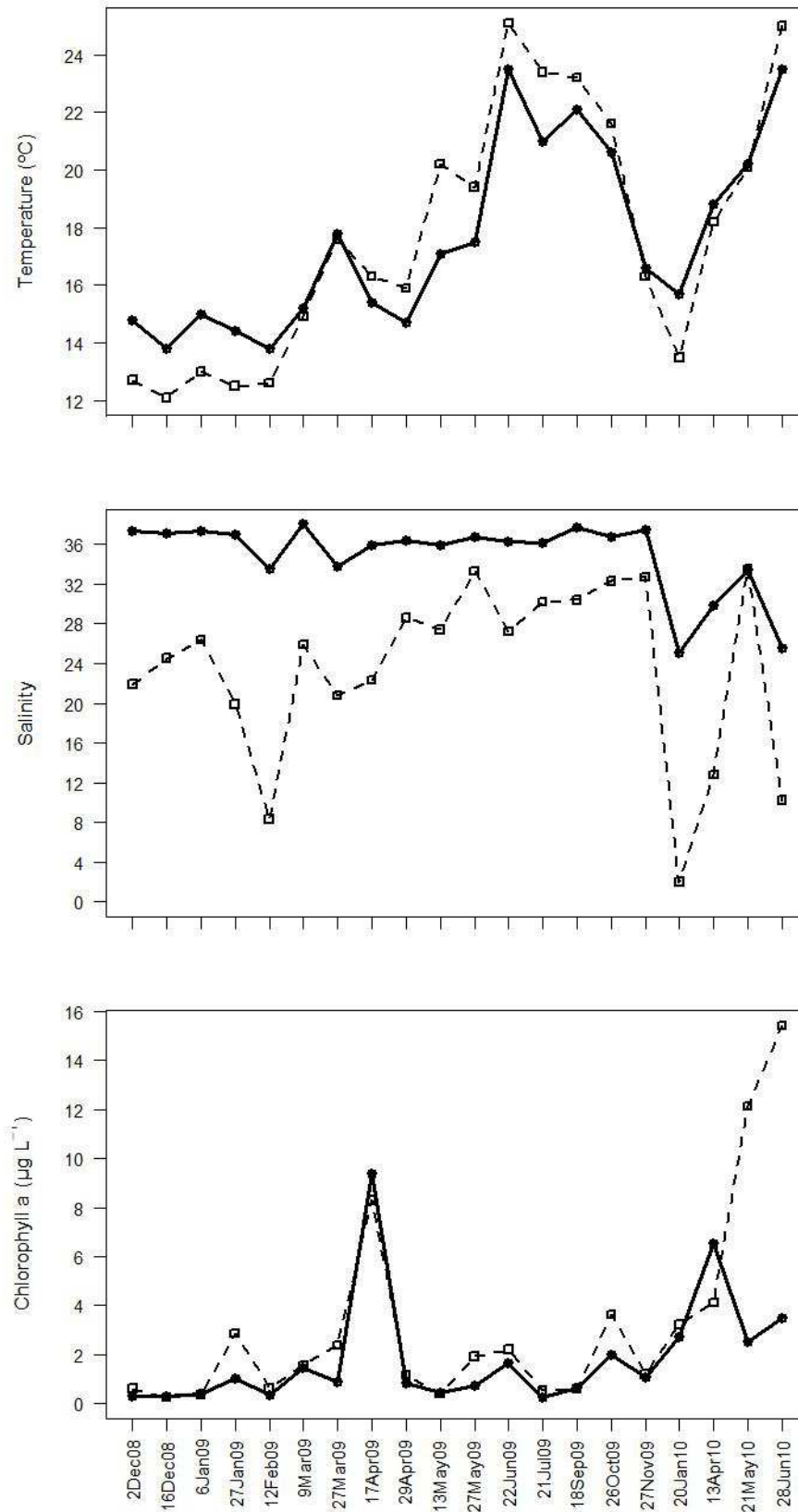
Figure 2 - Seasonal variations in water temperature, salinity and chlorophyll *a* concentration in the two sampling stations; dashed line corresponds to inside Guadiana river estuary and solid line to adjacent coastal area.
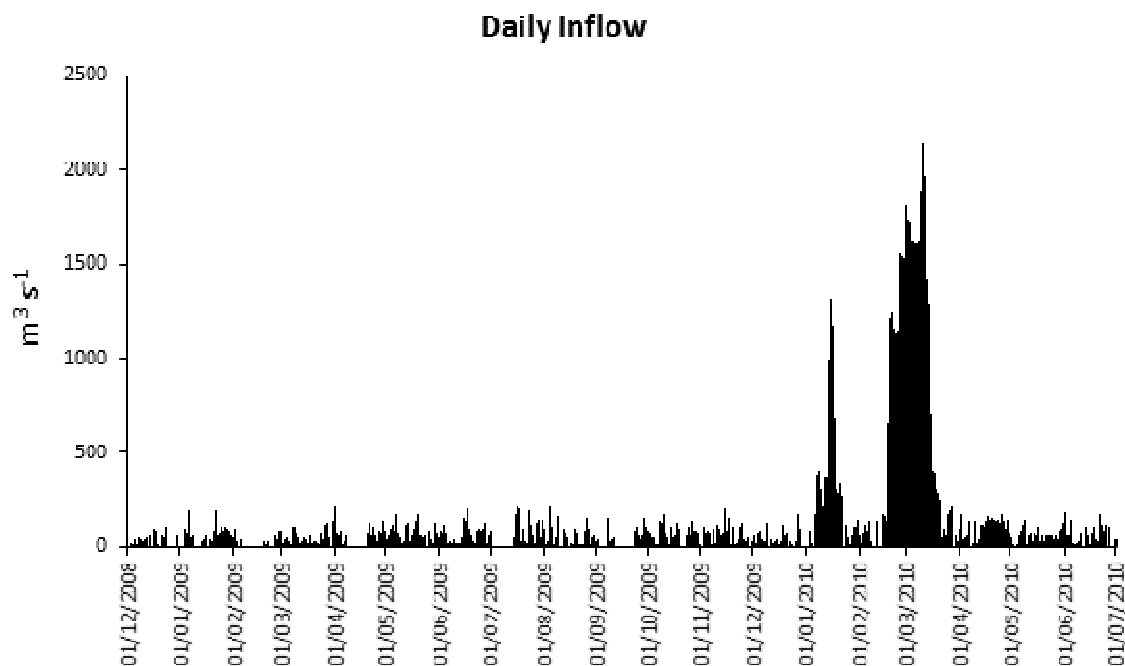
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**Daily Inflow**



Figure 3 – Daily freshwater discharge during the sampling period. Data was measured at the Pulo do Lobo hydrometric station and obtained from the Sistema Nacional Informação Recursos Hídricos (http://snirh.pt/)

## 3.2. Species abundance, EPR, HS and RNA:DNA ratio

Both species of *Acartia* occurred inside and outside the Guadiana estuary, at least some times during the sampling period, but *Acartia tonsa* was significantly more frequent and abundant inside the estuary while *Acartia clausi* was significantly more frequent and abundant in the coastal area (Fig. 5). *A. tonsa* total abundance, inside the estuary, was higher during February and March 2009, reaching 15918 ind. m$^{-3}$, while *A. clausi*, predominant in the coastal area, was usually less abundant than its congener, peaking in March 2009 with 8901 ind. m$^{-3}$ (Fig. 6). Generally, the abundance of the females of both species showed a similar pattern to total abundance, with maximum values in March 2009, with 1200 and 1004 ind. m$^{-3}$ for *A. tonsa* and *A. clausi*, respectively (Fig. 6). The EPR of both species peaked during spring months and was minimum during the winter. EPR of *A. tonsa* ranged from 0 to 26.5 ± 3.7 eggs female$^{-1}$ day$^{-1}$ in December 2008 and May 2010, respectively, and *A. clausi* from 0 to 25.1 ± 3.3 eggs female$^{-1}$ day$^{-1}$ for the same months. Hatching success varied between 66.7 - 93.3 % and 59.1 – 94.6 % for *A. tonsa* and *A. clausi*, respectively. HS was higher during warmer months for both species (Fig. 7).
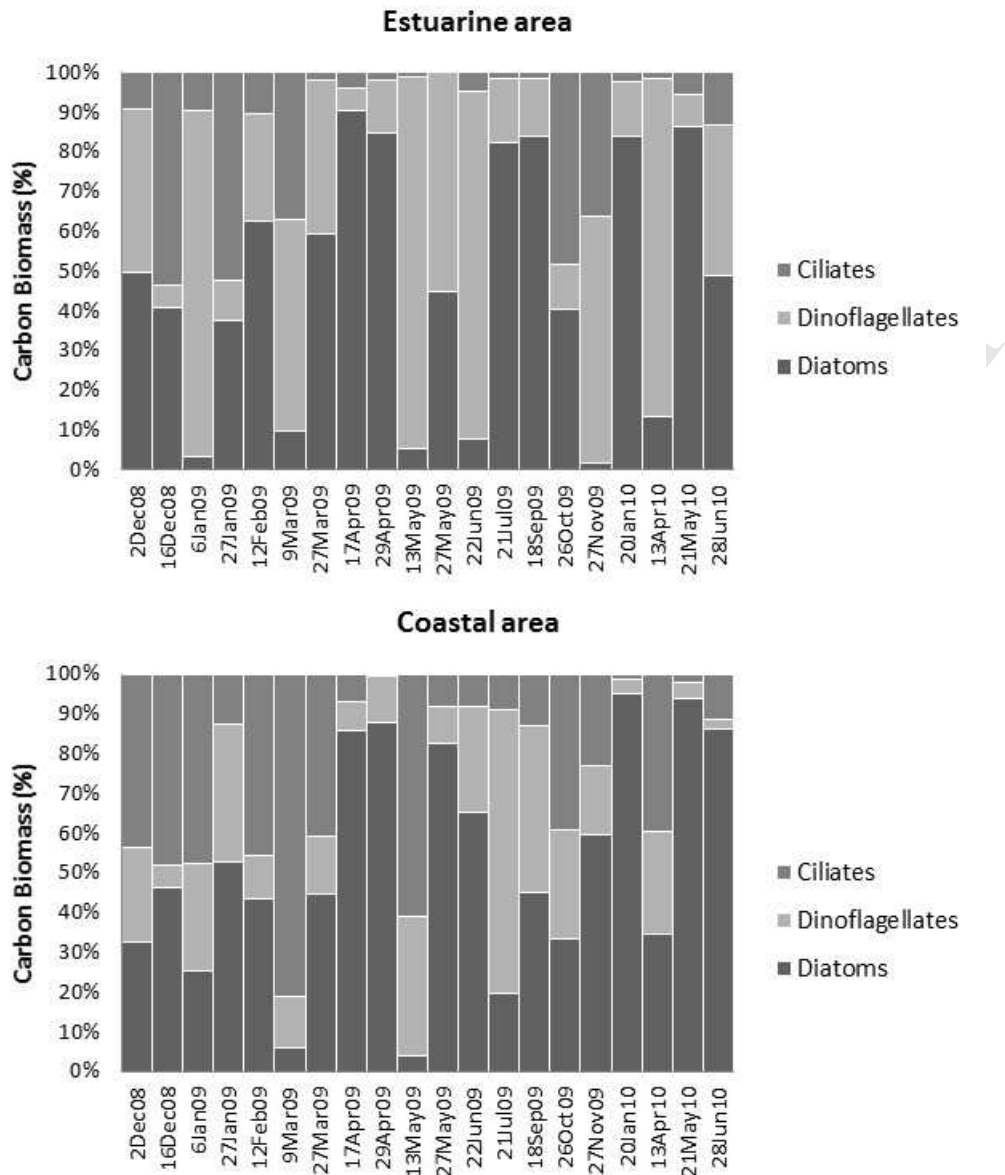
**Estuarine area**



**Coastal area**



Figure 4 - Carbon biomass of the evaluated microplankton groups at both stations. Data are represented as %.

The RNA:DNA ratio varied between 0.26-2.38 and 0.49-2.44 for *A. tonsa* and *A. clausi* females, respectively (Fig. 8). The highest value of the RNA:DNA ratio for both species occurred during May 2009.
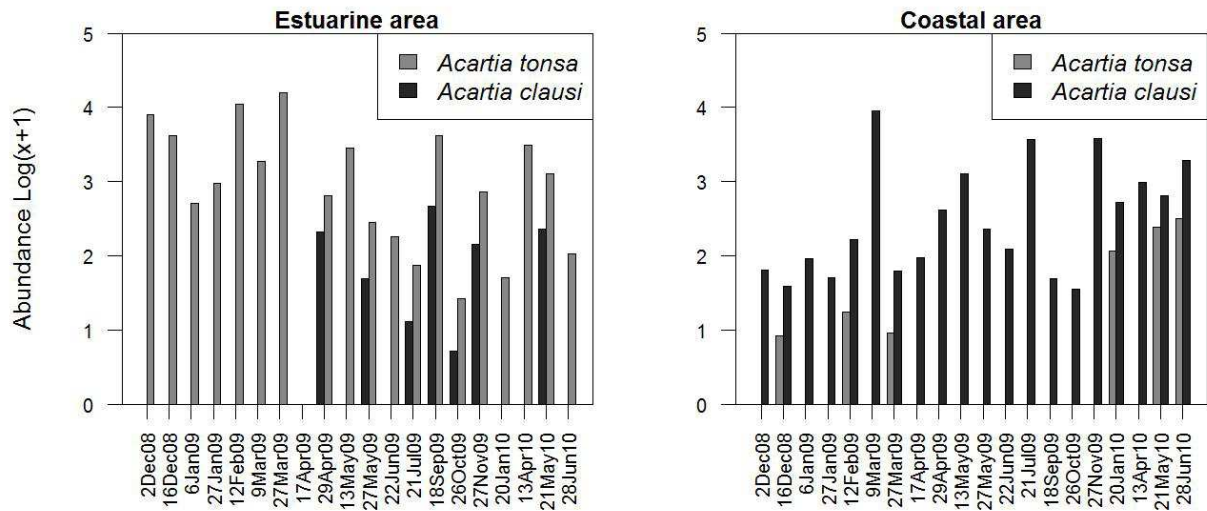
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Figure 5 – *Acartia tonsa* and *Acartia clausi* total abundance in the estuarine and coastal area stations; *A. tonsa* in grey bars and *A. clausi* in black bars; values are log transformed.
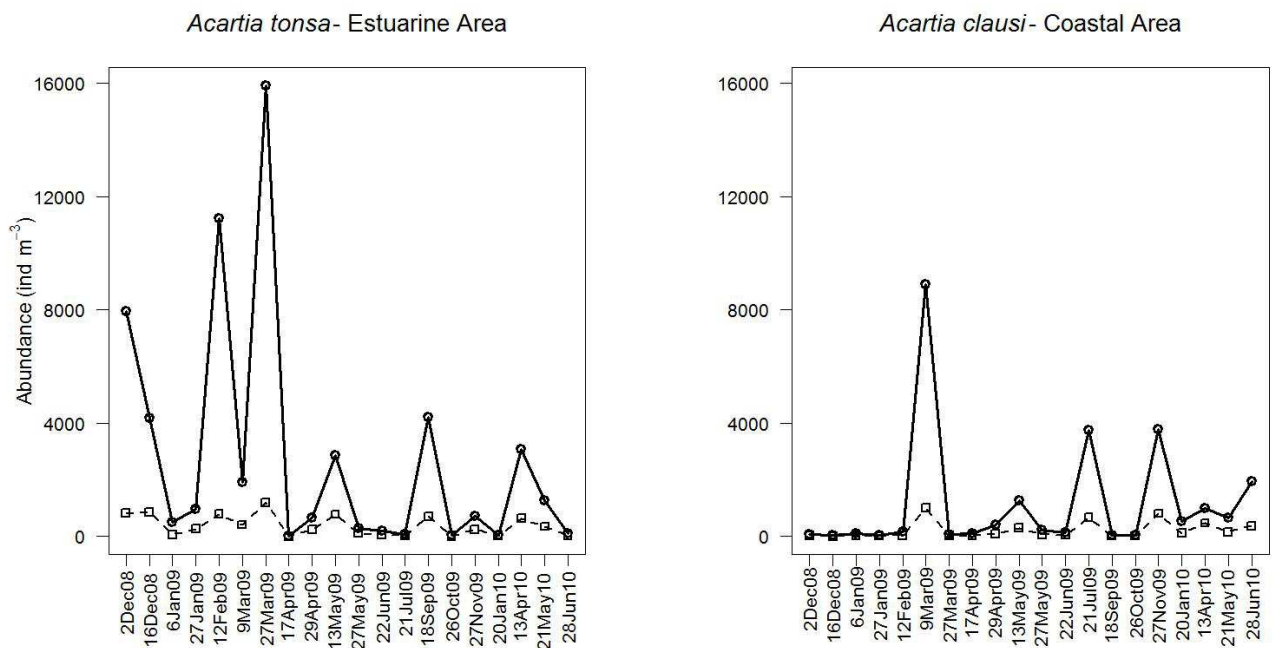


Figure 6 – *Acartia tonsa* and *Acartia clausi* total and females abundances in estuarine and coastal area stations; solid line is total abundance and dashed line is female abundance.
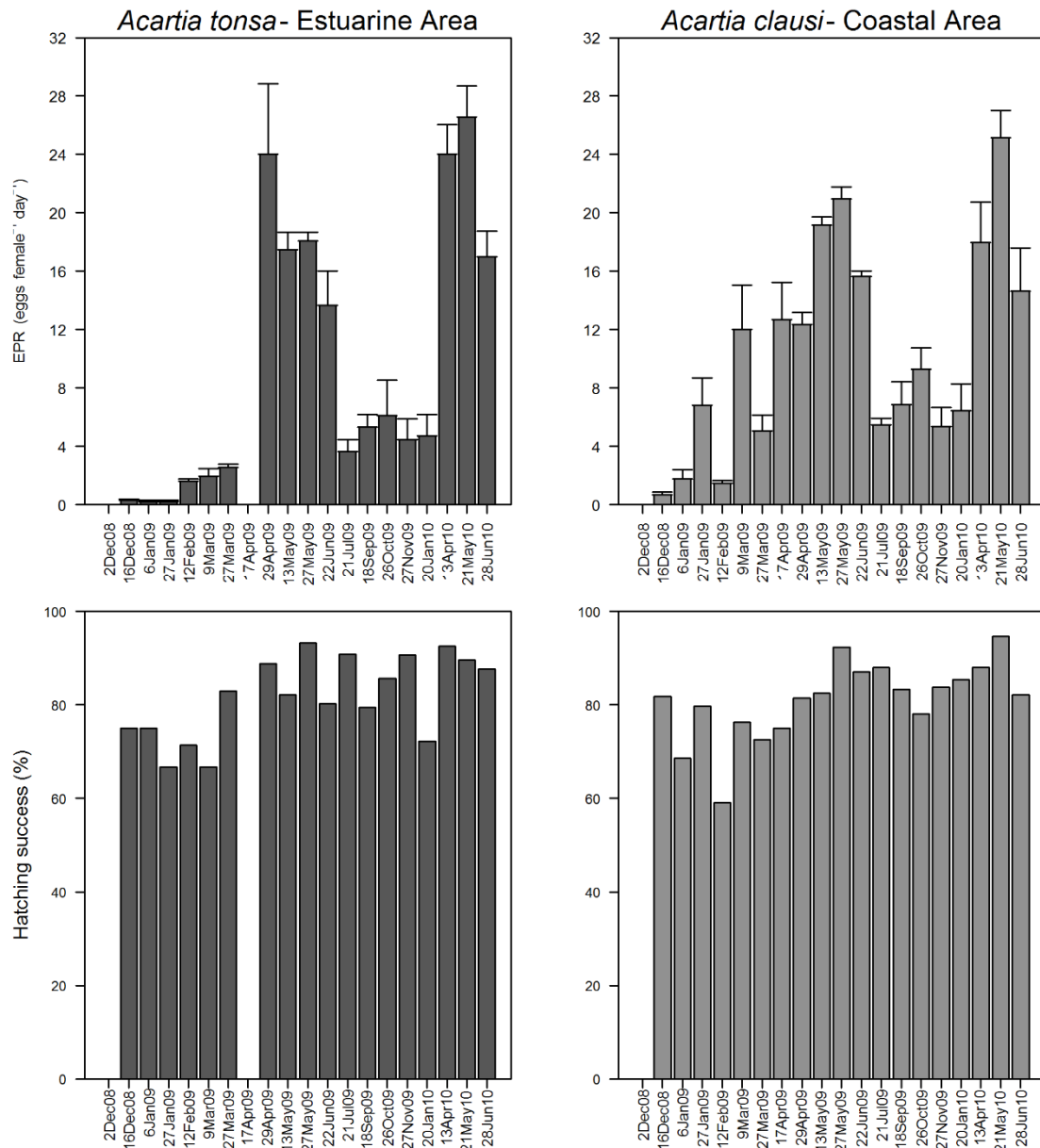
Figure 7 – Reproductive traits of *Acartia tonsa* and *Acartia clausi*; egg production rates (EPR) as eggs female$^{-1}$ day$^{-1}$ and hatching success (%). Error bars indicate ± standard deviation.

The analysis of the egg production rates of *Acartia* species in relation to the environmental variables showed that total EPR of *A. tonsa* was significantly and positively related to freshwater inflow, chlorophyll *a* and dinoflagellates abundance (Table I) while the EPR of *A. clausi* was not related to freshwater inflow (sampled mostly outside the river plume) being only significantly and positively related to dinoflagellates. Hatching success of both species was not explained by any variable used in the analysis ($p > 0.05$ for all the variables used). In relation to the RNA:DNA ratio, this index was significantly and positively related to EPR (Table I).
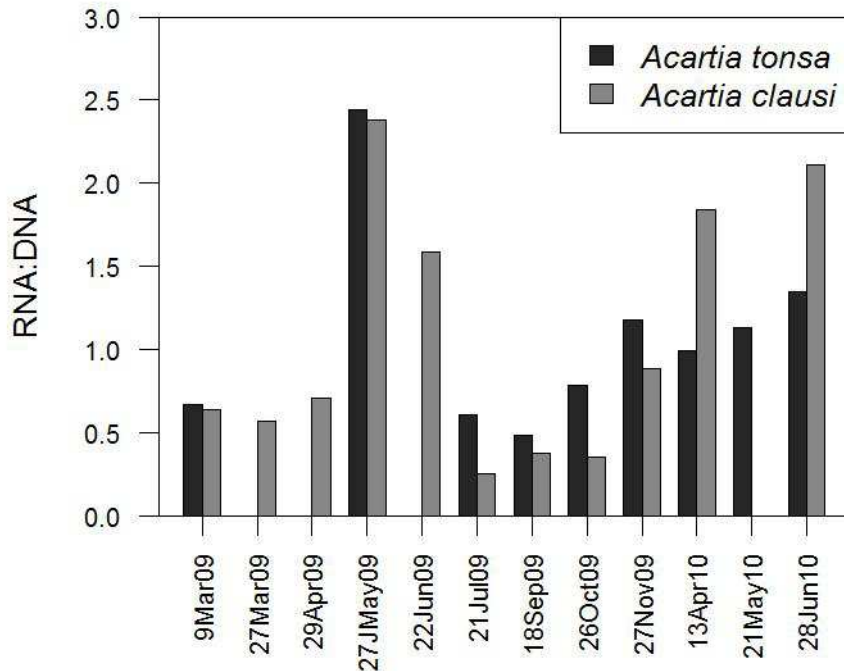
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Figure 8 – RNA:DNA ratios of *Acartia tonsa* and *Acartia clausi* females.

| Coefficients | Independent variables | *Acartia tonsa* EPR | *Acartia clausi* EPR | *Acartia* RNA:DNA |
|---|---|---|---|---|
| Negative Binomial | Temperature | 0.0694 | n.i. | |
| (logit) | Inflow | 0.602* | 0.4067 | |
| | Chlorophyll *a* | 0.086* | n.i. | |
| | Diatoms | n.i. | 0.1727 | |
| | Dinoflagellates | 0.8547*** | 0.4219*** | |
| | Ciliates | n.i. | n.i. | |
| | | | | |
| Gaussian | Temperature | | | n.i. |
| (indentity) | Diatoms | | | n.i. |
| | Dinoflagellates | | | n.i. |
| | Ciliates | | | n.i. |
| | EPR | | | 0.0553 ** |
| | | | | |
| | AIC | 110.4 | 127.8 | 35.36 |
| | LogLik | -48.8 | -58.4 | -13.5 |
| | DF | 8 | 8 | 8 |

Table I - Coefficients and significance (p-value) of each of the explanatory variables of the two GLMs (Negative Binomial and Gaussian) describing the seasonal variation of the egg production rates of *Acartia tonsa* and *Acartia clausi* and the RNA:DNA ratios of *Acartia*. Levels of significance are represented as ***p< 0.0001, **p< 0.001, *p< 0.01 and n.i. represents variables not included in the final model after backward stepwise regression. AIC is the Akaike Information Criterion, LogLik is the log-likelihood of the fitted model, DF are the degrees of freedom.

## 3.3. Females secondary production and recruitment

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Secondary production of the females of both *Acartia* species was higher during spring (*A. tonsa*: 364 ± 227.1 and *A. clausi*: 119 ± 116.4 µg C m$^{-3}$ day$^{-1}$) and lowest during autumn months (*A. tonsa*: 15.6 ± 20.0 and *A. clausi*: 44 ± 73.9 µg C m$^{-3}$ day$^{-1}$) (Table II). Similarly, the egg production rates presented its maxima and minima at the same seasons. Recruitment reached maximum values also during spring (*A. tonsa*: 320.7 ± 202.2 and *A. clausi*: 104 ± 101.9 µg C m$^{-3}$ day$^{-1}$), and was at its lowest during autumn and winter for *A. tonsa* (~13.7 ± 16.9 µg C m$^{-3}$ day$^{-1}$) and during autumn for *A. clausi* (37.1 ± 61.9 µg C m$^{-3}$ day$^{-1}$) (Table II). Hatching success was very similar between seasons for both species, reaching the lowest values during winter (Table II).

| Species | Season | No. Females (females m$^{-3}$) | SD | EPR (eggs fem.$^{-1}$ d$^{-1}$) | SD | HS (%) | SD | SP (µC m$^{-3}$ d$^{-1}$) | SD | R (µC m$^{-3}$ d$^{-1}$) | SD |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Acartia tonsa* | winter | 306.6 | 277.9 | 1.7 | 1.6 | 70.4 | 3.3 | 19.6 | 22.3 | 13.7 | 15.7 |
| | spring | 549.3 | 369.9 | 18.8 | 8.0 | 88.2 | 4.3 | 364.0 | 227.1 | 320.7 | 202.2 |
| | summer | 203.0 | 293.6 | 9.9 | 5.6 | 84.6 | 4.8 | 57.2 | 65.8 | 46.1 | 52.0 |
| | autumn | 481.8 | 365.8 | 2.7 | 2.6 | 83.8 | 6.5 | 15.6 | 20.0 | 13.7 | 18.3 |
| *Acartia clausi* | winter | 239.6 | 384.1 | 5.7 | 3.9 | 73.8 | 9.2 | 103.6 | 189.7 | 79.5 | 144.6 |
| | spring | 160.1 | 154.0 | 16.2 | 6.2 | 83.8 | 7.7 | 119.2 | 116.4 | 104.1 | 101.9 |
| | summer | 274.2 | 264.6 | 10.6 | 4.5 | 85.1 | 2.4 | 98.8 | 84.3 | 83.7 | 70.2 |
| | autumn | 214.0 | 341.7 | 3.8 | 3.8 | 81.2 | 2.3 | 44.4 | 73.9 | 37.1 | 61.9 |

Table II – Seasonal reproductive traits and estimations of females secondary production and recruitment of *Acartia tonsa* and *Acartia clausi*. No. Females is the abundance of females, EPR is egg production rate, HS is hatching success, SP is the secondary production of females, R is recruitment. SD is standard deviation.

## 4. Discussion

### 4.1. RNA:DNA ratio as a proxy of EPR

The RNA:DNA ratio was significantly related to the egg production rate of *Acartia* species during the sampling period. This result agrees with previous studies of different copepod species such as *Acartia grani* (Saiz et al., 1998), *Acartia bifilosa* (Gorokhova, 2003) and *Calanus sinicus* (Ning et al., 2013). Consequently, there is more evidence that this index can be a good indicator of reproductive growth rate of copepods, resulting in the use of a less laborious method. Temperature, chlorophyll *a* and food availability did not influence the RNA:DNA index. Although the biosynthesis of proteins is influenced by temperature as any other chemical reaction, suggesting a temperature dependency of any index containing RNA, there is some discrepancy of previous results regarding temperature and RNA:DNA ratio relationship. Saiz et al. (1998) found a linear increase between EPR and RNA:DNA ratio for *A. grani* that was temperature dependent, although

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only two temperatures were used. Accordingly, for the copepod *Calanus finmarchicus* there was a reduction of the RNA:DNA ratio with temperature increase (Wagner et al., 2001). On the other hand, Ning et al. (2013) did not find any correlation between temperature and RNA:DNA ratios when studying the copepod *Calanus sinicus*, and Gorokhova (2003) verified that RNA indices of *A. bifilosa* were not significantly affected by temperature. All the results showing a correlation were conducted under laboratory experiments, except for the work of Ning et al. (2013) that was based on a field study just like the present study. The temperature range found in the present study and used in the laboratory varied between 14.9 and 25 ºC, which seems to be adequate to infer the possible effect of this variable on the ratio. It appears that there is a complexity of the various environmental factors in the field capable of influencing the RNA:DNA ratio, masking any direct effect of temperature. For instance, Buckley et al. (2008) found that the best-fit meta-analysis model including RNA:DNA ratio, temperature and growth rate, the nucleic acids index was temperature dependent only for fishes less than fully fed and not when considering the well fed, suggesting that food may be an important factor for this dependency.

A lack of relation between RNA:DNA ratio and food type was also found, which indicates that EPR is a more sensitive indicator of food availability than the nucleic acids index. Although there was a significant relationship between both species EPR and the abundance of dinoflagellates, the RNA:DNA ratio was only related to EPR. Vehmaa et al. (2012) found different responses of EPR and RNA:DNA ratio when the copepod *Eurytemora affinis* was given several dominating phytoplankton species of spring blooms in the Baltic sea. Moreover, Speekmann et al. (2006) observed that *Acartia tonsa* EPR was influenced by the various mixed food types given to females, but not the RNA:DNA ratio. On the other hand, Nakata et al. (1994) found a positive relation of RNA:DNA ratio and egg productivity alongside with chlorophyll *a* concentration for *Paracalanus* sp. females. A possible explanation for the different effects of food on these two physiological parameters is the fact that EPR reflects growth while RNA:DNA ratio is a measure of growth and physiological condition. Thus, a certain type of food may be promoting a high egg production and at the same time causing low physiological condition reflected in the RNA:DNA ratio, and *vice versa*. Again, the present study aims to verify the influence of food availability found in nature on the RNA:DNA ratio, while most of the previous studies were mainly conducted under laboratory experiments, which may explain the variation in the results. Nevertheless, the present results show that RNA:DNA ratio is a good proxy to infer copepod reproduction performance.

### 4.2. Factors influencing reproduction

This study presents the first description of the reproduction dynamics of *Acartia clausi* and *Acartia tonsa* at southern Iberia, being one of the few studies analyzing copepod secondary production

along the entire Portuguese coast (e.g. Pastorinho et al., 2003; Vieira et al., 2003; Leandro et al., 2007). Chícharo et al. (2003) estimated the egg production of *Calanus helgolandicus* off the northwest coast of Portugal, whereas all other studies of this area were based in cohort analysis or indirect inference growth rate models, instead of the in situ egg production rates method used herein.

Both species are the most abundant copepods in the studied area and belong to one of the most studied genera around the world. Due to their different physiology and biology, they seem to occupy different habitats, in this case *A. tonsa* is present mainly inside the estuary, while *A. clausi* is the predominant species in the adjacent coastal area, although, depending on the water conditions along the estuary they co-occur. Chicharo et al. (2006b) showed that plankton productivity at the estuary varied with freshwater discharge and reflected associated modifications in planktonic assemblages. This may explain the advection of *A. clausi* from inside the estuary to the adjacent coastal waters, once this species presents an optimal physiological condition at higher salinity values (Castro-Longoria, 2003). Although a significant increase in freshwater discharge may in a short term negatively affect the distribution and abundance of those species, the sudden increase in river discharge, if of short duration (a freshwater pulse), may affect positively the zooplankton abundance, especially copepods. Copepods are selective feeders (Reynolds, 1984) and benefit from an availability of a more diverse prey assemblage, that result after the pulse due to nutrient increase, that promotes phytoplankton diversity and the suppression of competitive exclusion processes, owing to the changes in physicochemical conditions and to top-down control.

The egg production rates determined here for both congeneric species are in accordance to other studies conducted for *A. clausi* (Uriarte et al., 2005; Boyer et al., 2013; Üstün and Bat, 2014) and for *A. tonsa* (Kleppel, 1992; Kleppel and Hazzard, 2000). Once they often occupy different habitats, the environmental parameters that influenced the reproduction were different for each species. There was a positive relationship between the egg production rates of *A. tonsa* and the freshwater inflow, chlorophyll *a* and dinoflagellates abundance, while *A. clausi* was only related to dinoflagellate biomass.

Salinity within the estuary and in the immediate neighborhood coastal area is regulated by the tidal cycle and the amount of freshwater reaching the coastal area, itself regulated by the dams that exist upstream the Guadiana river, especially by the Alqueva dam. The water flow increases when the dam reaches highest levels during the winter, consequent of higher rainfall, which provides a nutrient enrichment to downstream waters. This directly affects the primary production which constitutes the food source necessary for copepods metabolic maintenance, including their reproductive success. The construction of the Alqueva dam has changed the river inflow

introducing major changes in the phytoplankton composition and abundance that has decreased when compared to pre-filling period data (Domingues et al., 2014). Present results showed that there is a relationship between the egg production rates of *A. tonsa* and the freshwater inflow, suggesting that it has a positive impact on the reproductive performance of this species that lives preferentially inside the estuary, while it apparently had no effect on *A. clausi* reproductive performance, which is more abundant in the coastal waters. This may suggest that the productivity of one of the most abundant species inside the estuary was probably higher and more constant throughout the year, when there was a natural freshwater inflow before the dam construction. Furthermore, according to Domingues et al. (2012), another consequence of the Alqueva dam construction is the decrease of phytoplankton abundance and delay of the phytoplankton bloom, occurring now in late spring/early summer instead of early spring. The decrease of phytoplankton abundance might have decreased the secondary production in the estuary, as shown by the positive relationship between chlorophyll *a* concentration and EPR of *A. tonsa* while the delay of the phytoplankton bloom might have caused a temporal shift in the reproduction peak time of *A. tonsa*. This temporal shift might have as consequence a mismatch of pelagic fish spawning and plankton productivity. In fact, deleterious impacts in higher trophic levels were found by Morais et al. (2009) due to the freshwater inflow changes, showing a decline in the abundance of anchovy larval stages, associated with a decrease of the estuarine productivity and to uncontrolled river discharges during the spring. In summary, the dam construction may have affected negatively the production of *A. tonsa* through a bottom-up effect, and consequently the higher trophic levels. To enable a successful management of dammed rivers, Chícharo et al. (2006b) developed a model for the Guadiana estuary, taking into consideration not only the quantity of freshwater but also the timing of the release of freshwater from the Alqueva dam. This would help to maintain all the trophic structure and minimize all the changes in the ecosystems downstream promoted by the retention of the nutrient enriched freshwater in the dam.

The freshwater inflow is negatively correlated with the salinity, meaning that lower salinity was favorable to *A. tonsa* reproduction. This species is usually confined to inner waters such as estuaries, probably due to food availability (Paffenhöfer and Stearns, 1988). It is expected that *A. tonsa* is adapted to high variations of environmental factors like salinity, due to tidal changes. However, Castro-Longoria (2003) found that this species has similar egg production rates at a range of salinity from 20 to 35, only decreasing when it reaches a salinity of 15. Moreover, experiments conducted with *A. tonsa* showed that egg production rates increased at salinities of 14 and 20, and decreased with lower (6 and 10) and higher salinities (30) (Peck and Holste, 2006). Values of salinity during the sampling period of the present study inside the estuary were always around or

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higher than 20, except for few months that reached lower values (February 2009 and January, April, June 2010) ranging from 2 to 12.8. Although the salinity registered during April and June 2010 was approx. 10-12, the egg production rates were high, due to the fact that freshwater inflow increased in the beginning of the year, which induced the increase of primary production in the lower estuary. Consequently, in this case scenario, it is the freshwater inflow and not the salinity that mostly impacts the EPR of *A. tonsa*, with the increase of nutrients and consequently primary producers, since the secondary production was kept high despite the salinity decrease.

*Acartia clausi* egg production rate was not affected by the freshwater inflow and consequently by salinity, due to the fact that sampling was conducted in the adjacent coastal area outside the influence of the river plume, as shown by the constant values of salinity registered throughout the sampling period (~ 35).

Many previous studies have observed that the main factors that influence *Acartia* fecundity are chlorophyll *a* as food proxy (Uye, 1981; Jung et al., 2004; Pagano et al., 2004; Kimmerer et al., 2005) and temperature (Uye, 1981; Ara, 2001; Castro-Longoria, 2003; Boyer et al., 2013). However, this relationship was not always confirmed in field studies in the case of food availability (White and Roman, 1992, Uriarte et al., 1998; Boyer et al., 2013) and temperature (Hay, 1995; Rodriguez et al., 1995; Gómez-Gutiérrez et al., 1999). Chlorophyll *a* was only significantly related to *A. tonsa* egg production rate, while temperature did not influence the fecundity of both species. This probably indicates that food quantity may be the main factor influencing the reproduction of *A. tonsa* in this area. Diatoms were present in high abundance during all the sampling period, meaning that they were not a limiting factor for the *Acartia* productivity, although probably being an important prey for both species. *Acartia* genera feed not only on phytoplankton but also on mixotrophic and heterotrophic microplankton such as ciliates and dinoflagellates (Rollwagen-Bollens and Penry, 2003; Dutz and Peters, 2008; Fileman et al., 2010). The factor that most correlated with the egg production rate of both species was the dinoflagellates biomass. Food quality has been pointed out as one of the main triggers of copepods fecundity (Jónasdóttir and Kiørboe; 1996; Kleppel et al., 1998), such as type, size, shape, and nutrient content. Vehmaa et al. (2011) showed that *Acartia bifilosa* feeding on a dinoflagellate had higher egg production rate than feeding on a diatom, probably due to a higher fatty acids ratio contained in the dinoflagellate that is essential to maximize the fecundity of females (Evjemo et al., 2008). High egg production rate was also found for *A. tonsa* during summer when there was a high proportion of different dinoflagellates and a bloom of *Prorocentrum micans* during autumn in the Baltic Sea (Schmidt et al., 1998). Therefore, prey quality and not only prey concentration might be responsible for the secondary production off the Guadiana estuary.

Hatching success of both species was not related to any environmental variable, and showed no trend throughout the sampling period. This is in agreement with several other studies that found no relationship between hatching success and environmental factors such as temperature, salinity and chlorophyll *a* (e.g. *Acartia lilljeborgi*, Ara, 2001; *Acartia clausi*, Uriarte et al., 2005). In contrast, salinity and temperature have been found to significantly impact the viability of eggs of *A. tonsa* and *A. clausi*, respectively (Peck and Holste, 2006; Holste and Peck, 2006; Boyer et al., 2013). The percentage of hatched eggs found in the present study is considerably high and very similar to other results presented for the same species (Ambler, 1985; Ara, 2001; Uriarte et al., 2005). Uriarte et al. (2005) studied the reproduction of *A. clausi* in two estuaries of northern Spain, and explained the lower hatching success values registered in Bilbao estuary with hypoxic conditions. Although the dissolved oxygen in the water was not measured in the present study, the Guadiana lower estuary and adjacent coastal area are not organically enriched zones where anoxic conditions are frequent (e.g. Garel and Ferreira, 2015), which is reflected by the frequently high hatching success obtained. Hatching success can be highly influenced by the food quality (Arendt et al., 2005), although in the present study it did not reveal any relationship with the main groups of microplankton. The fact that the hatching success was high almost in all sampling dates could be due to a good nutritional environment favorable to this reproductive rate.

### 4.3. Female secondary production and recruitment

In the present study, the secondary production was estimated considering only the females, a fraction of the population studied, that corresponds to an underestimation of the total production of the species. In order to calculate the total production, the biomass of each developmental stage should have been determined, which would involve different sampling techniques that were not conducted. According to Poulet el al. (1995), the egg production method has some technical and practical advantages such as the short incubation time, replicability and accuracy of the measurements of biomass and fecundity, and simplification in the identification, similar to those used to estimate primary production. Furthermore, some authors have developed models that are frequently used to infer indirectly the copepods/zooplankton productivity, based on body weight, temperature or food (e.g. Ikeda and Motoda, 1978; Huntley and Lopez, 1992; Hirst and Bunker, 2003), although none of them have been accepted as standard methods for secondary production determination, mainly due to the existence of several factors controlling it.

In fact, when comparing the present results with those from other studies that estimated the secondary production of the same *Acartia* species, using different methods and developmental stages, there is not a great discrepancy (Table III). Leandro et al. (2014) obtained the juvenile secondary production of both species in Ria de Aveiro by combining in situ data on abundance with

specific temperature-dependent growth models, showing similar values for *A. clausi* than the ones found here for females production. In the Mondego estuary, both species secondary production estimated by cohort analysis presented also similar values to the ones estimated in the present study (Pastorinho et al., 2003; Vieira et al., 2003). Therefore, these results indicate that if the total secondary production had been estimated in the present study, most probably it would have reached higher or equal values than the studies referred in table III, suggesting that the Guadiana estuary is a very productive system.

| Species | Location | Method | Production | Reference |
|---------|----------|--------|------------|-----------|
| *Acartia tonsa* | Mondego Estuary (Portugal) | Cohort analysis | 0.12[a] | Pastorinho et al. (2003) |
| | Ria de Aveiro (Portugal) | Growth rate approach (temperature-dependent growth model) | 1.14 | Leandro et al. (2014) |
| | Patos Lagoon Estuary (Brasil) | Hirst and Bunker (2003) growth model | 0.4-3.65 | Muxagata et al. (2012) |
| | Westerschelde Estuary (The Netherlands) | Growth rate approach (temperature-dependent growth model) | 1.9[b] | Escaravage and Soetaert (1995) |
| | Guadiana Estuary (Portugal) | Egg production rate method | 0.143 | Present study |
| *Acartia clausi* | Mondego Estuary (Portugal) | Cohort analysis | 0.17[a] | Vieira et al. (2003) |
| | Ria de Aveiro (Portugal) | Growth rate approach (temperature-dependent growth model) | 0.068 | Leandro et al. (2014) |
| | Guadiana Estuary (Portugal) | Egg production rate method | 0.101 | Present study |

Table III - Published data on the average daily secondary production (mg C m$^{-3}$ day$^{-1}$) of *Acartia tonsa* and *Acartia clausi* including the present results. a. based on annual average and assuming 365 days per year; b. assuming a carbon-dry weight conversion of 0.5.

Zooplankton plays a key role on transferring energy to higher trophic levels, and copepods (mainly nauplii and copepodites) are an important food source to fish larvae and some adult pelagic fish (Morote, et al.; 2010, Garrido et al., 2015). Therefore, is extremely important to understand the synchrony between the copepods secondary production/recruitment and the fish larvae recruitment (Cushing, 1995). Early stages of pelagic fish *Sardina pilchardus* and *Engraulis encrasicolus* are abundant in the Guadiana estuary and adjacent coastal areas (Faria et al., 2006; Gonçalves et al., 2015), and previous studies have found that *Acartia* spp. is included in their diets (Garrido et al., 2008, 2015; Borme et al., 2009; Costalago et al., 2014). In addition, feeding experiments conducted with *S. pilchardus* larvae showed that higher ingestion rates were reached with high prey concentrations, consisting in *Acartia grani* nauplii and copepodites, suggesting that this species is adapted to forage within dense prey patches (Caldeira et al., 2014). Therefore, it is important that the time of highest copepod production/recruitment matches the time of pelagic fish recruitment. In the Guadiana River, *E. encrasicolus* larvae are more abundant in spring and summer while *S. pilchardus* larvae start to be abundant in winter, peaking during spring and summer (Faria et al.,

2006; Gonçalves et al., 2015). The present results showed higher *Acartia* production/recruitment during spring and summer, exhibiting a good synchrony with the pelagic fish larvae foraging, being a potential key prey to monitor food availability for these important fishery resources.

## 5. Conclusion

There was a positive relationship between RNA:DNA ratio and egg production rate of *Acartia* species, indicating this biochemical index as a good proxy for fecundity and a less laborious method to be used in the future to infer secondary productivity. The fecundity of two of the most abundant zooplankton species that occur in the lower part of the Guadiana estuary and the adjacent coastal area, the congeners *Acartia tonsa* and *A. clausi* are influenced by different environmental variables. Egg production rate of *Acartia tonsa* was related to freshwater inflow, chlorophyll *a* and dinoflagellates biomass, while the egg production of *Acartia clausi* was mainly related to dinoflagellate availability. Freshwater discharges induced higher productivity downstream with nutrient enrichment, justifying its positive influence on the amount of eggs laid by *A. tonsa*. However, changes in inflow regime which had an impact on phytoplankton communities may have altered copepods reproduction. Dinoflagellates seem to be the optimal food item that influences the reproduction of both *Acartia* species in the studied area, although diatoms are available in high concentrations during the year. This probably is due to their higher nutritional composition, promoting higher egg production rates. Hatching success was constantly high during the sampled period and was not related to any environmental variable, probably due to a lack of any limiting factor. Female secondary production and recruitment of both *Acartia* species showed higher values during spring and summer, exhibiting a synchrony with the recruitment of pelagic fish inhabiting the Guadiana estuary, which means they can be used as indicators of food availability for their early development stages.

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