UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS

Copepod communities and secondary production in Portuguese coastal waters

" Documento Definitivo"

Doutoramento em Ciências do Mar

Joana Maria dos Reis Franco Cruz

Tese orientada por:
Professor Doutor Pedro Miguel Alfaia Barcia Ré
Doutor António Miguel Piecho de Almeida Santos

Documento especialmente elaborado para a obtenção do grau de doutor

2019

UNIVERSIDADE DE LISBOA

FACULDADE DE CIÊNCIAS

Ciências
ULisboa

Copepod communities and secondary production in Portuguese coastal waters

Doutoramento em Ciências do Mar

Joana Maria dos Reis Franco Cruz

Tese orientada por:

Professor Doutor Pedro Miguel Alfaia Barcia Ré

Doutor António Miguel Piecho de Almeida Santos

Júri:
Presidente:

- Doutora Maria Manuela Gomes Coelho de Noronha Trancoso, Professora Catedrática e Presidente do departamento de Biologia Animal da Faculdade de Ciências da Universidade de Lisboa

Vogais:

- Doutor Fernando Manuel Raposo Morgado, Professor Associado com Agregação
  Departamento de Biologia da Universidade de Aveiro
- Doutor Sérgio Miguel Franco Martins Leandro, Professor Adjunto
  Escola Superior de Turismo e Tecnologia do Mar do Instituto Politécnico de Leiria
- Doutora Carla Susana Ferreira Garrido Coelho, Investigadora Auxiliar
  Instituto Português do Mar e da Atmosfera (IPMA)
- Doutor Pedro Miguel Alfaia Barcia Ré, Professor Associado com Agregação
  Faculdade de Ciências da Universidade de Lisboa (orientador)
- Doutor José Pavão Mendes de Paula, Professor Associado com Agregação
  Faculdade de Ciências da Universidade de Lisboa

Documento especialmente elaborado para a obtenção do grau de doutor

Esta tese teve o financiamento da Fundação para a Ciência e Tecnologia

SFRH/BD/28198/2006

2019

Previous note

According with the Article 25 nr. 2 of the Post-graduate Studies Regulation (Diário da República Nº 57 de 2015, 2ª série de 23 de Março de 2015), this thesis includes papers published (Chapter 2 to 5) in collaboration with other co-authors.

The leading author of the papers of this thesis, was responsible for sampling design and planning, sample collection, laboratory processing, data and statistical analysis and manuscript writing.

The candidate is the second author of the paper "Effects of temperature, food type and concentration on the grazing of the calanoid copepod***, Centropages chierchiae***", included in the Chapter 2, and was co-responsible for the experimental design, execution, data processing and for helping on the writing of the manuscript.

This thesis had the financial support of the *Fundação para a Ciência e Tecnologia* through PhD grant (SFRH/BD/28198/2006).

Lisboa, Outubro de 2019

Joana Maria dos Reis Franco Cruz

ACKNOWLEDGMENTS

Gostaria de agradecer a todos que de algum modo contribuíram para a realização desta tese, ao longo destes anos.

Em primeiro lugar, gostaria de agradecer aos meus orientadores, ao Doutor António Miguel Santos por aceitar ser meu orientador, ter lançado o desafio de realizar o Doutoramento e a oportunidade de iniciar a minha investigação no mundo do plâncton, por todo o apoio científico e incentivo dado ao longo destes anos. Ao Professor Pedro Ré, por aceitar ser meu orientador, por todo o apoio científico, logístico e incentivo para concretizar este desafio.

À Professora Alexandra Teodósio por me aceitar no seu grupo de trabalho, dando-me todo o apoio para a realização do trabalho prático, pelo apoio científico, incentivo, motivação e amizade durante todo este percurso.

À Susana Garrido por todo o apoio dado ao longo destes anos, principalmente o apoio científico relativamente ao planeamento de experiências, a sua concretização, e discussão final. Pela amizade e incentivo para conseguir terminar esta etapa.

Ao Radhouan Ben-Hamadou, o meu primeiro contacto no CCMAR, que me ajudou na integração de um novo ambiente, por toda amizade e disponibilidade sempre demonstradas ao longo do tempo.

Ao Professor Luís Chícharo por me aceitar no seu grupo de trabalho, e ajudar-me sempre que era necessário.

À Fátima Quintela por me ensinar a identificar zooplâncton, com todo o seu entusiasmo e motivação, por estar sempre pronta a ajudar em tudo o que era preciso e pela amizade demonstrada.

À Cátia Bartilotti que sempre esteve ao meu lado e ajudou-me nas correções finais, pela amizade e motivação demonstrada ao longo de todo este percurso.

À Doutora Antonina dos Santos pela ajuda nos primeiros passos no mundo do zooplâncton.

Aos meus colegas e amigos de laboratório do CCMAR, Renata Gonçalves, David Piló, Pedro Range por me ajudarem nas saídas de campo, no laboratório, pela amizade e motivação. À Vânia Baptista pela amizade e toda a motivação demonstrada nesta etapa final.

Ao Senhor Antero pela ajuda nas saídas de campo ao estuário do rio Guadiana, pela disponibilidade e amizade ao longo destes anos.

Ao laboratório de fitoplâncton do IPMA, em especial a Doutora Teresa Moita, por ter permitido a utilização dos microscópios de inversão, e à Sofia Palma e Alexandra Silva por me ajudarem na identificação, quando desconhecia alguma espécie.

Ao Rui Rosa e à Marta Pimentel por terem proporcionado as instalações e equipamento do Laboratório Marítimo da Guia, para medir o metabolismo dos copépodes durante as experiências e por toda a ajuda prestada.

A todos os meus amigos que sempre me ajudaram e incentivaram ao longo destes anos.

À minha família, que está sempre presente e sempre pronta a ajudar quando é preciso, por toda a paciencia e amizade demonstrada, sem ela tudo seria mais difícil de concretizar.

ABSTRACT

The study of zooplankton communities and estimates of copepod secondary production are of great importance to infer about 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 to eliminate time consuming experimental analyses. This thesis aims at improving the knowledge on copepods reproduction, with special emphasis on estimations of secondary production, and its relationship with environmental factors. For this purpose, different approaches (experimental and field studies) and time scales were used to study: 1) the effects of temperature and food on the physiology and metabolism of the climate change indicator copepod species *Centropages chierchiae*; 2) the effect of abiotic and biotic parameters on the production of the ubiquitous copepod genus *Acartia* in an interannual temporal scale (Guadiana estuary and adjacent coastal waters), as well as during a short temporal scale (Ria Formosa lagoon); 3) the effect of the environmental conditions on the mesozooplanktonic community in Ria Formosa lagoon; and, 4) the relationship between the biochemical method "RNA:DNA ratio" and the egg production rates of the genus *Acartia*, to infer if this could be a good method to estimate secondary production. Results showed that temperature influenced the feeding, reproductive and respiration rates of the calanoid *C. chierchiae*, with an optimal temperature at 19ºC for feeding and reproduction, while respiration increased exponentially with temperature and was sex independent. Reproduction rates and hatching success were influenced by different type of algae, a diatom and a dinoflagellate, respectively, and increased with food concentration. Food intake was always higher than the metabolic demands, except for the highest temperature tested (24ºC). During the interannual study in Guadiana estuary, the egg production rate (EPR) of *Acartia tonsa* was positively related with chlorophyll *a* concentration, freshwater inflow and dinoflagellates biomass, while *Acartia clausi* was only related to dinoflagellates. In the intensive field study conducted in Ria Formosa lagoon during summer, EPR of *A. clausi* was influenced by salinity and ammonia concentration. Moreover, salinity was the main factor affecting the short-term variability of mesozooplankton community, followed by tidal phase, semilunar cycle, temperature and food type. Estimates of *Acartia* females' secondary production of both ecosystems were comparable to values of total production obtained in previous studies for other regions, indicating them as high productive areas. The biochemical index RNA:DNA was positively related to EPR in both *in situ* studies, indicating that it is a

good proxy to infer copepod production. In conclusion, simple methods such as the egg production rate and the RNA:DNA ratio should be considered to estimate copepods secondary production in future studies using different species, taking into account the environmental factors that might influence them.

Keywords: Zooplankton, Copepoda, egg production rate, RNA:DNA ratio, secondary production

RESUMO

O zooplâncton tem um papel fundamental na teia trófica, promovendo o fluxo de energia entre os produtores primários e os consumidores de ordem superior. Deste modo, o estudo das comunidades zooplanctónicas e da produção secundária são bastante importantes para inferir sobre o fluxo global da matéria orgânica nos ecossistemas aquáticos e as respostas específicas das espécies de zooplâncton à variabilidade ambiental. Dentro do zooplâncton, os copépodes representam o grupo dominante, sendo o mais estudado em termos de produção secundária. Contudo, ainda não existe um método de rotina para determinar a produção secundária dos copépodes, de modo a eliminar análises experimentais morosas e difíceis de executar. Adicionalmente, o facto de os copépodes apresentarem um ciclo de vida curto e serem poiquilotérmicos fazem deles bons indicadores de alterações climáticas.

Deste modo, a presente tese teve como objetivo melhorar o conhecimento da reprodução dos copépodes, mais precisamente das estimativas da produção secundária, e a sua relação com os fatores ambientais. Para este propósito, diferentes abordagens (experimental e estudos de campo) e escalas temporais foram utilizadas para estudar: 1) os efeitos da temperatura e alimentação na fisiologia e metabolismo de uma espécie de copépode indicadora de alterações climáticas, o *Centropages chierchiae*; 2) o efeito dos parâmetros abióticos e bióticos na produção do género de copépode cosmopolita *Acartia* numa escala temporal interanual (estuário do rio Guadiana e águas costeiras adjacentes), bem como numa curta escala de tempo (sistema lagunar da Ria Formosa); 3) o efeito das condições ambientais na comunidade mesozooplanctónica do sistema lagunar da Ria Formosa; e 4) a relação entre o método bioquímico, o rácio RNA:DNA e a taxa de produção de ovos do género *Acartia*, de modo a inferir se este será um bom método para estimar a produção secundária.

As experiências realizadas com a espécie de copépode *C. chierchiae* mostraram que a temperatura influenciou as taxas de alimentação, reprodução e respiração, com uma temperatura ótima de 19ºC para a alimentação e reprodução, enquanto que a respiração aumentou exponencialmente com a temperatura sem diferenças entre os sexos. A taxa de eclosão dos ovos foi significativamente inferior com a temperatura de 13ºC relativamente às outras temperaturas testadas (19 e 24ºC). As taxas de reprodução e eclosão foram influenciadas por diferentes tipos de algas, uma espécie de diatomácea (*Phaeodactylum tricornutum*) e uma de dinoflagelado (*Gymnodinium* sp.), respetivamente, indicando diferentes necessidades em

termos de alimento. Relativamente à concentração e ao tipo de alimento, esta espécie apresentou elevadas taxas de ingestão para ambos os grupos (diatomáceas e dinoflagelados), e o seu comportamento seletivo de alimentação foi dependente da concentração de alimento, com presas maiores (*Ditylum brightwellii*) a serem selecionadas quando fornecidas em baixas concentrações. O alimento ingerido foi sempre superior às necessidades metabólicas, exceto para a maior temperatura (24ºC), o que significa que num cenário de aumento de temperatura no futuro, esta espécie terá de consumir mais alimento por unidade de tamanho corporal de modo a manter as suas necessidades metabólicas. Estes resultados confirmam a sensibilidade do copépode *C. chierchiae* para as variações de temperatura ajudando na compreensão da expansão bem-sucedida desta espécie para latitudes mais a norte.

Durante o estudo interanual no estuário do rio Guadiana, a taxa de produção de ovos da espécie de copépode *Acartia tonsa* foi positivamente relacionada com a concentração da clorofila *a*, caudal de água doce, e com a biomassa de dinoflagelados, enquanto que a espécie *Acartia clausi*, localizada nas águas costeiras adjacentes foi unicamente relacionada com os dinoflagelados. O caudal de água doce induziu o aumento da produtividade no baixo estuário com o enriquecimento de nutrientes nesta área. Adicionalmente, os dinoflagelados parecem ser o alimento óptimo que influencia a reprodução de ambas as espécies, provavelmente devido à sua melhor composição nutricional. Por outro lado, no estudo intensivo realizado na Ria Formosa durante o Verão, a taxa de produção de ovos da espécie *A. clausi* foi influenciada pela salinidade e a concentração de amónia. A amónia esteve presente na água em baixas concentrações durante todo estudo, o qual é normalmente registado em muitas áreas eutróficas, sugerindo uma boa adaptação desta espécie a estas condições. A taxa de eclosão dos ovos das espécies *A. clausi* e *A. tonsa* no estuário do rio Guadiana, foi relativamente alta durante todo o período de amostragem, e não esteve relacionada com nenhum fator ambiental, provavelmente por nenhum ser limitante.

No estuário do rio Guadiana, a produção secundária das fêmeas e o recrutamento de ambas as espécies de *Acartia* apresentaram valores superiores durante a primavera e o verão, exibindo uma sincronia com o recrutamento de peixes pelágicos que habitam este ecossistema, o que significa que podem ser bons indicadores para a disponibilidade de alimento para as suas fases iniciais de desenvolvimento. As estimativas da produção secundária das fêmeas do género *Acartia* em ambos os ecossistemas foram semelhantes a valores da produção total obtidos anteriormente para outros sistemas, indicando que os ecossistemas estudados são áreas

altamente produtivas. O índice bioquímico RNA:DNA foi positivamente relacionado com a taxa de produção de ovos do género *Acartia* em ambos os estudos realizados *in situ*, indicando ser um bom indicador para inferir a produção de copépodes. No entanto, o modelo obtido para a Ria Formosa incluiu também a temperatura, uma vez que este parâmetro tem um forte efeito em reações bioquímicas como a biossíntese proteica, sugerindo uma dependência da temperatura em qualquer índice contendo RNA. O facto de não ter havido nenhuma limitação quanto ao alimento disponível durante o estudo na Ria Formosa, pode ter evidenciado a influência da temperatura no rácio bioquímico.

Relativamente à comunidade mesozooplanctónica da Ria Formosa, diversos fatores ambientais influenciaram diferentes *taxa*. A salinidade foi um dos principais factores que afetou a abundância e variabilidade de várias espécies de copépodes e larvas de Cirripedia. A salinidade óptima que favoreceu abundâncias mais elevadas foi de aproximadamente 34.5-35, verificando-se um decréscimo com o aumento da salinidade. A ocorrência de verões mais quentes e secos no futuro devido a possíveis alterações climáticas irá promover um aumento da salinidade no sistema lagunar da Ria Formosa, o qual poderá ser amenizado com eventos pontuais de descargas submarinas de águas subterrâneas na área de estudo. Os copépodes neríticos e as larvas de decápodes parecem ter sido favorecidos pelas marés vazantes, usando estas correntes para serem transportadas para o mar. O ciclo semilunar (coeficiente de maré), temperatura e disponibilidade de alimento foram também importantes fatores na composição das comunidades zooplanctónicas. Os *blooms* de dinoflagelados tóxicos, comuns na Ria Formosa durante o verão, não apresentaram efeitos nefastos na abundância de alguns *taxa* (Apendicularia e *Penilia avirostris*).

Concluindo, métodos mais simples tais como a taxa de produção de ovos e o índice bioquímico RNA:DNA deverão ser considerados para estimar a produção secundária em estudos futuros usando diferentes espécies, e tendo em conta os fatores ambientais que poderão influenciá-los. Com os resultados apresentados na presente tese, espera-se uma melhoria no conhecimento das espécies e comunidades zooplanctónicas das águas costeiras Portuguesas, nomeadamente nas possíveis respostas destes organismos a futuras alterações antropogénicas e climáticas. Adicionalmente, estudos futuros deverão assentar no desenvolvimento de modelos para estimar a produção secundária através de deteção remota, utilizando dados de satélite de temperatura superficial e clorofila *a* juntamente com os dados das taxas de produção de ovos obtidos no

presente trabalho para validação do modelo, contribuindo para uma melhor gestão de recursos pesqueiros e monitorização de alterações climáticas.

Palavras-chave: Zooplâncton, Copepoda, taxa de produção de ovos, rácio RNA:DNA, produção secundária,

TABLE OF CONTENTS

ACKNOWLEDGMENTS ..... **IV**

ABSTRACT ..... **VI**

RESUMO ..... **VIII**

TABLE OF CONTENTS ..... **XII**

LIST OF FIGURES ..... **XVI**

LIST OF TABLES ..... **XX**

CHAPTER 1 ..... **1**

GENERAL INTRODUCTION **1**

1.1 IMPORTANCE OF ZOOPLANKTON AND SECONDARY PRODUCTION ESTIMATIONS 3
1.2 ENVIRONMENTAL INFLUENCE ON COPEPODS PHYSIOLOGY AND METABOLISM 4
1.3 METHODS TO ESTIMATE SECONDARY PRODUCTION: THE EGG PRODUCTION RATE METHOD 5
1.4 RNA:DNA RATIO METHOD AS A PROXY OF EGG PRODUCTION RATES 7
1.5 AIMS AND OUTLINE OF THE THESIS 9
1.6 REFERENCES 11

CHAPTER 2 ..... **21**

2.1 INTRODUCTION 24
2.2 MATERIALS AND METHODS 25
2.2.1 COPEPOD AND MICROALGAE CULTURES 25
2.2.2 UNIALGAL FEEDING EXPERIMENTS AND TEMPERATURE EFFECTS 26
2.2.3 PLURIALGAL FEEDING EXPERIMENTS AND SELECTIVE BEHAVIOUR 27
2.2.4 DATA ANALYSIS 28
2.3 RESULTS 30
2.3.1 UNIALGAL FEEDING EXPERIMENTS AND TEMPERATURE EFFECTS 30
2.3.2 PLURIALGAL FEEDING EXPERIMENTS AND SELECTIVE BEHAVIOUR 35
2.4 DISCUSSION 37
2.4.1 FEEDING RATES 37
2.4.2 TEMPERATURE DEPENDENCE OF FEEDING RATES 39
2.4.3 PLURIALGAL EXPERIMENTS AND SELECTIVE FEEDING 40
2.5 CONCLUSIONS 42
2.6 ACKNOWLEDGEMENTS 42
2.7 REFERENCES 43

CHAPTER 3 ..... **49**

3.1 INTRODUCTION 52
3.2 MATERIALS AND METHODS 53
3.2.1 COLLECTION AND ORGANISMS' CULTURES 53
3.2.2 REPRODUCTION EXPERIMENTS: EFFECT OF TEMPERATURE AND FOOD 54
3.2.3 RESPIRATION EXPERIMENTS: EFFECT OF TEMPERATURE AND SEX 55
3.2.4 ENERGY REQUIREMENTS CALCULATIONS 56
3.2.5 STATISTICAL ANALYSIS 57

3.3 RESULTS 57
3.3.1 EFFECTS OF TEMPERATURE AND FOOD ON REPRODUCTION 57
3.3.2 EFFECTS OF TEMPERATURE AND GENDER ON RESPIRATION 61
3.3.3 ENERGY REQUIREMENTS 62
3.4 DISCUSSION 64
3.4.1 EFFECTS OF TEMPERATURE ON REPRODUCTION 64
3.4.2 EFFECTS OF FOOD ON REPRODUCTION 66
3.4.3 EFFECTS OF TEMPERATURE AND GENDER ON RESPIRATION RATES 67
3.4.4 ENERGY REQUIREMENTS 68
3.4.5 *CENTROPAGES CHIERCHIAE* AS A CLIMATE CHANGE INDICATOR 69
3.5 ACKNOWLEDGMENTS 70
3.6 REFERENCES 71

CHAPTER 4 ..... **79**

4.1 INTRODUCTION 82
4.2 MATERIALS AND METHODS 84
4.2.1 STUDY AREA AND SAMPLING 84
4.2.2 EGG PRODUCTION AND HATCHING SUCCESS EXPERIMENTS 86
4.2.3 BIOMASS AND SECONDARY PRODUCTION DETERMINATION 86
4.2.4 RNA:DNA RATIO ANALYSIS 87
4.2.5 DATA ANALYSIS 88
4.3 RESULTS 89
4.3.1 HYDROGRAPHY AND FOOD ENVIRONMENT 89
4.3.2 SPECIES ABUNDANCE, EPR, HS AND RNA:DNA RATIO 92
4.3.3 FEMALES SECONDARY PRODUCTION AND RECRUITMENT 97
4.4 DISCUSSION 98
4.4.1 RNA:DNA RATIO AS A PROXY OF EPR 98
4.4.2 FACTORS INFLUENCING REPRODUCTION 100
4.4.3 FEMALES SECONDARY PRODUCTION AND RECRUITMENT 104
4.5 CONCLUSIONS 106
4.6 ACKNOWLEDGMENTS 106
4.7 REFERENCES 107

CHAPTER 5 ..... **117**

5.1 INTRODUCTION 120
5.2 MATERIALS AND METHODS 122
5.2.1 STUDY AREA 122
5.2.2 SAMPLING 122
5.2.3 EGG PRODUCTION EXPERIMENTS 124
5.2.4 RNA:DNA RATIO ANALYSIS 125
5.2.5 DATA ANALYSIS 126
5.3 RESULTS 127
5.3.1 ENVIRONMENTAL CONDITIONS AND FOOD AVAILABILITY 127
5.3.2 MESOZOOPLANKTON BIOMASS, DIVERSITY AND ABUNDANCE: ENVIRONMENTAL CONDITIONS INFLUENCE 131
5.3.3 *ACARTIA CLAUSI* EPR, RNA:DNA RATIO AND SECONDARY PRODUCTION 136
5.4 DISCUSSION 138
5.4.1 MESOZOOPLANKTON COMMUNITY: INFLUENCE OF ENVIRONMENTAL CONDITIONS 138
5.4.2 *ACARTIA CLAUSI* PRODUCTION AND RNA:DNA RATIO: INFLUENCE OF ENVIRONMENTAL CONDITIONS 141

5.5 CONCLUSIONS 143
5.6 ACKNOWLEDGEMENTS 145
5.7 REFERENCES 145

CHAPTER 6 ..... **155**

6.1 GENERAL CONCLUSIONS 157
6.2 REFERENCES 162

LIST OF FIGURES

Fig. 2.1 - Clearance rates (mL cop^{-1} day^{-1}) of *Centropages chierchiae* feeding on *Phaeodactylum tricornutum* (filled circles) and *Gymnodinium* sp. (open circles) in the experiments conducted at 8, 13, 19 and 24ºC.....31

Fig. 2.2 - Ingestion rates (µg C cop^{-1} day^{-1}) of *Centropages chierchiae* feeding on *Phaeodactylum tricornutum* (filled circles) and *Gymnodinium* sp. (open circles) in the experiments conducted at 8, 13, 19 and 24ºC.....32

Fig. 2.3 - Pairwise comparison of ingestion (left panel) and clearance rates (right panel) between each level of the factor temperature (8, 13, 19, 24ºC). Differences in mean values and 95% confidence intervals of the Tukey test for unequal sample size are shown. A given pairwise comparison of means is significant if the confidence intervals do not overlap the 0 difference. Only data from experiments conducted at food concentrations < 410.0 µg C L^{-1} are used.....33

Fig. 2.4 - Functional feeding response of *Centropages chierchiae* at 19ºC using *Gymnodinium* and *Phaeodactylum tricornutum* as prey. Data of both algae are pooled since no significant differences were found of the feeding rates for both algae (Table 2.2). Ingestion (I; µg C cop^{-1} day^{-1}) in the upper panel, and clearance (F; mL cop^{-1} day^{-1}) in the lower panel.....34

Fig. 2.5 - Box-plots of *Centropages chierchiae* weight-specific ingestion rates expressed as daily ration (%BC, % of body carbon ingested day^{-1}) at the four different temperatures studied (only data from experiments conducted at food concentrations < 410.0 µg C L^{-1} are shown). Median values are indicated by the horizontal line in the boxes.....35

Fig. 2.6 - Electivity index (Ei) for the (plurialgal) selective feeding experiment, using a mixed assemblage of four prey (*Ditylum brightwellii, Gymnodinium* sp., *Phaeodactylum tricornutum* and *Rhodomonas baltica*) at three total prey concentrations (low: 56.1 ± 3.39, inter: 454.7 ± 12.99 and high: 1107.9 ± 237.08 µg C L^{-1}). Each prey level has three replicates. The dotted line represents the threshold level (i.e. *Ei* = 0) at which a given prey is neither selected nor avoided.....37

Fig. 3.1 – Egg production rates (EPR, eggs $female^{-1}$ day^{-1}) of *Centropages chierchiae* at different temperatures fed with two algae, *Gymnodinium* sp. and *Phaeodactylum tricornutum*,

during 5 days; black circles are for algae concentration of 60 µg C L^{-1} and white of 300 µg C L^{-1}. Error bars indicate standard deviation.....59

Fig. 3.2 – Hatching success (HS, %) of Centropages chierchiae at different temperatures fed with two algae, Gymnodinium sp. and Phaeodactylum tricornutum, during 5 days; black bars are for algae concentration of 60 µg C L^{-1} and grey for 300 µg C L^{-1}. Error bars indicate standard deviation.....60

Fig. 3.3 - Variation of mean respiration rates of *Centropages chierchiae* males (squares) and females (circles) for different temperatures. Each point represents the mean value of replicates and error bars indicate standard deviation. The dashed line corresponds to the fitted exponential function for males and the continuous for females, in equations T is the temperature (ºC) and R is the respiration rate (µL O_2 mg^{-1} DW h^{-1}).....61

Fig. 3.4 - Boxplots of the daily ration of Centropages chierchiae (expressed as percentage of body carbon) at the four different temperatures analysed using a) *Phaeodactylum tricornutum* and b) *Gymnodinium* sp.; white boxplots are for feeding rates experiments results (Garrido et al., 2013) and black for present work.....63

Fig. 4.1 – 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.....85

Fig. 4.2 - 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.....91

Fig. 4.3 – 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/).....92

Fig. 4.4 - Carbon biomass of the evaluated microplankton groups at both stations. Data are represented as %.....93

Fig. 4.5 – *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.....94

Fig. 4.6 – *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.....94

Fig. 4.7 – 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.....95

Fig. 4.8 – RNA:DNA ratios of *Acartia tonsa* and *Acartia clausi* females….....….....96

Fig. 5.1 – Map of the studied area with the fixed sampling station located in the western part of Ria Formosa Coastal lagoon (light grey triangle).....123

Fig. 5.2 - Environmental parameters measured in Ria Formosa lagoon during the sampling days from July 28th (28J) to September 3rd (03S), 2009, at 11h00 – black line, and 16h00 – grey line. (Temperature ºC, Salinity, Dissolved Oxygen mg L^{-1}, pH, NH4 – ammonia mg L^{-1}, NO2 – nitrates mg L^{-1}, NO3 – nitrites mg L^{-1}, PO4 – phosphates mg L^{-1}, Si – silica mg L^{-1}).....128

Fig. 5.3 - Carbon content (µg C L^{-1}) of the main microplankton groups identified in Ria Formosa from July 28th (28J) to September 3rd (03S), 2009, at 11h00 – black bar and 16h00 – grey bar. Note the change of scale in the y-axis.....130

Fig. 5.4 - Mesozooplankton biomass (mg m^{-3}), species richness and Shannon-Wiener diversity index in Ria Formosa lagoon from July 28th (28J) to September 3rd (03S), 2009, at 11h00 – black bar and 16h00 – grey bar.....132

Fig. 5.5 – Abundances (ind.m^{-3}) of the main mesozooplankton taxa in Ria Formosa lagoon from July 28th (28J) to September 3rd (03S), 2009, at 11h00 – black bar and 16h00 – grey bar. Note the change of scale in the y-axis.....133

Fig. 5.6 - Abundances (ind.m^{-3}) of the main mesozooplankton species in Ria Formosa lagoon from July 28th (28J) to September 3rd (03S), 2009, at 11h00 – black bar and 16h00 – grey bar. Note the change of scale in the y-axis.....134

Fig. 5.7 – *Acartia clausi* females abundance (ind.m^{-3}) (a), egg production rates (eggs female^{-1} day^{-1}) (b), RNA:DNA ratio (c) and females secondary production (µg C m^{-3} day^{-1}) (d) in Ria Formosa lagoon during twelve sampling days from 28th July (28J) to 3rd September (03S), 2009…….137

LIST OF TABLES

Table 2.I - Size and estimated carbon content of *Centropages chierchiae* and of the prey species used. Carbon content was estimated from the equations provided by van der Lingen (van der Lingen, 2002) and Smayda (Smayda, 1965) for *C. chierchiae* and microalgae, respectively. [a] Prossome length, [b] Mean diameter, [c] Minor and major axes.....26

Table 2.2 - ANCOVA analysis of the effect of prey concentration (µg C L^{-1}), temperature and prey type (*Gymnodinium* sp. and *Phaeodactylum tricornutum*) on the ingestion and clearance rates of *Centropages chierchiae*.....33

Table 2.3 - Ingestion (I; µgC cop^{-1} day^{-1}) and clearance rates (F; mL cop^{-1} day^{-1}) of *Centropages chierchiae* in the plurialgal experiment. Total and prey-specific food concentrations (µg C L^{-1}) are shown. rho: *Rhodomonas baltica*; gym: *Gymnodinium* sp.; pha: *Phaeodactylum tricornutum*; dyt: *Dytilum brightwellii*.....36

Table 3.1 - Results of a three-way ANOVA performed to test for temperature, algae species and concentration effects on the egg production rates and hatching success of *Centropages chierchiae*. Significant values are given in bold. EPR – egg production rate (eggs $female^{-1}$ day^{-1}), HS – hatching success (%), Temp – temperature, Alga – food type, Conc – food concentration.……...…….58

Table 3.2 - Results of a two-way ANOVA performed to test for temperature and sex effects on the oxygen rates of *Centropages chierchiae*. Significant values are given in bold. Temp – temperature.....62

Table 3.3 – Results of respiration rates experiments for the temperatures tested: F – females, M – males, Temp – temperature, n – number of replicates, showing mean values and standard deviation of copepods dry weight (µg), oxygen consumption (µlO_2 mg^{-1}DW h^{-1}) and food intake (µgC day^{-1}) and daily ration (% body carbon) for *Phaeodactylum tricornutum* and *Gymnodinium* sp.....64

Table 3.4 – Published data on the respiration rates of *Centropages typicus* including the present results for *Centropages chierchiae*.....68

Table 4.1 - 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.....97

Table 4.2 – 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.....98

Table 4.3 - 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.....105

Table 5.1 - Mean abundances (ind.m^{-3}) of mesozooplankton taxa and mean carbon content (µg C L^{-1}) of the main microplankton taxa identified in Ria Formosa lagoon from July 28th (28J) to September 3rd (03S), 2009.....129

Table 5.2 - Results of Generalized Additive Mixed Models (GAMMs), species richness (SR) and abundance of mesozooplankton taxa in ind. m^{-3} (Copep – Copepoda; *Parac. – Paracalanus* spp.; *Acart. – Acartia clausi*; *Centrop. – Centropages chierchiae*; *Oith – Oithona* spp.; *Euterp. – Euterpina acutifrons*; *Evad. – Evadne* spp.; *Penil. – Penilia avirostris*; Append. – Appendicularia; Decap. – Decapoda larvae; Cirrip. – Cirripedia larvae) with indication of significant explanatory variables: tidal phase as a factorial parameter (F – Flood; H – High; L – Low) and smooth terms (T – temperature ºC; S – salinity; TC – tidal coefficient m; Diat – diatoms µg C L^{-1}; Scripp – *Scrippsiella trochoidea* µg C L^{-1}; Proroc - *Prorocentrum* spp. µg C L^{-1}; Gymnc– *Gymnodinium catenatum* µg C L^{-1}. AIC is the Akaike Information Criterion. (Signif. codes '***' $p < 0.001$ '**' $p < 0.01$ '*' $p < 0.05$).....136

Table 5.3 - Coefficients and significance (p-value) of each of the explanatory variables of the Generalized Linear Models (GLMs - Gaussian) describing the variation of the egg production rates (EPR) and the RNA:DNA ratios of *Acartia clausi*. NH_4 represents ammonia. 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 is the degrees of freedom.....138

Chapter 1

General Introduction

1.1 Importance of zooplankton and secondary production estimations

Zooplankton plays an important role in food webs, transferring organic energy between the primary producers and higher order consumers. They are able to shape phytoplankton biomass, production and species composition by grazing them, and also can influence some fisheries resources recruitment, based on several hypothesis stating the importance of zooplankton as prey to fish larvae during their first feeding period (e.g. Lasker 1978; Cushing, 1990; Agostini and Bakun, 2002). Moreover, zooplankton contributes to the vertical flux of organic matter in the water column (Kiorboe, 1998), playing an important role in the biogeochemistry of nitrogen and carbon cycles (Hays et al., 1997; Cavan et al., 2017), more specifically in the biological pump of carbon to the bottom of the ocean. All their waste-products and exoskeletons will fuel the benthic community and their grazing influences the nutrients availability distribution and consequently the distribution and composition of benthic and pelagic organisms. As they perform diel vertical migrations, all the food particles consumed during the night in upper ocean layers will be egested during the day at higher depths, helping to remove most of particulate organic carbon from upper mesopelagic layers where recycling is more intense (Martin et al., 1987; Cavan et al., 2015). Their role in carbon dioxide sequestration in deeper layers removing it from the sea surface and atmosphere has a huge impact on the anthropogenic and climate changes. In summary, zooplankton constitutes the main interface between the cascades of bottom-up and top-down effects (Sommer, 2008), and therefore is involved in several fundamental ecosystem processes that regulates the abiotic and biotic environment. That is why is so important to understand the distribution and composition of zooplankton communities, to know how zooplankton growth and trophic transfer efficiency is regulated in order to comprehend the structure of the pelagic food webs.

The amount of energy transferred to and supporting higher trophic levels is quantified by estimating the zooplanktonic secondary production. For a long time, researchers have been trying to find the right method to measure secondary production, due to the importance of zooplanktonic organisms as the main food source of many marine resources, that would help to predict and manage important fish stocks (Huntley and Lopez, 1992). Moreover, in terms of ecological importance, it is also an indicator of zooplanktonic organisms' physiology and nutrition condition (Kimmerer, 1987). However, until now, no standard method has been adopted for estimating secondary production, and more effort must be made to find a universal methodology to such an important issue. Among the zooplankton constituents, copepods are the dominant group representing between 55 to 90% of the total mesozooplankton abundance

in marine pelagic systems (Longhurst, 1985). Therefore, most of the studies conducted to estimate secondary production were applied to copepods, being time, site and species-specific (Runge and Roff, 2000).

1.2 Environmental influence on copepods physiology and metabolism

Zooplanktonic organisms are driven by biotic and abiotic factors, responding rapidly to environmental changes since many species are fast-growing and with short life cycles (Hays et al., 2005). Also, zooplankton are poikilothermic meaning that they are sensitive to temperature fluctuations which affects their physiological processes and induces to shifts in their communities (Richardson, 2008). Physiological processes such as feeding, reproductive and respiration rates are influenced by environmental shifts in temperature and many other factors (Mauchline, 1998). Respiration rates in terms of oxygen consumption are considered an index of copepods metabolism and are highly positively dependent on temperature and body mass (Ikeda, 1985; Ikeda et al., 2001). Nevertheless, some estuarine and ubiquitous copepod species presented no effect of temperature on respiration rates, revealing a possible adaptation to rapid temperature fluctuations (Gaudy et al., 2000; Hiromi et al., 1988).

Feeding is the main way to transfer energy through the food webs and the main source to production and zooplanktonic activity (Båmstedt et al., 2000). Moreover, the knowledge on feeding rates and feeding behaviour is fundamental to understand how copepods species cope with environmental variability. Copepods feeding is usually expressed as ingestion rate (the amount of food ingested by a consumer per unit time), clearance rate (the volume of water cleared of food by a consumer per unit time) or daily ration (mass of food ingested per day expressed as the percentage of body mass of the consumer). The main factors that control these feeding rates are food availability, body weight and in a lesser extent temperature (Saiz and Calbet, 2011). Although it would be expected that feeding rates would increase with higher temperatures, following the same temperature dependence as metabolic processes, the meta-analysis of Saiz and Calbet (2011) has demonstrated in field studies that temperature induces a minor effect compared to other factors.

Copepods reproductive rates (number of eggs per female per day) and hatching success (total percentage of eggs hatched) are highly dependent on food availability (Mauchline, 1998). Although many studies showed a positive relation between food quantity and reproduction (e.g. Uye, 1988; Pagano et al., 2004), some others, especially field studies, revealed no correlation

between both (e.g. Stearns et al., 1989; Boyer et al., 2013). This fact changed the focus of research to food quality instead of only food quantity, as a major contributor to reproduction. As it was previously thought, autotrophic diatoms were the main food source of copepods, however, further studies showed that other heterotrophic protists such as ciliates and dinoflagellates are more important food preys with better quality supporting an important fraction of copepods egg production (Calbet and Saiz, 2005; Vehmaa et al., 2011). Food quality consists on different essential diet components as fatty acids (Jónasdóttir, 1994; Broglio et al., 2003), sterols (Ederington et al., 1995), proteins (Kleppel and Hazzard, 2000) or amino acids (Kleppel et al., 1998). Furthermore, these essential diet components consumed by copepods will be in turn crucial to zooplanktivorous fish growth and their larval development (Desvilettes et al. 1997; Garrido et al., 2012). Another major factor that as a strong influence on copepods egg production rate is temperature (e.g. Halsband & Hirche 2001; Maps et al. 2005). However, there are some incongruences on this factor influence as many field studies failed to show a direct correlation with copepods reproduction (e.g. Bautista et al. 1994; Hay, 1995), probably due to other factors overriding the effect of temperature. In some environments, such as estuaries and coastal lagoons, salinity shifts can also control copepods egg production rates (Castro-Longoria 2003; Calliari et al., 2006).

In summary, there are still some controversial results in the existing literature related to the influence of abiotic and biotic parameters on copepods physiological processes. With the constant climatic and anthropogenic changes, it is crucial to understand how marine copepods interact with all environmental factors, conducting further experiments and field studies.

1.3 Methods to estimate secondary production: the egg production rate method

Several methods have been developed over the last century to estimate secondary production, such as the cohort analysis (Bougis, 1974), cumulative growth (Omori and Ikeda, 1976) and turnover (Rigler and Downing, 1984). However, all these methods are time consuming and very difficult to perform. Besides the latter methods, researchers started developing models using large available datasets, to simplify the estimation of copepods secondary production. Huntley and Lopez (1992) have developed a model using temperature as the main factor influencing growth, since all enzymatic reactions occurring during protein synthesis of poikilotherms marine animals are temperature dependent. However, there can be certain limitations on this model, once it does not consider the influence of food quality and

quantity on growth, leading to an overestimation of secondary production when food is a limiting factor. The model proposed by Hirst and Lampitt (1998) has added the body weight besides the temperature, achieving lower values than the previous model as expected. Furthermore, Hirst and Bunker (2003) came up with another model which considered an additional important factor, food in terms of chlorophyll *a*, besides temperature and body weight. Although all these models are simple to use, they can have certain limitations and the appropriate conditions to apply them must be verified, such as the the time periods and regions by knowing the life history of the studied copepod community (Runge and Roff, 2000).

Secondary production of a certain species is usually calculated by multiplying the biomass with the instantaneous growth rate of that population and it is called the growth rate approach. Thus, given that copepods life cycle presents 13 stages (egg, 6 naupliar stages, 5 copepodite stages and adult stage) it is necessary to determine the biomass and growth rate for each stage. In order to obtain a more accurately *in situ* growth of each development stage, there is the need of incubating individually each stage during ~24h with high replicability, which is time, site and species specific, becoming a very laborious method and difficult to achieve. Therefore, there was a need to simplify this method and it was suggested the so-called egg production rate method (Berggreen et al., 1988). This method has two assumptions, the first is that all stage-specific growth rates are equal for a given species and second that the egg production rate is equivalent to the growth of adult females (Poulet et al., 1995). Since adult copepods do not experience somatic growth, all the energy is channelled into egg production by adult females, which reflects the reproductive growth. In that way, the secondary production is calculated by summing the product between the specific egg production rate and the biomass of each development stage. Many studies have shown that the growth rates of all development stages are not always equivalent between them when food is a limiting factor in the environment, which is contradictory to the first assumption of this method (Landry, 1978; Peterson et al., 1991; Peterson and Hutchings, 1995). Also, Hirst and Bunker (2003) have found that under *in situ* conditions the growth rate of juveniles is higher than females' egg production, probably due to different food requirements for each stage to grow. Moreover, the identification and enumeration of the juvenile fraction to obtain their biomass is extremely difficult and requires different zooplankton gears with different mesh sizes, which is time consuming and non-practical. For all these reasons described, Poulet et al. (1995) suggested a simple and easier way to estimate secondary production, considering only the females biomass fraction. Even though it is an underestimation of the total secondary production, this method brings several

advantages such as short incubation time, replicability and accuracy of the measurements of biomass and fecundity and easy identification of adult females and species (Poulet et al., 1995).

Estimation of copepods recruitment is also an important measurement that is calculated multiplying the hatching success by the female's secondary production (Poulet et al., 1995). Hatching success corresponds to the percentage of nauplii that will hatch, i.e. the percentage of viable eggs. In terms of the fraction of secondary production that is transferred to higher trophic levels, the determination of the survival rate of the eggs is necessary to comprehend the energy flow through the food web (Ianora and Poulet, 1993). Additionally, the knowledge of the total number of copepods nauplii hatched is important to the survival of early life stages of many fishery resources since they are an important food source to these organisms (Garrido et al., 2015)

1.4 RNA:DNA ratio method as a proxy of egg production rates

Biochemical and radiochemical methods have been developed in last decades to measure zooplankton productivity, in a way of achieving a standardized technique as the ^{14}C used to measure primary production (Runge and Roff, 2000). These methods would allow a faster and more instantaneously estimation of growth and the immediate past physiological state of the organisms with precision, sensitivity and replicability, compared to the traditional incubations such as artificial cohort and egg production rates methods. Radiochemical rates of synthesis of specific macromolecules (Roff et al. 1994), enzyme activities such as DNA polymerase (Sapienza and Mague 1979), chitobiase (Espie and Roff 1995) or aspartate transcarbamylase (Bergerron, 1983) and nucleic acid content (Sutcliffe 1965; Chícharo and Chícharo, 2008) are all potential methods but none have been routinely adopted as an index of both somatic and germinal productivity.

The analysis of the nucleic acids contents in invertebrates started in 1960's (Stutcliffe, 1965), and since then has been developed as a potential measurement of growth rates, using not only the quantification of RNA and DNA concentrations but also their ratios (e.g. Ota and Landry, 1984; Wagner et al., 2001; Yebra et al., 2011). The RNA:DNA ratio is based on the fact that DNA content within the cells remains constant and the RNA concentration increases with protein synthesis increment (Bulow, 1987), varying with age, body size, ontogenetic development stage, related to changing environmental conditions (Bulow, 1970). Therefore,

individual organisms that are well fed and metabolic active present higher ratios than starving and metabolic inactive organisms. The RNA:DNA ratios have been applied to several types of aquatic organisms such as phytoplankton (Dortch et al., 1983), zooplankton (Wagner et al., 1998; Gorokhova and Kyle, 2002; Ikeda et al., 2007), crustaceans (Chícharo et al., 2007; Amaral et al., 2009), bivalves (Grémare and Vétion, 1994; Chícharo et al., 2001), cephalopods (Clarke et al., 1989; Sykes et al., 2004), and fish larvae (Buckley, 1984; Caldarone et al., 2003; Chícharo et al. 2003), juvenile and adults (Bulow, 1970; Thorpe et al., 1982), providing a direct measurement of recent growth and physiological condition in the field.

Regarding copepods, many studies have attempted to correlate RNA:DNA ratios with growth, both somatic and reproductive, whereas some have been successful (Nakata et al., 1994; Saiz et al., 1998; Wagner et al., 2001) while others have found a poor relationship between the nucleic acids contents and growth rates (Dagg and Littlepage, 1972; Ota and Landry, 1984). Until now, the RNA:DNA ratio was used as an indicator of growth (Elser et al., 2000; Wagner et al., 2001), nutritional condition (Wagner et al., 1998; Vehmaa et al., 2012), dormant condition (Kobari et al., 2013), egg viability (Hogfors et al., 2011) and egg production rate (Nakata et al., 1994; Saiz et al., 1998; Gorokhova, 2003). The continuously effort of investigating the use of RNA:DNA ratios as a proxy of copepods reproduction is essential to overcome some issues related to the egg production rates methodology. The use of artificial incubations that may not reflect the *in situ* food and environmental conditions, the time consuming which will not allow a more intensive sampling using several species are the main problems when applying the egg production rate method.

Environmental factors such as food quantity and quality, temperature and salinity have been shown to strongly affect the nucleic acids content of copepods (Saiz et al., 1998; Wagner et al., 2001; Calliari et al., 2006). However, there are still some incongruences with the results found in previous works. For instance, Saiz et al. (1998) found a good linear relationship between egg production rates and RNA:DNA ratios of *Acartia grani*, that was temperature dependent, while nucleic acid values of *Acartia bifilosa* were not temperature dependant, only changing in response to food concentration (Gorokhova, 2003). Moreover, experiments conducted with *Acartia sinjiensis* suggested that food quality has a poor influence on the relationship of nucleic acid contents and egg production, while temperature should be used in models using nucleic acid indices as a proxy of egg production rates (Gusmão and McKinnon, 2011). During a field study, Nakata et al. (1994) showed a good correlation between RNA:DNA ratios and egg production in *Paracalanus* sp. and an increase of both related to higher

phytoplankton biomass near the frontal waters of Kurshio current. On the other hand, Ning et al. (2013) also obtained a positive correlation between egg production rates and RNA:DNA ratios for the calanoid copepod *Calanus sinicus*, but no relationship with food (chlorophyll a concentration) or temperature.

Most of the works showing a positive correlation between RNA:DNA ratios and egg production rates were conducted under laboratory experimental conditions, instead of directly measure the nucleic acids contents of wild caught copepods. Even though laboratory experiments are important to develop models relating nucleic acid ratios with egg production rates and inherent environmental factors influence, inference of this relationship using direct field measurements would allow a more realistic understanding of these physiological traits correlation. As it seems to be species specific, more studies should be conducted with different species and in different environments to achieve powerful models to implement the use of nucleic acids contents as a proxy of copepods productivity.

1.5 Aims and outline of the thesis

The general aim of this thesis was to improve the knowledge on copepods reproduction, more precisely on estimations of secondary production, and the relationship with environmental factors. Until now there is no routine method to estimate secondary production, being this study a reinforcement on the research of simple methods that give us a direct *in situ* measurement such as the egg production rate method. Moreover, this thesis aims at finding more evidence of the positive correlation between the egg production rate and biochemical methodologies, such as the nucleic acids ratios analysis, to infer if this could be a good method to estimate secondary production. The main target species are small copepods (*Centropages chierchiae*, *Acartia clausi* and *Acartia tonsa*) inhabiting Portuguese coastal areas, such as the Ria Formosa lagoon system and the lower part of the Guadiana river estuary and adjacent coastal waters. Several approaches and different spatio-temporal scales were used on the methods of each work, to study the variability of copepod's species physiology. The 2 studies with the calanoid copepod *C. chierchiae* are based on manipulative laboratory experiments, while the calanoid copepods belonging to the genus *Acartia* were studied *in situ* in an interannual scale (monthly sampling frequency), as well as during the most productive season in a shorter temporal scale (daily sampling frequency). Regarding the Portuguese coastal waters, the research on estimations of copepods secondary production is very scarce, including only the studies conducted in

Mondego river estuary (Vieira et al., 2003a,b; Pastorinho et al., 2003) using the cohort analysis and in Ria de Aveiro using a combination of *in situ* data on abundance with specific temperature-dependent growth models (Leandro et al. 2014).

This thesis is divided in 6 chapters, and the specific objectives are:

- The current chapter (Chapter 1) is the General Introduction where the scientific background of the main themes studied in this thesis is described and discussed.

- Chapter 2 intends to contribute to understanding the effect of temperature and prey type and concentration on the functional response and the selective feeding behaviour of the copepod *C. chierchiae*, with the goal of improving the knowledge of its capacity to deal with environmental variability.

- Chapter 3 aims to understand the physiological state of *C. chierchiae* under different environmental conditions and how this can relate to climate changes. With this purpose, we investigated the effects of temperature, food quality and quantity on the egg production and hatching success, as well as the effect of temperature and gender on metabolic rates for this calanoid copepod. Furthermore, the metabolic energy demands were estimated at different temperatures in relation to the prey concentration and compared to the estimations of daily ration.

- Chapter 4 aims at determining the seasonal and spatial variability of *A. tonsa* and *A. 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; measure RNA:DNA ratios in order to determine if it can be considered a good proxy for secondary production.

- Chapter 5 aims to analyze the short-term variability of the mesozooplanktonic assemblage structure in a temperate coastal lagoon (Ria Formosa) during the peak production period that occurs during the summer. For that, we investigate the correlation between the mesozooplankton and the environmental conditions (abiotic and biotic parameters). Particularly, the production (egg production rates and females' secondary

production) and physiological condition (RNA:DNA ratio) of *A. clausi* are assessed in relation to the main hydrological parameters and phytoplanktonic prey availability.

- The main conclusions of the thesis are described on chapter 6, integrating all the results of the studies (papers) presented.

1.6 References

Agostini, V.N., Bakun, A. (2002) 'Ocean triads' in the Mediterranean Sea: physical mechanisms potentially structuring reproductive habitat suitability (with example application to European anchovy, *Engraulis encrasicolus*). *Fisheries Oceanography*, 11: 129-142.

Amaral, V., Penha-Lopes, G, Paula, J. (2009) RNA/DNA ratio of crabs as an indicator of mangrove habitat quality. *Aquatic Conservation: Marine and Freshwater Ecosystems*, 19: S56-S62.

Båmstedt, U., Gifford, D.J., Irigoien, X., Atkinson, A., Roman, M. (2000) Feeding. In Harris, R., Wiebe, P., Lenz, J., Skjoldal, H.R., Huntley, M. (eds), ICES Zooplankton Methodology Manual. Academic Press, London, pp. 401-454.

Bautista, B., Harris, R.P., Rodriguez, V., Guerrero, F. (1994) Temporal variability in copepod fecundity during two different spring bloom periods in coastal waters off Plymouth (SW England). *Journal of Plankton Research*, 16: 1367-1377.

Bergerron, J.,-P.R. (1983) Approche systémique des potentialités de production secondaire pélagique: l'évolution printanière en province néritique. *Oceanologica Acta*, Spécial(4): 15-19.

Berggreen, U., Hansen, B., Kiørboe, T. (1988) Food size spectra, ingestion and growth of the copepod Acartia tonsa during development: Implications for determination of copepod production. *Marine Biology*, 99: 341-352.

Bougis, P. (1974) Ecologic du plancton marin. II. Le zooplancton. Masson, Paris.

Boyer, S., Bouvy, M., Bonnet, D. (2013) What triggers *Acartia* species egg production in a Mediterranean lagoon? *Estuarine, Coastal and Shelf Science*, 117: 125-135.

Broglio, E., Jónasdóttir, S.H., Calbet, A., Jakobsen, H.H., Saiz, E. (2003) Effect of heterotrophic

versus autotrophic food on feeding and reproduction of the calanoid copepod *Acartia tonsa*: relationship with prey fatty acid composition. *Aquatic Microbial Ecology*, 31: 267-278.

Buckley, L.J. (1984) RNA:DNA ratio: an index of larval fish growth in the sea. *Marine Biology*, 80: 291-298.

Bulow, J.F. (1970) RNA-DNA ratios as indicators of recent growth rates of fish. *Journal of the Fisheries Research Board of Canada*, 27: 2343-2349.

Bulow, J.F. (1987) RNA-DNA ratios as indicators of growth rates in fish: A review. In The age and growth of fish; Summerfelt, R.C., Hall, G.E. Eds.; The Iowa State University Press: Ames, Iowa, pp. 45-64.

Calbet, A., Saiz, E. (2005) The ciliate-copepod link in marine ecosystems. *Aquatic Microbial Ecology*, 38: 157-167.

Caldarone, E.M., Onge-Burns, J.M.St., Buckley, L.J. (2003) Relationship of RNA/DNA ratio and temperature to growth in larvae of Atlantic cod, *Gadus morhua*. *Marine Ecology Progress Series*, 262: 229-421.

Calliari, D., Andersen, C.M., Thor, P., Gorokhova, E., Tiselius, P. (2006) Salinity modulates the energy balance and reproduction success of co-occuring copepods *Acartia tonsa* and *A. clausi* in different ways. *Marine Ecology Progress Series*, 312: 177-188.

Castro-Longoria, E. (2003) Egg production and hatching success of four *Acartia* species under different temperature and salinity regimes. *Journal of Crustacean Biology*, 23: 289-299.

Cavan, E.L., Le Moigne, F.A.C., Poulton, A.J., Tarling, G.A., Ward, P., Daniels, C.J., Fragoso, G.M., Sanders, R.J. (2015) Attenuation of particulate organic carbon flux in the Scotia Sea, Southern Ocean, is controlled by zooplankton fecal pellets. *Geophysical Research Letters*, 42: 821-830.

Cavan, E.L., Henson, S.A., Belcher, A., Sanders, R. (2017) Role of zooplankton in determining the efficiency of the biological carbon pump. *Biogeosciences*, 14: 177-186

Chícharo, M.A., Chícharo, L. (2008) RNA:DNA ratio and other nucleic acid derived indices in marine ecology. *International Journal of Molecular Sciences*, 9: 1453-1471.

Chícharo, L., Chícharo, M.A., Alves, F., Amaral, A., Pereira, A., Regala, J. (2001) Diel variation of the RNA:DNA ratios in *Crassostrea angulata* (Lamarck) and *Ruditapes decussatus*

(Linnaeus 1758) (Mollusca, Bivalvia). *Journal of Experimental Marine Biology and Ecology*, 259: 121-129.

Chícharo, M.A., Esteves, E., Santos, A.M.P., dos Santos, A., Peliz, A., Ré, P. (2003) Are sardine larvae caught off northern Portugal in winter starving? An approach examining nutritional conditions. *Marine Ecology Progress Series*, 257: 303-309.

Chícharo, M.A., Chícharo, L., Amaral, A., Morais, P. (2007) Sex effect on ratios and concentrations of DNA and RNA three in marine organisms. *Marine Ecology Progress Series*, 332: 241-245.

Clarke, A., Rodhouse, P.G., Holmes, L.J., Pascoe, P.L. (1989) Growth rate and nucleic acid ratio in cultured cuttlefish, Sepia officinalis (Mollusca: Cephalopda). *Journal of Experimental Marine Biology and Ecology*, 133: 229-240.

Cushing, D.H. (1990) Plankton production and year class strength in fish populations: an update of the match/mismatch hypothesis. *Advances in Marine Biology*, 26: 249-293.

Dagg, M.J., Littlepage, J.L. (1972) Relationships between growth rate and RNA, DNA, protein and dry weight in *Artemia salina* and *Euchaeta elongata*. *Marine Biology*, 17: 162-170.

Desvilettes, C., Bourdier, G., Breton, J.C. (1997) Changes in lipid class and fatty acid composition during development in pike (*Esox lucius* L) eggs and larvae. *Fish Physiology and Biochemistry*, 16: 381-393.

Dortch, Q., Roberts, T.L., Clayton, J.R.Jr., Ahmed, S.I. (1983) RNA/DNA ratios and DNA concentrations as indicators of growth rate and biomass in planktonic organisms. *Marine Ecology Progress Series*, 13: 61-71.

Ederington, M.C., McManus, G.B., Harvey, H.R. (1995) Trophic transfer of fatty acids, sterols, and a triterpenoid alcohol between bacteria, a ciliate and the copepod *Acartia tonsa*. *Limnology and Oceanography*, 40: 860-867.

Elser, J.J., Sterner, R.W., Gorokhova, E., Fagan, W.F., Markow, T.A., Cotner, J.B., Harrison, J.F., Hobbie, S.E., Odell, G.M., Weider, L.W. (2000) Biological stoichiometry from genes to ecosystems. *Ecology Letters*, 3: 540-550.

Espie, P.J., Roff, J.C. (1995) Characterization of chitobiase from *Daphnia magna* and its relation to chitin flux. *Physiological Zoology*, 68: 727-748.

Garrido, S., Saiz, E., Peters, J., Ré, P., Alvarez, P., Cotano, U., Herrero, D.L., Martínez de

Murguía, A., Irigoien, X. (2012) Effect of food type and concentration on growth and fatty acid composition of early larvae of the anchovy (*Engraulis encrasicolus*) reared under laboratory conditions. *Journal of Experimental Marine Biology and Ecology*, 434-435: 16-24.

Garrido, S., Silva, A., Pastor, J., Dominguez, R., Silva, A.V., Santos, A.M. (2015) Trophic ecology of pelagic fish species off the Iberia: diet overlap, cannibalism and intraguild predation. *Marine Ecology Progress Series*, 539: 271-285.

Gaudy, R., Cervetto, G., Pagano, M. (2000) Comparison of the metabolism of *Acartia clausi* and *A. tonsa*: influence of temperature and salinity. *Journal of Experimental Marine Biology and Ecology*, 247: 51-65.

Gorokhova, E. (2003) Relationships between nucleic acid levels and egg production rates in *Acartia bifilosa*: implications for growth assessment of copepods in situ. *Marine Ecology Progress Series*, 262: 163-172.

Gorokhova, E., Kyle, M. (2002) Analysis of nucleic acids in *Daphnia*: development of methods and ontogenetic variations in RNA–DNA content. *Journal of Plankton Research*, 24: 511-522.

Grémare, A., Vétion, G. (1994) Comparison of several spectrofluorometric methods for measuring RNA and DNA concentrations in the deposit-feeding bivalve *Abra ovata*. *Comparative Biochemistry and Physiology Part B*, 107B, 297-308.

Gusmão, L.F.M, McKinnon, A.D. (2011) Nucleic acid indices of egg production in the tropical copepod *Acartia sinjiensis*. *Journal of Experimental Marine Biology and Ecology*, 396: 122-137.

Halsband, C., Hirche, H.J. (2001) Reproductive cycles of dominant calanoid copepods in the North Sea. *Marine Ecology Progress Series*, 209: 219-229.

Hays, G.C., Harris, R.P., Head, R.N. (1997) The vertical nitrogen flux caused by zooplankton diel vertical migration. *Marine Ecology Progress Series*, 160: 57-62.

Hay, S. (1995) Egg production and secondary production of common North Sea copepods: Field estimates with regional and seasonal comparisons. *ICES Journal of Marine Science*, 52: 315-327.

Hays, G.C., Richardson, A.J., Robinson, C. (2005) Climate change and plankton. *Trends in*

Ecology and Evolution, 20: 337-344.

Hiromi, J., Nagata, T., Katoda, S. (1988) Respiration of the small planktonic copepod *Oithona davisae* at different temperatures. *Bulletin of Plankton Society of Japan*, 35: 143-148.

Hirst, A.G., Lampitt, R.S. (1998) Towards a global model of in situ weight-specific growth in marine planktonic copepods. *Marine Biology*, 132: 247-257

Hirst, A.G, Bunker, A.J. (2003) Growth of marine planktonic copepods: Global rates and patterns in relation to chlorophyll a, temperature, and body weight. *Limnology and Oceanography*, 48: 1988-2010

Hogfors, H., Holmborn, T., Hajdu, S., Gorokhova, E. (2011) Does female RNA content reflect viable egg production in copepods? A test with the Baltic copepod *Acartia tonsa*. *Journal of Plankton Research*, 33: 1460-1463.

Huntley, M.E., Lopez, M.D.G. (1992) Temperature-dependent production of marine copepods: A global synthesis. *The American Naturalist*, 140: 201-242.

Ianora, A., Poulet, S.A. (1993) Egg viability in the copepod *Temora stylifera*. *Limnology and Oceanography*, 38: 1615-1626.

Ikeda, T. (1985) Metabolic rates of epipelagic zooplankton as a function of body size and temperature. *Marine Biology*, 85: 1-11.

Ikeda, T., Kanno, Y., Ozaki, K., Shinada, A. (2001) Metabolic rates of epipelagic marine copepods as a function of body mass and temperature. *Marine Biology*, 139: 587-596.

Ikeda, T., San, F., Yamaguchi, A., Matsuishi, T. (2007) RNA:DNA ratios of calanoid copepods from the epipelagic through abyssopelagic zones of the North Pacific Ocean. *Aquatic Biology*, 1: 99-108.

Jónasdóttir, S.H. (1994) Effects of food quality on the reproductive success of *Acartia tonsa* and *Acartia hudsonica*: laboratory observations. *Marine Biology*, 121: 67-81.

Kimmerer, W.J. (1987) The theory of secondary production calculations for continuously reproducing populations. *Limnology and Oceanography*, 32: 1-13

Kiørboe, T. (1998) Population regulation and role of mesozooplankton in shaping marine pelagic food webs. *Hydrobiologia*, 363: 13-27.

Kleppel, G.S., Hazzard, S.E. (2000) Diet and egg production of the copepod *Acartia tonsa* in

Florida Bay. II. Role of the nutritional environment. *Marine Biology*, 137: 111-121.

Kleppel, G.S., Burkart, C.A., Houchin, L. (1998) Nutrition and the regulation of egg production in the calanoid copepod *Acartia tonsa*. *Limnology and Oceanography*, 43: 1000-1007.

Kobari, T., Mori, H., Tokushige, H. (2013) Nucleic acids and protein content in ontogenetically migrating copepods in the Oyashio region as influenced by development stage and depth distribution. *Journal of Plankton Research*, 35: 97-104.

Landry, M.R. (1978) Population dynamics and production of a planktonic marine copepod, *Acartia clausii*, in a small temperate lagoon on San Juan Island; Washington. *Internationale Revue der gesamten Hydrobiologie*, 63: 77-119.

Lasker, R. (1978) The relation between oceanographic conditions, and larval anchovy food in the California Current: identification of factors contributing to recruitment failure. Rapports et Proces-verbaux des Réunions. *Conseil International pour l'Éxploration de la Mer*, 173: 212-230.

Leandro, S.M., Tiselius, P., Marques, S.M, Avelelas, F., Correia, C., Sá, P., Queiroga, H. (2014) Copepod production estimated by combining in situ data and specific temperature-dependent somatic growth models. *Hydrobiologia*, 741: 139-152.

Longhurst, A. (1985) The structure and evolution of plankton communities. *Progress in Oceanography*, 15: 1-35.

Maps, F., Runge, J.A., Zakardjian, B., Joly, P. (2005) Egg production and hatching success of *Temora longicornis* (Copepoda, Calanoida) in the southern Gulf of St. Lawrence. *Marine Ecology Progress Series*, 285: 117-128.

Martin, J.H., Knauler, G., Karl, D., Broenkow, W. (1987) VERTEX: carbon cycling in the north east Pacific. *Deep-Sea Research Part A,* 34: 267-285.

Mauchline, J. (1998) The biology of calanoid copepods. *Advances in Marine Biology*, 33: 1-710.

Nakata, K., Nakano, H., Kikuchi, H. (1994) Relationship between egg productivity and RNA/DNA ratio in *Paracalanus* sp. In the frontal waters of the Kurshio. *Marine Biology*, 119: 591-596.

Ning, J., Li, C., Yang, G., Wan, A., Sun, S. (2013) Use of RNA:DNA ratios to evaluate the condition and growth of the copepod *Calanus sinicus* in the Southern Yellow Sea. *Deep-*

Sea Research Part II, 97: 109-116.

Omori, M., Ikeda, T. (1976) Methods in marine zooplankton ecology. Ed. by John Wiley, New York.

Ota, A.Y., Landry, M.R. (1984) Nucleic acids as growth rates indicators for early development stages of *Calanus pacificus* Brodsky. *Journal of Experimental Marine Biology and Ecology*, 80: 147-160.

Pagano, M., Kouassi, E., Arfi, R., Bouvy, M., Saint-Jean, L. (2004) In situ spawning rate of the calanoid copepod *Acartia clausi* in a tropical lagoon (Ebrié, Côte d'lvoire): diel variations and effects of environmental factors. *Zoological Studies*, 43: 244-254.

Pastorinho, R., Vieira, L., Ré, P., Pereira, M., Bacelar-Nicolau, P., Morgado, F., Marques, J.C., Azeiteiro, U. (2003) Distribution, production, histology and histochemistry in *Acartia tonsa* (Copepoda: Calanoida) as means for life history determination in a temperate estuary (Mondego estuary, Portugal). *Acta Oecologica*, 24: S259-S273.

Peterson, W.T., Hutchings, L. (1995) Distribution, abundance and production of the copepod *Calanus agulhensis* on the Agulhas Bank in relation to spatial variations in hydrography and chlorophyll concentration. *Journal of Plankton Research*, 17: 2275-2295.

Peterson, W.T., Tiselius, P., Kiørboe, T. (1991) Copepod egg production, moulting and growth rates and secondary production, in the Skagerrak in August 1988. *Journal of Plankton Research*, 13: 131-154.

Poulet, S.A., Ianora, A., Laabir, M., Klein Bretler, W.C.M. (1995) Towards the measurement of secondary production and recruitment in copepods. *ICES Journal of Marine Science*, 52: 359-368.

Richardson, A.J. (2008) In hot water: zooplankton and climate change. *ICES Journal of Marine Science*, 65: 279-295.

Rigler, F.H., Downing, J.A. (1984) The calculation of secondary production. In Manual on methods for the assessment of secondary production in fresh waters, pp. 19- 58, 2nd ed. Ed. by J. A. Downing and F. H. Rigler. Blackwell, Oxford.

Roff, J.C., Kroetsch, J.T., Clarke, A.J. (1994) A radiochemical method for secondary production in planktonic crustacea based on rate of chitin synthesis. *Journal of Plankton Research*, 16: 961-976.

Runge, J.A., Roff, J.C. (2000) The measurement of growth and reproductive rates. In Harris, R., Wiebe, P., Lenz, J., Skjoldal, H.R., Huntley, M. (eds), ICES Zooplankton Methodology Manual. Academic Press, London, pp. 401-454.

Saiz, E., Calbet, A., Fara, A., Berdalet, E. (1998) RNA content of copepods as a tool for determining adult growth rates in the field. *Limnology and Oceanography*, 43: 465-470.

Saiz, E., Calbet, A. (2011) Copepod feeding in the ocean: scaling patterns, composition of their diet and the bias of estimates due to microzooplankton grazing during incubations. *Hydrobiologia*, 666: 181-196.

Sapienza, C., Mague, T.H. (1979) DNA polymerase activity and growth rates in *Artemia salina* nauplii. *Limnology and Oceanography*, 24: 572-576

Sommer, U. (2008) Trophic cascades in marine and freshwater plankton. *International Review of Hydrobiology*, 93: 506-516.

Stearns, D.E., Tester, P.A., Walker, R.L. (1989) Diel changes in the egg production rate of *Acartia tonsa* (Copepoda, Calanoida) and related environmental factors in two estuaries. *Marine Ecology Progress Series*, 52: 7-16.

Sutcliffe, W.H.Jr. (1965) Growth estimates from ribonucleic acid content in some small organisms. *Limnology and Oceanography*, 10(Suppl): R253-R258.

Sykes, A., Domingues, P., Andrade, J.P. (2004) Nucleic acid derived indices or instantaneous growth rate as tools to determine different nutritional condition in cuttlefish (*Sepia officinalis*, Linnaeus 1758) hatchlings. *Journal of Shellfish Research*, 23: 585-591.

Thorpe, J.E., Talbot, C., Villarreal, C. (1982) Bimodality of growth and smolting in Atlantic salmon, *Salmo salar L. Aquaculture*, 28: 123-132.

Uye, S. (1981) Fecundity studies of neritic calanoid copepods *Acartia clausi* Giesbrecht and *A. steueri* Smirnov: a simple empirical model of daily egg production. *Journal of Experimental Marine Biology and Ecology*, 50: 255-271.

Vehmaa, A., Larsson, P., Vidoudez, C., Pohnert, G., Reinikainen, M., Engström-Öst, J. (2011) How will increased dinoflagellate:diatom ratios affect copepod egg production? - A case study from the Baltic Sea. *Journal of Experimental Marine Biology and Ecology*, 401: 134-140.

Vehmaa, A., Kremp, A., Tamminen, T., Hogfors, H., Spilling, K., Engström-Öst, J. (2012)

Copepod reproductive success in spring-bloom communities with modified diatom and dinoflagellate dominance. *ICES Journal of Marine Science*, 69: 351-357.

Vieira, L., Morgado, F., Ré, P., Nogueira, A., Pastorinho, R., Pereira, M., Bacelar-Nicolau, P., Marques, J.C., Azeiteiro, U.M. (2003a) Population dynamics of Acartia clausi from a temperate estuary (Mondego Estuary, Western Portugal). *Invertebrate Reproduction and Development*, 44: 9-15.

Vieira, L., Azeiteiro, U., Ré, P., Pastorinho, R., Marques, J.C., Morgado, F. (2003b) Zooplankton distribution in a temperate estuary (Mondego estuary southern arm: Western Portugal). *Acta Oecologica*, 24: 163-173

Wagner, M., Durbin, E., Buckley, L. (1998) RNA:DNA ratios as indicators of nutritional condition in the copepod *Calanus finmarchicus*. *Marine Ecology Progress Series*, 162: 173-181.

Wagner, M.M., Campbell, R.G., Boudreau, C.A., Durbin, E.G. (2001) Nucleic acids and growth of *Calanus finmarchicus* in the laboratory under different food and temperature conditions. *Marine Ecology Progress Series*, 221: 185-197.

Yebra, L., Berdalet, E., Almeda, R., Pérez, V., Calbet, A., Saiz, E. (2011) Protein and nucleic acid metabolism as proxies for growth and fitness of *Oithona davisae* (Copepoda, Cyclopoida) early developmental stages. *Journal of Experimental Marine Biology and Ecology*, 406: 87-94.

Chapter 2

Effects of temperature, food type and food concentration on the grazing of the calanoid copepod *Centropages chierchiae*

Susana Garrido, Joana Cruz, A. Miguel P. Santos, Pedro Ré, Enric Saiz

Journal of Plankton Research (2013) 35(4): 843-854

Effects of temperature, food type and food concentration on the grazing of the calanoid copepod *Centropages chierchiae*

Abstract

Laboratory experiments were conducted to study the combined effect of temperature (8, 13, 19 and 24ºC), food type and food concentration on the grazing rates of the adult stages of the calanoid copepod *Centropages chierchiae*. As prey, the diatom *Phaeodactylum tricornutum* and the dinoflagellate *Gymnodinium* sp. (both ca. 15 µm cell diameter) were used at a range of carbon concentrations similar to the ones experienced in nature (6.4 to 393.8 µg C L^{-1}). Ingestion rates increased linearly with food concentration and did not differ between prey types. When comparing the effect of temperature, highest clearance and ingestion rates were obtained at 19ºC, whereas no difference was observed among the other temperatures. Daily rations varied between 1.2 and 183.5% body carbon day^{-1}. Additional experiments were conducted to study the selective feeding behaviour of *C. chierchiae* when offered a mixture of different prey types. Selective feeding was dependent on food concentration; at low food levels, large cells were selected (*Ditylum brightwellii*), whereas at medium and high food concentrations no clear selection patterns were observed. In contrast to other studies, no positive selection of dinoflagellates over other algal food was found.

Keywords: Functional response, *Centropages chierchiae*, clearance rate, selective feeding

2.1 Introduction

The calanoid copepod *Centropages chierchiae* typically inhabits tropical and subtropical waters of the eastern Atlantic Ocean, the Mediterranean Sea and the western Indian Ocean (Razouls, 1996). From the northern limit of its distribution south to the Bay of Biscay, *C. chierchiae* occurs preferentially during the warm summer months (Lindley and Daykin, 2005), whereas during the rest of the year it can also be found in high concentrations off western Iberia (Sobrinho-Gonçalves et al., 2013). This copepod species constitutes one of the most important prey items in the diet of small pelagic fish in the productive, upwelling waters off the Iberian Peninsula, such as adult sardines (Garrido et al., 2008) and anchovies (Plouvenez and Champalbert, 1999). A better understanding of the dynamics of the coastal food webs that sustain small pelagic fish stocks involves not only knowing the kinds and amounts of food required for fish larval survival, but also aspects of the biology and ecology of the key organisms upon which fish feed (Turner, 1984). Additionally, the implications that fish pressure on certain key copepod species may have shaping the trophic web structure are relevant. Despite its importance, however, information on the biology and ecology of this copepod species is lacking, in particular when compared with the better studied congeneric species *Centropages hamatus* and *Centropages typicus* (e.g. Kiørboe et al., 1982; Carlotti et al., 1997; Calbet et al., 2007).

In addition to the importance of *Centropages chierchiae* in southern Atlanto-European waters, this species has expanded its distribution northward during the last decade. Before 1988, *C. chierchiae* occurred rarely in the Bay of Biscay, Celtic Sea and English Channel, whereas since 2000 it is frequently found and at greater abundance in these areas (Lindley and Daykin, 2005). This northward shift in the eastern North Atlantic Ocean and European shelf seas has been associated with an increase in water temperature in these areas (Lindley and Daykin, 2005). Range shifts exhibited by zooplankton in response to global warming are reported as among the fastest and largest of any marine or terrestrial group (Richardson, 2008). Such ecosystem modifications arising from changes in the distribution of species at the base of the marine food web may have dramatic impacts for exploited resources, as indicated for cod in the North Sea (Beaugrand et al., 2003).

In this study, we aim to provide basic ecophysiological knowledge on the feeding rates and behaviour of *Centropages chierchiae*, with the goal of improving our understanding of its

capability to cope with environmental variability. Understanding the physiological limits and plasticity of an organism are essential traits required in order to forecast its direct impact on the trophic food webs of newly colonized ecosystems. We will focus on two of the major factors driving copepod feeding in the oceans, temperature and food type and availability (Saiz and Calbet, 2011). We also studied the selective feeding behaviour in the presence of different prey types provided at different concentrations. The rate of food intake of one prey may be affected by the presence of alternative prey choices. Because of time restrictions due to handling of alternative prey, selectivity patterns and/or behavioural switches may occur associated with changes in the relative prey abundances (Kiørboe et al., 1996; Gentleman et al., 2003). Copepods can discriminate between different types of food and select preferred food items. Usually, larger particles are positively selected over small cells (e.g. Frost, 1972; Meyer et al., 2002), although discrimination between similar-sized items has also been reported (e.g. Fernández, 1979; Henriksen et al., 2007). Other, more complex feeding behaviours have been described for copepods, where selection depends on the interplay between cell size and abundance (e.g. Wilson, 1973; Kiørboe et al., 1996).

To our knowledge, this is the first time that the functional response of *Centropages chierchiae* has been described in relation to temperature, and one of the few works studying such relationships for this genus (Calbet et al., 2007).

2.2 Materials and methods

2.2.1 Copepod and microalgae cultures

Zooplankton samples were collected in coastal waters near Lisbon (western Iberian Peninsula) during November 2009 and July 2010, with a 200-µm mesh size WP2 net with a plastic bag as cod end to avoid copepod damage. The samples were immediately transported to the laboratory, and *Centropages chierchiae* individuals were sorted under the stereomicroscope. The sorted copepods were kept in culture in 30 L tanks with filtered, autoclaved seawater (35 salinity) at 20ºC, and fed a mixture of different microalgae at saturated conditions (matching those used in the feeding experiments, described below).

Two sets of experiments were conducted on adult *Centropages chierchiae*: (i) unialgal experiments to study the effect of temperature on the feeding rates, using the diatom *Phaeodactylum tricornutum* (~17.0 x 2.3 µm) and the dinoflagellate *Gymnodinium* sp. (~14 µm) as prey, and (ii) plurialgal experiments to study the feeding selectivity behaviour. The size of the prey used in the selective feeding experiments ranged between 8 and 50 µm, and comprised the cryptophyte *Rhodomonas baltica*, the dinoflagellate *Gymnodinium* sp. and the diatoms *P. tricornutum* and *Ditylum brightwellii*. All microalgae used as prey were obtained from cultures growing exponentially at 19ºC at a 12:12 h light/ dark regime in the f/2 medium. Phytoplankton cell size (n = 30) and copepod prosome length (n = 80) were measured from digital pictures using the software Visilog Expert 6.300 (Table 2.1). The carbon content of microalgae and copepods were estimated using the equations of, respectively, Smayda (1978) and van der Lingen (van der Lingen, 2002) (Table 2.1).

Table 2.I - Size and estimated carbon content of *Centropages chierchiae* and of the prey species used. Carbon content was estimated from the equations provided by van der Lingen (van der Lingen, 2002) and Smayda (Smayda, 1965) for *C. chierchiae* and microalgae, respectively. [a] Prossome length, [b] Mean diameter, [c] Minor and major axes.

Species	**Size (µm)**	**Carbon content (µg)**
Centropages chierchiae adult Female	1545.6 ± 80.89SE[a]	17.3
Centropages chierchiae adult Male	1507.2 ± 49.72SE[a]	16.1
Dytilum brightwellii	50[b]	5.1×10^{-3}
Rhodomonas baltica	8[b]	7.8×10^{-5}
Gymnodinium sp.	14[b]	2.2×10^{-4}
Phaeodactylum tricornutum	17 x 2.3[c]	1.5×10^{-5}

2.2.2 Unialgal feeding experiments and temperature effects

Unialgal experiments were conducted at four different temperatures (8, 13, 19 and 24ºC), using as prey *Gymnodinium* sp. and *Phaeodactylum tricornutum* at concentrations between 29.0 and 1790.0 cells mL^{-1}, equivalent to 6.4 to 393.8 µg C L^{-1}. These food concentrations were chosen with the aim of mimicing the natural range of dinoflagellate and

diatom densities typically experienced by *Centropages chierchiae* during summer and autumn in the waters off the central western Portuguese coast, as reported by Silva et al. (2009). In the case of the experiments conducted at 19ºC, the upper bound of the range was further expanded to food concentrations (6.4 and 457.5 µg C L^{-1}) higher than those found in the wild, to be able to describe the functional response of this species at the temperature when its abundance peaks in Iberian waters.

The copepods and microalgae were acclimated to the experimental temperature and light conditions for at least 24 h prior to the beginning of the feeding experiments. Experiments were conducted in 600-mL Pyrex screw-cap bottles filled with filtered seawater enriched with the f/2 medium, which were amended with aliquots of the prey stock cultures in order to achieve the desired experimental concentrations. Five bottles were set for each prey concentration, two acting as control bottles (no copepods added) and three acting as experimental ones (in which three adult females and two adult males were added); the sex ratio used in the incubations is similar to the average sex ratio of adult copepods in the sea, as reported by Hirst and Kiørboe (2002). The bottles were rotated at 1 rpm on a plankton wheel, and incubation ended after 24 h. Phytoplankton samples were collected at the start and end of the experiments, preserved with 2% Lugol's solution and counted by triplicate using a Sedgewick-Rafter counting chamber under an inverted microscope. The number of dead and live copepods at the end of the incubation was also recorded.

2.2.3 Plurialgal feeding experiments and selective behaviour

The plurialgal feeding experiments were conducted by offering the copepods simultaneously four prey species (*Gymnodinium* sp., *Phaeodactylum tricornutum*, *Rhodomonas baltica* and *Ditylum brightwellii*), at three prey mixture concentrations (respectively 56.1, 454.7 and 1107.9 µg C L^{-1}). These experiments were conducted only at 19ºC, and the remaining procedures were similar to the ones followed for the unialgal experiments, using two control and three experimental (three females and two males added) bottles for each mixture concentration tested.

2.2.4 Data analysis

Calculations of average food concentration, clearance and ingestion rates followed the equations of Frost (1972). Weight-specific ingestion rates were estimated as daily ration (i.e. percentage of body mass ingested daily), taking into account the carbon content of the microalgae and the copepod. Prior to calculations, we compared the prey growth rates between experimental (prey with added copepods) and control (prey without copepods) bottles using t-tests. Only when (apparent) growth rates were significantly lower in the experimental bottles were calculations done (i.e. feeding took place). An exponential decay equation was fitted to the functional response of clearance rates

$$F = F_{max} \times e^{-bC}$$

where F_{max} is the maximum clearance rate, b a preyspecific constant and C the food concentration (µg C L^{-1}).

In the case of ingestion rates, the functional response was fitted to the Ivlev equation (Ivlev, 1961)

$$I = I_{max} \times (1 - e^{-aC})$$

where I_{max} is the maximum ingestion rate, a is a constant describing the rate at which ingestion I approaches the maximum, and C is the food concentration (µg C L^{-1}). In both cases, fitting was done on non-transformed data using an iterative non-linear regression routine. Additionally, to compare the ability of *Centropages chierchiae* to approach saturation under the different diets, we estimated the half-saturation rate concentration (Isari et al., 2011), which is the concentration at which ingestion equals half of the ingestion maximum rate:

$$\frac{CI_{max}}{2} = \frac{\ln(0.5)}{-\alpha}$$

In the unialgal experiments, differences between the feeding rates obtained with the two algae and the four temperatures tested were determined using the analysis of covariance (ANCOVA). The ANCOVA was constructed with the initial prey concentration, prey type (*Gymnodinium* or *Phaeodactylum*) and temperature (8, 13, 19, 24ºC) as predictor variables, and ingestion rate as dependent variable (using both per individual and carbon-specific rates). The comparison of feeding rates under different temperature regimes was done using data only from the common range of initial prey concentrations (i.e. it did not include the extended range of food concentrations used in the experiments conducted at 19ºC). Multiple comparisons of means between pairs of categories were tested *a posteriori* using the Tukey test for unequal sample sizes at a confidence level of $P < 0.05$.

To evaluate if there was prey selection in the plurialgal experiments, selectivity was determined using the Vanderploeg and Scavia relativized electivity index (E') (Vanderploeg and Scavia, 1979), calculated as

$$Ei = \frac{Wi - 1/n}{Wi + 1/n}$$

where n is the number of prey types, i is prey type i, and Wi is the selectivity coefficient for prey type i calculated as:

$$Wi = \frac{Ri/Pi}{\sum_{i=1}^{n} Ri/Pi}$$

where Ri is the proportion of prey type i ingested, and Pi is proportion of prey type i in the water. Ei values range between +1 and -1; values close to 1 indicate positive selection, close to 0 represent 'random' selection and negative values close to -1 indicate avoidance.

All graphics and statistical analyses were performed using the open source software R version 2.9.2 (R Development Core Team; www.r-project.org).

2.3 Results

2.3.1 Unialgal feeding experiments and temperature effects

The clearance rates of *Centropages chierchiae* in the unialgal experiments, fed either *Gymnodinium* sp. or *Phaeodactylum tricornutum*, were very variable and did not show a clear relationship with food availability, ranging from 6.3 to 150.0 mL cop^{-1} day^{-1}. Maximum clearance rates increased from 8 to 19ºC (69.3 ± 11.82, 93.9 ± 11.71 and 116.1 ± 10.47 SE mL cop^{-1} day^{-1} at, respectively, 8, 13 and 19ºC) and decreased at 24ºC (62.1 ± 6.14 mL cop^{-1} day^{-1}) (Fig. 2.1). Maximum clearance rates were all significantly different (*t*-tests using the mean and SE obtained from the iterative non-linear regressions at $P < 0.005$). Ingestion rates of *C. chierchiae* in the unialgal experiments ranged between 0.2 and 28.9 µg C cop^{-1} day^{-1} (Fig. 2.2) and increased linearly with food concentration at all temperatures tested. Maximum individual ingestion rates were 24.4 ± 2.59, 20.9 ± 3.05, 32.1 ± 1.83 and 7.5 ± 0.73 SE µg C day^{-1} at, respectively, 8, 13, 19 and 24ºC (Fig. 2.2). Maximum ingestion rates were all significantly different (*t*-tests using the mean and SE obtained from the iterative non-linear regression at $P < 0.005$) except those obtained at 8 and 13ºC.

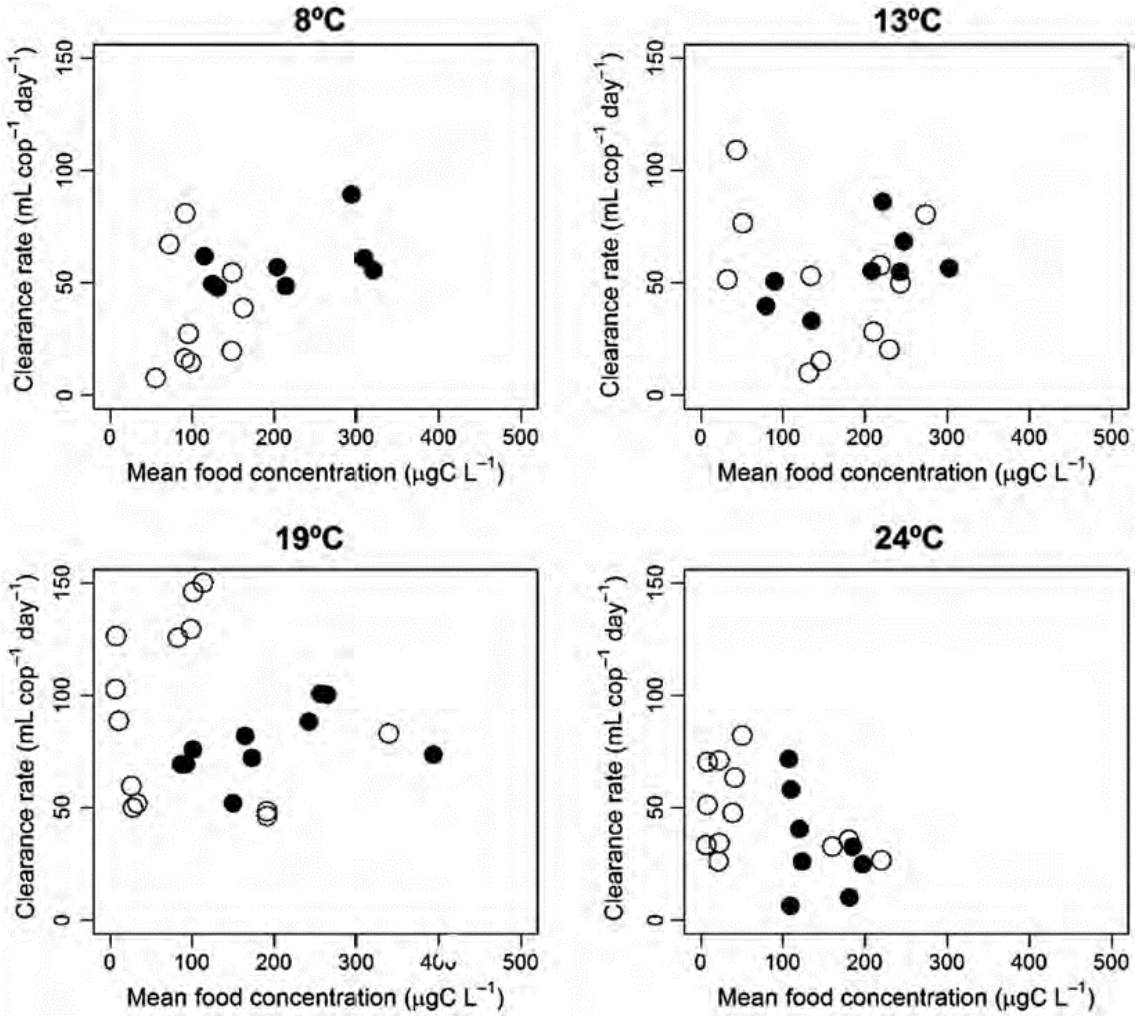

Fig. 2.1 - Clearance rates (mL cop^{-1} day^{-1}) of *Centropages chierchiae* feeding on *Phaeodactylum tricornutum* (filled circles) and *Gymnodinium* sp. (open circles) in the experiments conducted at 8, 13, 19 and 24ºC.

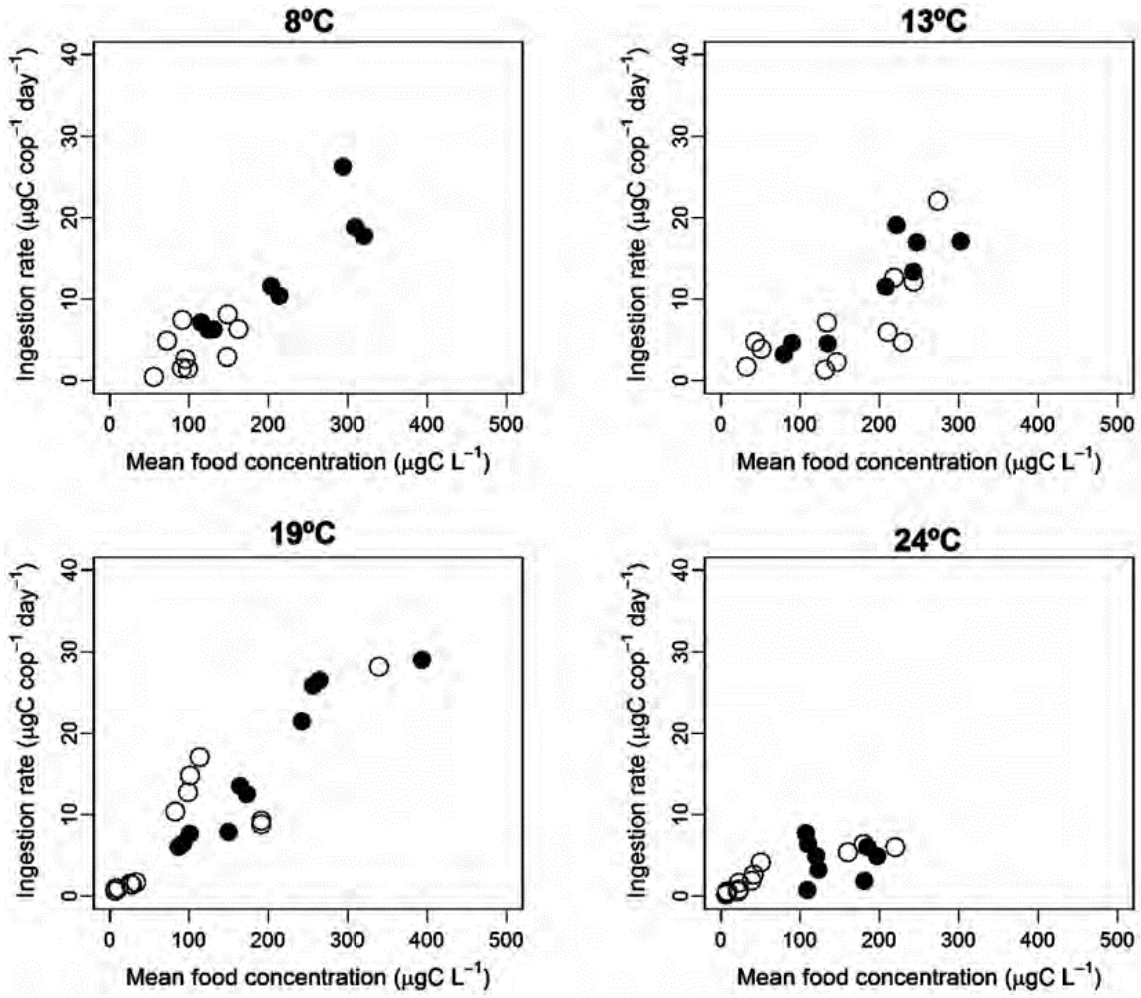

Fig. 2.2 - Ingestion rates (µg C cop^{-1} day^{-1}) of *Centropages chierchiae* feeding on *Phaeodactylum tricornutum* (filled circles) and *Gymnodinium* sp. (open circles) in the experiments conducted at 8, 13, 19 and 24ºC.

The ingestion rates of *Centropages chierchiae* on *Gymnodinium* sp. and *Phaeodactylum tricornutum* in the unialgal experiments conducted at four temperatures showed no significant effect of prey type, whereas there were significant differences for temperature and prey concentration (Table 2.2). There was also no significant interaction between temperature and food concentration (ANCOVA test, F = 1.239, *P* = 0.301), indicating that the slopes of the linear relationship between food concentration and ingestion were similar across temperatures. Ingestion rates obtained at 19ºC were significantly higher than those achieved at the other temperatures, which did not show significant differences among them (a posteriori Tukey test, Fig. 2.3).

Table 2.2 - ANCOVA analysis of the effect of prey concentration (µg C L^{-1}), temperature and prey type (*Gymnodinium* sp. and *Phaeodactylum tricornutum*) on the ingestion and clearance rates of *Centropages chierchiae*.

Response variable	**Independent variables**	**DF**	**F-value**	***P*-value**
Ingestion rate	Initial concentration (µgC L^{-1})	1	338.1	<0.0001
	Temperature	3	11.2	<0.0001
	Prey type	1	0.3	0.6164
Clearance rate	Initial concentration (µgC L^{-1})	1	338.1	0.9860
	Temperature	3	11.2	<0.0001
	Prey type	1	0.3	0.9674

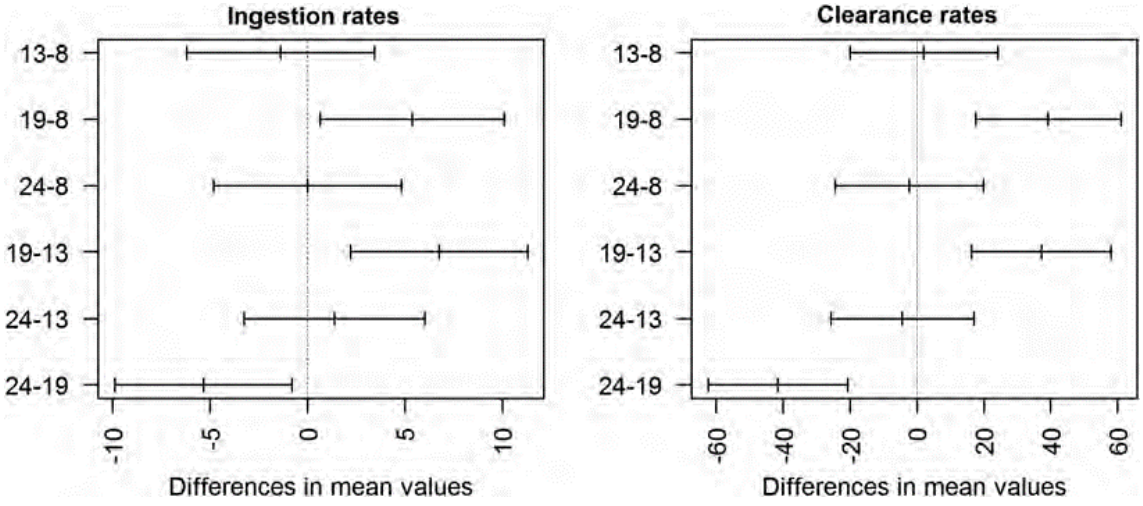

Fig. 2.3 - Pairwise comparison of ingestion (left panel) and clearance rates (right panel) between each level of the factor temperature (8, 13, 19, 24ºC). Differences in mean values and 95% confidence intervals of the Tukey test for unequal sample size are shown. A given pairwise comparison of means is significant if the confidence intervals do not overlap the 0 difference. Only data from experiments conducted at food concentrations < 410.0 µg C L^{-1} are used.

Similarly to ingestion rates, clearance rates of *C. chierchiae* showed no significant differences between prey types and significant effects of temperature, with higher clearance rates at 19ºC when compared with the other temperatures; in this case, however, no significant relationship with food concentration was found (Table 2.2, Fig. 2.3).

As no differences were found regarding prey type (*Gymnodinium* vs. *Phaeodactylum tricornutum)*, the broader range data set for feeding rates conducted at 19ºC was analysed by pooling data for both prey types (Fig. 2.4). The maximum clearance rate was 150 mL^{-1} cop^{-1} day^{-1}, whereas at prey concentrations > 400 µg C L^{-1} ingestion rates reached a plateau (Imax: 37.9+ 3.58 SE µg C cop^{-1} day^{-1}). The half-saturation concentration was 206 µg C L^{-1}.

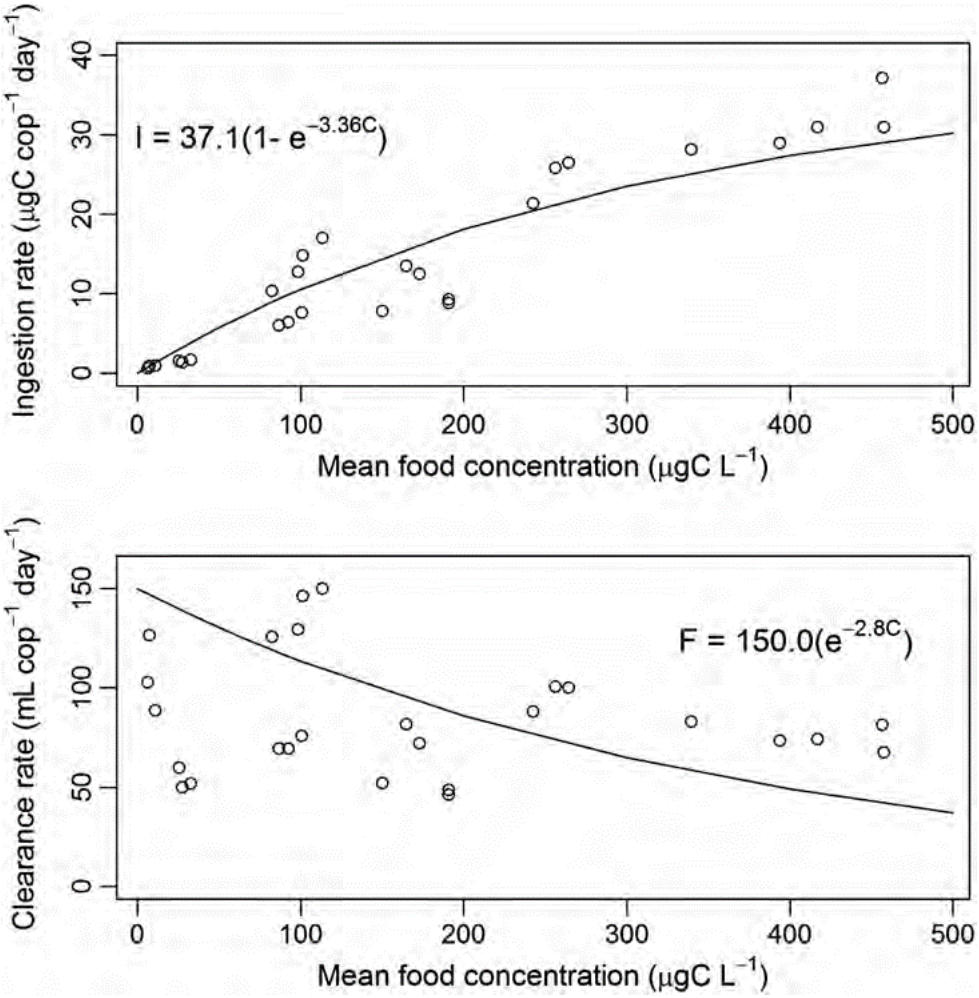

Fig. 2.4 - Functional feeding response of *Centropages chierchiae* at 19ºC using *Gymnodinium* and *Phaeodactylum tricornutum* as prey. Data of both algae are pooled since no significant differences were found of the feeding rates for both algae (Table 2.2). Ingestion (I; µg C cop^{-1} day^{-1}) in the upper panel, and clearance (F; mL cop^{-1} day^{-1}) in the lower panel.

In terms of daily rations, feeding rates varied between 1.2 and 183.5% body carbon day^{-1} (Fig. 2.5). Similarly to the per-individual feeding rates, daily rations showed no significant effect of prey type (ANCOVA test, $P = 0.63$), whereas significant effects of prey concentration ($P < 0.0001$) and temperature ($P < 0.0001$) were found. Daily ration was significantly higher at 19ºC and similar among the other temperatures tested (Fig. 2.5; Tukey test, data not shown).

Mean daily rations (± SE) estimated within the range of prey concentrations common to all temperatures (< 410 µgC L^{-1}) were 48.7 ± 41.38, 52.4 ± 38.33, 73.2 ± 48.83 and 19.5 ± 19.56 % body carbon ingested day^{-1} at, respectively, 8, 13, 19 and 24ºC.

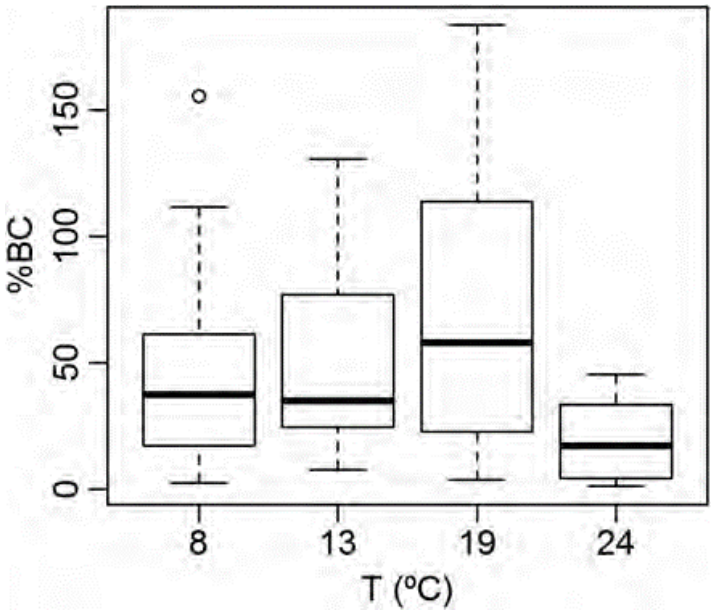

Fig. 2.5 - Box-plots of *Centropages chierchiae* weight-specific ingestion rates expressed as daily ration (%BC, % of body carbon ingested day^{-1}) at the four different temperatures studied (only data from experiments conducted at food concentrations < 410.0 µg C L^{-1} are shown). Median values are indicated by the horizontal line in the boxes.

2.3.2 Plurialgal feeding experiments and selective behaviour

When offered a mixture of different prey types, the total carbon ingestion rates of *Centropages chierchiae* adults varied from 4.7 ± 1.56 µg C cop^{-1} day^{-1} at the lowest concentration used (56.1 µg C L^{-1}) to 18.2 ± 3.03 µg C cop^{-1} day^{-1} at the intermediate concentration (454.7 µg C L^{-1}) and up to 85.6 ± 15.31 µg C cop^{-1} day^{-1} when at the highest prey concentration (1107.9 µg C L^{-1}) (Table 2.3). Maximum clearance rates (159 ± 87.20 µL cop^{-1} day^{-1}) were obtained at the low concentration treatment when fed the diatom *Ditylum brightwellii*. Regarding the electivity index, there was a shift in selectivity patterns as food concentration increased (Fig. 2.6). In the experiments performed at the lowest food concentration, the large diatom *Dytilum brightwellii* was selected over the other smaller algae; however, at the intermediate and high food concentrations, the electivity index for *D.*

brightwellii indicated no selection or weak avoidance (below threshold line; Fig. 2.6). The other prey types were not consistently selected nor avoided at any of the prey concentrations used (Fig. 2.6).

Table 2.3 - Ingestion (I; µg C cop^{-1} day^{-1}) and clearance rates (F; mL cop^{-1} day^{-1}) of *Centropages chierchiae* in the plurialgal experiment. Total and prey-specific food concentrations (µg C L^{-1}) are shown. rho: *Rhodomonas baltica*; gym: *Gymnodinium* sp.; pha: *Phaeodactylum tricornutum*; dyt: *Dytilum brightwellii*.

Total prey concentration	**Prey**	**Species-specific prey concentration**	**I**	**F**
56.1 ± 3.39	Dyt	13.6 ± 6.25	2.4 ± 1.61	159. ± 87.20
	Gym	3.3 ± 0.50	0.2 ± 0.12	56 ± 30.79
	Pha	37.8 ± 3.43	2.1 ± 0.25	54 ± 1.89
	Rho	1.43 ± 0.14	0.1 ± 0.09	58 ± 56.37
454.7 ± 12.99	Dyt	128.6 ± 6.25	2.5 ± 0.23	19 ± 3.00
	Gym	41.1 ± 0.50	1.4 ± 0.67	33 ± 13.66
	Pha	268.0 ± 5.81	13.9 ± 3.34	51 ± 13.40
	Rho	16.99 ± 0.14	0.4 ± 0.33	24 ± 19.89
1107.9 ± 237.08	Dyt	297.8 ± 46.31	18.8 ± 5.60	63 ± 14.86
	Gym	229.7 ± 123.02	17.6 ± 8.25	88 ± 59.02
	Pha	380.1 ± 22.62	29.0 ± 8.81	75 ± 18.21
	Rho	200.2 ± 160.2	20.2 ± 11.66	121 ± 87.37

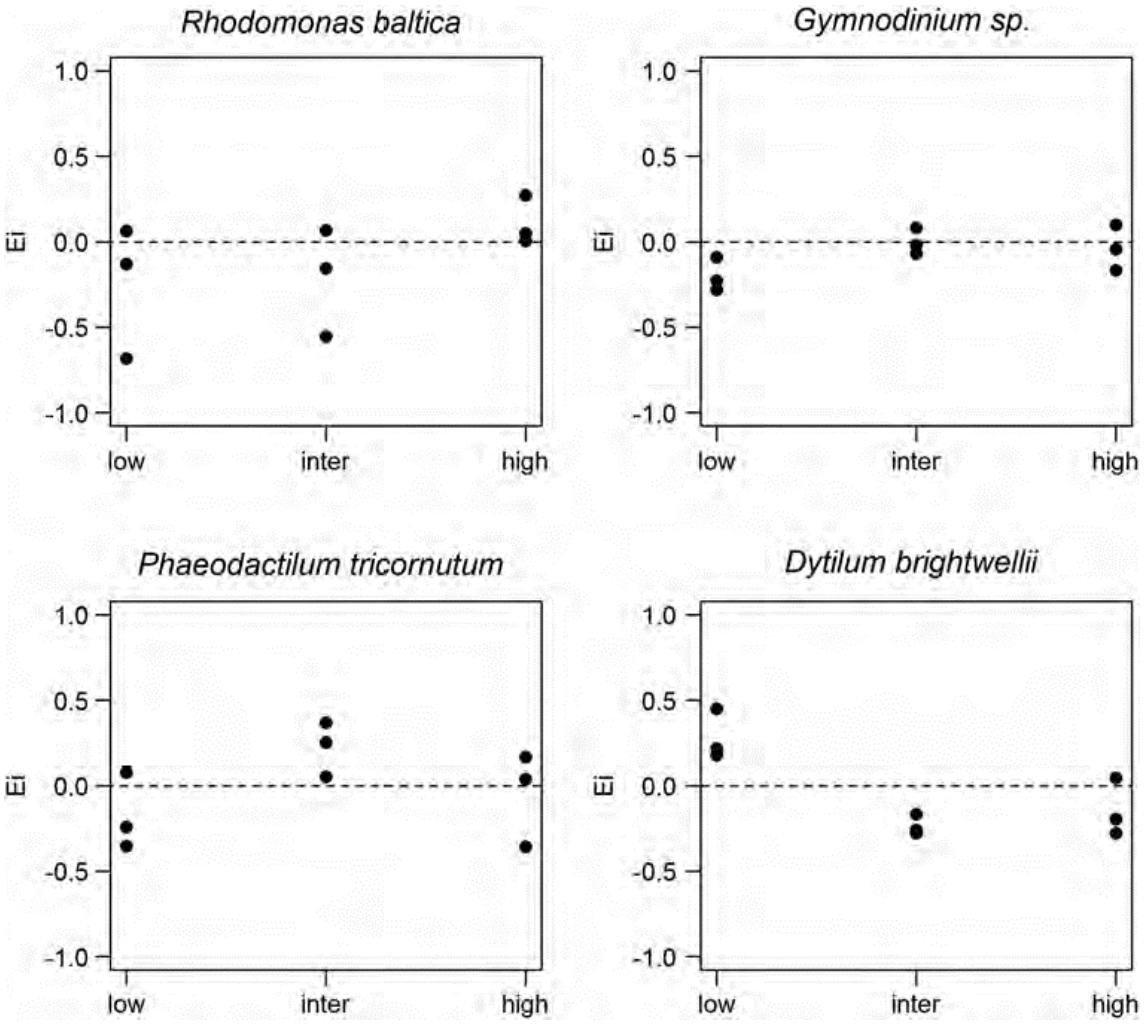

Fig. 2.6 - Electivity index (Ei) for the (plurialgal) selective feeding experiment, using a mixed assemblage of four prey (*Ditylum brightwellii*, *Gymnodinium* sp., *Phaeodactylum tricornutum* and *Rhodomonas baltica*) at three total prey concentrations (low: 56.1 ± 3.39, inter: 454.7 ± 12.99 and high: 1107.9 ± 237.08 µg C L^{-1}). Each prey level has three replicates. The dotted line represents the threshold level (i.e. *Ei* = 0) at which a given prey is neither selected nor avoided.

2.4 Discussion

2.4.1 Feeding rates

The feeding rates of *Centropages chierchiae*, in terms of carbon ingested per individual, obtained in this work ranged from 0.2 to 37.9 µg C ind^{-1} day^{-1}. The mean ingestion rate across temperatures was 3.0 µg C ind^{-1} day^{-1} at low food concentrations (< 100 µg C L^{-1}) and 12.4 µg C ind^{-1} day^{-1} at high food concentrations (100 – 500 µg C L^{-1}). Our estimated ingestion rates are consistent with in situ determinations of *C. chierchiae* feeding rates at 11ºC in the Bay of

Biscay at (natural) low prey concentrations (Vincent and Hartmann, 2001), and slightly lower than those previously reported in the laboratory in experiments conducted at 15ºC (Schnack, 1983). The ingestion rates obtained here for *C. chierchiae* also fall within the range of values typically reported for similarly sized copepods (a copepod with body mass of 16 µg C, like *C. chierchiae*, is expected to eat 10–50 µg C ind^{-1} day^{-1}; Saiz and Calbet, 2011). In spite of the low food concentrations tested in our experiments (6.4 µg C L^{-1}), no zero feeding was observed, in agreement with the observations of Schnack (1983) who reported feeding by *C. chierchiae* at concentrations as low as 0.9 µg C L^{-1}.

The clearance rates of *Centropages chierchiae* obtained in the unialgal experiments were quite variable and ranged between 6.3 and 150.0 mL^{-1} cop^{-1} day^{-1}. The expected decline in clearance rate with increasing food availability was only observed for the 19ºC data set. At the other temperatures, the narrow range of (low) food concentrations used very likely precluded the identification of clearer declining trends. Overall, the clearance rates obtained in this study were lower than those reported by Schnack (1983) for *C. chierchiae* fed the diatom *Thalassiosira partheneia* (offered as single cell, 24–340 mL cop^{-1} day^{-1}) and the dinoflagellate *Scrippsiella trochoidea* (192–570 mL cop^{-1} day^{-1}) at low food concentrations (< 100 µg C L^{-1}). This was quite unexpected because *T. partheneia* given as single cells is much smaller (9 µm diameter) and *S. trochoidea* is similarly sized (20 µm diameter) to the prey we have used to describe the functional response of *C. chierchiae*. Moreover, our experiments were conducted at higher temperatures (19 and 24ºC) than the experiments described in Schnack (1983) (15ºC), and therefore one may expect higher rates in our study. The experiments described by Schnack (1983) only lasted 8 – 10 h during daylight, so daily rations might be underestimated, assuming that copepods might show a diel rhythm and eat more during the night (Saiz et al., 1992; Calbet et al., 1999). Differences in feeding rates between experiments can be explained by several factors such as the physiological condition and previous feeding history of the copepods, as well as the composition of the prey (Huntley, 1988), which are difficult to repeat between experiments. In the review by Calbet et al. (2007) on *Centropages* feeding, clearance rates obtained for congeneric species in different studies also showed highly variable results, even if restricted to the same species. For example, clearance rates of *Centropages typicus* reported in the literature can vary from a few mL up to 1221 mL cop^{-1} day^{-1} (Calbet et al., 2007). Using *Phaeodactylum tricornutum* as prey, Gaudy (1974) reported clearance rates in the range 0.5–2.5 mL cop^{-1} day^{-1} for *C. typicus* at 18ºC, which is much lower than our values; conversely, our

clearance rates are similar to those found for *C. typicus* feeding on the same algae in a different study (3.7–21 mL cop^{-1} day^{-1}; Tomasini and Mazza, 1978).

The range of daily ration values found in our study (1.2–183.5% body carbon ingested day^{-1}) reaches the upper bound of feeding rates reported for copepods feeding at intermediate and high concentrations of food (Saiz and Calbet, 2011). In the only previous feeding experiment conducted in the laboratory with *Centropages chierchiae*, Schnack (1983) determined maximum daily food intakes in the range of 29 to 56% body carbon. However, these results are difficult to compare with ours, as one would expect much lower rates since these experiments were conducted at very low food concentration (ca. 55 µg C L^{-1}). Also, the *C. chierchiae* used differed in size, having larger body mass (28 µg cop^{-1}) than the ones in our study (16.9 µg cop^{-1}). Regarding other congeneric species, our daily rations were slightly higher than those reported for *Centropages hamatus* (e.g. 85% body carbon ingested day^{-1} at 15ºC; Kiørboe et al., 1982), and similar to the ones reported for *Centropages typicus* [from ca. 50% body carbon ingested day^{-1} at low food concentrations to values up to 380% body carbon ingested day^{-1} at high food concentrations (Gaudy, 1974)]. In general, the feeding rates of *Centropages chierchiae* determined in this study are in the upper range of those previously determined for congeners and other copepod species (Calbet et al. 2007, Saiz and Calbet, 2011), which might represent an important advantage for *C. chierchiae* when competing for food with co-habiting copepod species.

2.4.2 Temperature dependence of feeding rates

In our study, feeding rates increased significantly with temperature up to 19ºC and then decreased at 24ºC, following a dome-shaped pattern (Fig. 2.5). Such patterns are the result of optimal temperature windows driven by species-specific physiological tolerance to temperature, and have been observed in several copepod species, both in the upper bound (too high temperature, e.g. *Calanus helgolandicus* and *Calanus finmarchicus*, Møller et al., 2012; *Temora stylifera*, Thébault, 1985) or the lower bound (*Oithona davisae*, Almeda et al., 2010b). Our results suggest that 19ºC falls in the range of optimal temperatures for *Centropages chierchiae*, likely as the result of an adaptation of the species to the prevailing habitat conditions. This hypothesis is indeed confirmed by the fact that this temperature corresponds

to the mean temperature in surface waters off western Iberia during the warm months when *Centropages chierchiae* peaks its abundance (Sobrinho-Gonçalves et al., 2013). In terms of temperature dependence, the Q_{10} values for maximum clearance rates obtained here for *Centropages chierchiae* (Q_{10} = 1.6) were similar to those described for *Acartia hudsonica* (Q_{10} = 1.8; Durbin and Durbin, 1992), lower than the Q_{10} = 3.9 and Q_{10} = 2.45 obtained for the ingestion rates of, respectively, *Centropages hamatus* (Kiørboe et al., 1982) and *Oithona davisae* (Almeda et al., 2010a), and within the range of variation of the temperature dependence of ingestion rates for *Temora stylifera* (Q_{10}:1.2–4.4), *Calanus helgolandicus* (Q_{10}:0.78–4.3) and *Clausocalanus arcuicornis* (Q_{10}:1.28–2.56) (Fernández, 1978). In this regard, some of the very high Q_{10} values found in laboratory experiments reported in the literature might be a consequence of a response to rapid changes in temperature without enough time for conditioning (thermal stress). Empirical analyses of copepod feeding field data (Peters and Downing, 1984; Saiz and Calbet, 2011) have shown that, compared with other variables such as food concentration, temperature had a weaker control over copepod feeding rates, likely as a consequence of physiological adaptation to habitat condition. This fact may suggest that the populations invading the North Atlantic in recent years might be better adapted to colder waters than the population we worked with, caught off western Iberia. Examples of physiological adaptation in copepods have been well studied in the calanoid *Eurytemora affinis* (e.g. Ketzner and Bradley, 1982; Petersen and Lee, 2003). There is also evidence that co-existing copepod species, as demonstrated for some *Temora* and *Centropages* species, may have more similarities in their abundance, body size and reproduction cycles than congeners living in different habitats (Halsband-Lenk et al., 2004).

2.4.3 Plurialgal experiments and selective feeding

Feeding rates of copepods depend on a variety of factors such as body mass, temperature, food type, quality and concentration, and also previous feeding history (Mullin, 1963; Price and Paffenhöfer, 1985; Saiz and Calbet, 2011). Although copepod feeding has been a key subject in zooplankton research for decades, one of the aspects that is less studied is the mechanisms involved in prey selection (e.g. Cowles et al., 1988; DeMott, 1988), particularly when copepods are offered a variety of prey as occurs in nature (e.g. Vanderploeg et al., 1984). Field studies of copepod feeding, in which they are exposed to a range of natural prey, are

relatively scarce in the literature (Saiz and Calbet, 2007) and have the drawback that complex interactions between various factors such as prey concentration, size, motility and composition (Kleppel, 1993) are difficult to discern within a natural assemblage. On the other hand, laboratory experiments of copepod feeding are typically conducted with single prey suspensions, and very few studies have used multiple prey approaches (e.g. Donaghay and Small, 1979; Kiørboe et al., 1996; Meyer et al., 2002). Generally, copepods are described as being able to selectively ingest large cells over small cells (e.g. Frost, 1972). In this regard, Vincent and Hartmann (2001) reported that in feeding incubation experiments with natural prey assemblages, *Centropages chierchiae* cleared large ciliates (< 40 µm) at higher rates than small ciliates. In our study, however, we have found that across a range of prey sizes between 5 and 50 µm, the pattern of preysize selectivity for this copepod species depended on prey abundance. For instance, when food availability was low *C. chierchiae* cleared the largest prey (*Ditylum brightwellii*) at higher rates than the smaller cells, in agreement with the observations by Vincent and Hartmann (2001). However, at intermediate and high prey concentrations, there was no clear positive selectivity for any of the prey. Food-concentration-dependent selectivity patterns have also been described for *Centropages brachiatus* (Cowles, 1979) and the group *Para-Pseudocalanus* (Fileman et al., 2007). The fact that in our experiments *Rhodomonas baltica* was, in most cases, not positively selected, is probably a consequence of its small size, likely below optimum, in agreement with a general preference for > 10 µm prey reported for the congeneric *Centropages typicus* (Tomasini and Mazza, 1978).

General patterns of copepod feeding show that ciliates are strongly selected prey, followed by dinoflagellates, while selective feeding patterns on diatoms may range from negative to positive selection (Saiz and Calbet, 2011). Vincent and Hartmann (2001) also observed that *Centropages chierchiae* cleared dinoflagellates (4.9 mL cop^{-1} day^{-1}) and ciliates (4.3 mL cop^{-1} day^{-1}) at higher rates than phytoplankton cells (0.7 mL cop^{-1} day^{-1}); these authors also highlighted the preference for dinoflagellates exhibited by *C. chierchiae* when compared with parallel incubations conducted with *Calanus helgolandicus* and *Temora longicornis*. In this regard, Schnack (1983) also found that the clearance rates of *C. chierchiae* were higher for the dinoflagellate *Scrippsiella trochoidea* than for the diatom *Thalassiosira partheneia*; we think that this latter result, however, might be biased by the larger size of the dinoflagellate in that study. Our results did not support any preference for dinoflagellates over other chlorophyll pigmented cells by *C. chierchiae*, and actually showed that similarly sized diatoms and dinoflagellates were cleared at similar rates. From an ecological point of view, diatoms

represent the major component of the phytoplankton biomass available from the summer months to early autumn in the productive upwelling region off the western Iberian Peninsula (Silva et al., 2009), when *C. chierchiae* abundance peaks (Sobrinho-Gonçalves et al. 2013). Therefore, it would make sense that this copepod species might benefit from the high abundance of diatoms in that period of the year. In fact, in productive ecosystems, the contribution of diatoms to the diet of copepods is higher than in oligotrophic environments, where it is considered to be very low (Saiz and Calbet, 2011).

2.5 Conclusions

This study has improved our understanding of the functional response and feeding behaviour of the calanoid copepod *Centropages chierchiae*, particularly under food concentrations similar to the ones experienced in situ. Our results show that this copepod species has high feeding rates for both diatoms and dinoflagellates, and that its selective-feeding behaviour is dependent on food concentration, larger prey being selected only at low food concentrations. Feeding rates were temperature dependent, showing a dome-shaped response with optimal temperature values at 19ºC, similar to the mean temperature in surface waters off western Iberia during the warm months when *C. chierchiae* peaks in abundance.

2.6 Acknowledgements

This work was funded by the Portuguese Science and Technology Foundation (FCT) under project VITAL (PTDC/MAR/111304/2009). The project MODELA (FCT PTDC/MAR/098643/2008) also partially funded this work. S.G. is supported by FCT through a Post-Doctoral Fellowship (SFRH/BPD/38332/2007) and J.C through a Doctoral Fellowship (SFRH/BD/28198/2006). E.S. is supported by project (CTM2011-23480) from the Spanish Ministry of Economy and Competitively. The authors wish to thank the Oceana´rio de Lisboa and IPMA (former INRBIPIMAR) for helping collecting the copepods. Thanks are due to M.A. Chícharo and E. Serrão for providing the facilities to conduct the experiments at the lowest temperatures and to S. Palma for helping culture the microalgae. The microalgae Rhodomonas baltica and Ditylum brightwellii were provided by the Assemble Program (grant 227799)

funded under the European Community - Research Infrastructure Action under the FP7 'Capacities'.

2.7 References

Almeda, R., Augustin, C., Alcaraz, M., Calbet, A., Saiz, E. (2010a) Feeding rates and gross growth efficiencies of larval developmental stages of *Oithona davisae* (Copepoda, Cyclopoida). *Journal of Experimental Marine Biology and Ecology*, 387: 24-35.

Almeda, R., Calbet, A., Alcaraz, M., Yebra, L., Saiz, E. (2010b) Effects of temperature and food concentration on the survival, development and growth rates of naupliar stages of *Oithona davisae* (Copepoda, Cyclopoida). *Marine Ecology Progress Series*, 410: 97-109.

Beaugrand, G., Brander, K.M., Lindley, J.A., Souissi, S. and Reid, P.C. (2003) Plankton effect on cod recruitment in the North Sea. *Nature*, 426: 661-664.

Calbet, A., Carlotti, F., Gaudy, R. (2007) The feeding ecology of the copepod Centropages typicus (Kroyer). *Progress in Oceanography*, 72: 137-150.

Calbet, A., Saiz, E., Irigoien, X., Alcaraz, M., Trepat, I. (1999) Food availability and diel feeding rhythms in the marine copepods *Acartia grani* and *Centropages typicus*. *Journal of Plankton Research*, 21: 1009-1015.

Carlotti, F., Rey, C., Javanshir, A., Nival, S. (1997) Laboratory studies on egg and faecal pellet production of *Centropages typicus*: Effect of age, effect of temperature, individual variability. *Journal of Plankton Research*, 19: 1143-1165.

Cowles, T.J. (1979) The feeding response of copepods from the Peru upwelling system: food size selection. *Journal of Marine Research*, 37: 601-622.

Cowles, T.J., Olson, R.J., Chisholm, S.W. (1988) Food selection by copepods: discrimination on the basis of food quality. *Marine Biology*, 100: 41-49.

DeMott, W.R. (1988) Discrimination between algae and artificial particles by freshwater and marine copepods. *Limnology and Oceanography*, 33: 397-408.

Donaghay, P.L., Small, L.F. (1979) Food selection capabilities of the estuarine copepod *Acartia clausi*. *Marine Biology*, 52: 137-146.

Durbin, E.G., Durbin, A.G. (1992) Effects of Temperature and Food Abundance on Grazing and Short-Term Weight Change in the Marine Copepod *Acartia hudsonica*. *Limnology and Oceanography*, 37: 361-378.

Fernández, F. (1978) Metabolismo y alimentación en copépodos planctónicos del Mediterráneo: Respuesta a la temperatura. *Investigación Pesquera*, 42: 97-139.

Fernández, F. (1979) Particle selection in the nauplius of *Calanus pacificus*. *Journal of Plankton Research*, 1: 313-328.

Fileman, E., Smith, T., Harris, R. (2007) Grazing by *Calanus helgolandicus* and *Para-Pseudocalanus* spp. on phytoplankton and protozooplankton during the spring bloom in the Celtic Sea. *Journal of Experimenal Marine Biology and Ecology*, 348: 70-84.

Frost, B.W. (1972) Effects of size and concentration of food particles on the feeding behavior of the marine planktonic copepod *Calanus pacificus*. *Limnology and Oceanography*, 17: 805-815.

Garrido, S., Ben-Hamadou, R., Oliveira, P.B., Cunha, M.E., Chícharo, M.A., van der Lingen, C.D. (2008) Diet and feeding intensity of sardine *Sardina pilchardus*: correlation with satellite-derived chlorophyll data. *Marine Ecology Progress Series*, 354: 245-256.

Gaudy, R. (1974) Feeding four species of pelagic copepods under experimental conditions. *Marine Biology*, 25: 125-141.

Gentleman, W., Leising, A., Frost, B., Strom, S., Murray, J. (2003) Functional responses for zooplankton feeding on multiple resources: a review of assumptions and biological dynamics. *Deep-Sea Research Part II*, 50: 2847-2875.

Halsband-Lenk, C., Carlotti, F., Greve W. (2004) Life-history strategies of calanoid congeners under two different climate regimes: a comparison. *ICES Journal of Marine Science*, 61: 709-720.

Henriksen, C.I., Saiz, E., Calbet, A., Hansen, B.W. (2007) Feeding activity and swimming patterns of *Acartia grani* and *Oithona davisae* nauplii in the presence of motile and non-motile prey. *Marine Ecology Progress Series*, 331: 119-129.

Hirst, A.G., Kiørboe, T. (2002) Mortality of marine planktonic copepods: global rates and patterns. *Marine Ecology Progress Series*, 230: 195-209.

Huntley, M. (1988) Feeding biology of *Calanus*: a new perspective. *Hydrobiologia*, 167/168: 83-99.

Ivlev, V.S. (1961) Experimental Ecology of Feeding Fishes. Yale University Press, New Haven.

Ketzner, P.A., Bradley, B.P. (1982) Rate of environmental change and adaptation in the copepod *Eurytemora affinis*. *Evolution*, 36: 298-306.

Kiørboe, T., Møhlenberg, F., Nicolajsen, H. (1982) Ingestion rate and gut clearance in the planktonic copepod *Centropages hamatus* (Liljeborg) in relation to food concentration and temperature. *Ophelia*, 21: 181-194.

Kiørboe, T., Saiz, E., Viitasalo, M. (1996) Prey switching behaviour in the planktonic copepod *Acartia tonsa*. *Marine Ecology Progress Series*, 146: 65-75.

Kleppel, G.S. (1993). On the diets of calanoid copepods. *Marine Ecology Progress Series*, 99: 183-195.

Lindley, J.A., Daykin, S. (2005) Variations in the distributions of *Centropages chierchiae* and *Temora stylifera* (Copepoda: Calanoida) in the north-eastern Atlantic Ocean and western European shelf waters. *ICES Journal of Marine Science*, 62: 869-877.

Meyer, B., Irigoien, X., Graeve, M., Head, R., Harris, R. (2002) Feeding rates and selectivity among nauplii, copepodites and adult females of *Calanus finmarchicus* and *Calanus helgolandicus*. *Helgoland Marine Research*, 56: 169-176.

Møller, E.F., Maar, M., S.H. Jónasdóttir, S.H., Nielsen, T.G., Tönnesson K. (2012) The effect of changes in temperature and food on the development of *Calanus finmarchicus* and *Calanus helgolandicus* populations. *Limnology and Oceanography*, 57: 211-220.

Mullin, M. (1963) Some factors affecting the feeding of marine copepods of the genus *Calanus*. *Limnology and Oceanogr*aphy, 8: 239-250.

Peters, R.H., Downing, J.A. (1984) Empirical analysis of zooplankton filtering and feeding rates. *Limnology and Oceanography*, 29: 763-784.

Petersen, C.H., Lee, C.E. (2003) Effects of developmental acclimation on adult salinity tolerance in the freshwater-invading copepod *Eurytemora affinis*. *Physiological and Biochemical Zoology*, 76: 296-301.

Plouvenez, S., Champalbert, G. (1999) Feeding Behaviour and Trophic Environment of *Engraulis encrasicolus* (L.) in the Bay of Biscay. *Estuarine, Coastal and Shelf Science*, 433: 177-191.

Price, H.J., Paffenhöfer, G.-A. (1985) Perception of food availability by calanoid copepods. *Archiv für Hydrobiologie–Beiheft, Ergebnisse der Limnologie*, 21: 115-124.

Razouls, C. (1996) Diversité et répartition geographique chez les copépodes pelagiques. 1. Calanoida. *Annales de l'Institut Oceanographique, Paris*, 71: 81-404.

R Development Core Team. (2011) R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/.

Richardson, A.J. (2008) In hot water: zooplankton and climate change. *ICES Journal of Marine Science*, 65: 279-295.

Saiz, E., Calbet, A. (2007) Scaling of feeding in marine calanoid copepods. *Limnolology and Oceanography*, 52: 668-675.

Saiz, E., Calbet, A. (2011) Copepod feeding in the ocean: scaling patterns, composition of their diet and the bias of estimates due to microzooplankton grazing during incubations. *Hydrobiologia*, 666: 181-196.

Saiz, E., Rodríguez, V., Alcaraz, M. (1992) Spatial distribution and feeding rates of *Centropages typicus* in relation to frontal hydrographic structures in the Catalan Sea (Western Mediterranean). *Marine Biology*, 112: 49-56.

Schnack, S.B. (1983) On the feeding of copepods on Thalassiosira partheneia from the Northwest African upwelling area. *Marine Ecology Progress Series*, 11: 49-53.

Silva, A., Palma, S., Oliveira, P.B., Moita, M.T. (2009) Composition and interannual variability of phytoplankton in a coastal upwelling region (Lisbon Bay, Portugal). *Journal of Sea Research*, 62: 238-249.

Smayda, T.J. (1978) From phytoplankters to biomass. In Phytoplankton Manual. Sournia, A. (Ed.). Unesco Monographs on Oceanographic Methodology, 6: 273-279.

Sobrinho-Gonçalves, L., Moita, M.T., Garrido, S., Cunha, M.E. (2013) Environmental forcing on the interactions of plankton communities across a continental shelf in the Eastern Atlantic upwelling system. *Hydrobiologia*, 713: 167-182.

Thebault, J.M., (1985) Etude expérimentale de la nutrition d'un copépode commun (*Temora stylifera* Dana). Effets de la température et de la concentration de nourriture. *Journal of Experimental Marine Biology and Ecology*, 93: 223-234.

Tomasini, J.A., Mazza, J. (1978) Feeding habits of 2 calanoid copepods (*Centropages typicus* and *Acartia clausi*) in nutritive medium with single alga. *Journal du Conseil/ Conseil Permanent International pour l'Exploration de la Mer*, 38: 154-179.

Turner, J. (1984) The feeding ecology of some zooplankters that are important prey items of larval fish. *NOAA Technical Report NMFS*, 7: 1-28.

van der Lingen, C.D. (2002) Diet of sardine *Sardinops sagax* in the Southern Benguela upwelling ecosystem. *South African Journal of Marine Science*, 24: 301-316.

Vanderploeg, H.A., Scavia, D. (1979) Two electivity indices for feeding with special reference to zooplankton grazing. *Journal of the Fisheries Research Board of Canada*, 36: 362-365.

Vanderploeg, H.A., Scavia, D., Liebig, J.R. (1984) Feeding rate of *Diaptomus sicilis* and its relation to selectivity and effective food concentration in algal mixtures and in Lake Michigan. *Journal of Plankton Research*, 6: 919-941.

Vincent, D., Hartmann, H.J. (2001) Contribution of ciliated microprotozoans and dinoflagellates to the diet of three copepod species in the Bay of Biscay. *Hydrobiologia*, 443: 193-204.

Wilson, D.S. (1973) Food size selection among copepods. *Ecology*, 54: 909-914.

Chapter 3

Reproduction and respiration of a climate change indicator species: effect of temperature and variable food in the copepod *Centropages chierchiae*

Joana Cruz, Susana Garrido, Marta S. Pimentel, Rui Rosa, A. Miguel P. Santos, Pedro Ré

Journal of Plankton Research (2013) 35(5): 1046-1058

Reproduction and respiration of a climate change indicator species: effect of temperature and variable food in the copepod *Centropages chierchiae*

Abstract

The abundance of the calanoid copepod *Centropages chierchiae* has increased at the northern limits of its distribution in recent decades, mainly due to oceanic climate forcing, suggesting this as a key species in monitoring climate change. Laboratory experiments were conducted to study the combined effect of temperature, food type and concentration on the egg production rate (EPR) and hatching success (HS) of *C. chierchiae*. Females were fed on two monoalgal diets (*Gymnodinium* sp. and *Phaeodactylum tricornutum*) at two food concentrations and at three different temperatures (13, 19, 24ºC). Respiration rates of both genders were measured at four different temperatures (8, 13, 19, 24ºC). EPR was significantly different between temperatures and food concentrations, the maximum EPR being attained when the copepods were exposed to high food levels and at 19ºC. Prey type significantly influenced EPR; feeding on *P. tricornutum* resulted in higher egg production than *Gymnodinium* sp. HS was significantly lower at 13ºC than at 19 and 24ºC and higher with *Gymnodinium* sp. Respiration rates were sex independent and increased exponentially with temperature. To maintain the basal metabolism, the minimum food intake of *P. tricornutum* ranged between 0.4 and 1.8 µg C and for *Gymnodinium* sp. between 0.03 and 0.13 µg C. Food intake was always higher than the metabolic demands, except for the highest temperature tested (24ºC). The present results confirm the sensitivity of *C. chierchiae* to temperature variations and may help understanding the successful expansion of its distribution towards northern latitudes.

Keywords*: Centropages chierchiae,* reproduction, respiration, temperature, food

3.1 Introduction

Copepods are the dominant component of marine mesozooplankton and the major link between primary production and higher trophic levels (Mauchline, 1998). Furthermore, the fact that they have short life-cycles and are poikilotherms makes them good indicators of climate changes (Hays et al., 2005). Ocean temperature has increased over the past century (Levitus et al., 2000) and is expected to increase 3-5ºC over the next century (IPCC, 2007). The responses exhibited by zooplankton to global warming are among the most rapid and greatest of any marine or terrestrial group (Richardson, 2008). Copepods have shown changes in their phenology, geographic distribution, community composition, with likely effects on ecosystem functioning (Edwards and Richardson, 2004; Mackas et al., 2007; Beaugrand et al., 2009). In the eastern North Atlantic Ocean and European shelf seas, calanoid copepods have moved northwards with the increase in water temperature (Lindley and Daykin, 2005).

The calanoid copepod *Centropages chierchiae* Giesbrecht, 1889 occurs in tropical and subtropical waters, restricted to the eastern Atlantic, Mediterranean and western Indian Ocean (Razouls, 1996). Data collected by the Continuous Plankton Recorder (CPR) survey from 1958 to 1999 has shown that this species was distributed in the Northeast Atlantic and western European continental shelf (CPR survey team, 2004). In the early years of the last century, *C. chierchiae* records in the northernmost limit of its distribution, i.e. around 50º N (Lysholm et al., 1945) were very scarce. Conversely, in recent decades its abundance and frequency has increased considerably in the Bay of Biscay, Celtic Sea and English Channel. Lindley and Daykin (2005) suggested that *C. chierchiae* has become a resident population in the Celtic sea (latitudes between 48º and 51º N) persisting even during the colder months (6-8ºC) (Garcia-Soto and Pingree, 2009). This northward migration has been shown to be related to long-term changes in temperature, its abundance being positively correlated with the strength of the shelf edge current and negatively with the North Atlantic Oscillation. Therefore, this species has been suggested as a key species to better understand the climate changes effects (Lindley and Daykin, 2005).

Centropages chierchiae is reported to occur in higher abundance between 13 and 20ºC and to be absent (or present in low abundance) below 13ºC (Bonnet et al., 2007). In northern Portuguese shelf waters, it is one of the most abundant zooplankters in late summer, when upwelling events take place (Fiúza, 1983), while it occurs with lowest abundance in winter

(Morgado et al., 2003; Queiroga et al., 2005; Sobrinho-Gonçalves et al., 2013). A recent study has shown that *C. chierchiae* is widespread in the western Iberian shelf and offshore generally in the upper 75 m (Sobrinho-Gonçalves et al., 2013). It is present all year round although its abundance is lowest in winter (mean 20 ind. m^{-3}), it increases in spring and summer (mean 73 and 76 ind. m^{-3}, respectively), and peaks in late summer (mean 185 individuals m^{-3}). Mean temperature during summer months off western Iberia when *C. chierchiae* peaks and upwelling events take place are usually around 19ºC; the values increase to 24ºC only on the south Portuguese coast, where upwelling is weaker and less frequent. *C. chierchiae* is considered an important prey of commercially important small pelagic fish such as *Engraulis encrasicolus* and *Sardina pilchardus* (Plouvenez and Champalbert, 1999; Garrido et al., 2008), inhabiting Iberian Atlantic waters. Only a few studies have focused so far on aspects of the biology of *C. chierchiae*, related to feeding selectivity (Vincent and Hartmann, 2001), distribution variability (Lindley and Daykin, 2005) or thermal niche (Bonnet et al., 2007).

The aim of this study is to understand the physiological state of *C. chierchiae* under different environmental conditions and how this can relate to climate change. With this aim, we investigated the effects of temperature, food quality and quantity on egg production and hatching success, as well as the effect of temperature and gender on metabolic rates. Furthermore, metabolic energy demands were estimated at different temperatures in relation to prey concentration and compared with estimates of daily ration. To our knowledge, this is the first attempt to measure respiration rates and reproductive traits of *C. chierchiae*.

3.2 Materials and methods

3.2.1 Collection and organisms' cultures

Centropages chierchiae adults used in the reproduction experiments were the progeny of copepods collected off southwestern Portugal in July 2010. Experiments were conducted in October 2010 most likely with individuals from the second and third generations reared in the laboratory starting from the local population. Although it would be preferable to use wild populations, using the second or third generation acclimated to laboratory conditions may not have significantly impacted our results in terms of genetic modifications or adaptations to

laboratory conditions. The copepods used to measure respiration rates were captured a few days before the beginning of the experiments in the same area in October 2011. At the time of collection, the temperature was 18ºC and the salinity was 35. On both sampling dates, two surface plankton tows were performed using a WP-2 net (0.26 m^2 mouth opening, 200 μm mesh size) with a transparent plastic bag as a codend for collecting copepods with minor damage. Samples were immediately taken to the laboratory where *C. chierchiae* were sorted from the rest of the plankton and placed in a 30 L tank acclimated at 19ºC (Salinity of 33, 14L:10D light/dark regime) with gentle aeration. Before the experiments, copepods were fed daily with a mixture of *Rhodomonas baltica*, *Dunaliella* sp., *Phaeodactylum tricornutum* and *Gymnodinium* sp. given *ad libidum*, while during the reproduction experiments only *P. tricornutum* and *Gymnodinium* sp. were used. Before feeding the copepods, about 30 % of the water in the tank was siphoned to maintain water quality (by removing organic debris, reducing ammonia and replenish trace elements) through both 45 and 150 μm sieves to prevent the removal of any eggs, nauplii, copepodites or adults. The content of the 150 μm sieve, mostly copepodites and adults, was put back in the tank while the content of 45 μm sieve, mostly containing faecal pellets was eliminated. At least once per month, the content retained in the 45 μm sieve was observed under a dissecting microscope in order to separate all the eggs and nauplii and begin a new culture.

Algae cultures were kept in 10 L flasks and grown in 0.2 μm filtered and UV sterilized sea water enriched with F/2 medium, under the same illumination regime and temperature as the copepods. The algae were kept in the exponential growth phase by frequent dilution of the culture. Concentrations of algal cultures were estimated using a Sedgwick-Rafter counting chamber.

3.2.2 Reproduction experiments: effect of temperature and food

Experiments to quantify the reproductive rate of *C. chierchiae* consisted on estimating daily egg production rate (EPR) and hatching success (HS) of copepods fed with high and low concentrations of two different algae, *Gymnodinium* sp. (dinoflagellate, size ~ 14 μm) and *Phaeodactylum tricornutum* (diatom, size 17 x 2.3 μm) at three different temperatures (13, 19 and 24ºC) over a 5-day period. The two algae used have a similar size and were chosen because

previous feeding experiments showed they were an adequate food for the calanoid copepod *C. chierchiae* consumed with high and similar clearance rates (Garrido et al., 2013). Temperature range was chosen according to *C. chierchiae* thermal niche (Bonnet et al., 2007). In each temperature treatment, the two algae used as food were given separately at two concentrations (60 and 300 µg C L^{-1}). These concentrations were chosen to mimic the natural range of dinoflagellate and diatom densities typically experienced by *C. chierchiae* during summer and autumn in waters off central western Portuguese coast (Silva et al., 2009). Prior to the experiments, the copepods were starved and acclimated during 24 h to the temperature and experimental conditions. Reproduction parameters were measured using triplicates of 600 ml experimental bottles for each temperature x food type x concentration treatment, with four adult copepods (three females and one male) in each bottle. We tried to select ripe females to use in the experiments. The bottles were sealed with no head space and placed on a rotating (1.5 rpm) plankton wheel. On each experimental day, the water in the bottles was sieved with 150 and 45 µm sieves, in order to count eggs and adult copepods, and to estimate EPR and HS. Adult copepods were immediately transferred to the bottles with renewed 0.2 µm filtered and UV sterilized sea water (to reduce the level of contamination) and the corresponding algae species to continue the experiments. Eggs were placed in petri dishes with fresh water at the same treatment temperature. After 48 or 72 h, depending on the temperature (since hatching time is longer at lower temperatures), the contents were fixed with 4 % formaldehyde and nauplii were counted using a stereoscope microscope to determine the percentage of hatched eggs.

3.2.3 Respiration experiments: effect of temperature and sex

Respiration experiments consisted of measuring oxygen consumption rates of individual female and male *Centropages chierchiae* at four different temperatures (8, 13, 19 and 24ºC). The lower temperature (8ºC) was chosen, assumed to be below copepod's optimal thermal tolerance boundaries. Oxygen consumption (µmol O_2 g^{-1} WW h^{-1}) measurements were performed according to Pimentel et al. (2012) and Rosa et al. (2012) and entailed an endpoint analysis using methods modified from Thuesen and Childress (1993) and Marsh and Manahan (1999). *C. chierchiae* were incubated in glass gas-tight 10 ml syringes filled with filtered (0.2 µm) and UV sterilized water. In order to compensate for a probable decrease in dissolved oxygen content in the water, the water used in the incubations was aerated prior to the beginning

of each experiment, as recommended by Ikeda et al. (2000). Control syringes without animals were run simultaneously, to correct eventual bacterial respiration. Syringes were placed in temperature controlled water baths (Lauda, Lauda-Königshofen, Germany) at the four different experimental temperatures. For each gender, at each experimental temperature, four syringes were used each one with one copepod in a volume of 2 ml. Water samples were taken from each syringe using a Hamilton gas-tight 500-mL syringe and were injected into a micro-respirometry chamber (MC100 Microcell, Strathkelvin). Oxygen concentrations were recorded with Clarke-type O_2 electrode connected to a multi-channel oxygen interface (Strathkelvin, North Lanarkshire, Scotland).

Duration of individual respiratory runs varied from 4 to 19 h. At the end of each experiment, copepods prosome length was measured. Data on dry weight were calculated according to the equation:

$$Log(DW) = 2.451Log(PL) - 6.103$$

where DW is dry weight and PL prosome length (Mauchline, 1998).

For each experiment, temperature dependence (Q_{10}) was determined using the standard equation:

$$Q_{10} = [R(T2)/R(T1)]^{10/(T2-T1)}$$

where R(T2) and R(T1) are the oxygen consumption rates at temperatures T2 and T1, respectively. In order to compare results with previous studies units were converted from μmol O_2 g^{-1} WW h^{-1} to μL O_2 mg^{-1} DW h^{-1} using the ideal gas law equation.

3.2.4 Energy requirements calculations

The energy required to maintain the routine metabolic rate estimated at the different experimental temperatures was calculated in terms of prey concentrations of *P. tricornutum* and *Gymnodinium* sp. The energetic values (Kcal d^{-1}) were estimated assuming a 4.7 Kcal $L^{-1}O_2$ (Childress and Nygaard, 1973). In order to calculate the calories contained in 1 g of wet weight

of *P. tricornutum*, the proximate composition (proteins, lipids and carbohydrates) determined by Fábregas et al. (1998) was used. The caloric content of *Gymnodinium* sp. used was twice the *P. tricornutum*, assuming that diatoms contain ca. half the caloric value of dinoflagellates of an equivalent volume (Hitchcock, 1982). The wet weights of a single cell of *P. tricornutum* and *Gymnodinium* sp. used were 2.86×10^{-10} and 1.8×10^{-8} g based on Raymont and Adams (1958) and Mansour et al. (2003), respectively. The number of algae necessary per day to sustain the metabolic requirements of the copepods was then calculated assuming an assimilation efficiency for *P. tricornutum* and *Gymnodinium* sp. of 21.5% (Gaudy, 1974) and 34% (Le Ruyet-Person et al., 1975), respectively. To calculate the required food intake (µg C day^{-1}) and daily ration (% body carbon), we used a carbon content of 1.5×10^{-5} and 2.2×10^{-4} µg C for *P. tricornutum* and *Gymnodinium* sp., respectively. Carbon content was estimated using equations given in Smayda (1978) for phytoplankton. The carbon content of each copepod was calculated using the formula $CC = 10^{(2.4492 \log PL - 6.0984)} \times 0.417$ (Halvorsen et al., 2001) where PL is the prosome length of copepods used in the experiments.

3.2.5 Statistical analysis

A multiway analysis of variance (multiway ANOVA) was used to study the effect of temperature, alga species and alga concentration on the reproduction parameters, EPR and HS. Differences between oxygen consumption for the experiments conducted at the four different temperatures and for both sexes were tested with a two-way ANOVA. When significant differences were found, a Tukey's honestly significant difference *post hoc* test was used for pair-wise comparisons of treatment means. Statistical analyses were carried out using the open source software R 2.14 (R Development Core Team 2009) and the significance level was set at $\alpha=0.05$.

3.3 Results

3.3.1 Effects of temperature and food on reproduction

The EPR of *Centropages chierchiae* showed significant differences between all the temperatures tested (Table 3.1, Tukey HSD; $P < 0.05$). EPR at 13ºC was very low for copepods fed on both phytoplankton species and at both concentrations, decreasing from 4.5 ± 1.8 and 7.6 ± 8.9 to 0 eggs female^{-1} day^{-1} on days 3 and 4 with *Gymnodinium* sp. and *P. tricornutum*, respectively. The EPR was higher at the other two temperatures (19 and 24ºC) analysed (Fig. 3.1). Food type and concentration significantly influenced EPR (Table 3.1), with *P. tricornutum* and higher algae concentration inducing a higher production.

Table 3.1 - Results of a three-way ANOVA performed to test for temperature, algae species and concentration effects on the egg production rates and hatching success of *Centropages chierchiae*. Significant values are given in bold. EPR – egg production rate (eggs female^{-1} day^{-1}), HS – hatching success (%), Temp – temperature, Alga – food type, Conc – food concentration.

Variable	**Factor**	**df**	***F***	***p*-value**
EPR	Temp	2	205.860	**<0.001**
	Alga	1	21.460	**<0.001**
	Conc	1	57.509	**<0.001**
	Temp:Alga	2	4.050	**0.02**
	Temp:Conc	2	13.896	**<0.001**
	Alga:Conc	1	0.710	0.40
	Temp:Alga:Conc	2	0.060	0.94
HS	Temp	2	92.471	**<0.001**
	Alga	1	15.249	**<0.001**
	Conc	1	0.090	0.77
	Temp:Alga	2	6.272	**0.01**
	Temp:Conc	2	1.849	0.18
	Alga:Conc	1	1.430	0.23
	Temp:Alga:Conc	2	0.380	0.54

EPR values reached a maximum of 36 ± 4 and 29 ± 14 eggs female^{-1} day^{-1} with *P. tricornutum* and *Gymnodinium* sp., respectively, at 19ºC and 300 µg C L^{-1} (Fig. 3.1). Although EPR varied through the experiments, there was a tendency to be higher at 19ºC and high prey concentrations. HS was significantly different between the three different temperatures (Table 3.1), specifically between 13-19ºC and 13-24ºC (Tukey HSD; $P < 0.05$). In fact, the percentage of nauplii hatched was reduced at lower temperatures (13ºC; ranging from 21 to 52%), and it

increased significantly at higher temperatures (between 55-95% at 19 and 24ºC) (Fig. 3.2). Food type significantly influenced HS (Table 3.1), with higher values obtained for copepods fed on *Gymnodinium* sp. (Fig. 3.2). When exposed to *P. tricornutum*, HS showed a decrease throughout the duration of the experiments at 19 and 24ºC (Fig. 3.2).

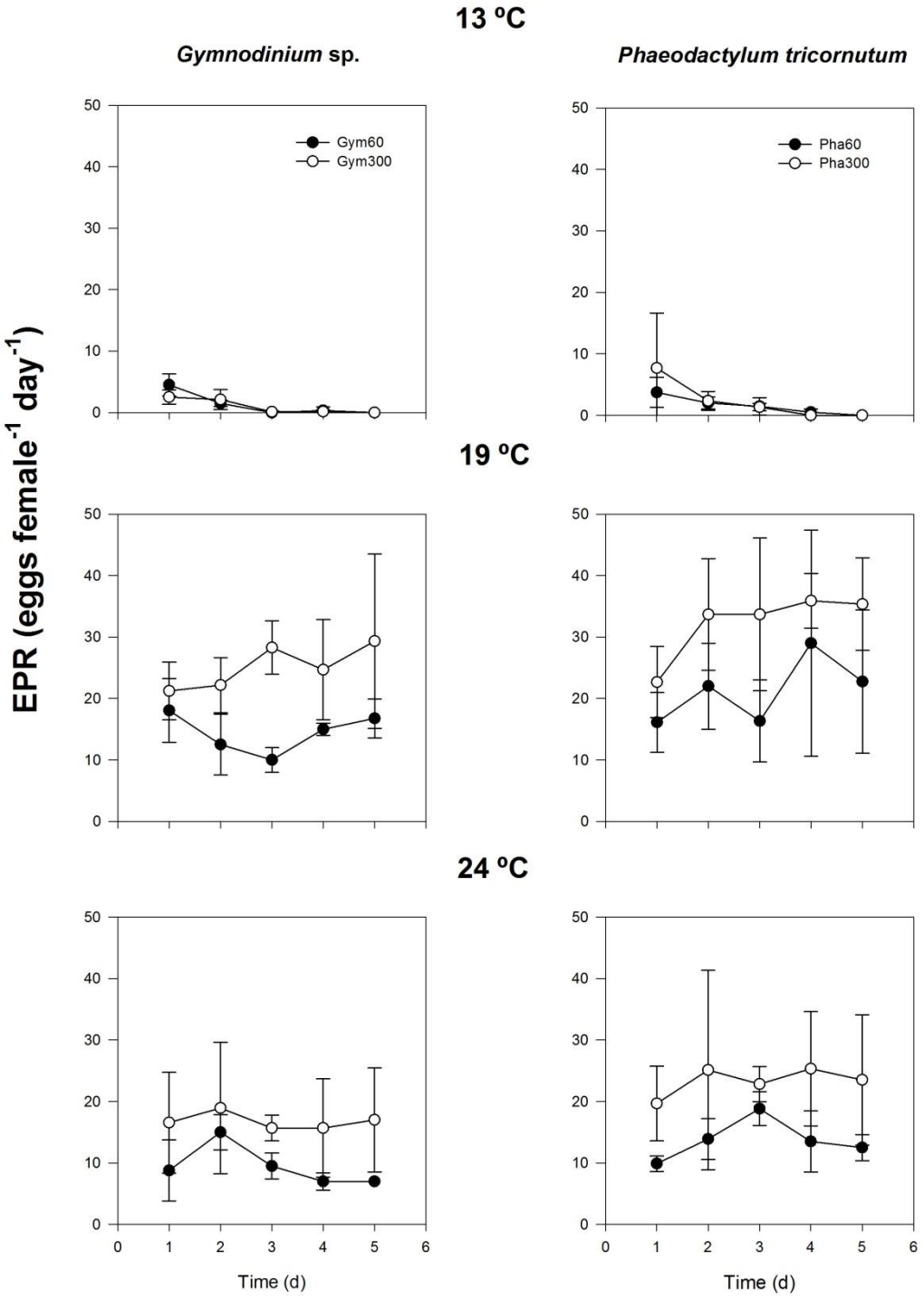

Fig. 3.1 – Egg production rates (EPR, eggs female^{-1} day^{-1}) of *Centropages chierchiae* at different temperatures fed with two algae, *Gymnodinium* sp. and *Phaeodactylum tricornutum*, during 5 days; black circles are for algae concentration of 60 µg C L^{-1} and white of 300 µg C L^{-1}. Error bars indicate standard deviation.

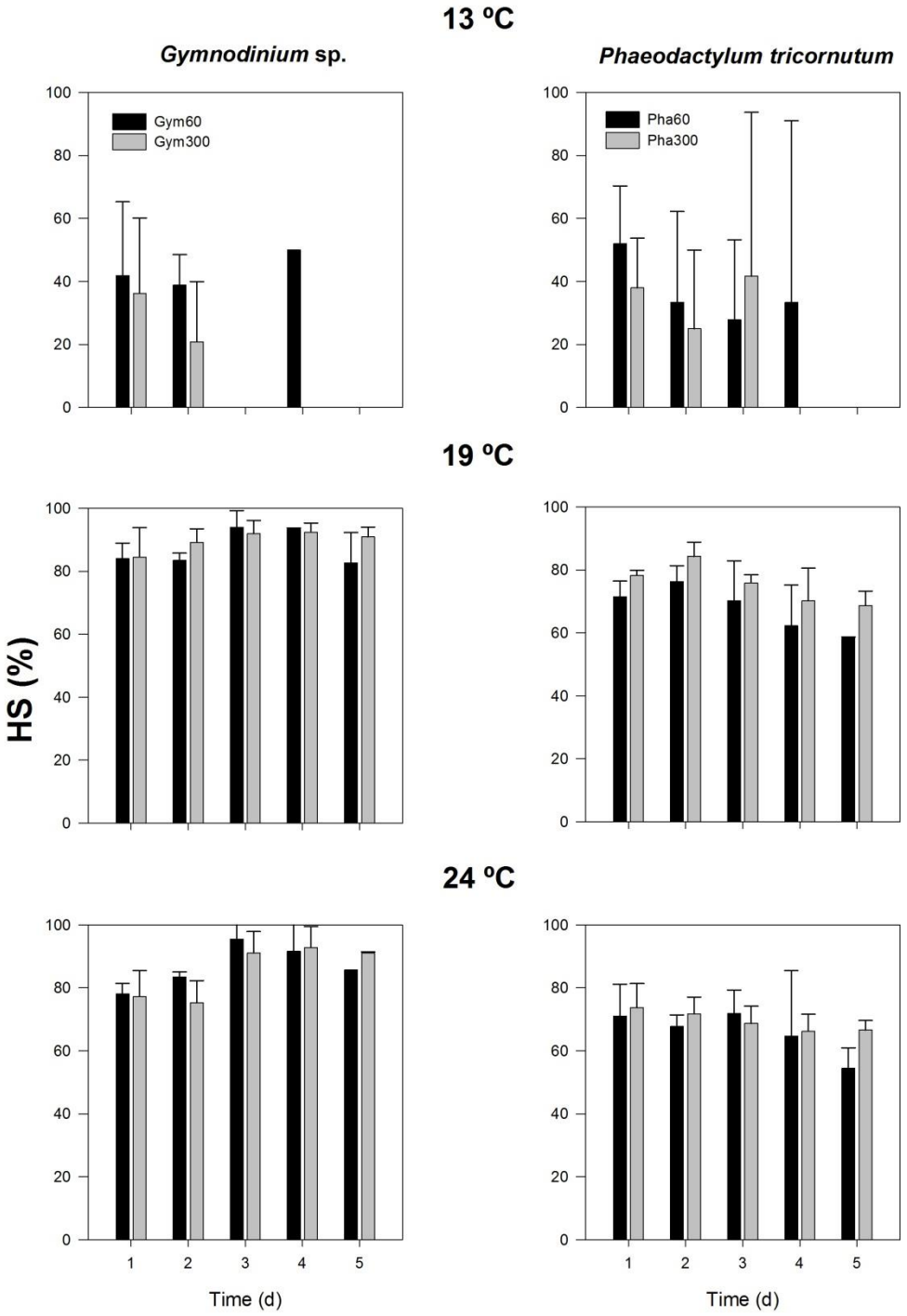

Fig. 3.2 – Hatching success (HS, %) of Centropages chierchiae at different temperatures fed with two algae, Gymnodinium sp. and Phaeodactylum tricornutum, during 5 days; black bars are for algae concentration of 60 µg C L^{-1} and grey for 300 µg C L^{-1}. Error bars indicate standard deviation.

3.3.2 Effects of temperature and gender on respiration

There was a significant relationship between respiration rates and temperature for both sexes of *C. chierchiae*, which was best fitted with an exponential function (R^2 = 0.83 and R^2 = 0.87 for females and males, respectively) (Fig. 3.3).

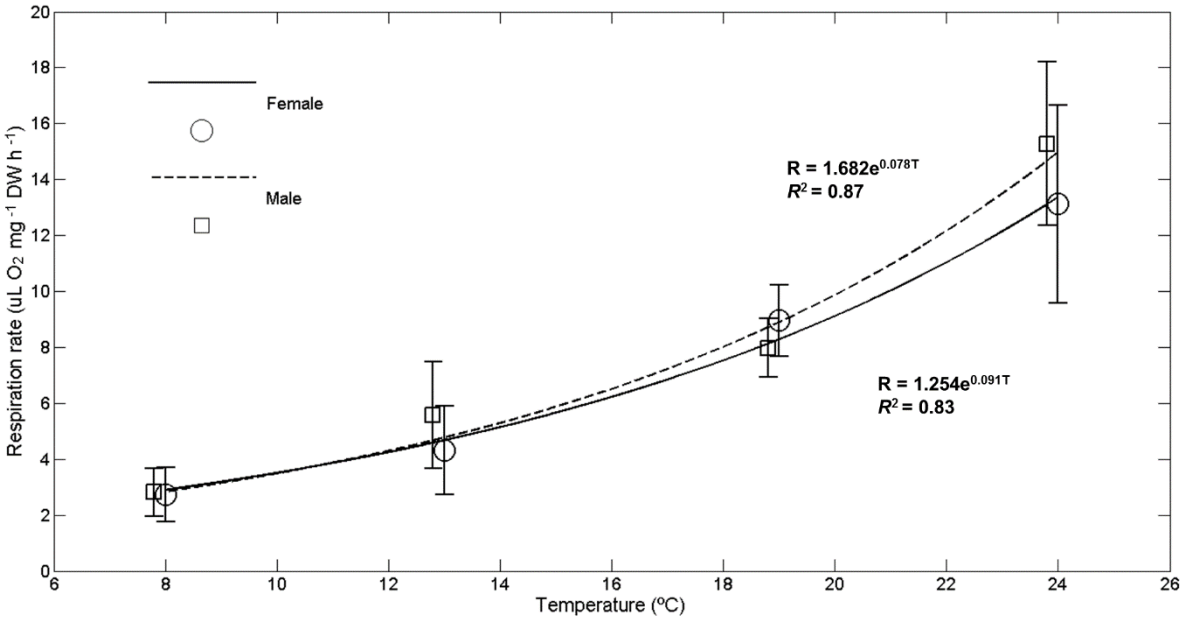

Fig. 3.3 - Variation of mean respiration rates of *Centropages chierchiae* males (squares) and females (circles) for different temperatures. Each point represents the mean value of replicates and error bars indicate standard deviation. The dashed line corresponds to the fitted exponential function for males and the continuous for females, in equations T is the temperature (ºC) and R is the respiration rate (µL O_2 mg^{-1} DW h^{-1}).

Two-way ANOVA analysis showed that temperature significantly influenced the respiration rates while the gender did not (Table 3.2). Oxygen consumption ranged between 2.7 ± 1.0 and 2.8 ± 0.9 µL O_2 mg^{-1} DW h^{-1} for females and males, respectively at 8ºC and 13.1 ± 3.5 and 15.3 ± 2.9 µL O_2 mg^{-1} DW h^{-1} at 24ºC (for an average body dry weights of 32.4 µg for females and 31.1 µg for males; Table 3.3). The Q_{10} values varied with temperature intervals and genders (Females: Q_{10} [8–13ºC] = 2.5; Q_{10} [13–19ºC] = 3.4; Q_{10} [19–24ºC] = 2.2; Males: Q_{10} [8–13ºC] = 3.9; Q_{10} [13–19ºC] = 1.8; Q_{10} [19–24ºC] = 3.7); yet when considering the entire range of temperature (8-24ºC) the thermal sensitivity values were 2.7 and 2.9 for females and males, respectively.

Table 3.2 - Results of a two-way ANOVA performed to test for temperature and sex effects on the oxygen rates of *Centropages chierchiae*. Significant values are given in bold. Temp – temperature.

Variable	Factor	df	*F*	*p*-value
Oxygen rate	Temp	3	50.363	**<0.001**
	Sex	1	0.812	0.377
	Temp:Sex	3	0.944	0.435

3.3.3 Energy requirements

The total carbon demands needed to assure growth, molting or reproduction are usually 5-10 times higher than the minimal requirements to maintain the basal metabolic rate (e.g. Perissinotto et al., 1997; Gaudy and Thibault-Botha, 2007). Figure 4 compares the daily ration estimated in complementary experiments (white boxes; Garrido et al., 2013), with the 10-folded daily ration obtained in the present work (grey boxes), showing that average daily ration obtained in the experiments was higher for all temperatures except for 24ºC. The energy needed to sustain minimal respiratory metabolic expenditure increased with temperature (Table 3.3). In order to achieve the respiratory requirements of *C. chierchiae*, the minimal food intake on *P. tricornutum* ranged on average from 0.4 to 1.8 µg C and *Gymnodinium* sp. from 0.03 to 0.13 µg C, which were equivalent to a range of 2.78 to 14.72 % and 0.21 to 1.09 % of the copepods body carbon, respectively (Table 3.3).

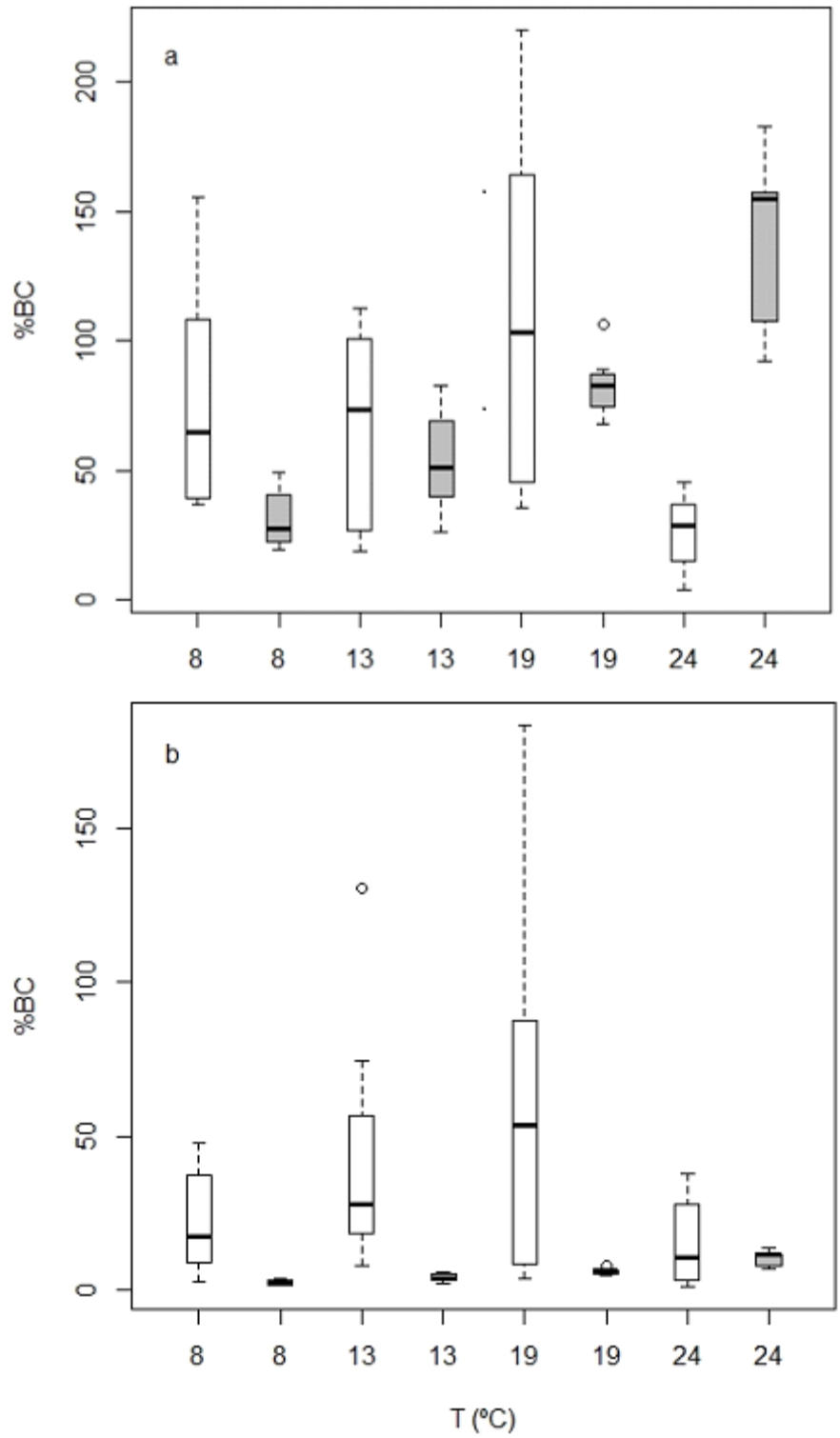

Fig. 3.4 - Boxplots of the daily ration of Centropages chierchiae (expressed as percentage of body carbon) at the four different temperatures analysed using a) *Phaeodactylum tricornutum* and b) *Gymnodinium* sp.; white boxplots are for feeding rates experiments results (Garrido et al., 2013) and black for present work.

Table 3.3 – Results of respiration rates experiments for the temperatures tested: F – females, M – males, Temp – temperature, n – number of replicates, showing mean values and standard deviation of copepods dry weight (µg), oxygen consumption (μlO_2 $mg^{-1}DW\ h^{-1}$) and food intake ($\mu gC\ day^{-1}$) and daily ration (% body carbon) for *Phaeodactylum tricornutum* and *Gymnodinium* sp.

Sex	Temp (ºC)	n	Dry weight (µg)	Oxygen consumption ($\mu lO_2 mgDW^{-1}h^{-1}$)	*Phaeodactylum tricornutum*		*Gymnodinium* sp.	
					($\mu gCday^{-1}$)	% Body Carbon	($\mu gCday^{-1}$)	% Body Carbon
F	8	5	34.1 ± 3.6	2.73 ± 0.96	0.40 ± 0.10	2.78 ± 0.97	0.033±0.011	0.24±0.09
	13	5	32.8 ± 6.2	4.31 ± 1.59	0.64 ± 0.25	4.31 ± 1.59	0.051±0.017	0.38±0.17
	19	4	30.2 ± 1.8	8.95 ± 1.29	1.13 ± 0.22	8.77 ± 1.26	0.083±0.016	0.65±0.09
	24	5	30.4 ± 3.2	13.13 ± 3.54	1.61 ± 0.53	12.65 ± 3.41	0.133±0.046	1.02±0.29
M	8	4	33.4 ± 1.5	2.81 ± 0.87	0.41 ± 0.13	2.86 ± 0.88	0.030±0.010	0.21±0.07
	13	4	31.7 ± 5.7	5.58 ± 1.90	0.73 ± 0.23	5.58 ± 1.90	0.054±0.017	0.41±0.14
	19	4	31.0 ± 3.5	7.98 ± 1.05	1.02 ± 0.05	7.81 ± 1.03	0.075±0.003	0.58±0.08
	24	4	29.1 ± 3.2	15.23 ± 2.93	1.80 ± 0.32	14.72 ± 2.82	0.133±0.023	1.09±0.21

3.4 Discussion

3.4.1 Effects of temperature on reproduction

Temperature significantly affected the fecundity of *C. chierchiae* females. The egg production was higher at 19ºC, intermediate at 24ºC and lower at 13ºC, in agreement with maximum feeding rates found at 19ºC (Garrido et al., 2013). The low productivity observed at 13ºC might explain the fact that this temperature determines the lower limit of optimal conditions for the distribution of this species. The higher production rate found at 19ºC agrees with the maximum abundance of *C. chierchiae* recorded off western Iberia during warmer months (Sobrinho-Gonçalves et al., 2013) when upwelling events provide rich food environments likely favourable to the reproduction of this species. The fact that *C. chierchiae* was able to exceed its northern limit of distribution in recent years seems to challenge the present results of reproductive performance at lower temperatures (13ºC). Lindley and Daykin (2005) suggested that by the late 1990's *C. chierchiae* has developed a resident population in the Celtic Sea and English Channel and the significant correlations between this species abundance and the minimum temperature also suggest that it persists in the plankton instead of

producing over-wintering eggs. However, in this area, higher abundances of this species are only found in summer when sea water temperature is generally higher than 13ºC, reaching values around 16-18ºC (Garcia-Soto and Pingree, 2009). Also, the population occurring in northern latitudes may have developed adaptation mechanisms to live permanently in lower temperatures that are sub-optimal for southern populations. The congeneric *Centropages typicus* is a very common species that occurs in the Mediterranean Sea and North Atlantic Ocean and is one of the most studied species of calanoid copepods. The seasonality of the abundance of both species is very similar in the English Channel and Celtic Sea (Lindley and Reid, 2002). Previous studies of *C. typicus* have shown that temperature significantly affects egg production. Smith and Lane (1985) observed that EPR was higher at 15ºC than at 10ºC, which were the only temperatures tested. On the other hand, experiments conducted by Carlotti et al. (1997) on the same species revealed that there were no differences of EPR between 15 and 20ºC. Halsband-Lenk et al. (2002) compared two distinct populations of *C. typicus* from the North Sea and Mediterranean Sea and found that the optimal temperature for spawning was 20ºC, but the lowest temperature at which females were able to produce eggs differed between the two populations, given that *C. typicus* from the North Sea were capable of spawning at 2ºC and Mediterranean population only started to produce eggs at 5ºC. In order to verify if the same could happen with *C. chierchiae*, comparative studies on the reproductive behavior between populations from northern and southern limits of its distribution are necessary.

Egg HS of copepods is temperature dependent (Holste and Peck, 2006; Hansen et al. 2010). Our results showed that HS in *C. chierchiae* was significantly lower at 13ºC than at 19 and 24ºC. The time used in the 13ºC experiment (72 hours) until all nauplii were counted following the work of Holste and Peck (2006), which suggests a low probability of copepods requiring more time to hatch. Smith and Lane (1985) showed that 95% of *C. typicus* eggs hatched within 48 hours at 15ºC but only 8% at 10ºC, with most eggs hatched within 72 hours. The lack of remating can also be ruled out since males were always added to the experimental bottles. Another hypothesis would be the production of resting eggs but Marcus (1996) and Lindley and Daykin (2005) did not observe any resting eggs production in *C. chierchiae*. Ianora et al. (1992) found no relationship between temperature and HS when studying the copepod *C. typicus* in the field. Regarding other calanoid genera such as the widespread *Acartia*, it was been shown that HS is highly influenced by temperature, and that no eggs were produced at temperatures below 10ºC (Castro-Longoria, 2003). Even though the underlying causes of embryonic death, when the development occurs under sub optimal conditions, are not known,

decreased membrane permeability, disequilibria of coupled enzyme reactions and limits imposed by kinetics and inactivation of enzyme proteins could be some of the responsible mechanisms (Rosa et al., 2012).

3.4.2 Effects of food on reproduction

Centropages chierchiae had high feeding rates when both *P. tricornutum* and *Gymnodinium* sp. were offered as monospecific diets in the feeding experiments conducted by Garrido et al. (2013). Here, a *P. tricornutum* diet induced higher EPR in *C. chierchiae* females than a *Gymnodinium* sp. diet, especially at higher cell concentrations. Similarly, *Centropages typicus* produced significantly more eggs when fed on a high density diet of *P. tricornutum* compared with low concentrations of the same alga (Razouls, 1981). EPR of *C. typicus* increases with food concentration, although there is a maximum concentration above which egg production remains constant or even decreases (Smith and Lane, 1985, Nival et al. 1990). Moreover, Gaudy (1971) found that the egg production of *C. typicus* fed on several diatoms' species, including *P. tricornutum* (~80 eggs female^{-1} day^{-1}), was higher than when fed on a flagellate-based diet. On the other hand, Miralto et al. (1995) found higher EPRs when *C. typicus* fed on *Thalassiosira rotula* (diatom) and *Gonyaulax polyedra* (dinoflagellate), around 50 eggs female^{-1} day^{-1}, rather than *P. tricornutum* on which females spawned on average 28 eggs female^{-1} day^{-1}. Fatty acids as an indicator of nutritional quality of phytoplankton, as well as nutritional components such as vitamins and amino acids, were not analyzed in the present work but may explain the differences on EPR achieved with the two algae.

Although EPR was higher when *C. chierchiae* fed on *P. tricornutum*, HS was found to be lower compared with *Gymnodinium* sp., indicating that these two processes may have different nutritional requirements to their success. Miralto et al. (1995) found that HS of *C. typicus* was higher using two species of dinoflagellates as prey rather than diatoms-based diets including *P. tricornutum*. The authors suggested a blockage of egg development due to the presence of intracellular chemical compounds in diatoms suggesting bottom-up control of recruitment in *C. typicus* populations. Miralto et al. (1999) reported the presence of three aldehydes compounds in diatoms as the cause of blocked embryonic development in copepods. Nevertheless, a study in which several diatoms were tested including *P. tricornutum* revealed

that this alga does not produce these deleterious compounds (Wichard et al., 2005). Egg viability was also lower when a *P. tricornutum* diet was given to several other calanoid species (Lee at al., 1999; Lacoste et al., 2001; Shin et al., 2003). In fact, *P. tricornutum* induced 100% blockage in hatching within 10 days in *Calanus helgolandicus* (Chaudron et al., 1996; Laabir et al., 1999). In the present work the 5-days experiments were not designed to detect long term effects and therefore such low results were not observed. On the other hand, Jónasdóttir and Kiørboe (1996) have suggested that high HS is achieved when eggs receive a favorable balance of required nutrients. They showed particularly low and rapid decline of the viability of *Acartia tonsa* eggs with *P. tricornutum* diets, reporting a positive correlation between hatching and fatty acid composition of prey. Furthermore, Shin et al. (2003) showed that egg viability of *Acartia omorii* declined with a *P. tricornutum* diet, suggesting that changes in egg viability of copepods are more closely related to the fatty acid composition of the eggs than to the production of reactive aldehydes blocking the development of copepod embryos proposed by other authors (Miralto et al., 1999).

Nival et al. (1990) proposed that *C. typicus* needs to feed on other particles (e.g. detritus or small metazoan) besides phytoplankton cells to achieve the maximal reproductive potential, because is not able to attain maximum production at high level of chlorophyll. In fact, Bonnet and Carlotti (2001) suggested that omnivory may be the best feeding strategy once mixed diets induced higher egg productivity. Including a different prey such as ciliates in future experiments would help to investigate a possible enhancing of *C. chierchiae* reproductive potential.

3.4.3 Effects of temperature and gender on respiration rates

Respiration rates of *C. chierchiae* increased with temperature following an exponential function which suggests a low capability to adapt to short-term temperature fluctuations. Most copepods show higher metabolic rates as temperature increases. However, some estuarine species can regulate their physiological activity in a wider temperature range in order to adapt to rapid temperature fluctuations (e.g. Gaudy et al., 2000). Hiromi et al. (1988) also found no effect of temperature on respiration activity of the ubiquitous species *Oithona davisae* over a wide temperature range. Our results agree with results of respiration rates found for *C. typicus* (see Table 3.4). The Q_{10} estimated for *C. chierchiae* here were highly variable, and for the entire

temperature range (8-24ºC), 2.7 and 2.9 for females and males, respectively. Similarly, Nival et al. (1974) observed an increase of *C. typicus* respiration off the Moroccan coast in the range from 13 to 23ºC with a Q_{10} value of 2.9.

Table 3.4 – Published data on the respiration rates of *Centropages typicus* including the present results for *Centropages chierchiae*.

Temperature (ºC)	Sex	Respiration rate ($\mu lO_2 mgDW^{-1}h^{-1}$)	Location	Reference
8, 13, 19, 24	F	2.7, 4.3, 8.9, 13.1	Portuguese West coast	Presente work (*Centropages chierchiae*)
	M	2.8, 5.6, 8.0, 15.2		
15	F	8.34	Woods Hole	Raymont, 1959
7-9	F	3.1	Gulf of Maine	Conover and Corner,1968
10, 14, 18, 21, 24	F	0.9, 1.2, 1.3, 3.4, 3.3	Moroccan coast	Champalbert and Gaudy, 1972
13, 15, 18, 20, 23	F	7.7, 9.7, 11.7, 13.8, 18.4	Moroccan coast	Nival et al., 1974
10, 14, 18, 20, 22	F	7.7, 9.7, 11.7, 13.8,18.4	Marseilles	Gaudy, 1973
10, 15, 20	F	3.1, 5.2, 6.0	Barcelona	Fernandez, 1978
	M	2.9, 4.6, 5.1		

Respiration rates were not influenced by the gender in *C. chierchiae*. Previous studies were not conclusive regarding the influence of sex on metabolism in *Centropages* species. Similarly to the present study, no differences were found in *C. typicus* between male and female respiration rates (Champalbert and Gaudy, 1972). Yet, Fernandez (1978) detected small differences with slightly higher values for females (3.095 – 6.023 μO_2 mg^{-1} DW h^{-1} for females and 2.874 – 5.103 μO_2 mg^{-1} DW h^{-1} for males; T: 10 – 20ºC). Also, Marshall and Orr (1966) found significantly higher values of female respiration rates of *Centropages hamatus* compared with males, with values two times higher for females than for males. Even though females were larger than males (females:1545.6 ± 80.9 µm, males: 1507.2 ± 49.7 µm), the difference of body mass between both genders are probably small enough not to cause differences of individual metabolic rates, which can explain the similar respiration rates found in this work.

3.4.4 Energy requirements

Respiration rates obtained in this work are the minimum carbon requirement of *C. chierchiae* given that copepods were in low activity and with no food during the experiments. Therefore, the food intake and daily ration estimated here are the values necessary to maintain the standard metabolism for the different temperatures tested. The average values of 10-folded minimum daily ration required were lower than average daily ration estimated from feeding experiments for both algae (Garrido et al., 2013), except at 24ºC results, meaning that food intake is usually in excess in terms of the energetic requirements. This means that algae concentrations provided in the feeding experiments are able to fulfill the total energetic requirements of *C. chierchiae* for the entire temperature range except for the highest temperatures. Average values of daily ration obtained at 24ºC do not seem to fulfill the energetic requirements. We were also able to compare the present findings with values of phytoplankton densities obtained in nature at a station located in the west Portuguese coast, area where *C. chierchiae* is found in higher abundances (Silva et al., 2009). Dinoflagellates and diatoms concentrations were determined for every season and the genera *Gymnodinium* was considered highly abundant during the summer period, the same as for *C. chierchiae*. On average, dinoflagellate concentration is 28 x 10^4 cells L^{-1}. Assuming such concentration of *Gymnodinium* sp., correspondent biomass of this species during this season would be 61 µg C L^{-1}. Making the same assumption, the 10-folded minimum carbon food intake would be 0.8 ± 0.1 µg C day^{-1} while the average food ingested during the experiments was 12.2 ± 3.3 µg C day^{-1} (Garrido et al., 2013), when *Gymnodinium* sp. is in concentrations similar to the one found by Silva et al. (2009), and for 19ºC (temperature more often registered during this season and area). This means that the ingested food, when *C. chierchiae* is exposed to a concentration of *Gymnodinium* sp. around 61 µg C L^{-1}, is higher than the food intake needed. Probably during the periods when this temperature occurs, dinoflagellates such as *Gymnodinium* sp. are sufficient to maintain *C. chierchiae* metabolism. Nonetheless, it seems that this dinoflagellate species is not the most efficient to guarantee a good production of *C. chierchiae*, as it was shown in the reproduction experiments.

3.4.5 *Centropages chierchiae* as a climate change indicator

The temperature range (8-24ºC) used in this study had a significant effect on the reproduction and respiration rates of *C. chierchiae*. The fact that the reproductive response was

highly temperature dependent may be indicative that the poleward movement of this copepod species is linked to climate change. More specifically, we suggest that such extended distribution is related to the ocean warming that has been occurring, in the northern European seas, for the past decades (MacKenzie and Schiedek, 2007). Moreover, in a future warming scenario, *C. chierchiae* will probably become more abundant in northern latitudes, while in the Iberian Peninsula coast will suffer a shift in its production time. It is worth noting that this warming effect may correspond to maximum temperature tested in this study (24ºC). As a consequence of the potential temporal population displacement, there may be a mismatch between *C. chierchiae* and their predators or preys. Although the thermal sensitivity data (Q_{10}) are within the normal temperature effects on metabolism, the projected near future warming (24ºC) will require *C. chierchiae* to consume more food per unit body size and feeding may be critical since this genera show high metabolic rates and low levels of metabolic reserves (Dagg, 1977). At low temperatures, food intake always exceeded the metabolic requirements for this species, which probably explains the northern shift of this species, even though EPRs were reduced when compared with higher temperatures. Based on the extremely low rates found with 13ºC, one may argue that the northern populations have probably adapted to colder temperatures, involving different life history traits and thermal stress tolerance. It would be of great interest to compare both *C. chierchiae* populations from the Iberian Peninsula and northern areas, in terms of their respiration physiology, reproductive biology and feeding ecology, in order to understand the phenotypic acclimation processes of this copepod species.

3.5 Acknowledgments

The authors wish to thank to the Oceanário de Lisboa and IPMA for helping collecting the copepods. Thanks are due to Maria Alexandra Chícharo and Ester Serrão for providing the facilities to conduct the experiments at the lowest temperatures and to Sofia Palma for helping culture the microalgae. The microalga *Rhodomonas baltica* was provided by the Assemble Program (grant 227799) funded under the European Community – Research Infrastructure Action under the FP7 "Capacities". The authors want to thank to Linda Holste and an anonymous reviewer for their comments and suggestions that contributed to improve the quality of the manuscript. This work was supported by the Portuguese Science and Technology Foundation (FCT) under project MODELA [PTDC/MAR/098643/2008], VITAL

[PTDC/MAR/111304/2009] and SYNERG [PTDC/MAR/0908066/2008]. J.C. is supported by FCT through a Doctoral Fellowship [SFRH/BD/28198/2006], S.G. through a Post-Doctoral Fellowship [SFRH/BPD/38332/2007] and R.R. through a Senior Research Position (Programa Ciência 2007).

3.6 References

Beaugrand, G., Luczak, C., Edwards, M. (2009) Rapid biogeographical plankton shifts in the North Atlantic Ocean. *Global Change Biology*, 15: 1790-1803.

Bonnet, D., Carlotti, F. (2001) Development and egg production in *Centropages typicus* (Copepoda: Calanoida) fed different food types: a laboratory study. *Marine Ecology Progress Series*, 224: 133-148.

Bonnet, D., Harris, R., Lopez-Urrutia, A., Halsband-Lenk, C., Greve, W., Valdes, L., Hirche, H.J., Engel, M., Alvarez-Ossorio, M.T., Wiltshire, K. (2007) Comparative seasonal dynamics of *Centropages typicus* at seven coastal monitoring stations in the North Sea, English Channel and Bay of Biscay. *Progress in Oceanography*, 72: 233-248.

Carlotti, F., Rey, C., Javanshir, A., Nival, S. (1997) Laboratory studies on the rates of egg production and fecal pellet production for the copepod *Centropages typicus*: individual variability, effect of age and effect of temperature. *Journal of Plankton Research*, 19: 1143-1165.

Castro-Longoria, E. (2003) Egg production and hatching success of four *Acartia* species under different temperature and salinity regimes. *Journal of Crustacean Biology*, 23: 289-299.

Champalbert, G., Gaudy, R. (1972) Étude de la respiration chez des copépodes de niveaux bathymétriques variés dans la région sud marocaine et canarienne. *Marine Biology*, 12: 159-169.

Chaudron, Y., Poulet, S.A., Laabir, M., Ianora, A.A., Miralto, A. (1996) Is hatching success diatom density-dependent? *Marine Ecology Progress Series*, 144: 185-193.

Childress, J.J., Nygaard, M.H. (1973) The chemical composition of midwater fishes as a function of depth of occurrence off southern California. *Deep-Sea Research*, 20: 1093-1109.

Conover, R.J., Corner, E.D.S. (1968) Respiration and nitrogen excretion by some zooplankton in relation to their life cycles. *Journal of the Marine Biological Association UK*, 48: 49-75.

CPR survey team (2004) Continuous Plankton Records: Plankton Atlas of the North Atlantic Ocean (1958–1999). II – Biogeographical charts. *Marine Ecolology Progress Series*, Supplement: 11-75.

Dagg, M. (1977) Some effects of patchy food environments on copepods. *Limnology and Oceanography*, 22: 99-107.

Edwards, M., Richardson, A.J. (2004) Impact of climate change on marine pelagic phenology and trophic mismatch. *Nature*, 430: 881-884.

Fábregas, J., Otero, A., Morales, E, Arredondo-Vega, B.O., Patiño, M. (1998) Modification of the nutritive value of *Phaeodactylum tricornutum* for *Artemia* sp. in semicontinuous cultures. *Aquaculture*, 169: 167-176.

Fernandez, F. (1978) Metabolismo y alimentacion en copepodos planctonicos del Mediterraneo: respuesta a la temperatura. *Investigación Pesquera*, 42: 97-139.

Fiúza, A.F.G. (1983) Upwelling patterns off Portugal. *In* Coastal upwelling. Its sediment records (Part A), pp. 85– 98. Ed. by A. E. Suess, and J. Thiede. Plenum Press, NY. 604 pp.

Garcia-Soto, C., Pingree, R.D. (2009) Spring and summer blooms of phytoplankton (SeaWiFS/MODIS) along a ferry line in the Bay of Biscay and western English Channel. *Continental Shelf Research*, 29: 1111-1122.

Garrido, S., Ben-Hamadou, R., Oliveira, P.B., Cunha, M.E., Chícharo, M.A., van der Lingen, C.D. (2008) Diet and feeding intensity of sardine *Sardina pilchardus*: correlation with satellite-derived chlorophyll data. *Marine Ecology Progress Series*, 354: 245-256.

Garrido, S., Cruz, J., Santos, A.M.P., Ré, P., Saiz, E. (2013) Effects of temperature, food type and concentration on the grazing of the calanoid copepod, *Centropages chierchiae*. *Journal of Plankton Research*, 35: 843-854.

Gaudy, R. (1971) Étude expérimentale de la ponte chez trios espèces de copepods pélagique (*Centropages typicus*, *Acartia clausi* et *Temora styilifera*). *Marine Biology*, 9: 65-70.

Gaudy, R. (1973) Les variations saisonnières de la respiration chez quatre espèces de copépodes pélagiques du Golfe de Marseille. *Netherlands Journal of Sea Research*, 7: 267-279.

Gaudy, R. (1974) Feeding four species of pelagic copepods under experimental conditions. *Marine Biology*, 25: 125-141.

Gaudy, R., Cervetto, G., Pagano, M. (2000) Comparison of the metabolism of *Acartia clausi* and *A. tonsa*: influence of temperature and salinity. *Journal of Experimental Marine Biology and Ecology*, 247: 51-65.

Gaudy, R., Thibault-Botha, D. (2007) Metabolism of *Centropages* species in the Mediterranean Sea and the North Atlantic Ocean. *Progress in Oceanography*, 72: 151-163.

Halsband-Lenk, C., Hirche, H.J., Carlotti, F. (2002) Temperature impact on reproduction and development of congener copepod populations. *Journal of Experimental Marine Biology and Ecology*, 271: 121-153.

Halvorsen, E., Hirst, A.G., Batten, S.D., Tande, K.S., Lampitt, R.S. (2001) Diet and community grazing by copepods in an upwelled filament off the NW coast of Spain. *Progress in Oceanography*, 51: 399-421.

Hansen, B.W., Drillet, G., Kozme, A., Madsen, K.V., Pedersen, M.F., Sørensen, T.F. (2010) Temperature effects on copepod egg hatching: does acclimatization matter? *Journal of Plankton Research*, 32: 305-315.

Hays, G.C., Richardson, A.J., Robinson, C. (2005) Climate change and marine plankton. *Trends in Ecology and Evolution*, 20: 337-344.

Hiromi, J., Nagata, T., Katoda, S. (1988) Respiration of the small planktonic copepod *Oithona davisae* at different temperatures. *Bulletin of the Plankton Society of Japan*, 35: 143-148.

Hitchcock, G.L. (1982) A comparative study of the size- dependent organic composition of marine diatoms and dinoflagellates. *Journal of Plankton Research*, 4: 363-377.

Holste, L., Peck, M. (2006) The effects of temperature and salinity on egg production and hatching success of Baltic *Acartia tonsa* (Copepoda: Calanoida): a laboratory investigation. *Marine Biology*, 148: 1061-1070.

Ianora, A., Mazzocchi, M.G., Grottoli, R. (1992) Seasonal fluctuations in fecundity and hatching success in the planktonic copepod *Centropages typicus*. *Journal of Plankton Research*, 14: 1483-1494.

Ikeda, T., Torres, J.J., Hernandez-Leon, S., Geiger, S.P. (2000) Metabolism. In Harris, R.P., Wiebe, P.H., Lenz, J., Skjoldal, H.R., Huntley, M. (eds) ICES zooplankton methodology manual. Academic Press, San Diego, CA, p 455-532.

IPCC (2007) Climate Change 2007: The Physical Science Basis: Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, New York.

Jónasdóttir, S.H., Kiørboe, T. (1996) Copepod recruitment and food composition: do diatoms affect hatching success? *Marine Biology*, 125: 743-750.

Laabir, M., Poulet, S.A., Cueff, A., Ianora, A. (1999) Effect of diet on levels of amino acids during embryonic and naupliar development of the copepod *Calanus helgolandicus.* *Marine Biology*, 134: 89-98.

Lacoste, A., Poulet, S.A., Cueff, A., Kattner, G., Ianora, A., Laabir, M. (2001) New evidence of the copepod maternal food effect on reproduction. *Journal of Experimental Marine Biology and Ecology*, 259: 85-107.

Lee, H.-W., Ban, S., And, Y., Ota, T., Ikeda, T. (1999) Deleterious effect of diatom diets on egg production and hatching success in the marine copepod *Pseudocalanus newmani*. *Plankton Biology and Ecology*, 46: 104-112.

Le Ruyet-Person, J., Razouls, C., Razouls, S. (1975) Biologie comparée entre espèces vicariantes et communes de copépodes dans un système néritique en Mediterranée et en Manche. *Vie et Milieu*, 25: 283-312.

Levitus, S., Antonov, J.I., Boyer, T.P., Stephens, C. (2000) Warming of the world ocean. *Science*, 287: 2225-2229.

Lindley, J.A., Daykin, S. (2005) Variations in the distributions of the *Centropages chierchiae* and *Temora stylifera* (Copepoda: Calanoida) in the north-eastern Atlantic Ocean and western European shelf waters. *ICES Journal of Marine Science*, 62: 869-877.

Lindley, J.A., Reid, P.C. (2002) Variations in the abundance of *Centropages typicus* and *Calanus helgolandicus* in the North Sea: deviations from close relationships with temperature. *Marine Biology*, 141: 153-165.

Lysholm, B., Nordgaard, O., Wiborg, K.F. (1945) Copepoda from the "Michael Sars" North Atlantic deep-sea expedition 1910. Reports on the Scientific Results of the "Michael Sars" North Atlantic Deep-sea Expedition, 1910, 5: 1-60.

Mackas, D.L., Batten, S.D., Trudel, M. (2007) Effects on zooplankton of a warming ocean: recent evidence from the Northeast Pacific. *Progress in Oceanography*, 75: 223-252.

MacKenzie, B.R., Schiedek, D. (2007) Daily ocean monitoring since the 1860s shows record warming of northern European seas. *Global Change Biology*, 13: 1335-1347.

Mansour, M.P., Volkman, J.K., Blackburn, S.I. (2003) The effect of growth phase on the lipid class, fatty acid and sterol composition in the marine dinoflagellate, *Gymnodinium* sp. in batch culture. *Phytochemistry*, 63: 145-153.

Marcus, N.H. (1996) Ecological and evolutionary significance of resting eggs in marine copepods: past, present, and future studies. *Hydrobiologia*, 320: 141-152.

Marsh, A.G., Manahan, D.T. (1999) A method for accurate measurements of the respiration rates of marine invertebrate embryos and larvae. *Marine Ecology Progress Series*, 184: 1-10.

Marshall, S.M., Orr, A.P. (1966) Respiration and feeding in some small copepods. *Journal of the Marine Biological Association UK*, 46: 513-530.

Mauchline, J. (1998) The biology of calanoid copepods. *Advances in Marine Biology*, 33: 1-710.

Miralto, A., Ianora, A., Poulet, S.A. (1995) Food type induces different reproductive responses in the copepod *Centropages typicus. Journal of Plankton Research*, 17: 1521-1534.

Miralto, A., Barone, G., Romano, G., Poulet, S. A., Ianora, A., Russo, G. L., Buttino, I., Mazzarella, G., Laabir, M., Cabrini, M., Giacobbe, M. G. (1999). The insidious effect of diatoms on copepod reproduction. *Nature*, 402: 173-176.

Morgado, F., Melo, R., Queiroga, H., Sorbe, J.-C. (2003) Zooplankton abundance in a coastal station off the Ria de Aveiro inlet (north-western Portugal): relation with tidal and day/night cycles. *Acta Oecologica*, 24: 175-181.

Nival, P., Malara, G., Charra, R., Palazzoli, I., Nival, S. (1974) Étude de la respiration et de l'excrétion de quelques copépodes planctoniques (Crustacea) dans la zone de remontée d'eau profonde des côtes marocaines. *Journal of Experimental Marine Biology and Ecology*, 15: 231-260.

Nival, S., Pagano, M., Nival, P. (1990) Laboratory study of the spawning rate of the calanoid copepod *Centropages typicus*: effect of fluctuating food concentration. *Journal of Plankton Research*, 12: 535-547.

Perissinotto, R., Pakhomov, E.A., McQuaid, C.D., Froneman P.W. (1997) In situ grazing rates and daily ration of Antarctic krill *Euphausia superba* feeding on phytoplankton at the Antarctic Polar Front and the Marginal Ice Zone. *Marine Ecology Progress Series*, 160: 77-91.

Pimentel, M.S., Trübenbach, K., Faleiro, F., Boavida-Portugal, J., Repolho, T., Rosa, R. (2012) Impact of ocean warming on the early ontogeny of cephalopods: a metabolic approach. *Marine Biology*, 159: 2051-2059.

Plouvenez, S., Champalbert, G. (1999) Feeding Behaviour and Trophic Environment of *Engraulis encrasicolus* (L.) in the Bay of Biscay. *Estuarine, Coastal and Shelf Science*, 433: 177-191.

Queiroga, H., Silva, C., Sorbe, J.-C., Morgado, F. (2005) Composition and distribution of zooplankton across an upwelling front on the northern Portuguese coast during summer. *Hydrobiologia*, 545: 195-207.

R Development Core Team (2009) R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. (available. from http://www.Rproject.org).

Raymont, J.E.G., Adams, M.N.E. (1958) Studies on the mass cultures of *Phaeodactylum*. *Limnology and Oceanography*, 2: 119-136.

Raymont, J.E.G. (1959) The respiration of some planktonic copepods. III. The oxygen requirements of some american species. *Limnology and Oceanography*, 4: 479-491.

Razouls, S. (1981) Étude expérimentale de la ponte de deux copépodes pélagiques *Temora stylifera* et *Centropages typicus*. I. Influences des conditions expérimentales. *Vie et Milieu*, 31: 195-204.

Razouls, C. (1996) Diversité et répartition geographique chez les copépodes pelagiques. 1. Calanoida. *Annales de l'Institut Océanographique, Paris*, 71: 81-404.

Richardson, A.J. (2008) In Hot Water: Zooplankton and Climate Change. *ICES Journal of Marine Science*, 65: 279-295.

Rosa, R., Pimentel, M.S., Boavida-Portugal, J., Teixeira, T., Trübenbach, K., Diniz, M.S. (2012) Ocean warming enhances malformations, premature hatching, metabolic suppression and oxidative stress in the early life stages of a keystone invertebrate. *PLOS One*, 7: e38283.

Shin, K., Jang, M.-C., Jang, P.-K., Ju, S.-J., Lee, T.-K., Chang, M. (2003) Influence of food quality on egg production and viability of the marine planktonic copepod *Acartia omorii*. *Progress in Oceanography*, 57: 265-277.

Silva, A., Palma, S., Oliveira, P.B., Moita, M.T. (2009) Composition and interannual variability of phytoplankton in a coastal upwelling region (Lisbon Bay, Portugal). *Journal of Sea Research*, 62: 238-249.

Smayda, T.J. (1978) From phytoplankters to biomass. In: Sournia, A. (ed) Phytoplankton manual. UNESCO, New York, p 273-279.

Smith, S.L., Lane, P.V.Z. (1985) Laboratory studies on the marine copepod *Centropages typicus*: egg production and development rates. *Marine Biology*, 85: 153-162.

Sobrinho-Gonçalves, L., Moita, M.T., Garrido, S., Cunha, M.E. (2013) Environmental forcing on the interactions of plankton communities across a continental shelf in the Eastern Atlantic upwelling system. *Hydrobiologia*, 713: 167-182.

Thuesen, E.V., Childress, J.J. (1993) Enzymatic activities and metabolic rates of pelagic chaetognaths: lack of depth-related declines. *Limnology and Oceanography*, 38: 935-948.

Vincent, D., Hartmann, H.J. (2001) Contribution of ciliated microprotozoans and dinoflagellates to the diet of three copepod species in the Bay of Biscay. *Hydrobiologia*, 443: 193-204.

Wichard, T., Poulet, S.A., Halsband-Lenk, C., Albaina, A., Harris, R., Liu, D.Y., Pohnert, G. (2005) Survey of the chemical defence potential of diatoms: screening of fifty one species for α,β,γ,δ-unsaturated aldehydes. *Journal of Chemical Ecology*, 31: 949-958.

Chapter 4

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

Joana Cruz, M. Alexandra Teodósio, Radhouane Ben-Hamadou, Luís Chícharo, Susana Garrido, Pedro Ré and A. Miguel P. Santos

Estuarine Coastal and Shelf Science (2017) 187: 96-109

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

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* species, by measuring their seasonal and spatial variability and the influence of environmental factors for *Acartia* sp. collected in the Guadiana river estuary. EPR of *Acartia tonsa* was positively related with chlorophyll *a* concentration, freshwater inflow and biomass of dinoflagellate, while *Acartia 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.

4.1 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 ecosystems (Hernández-Léon 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 hydrological 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 40ºC 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 (Paffenh€ofer 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 and McKinnon, 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 aspects, 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.

4.2 Materials and methods

4.2.1 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. 4.1). This estuary has the fourth largest catchment basin of the Iberian Peninsula (~67500 km2) and extends along approximately 70 km, with the lower 50 km constituting the southeastern border of Portugal with 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 ± 1096 m^3 s^{-1}: retrieved from http://www.snirh.pt), while since 2002 a more regular freshwater flow throughout the year has been occurring. The total volume of water retained in the river not reaching the estuary is estimated to be 13000 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 small 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. 4.1) 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). This sampling contributes to the ICES zooplankton monitoring programm (Chícharo et al., 2013).

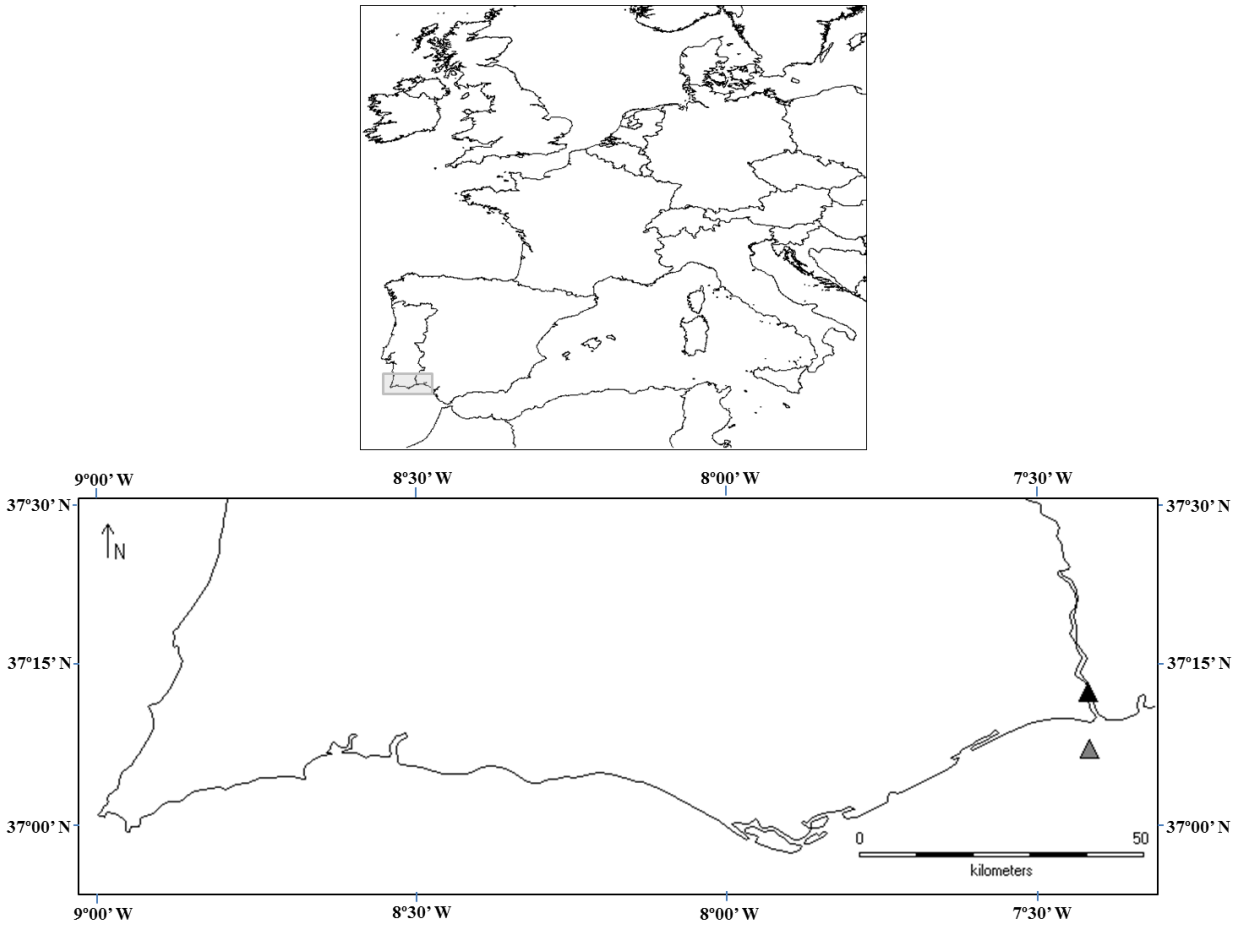

Fig. 4.1 – Map of the studied area with the two sampling stations, black triangle corresponds to inside Guadiana river estuary and grey triangle to adjacent coastal area.

Sampling was always carried out during daylight hours (9-15 h). 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 horizontal 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/).

4.2.2 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 h. 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 h at the same temperature in order to estimate the hatching success. At that period, all nauplii were counted.

4.2.3 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*:

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

and for *Acartia tonsa*:

$$\log(Wf) = 2.476 \times \log(PL)^{-0.698}$$ (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).

4.2.4 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 5-30 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 ± 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 37ºC 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).

4.2.5 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).

4.3 Results

4.3.1 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. 4.2), 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 and in the coastal area from 25.1 to 38 (Fig. 4.2). 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. 4.2). The freshwater inflow varied strongly between years, with maximum values in March 2010 (Fig. 4.3), 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 (Fig. 4.4). 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 ± 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 ± 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 ± 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 ± 30.2 μg C L^{-1}) and 2010 (May and June, carbon content: ~ 104 ± 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 ± 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 ± 6.1 μg C L^{-1}).

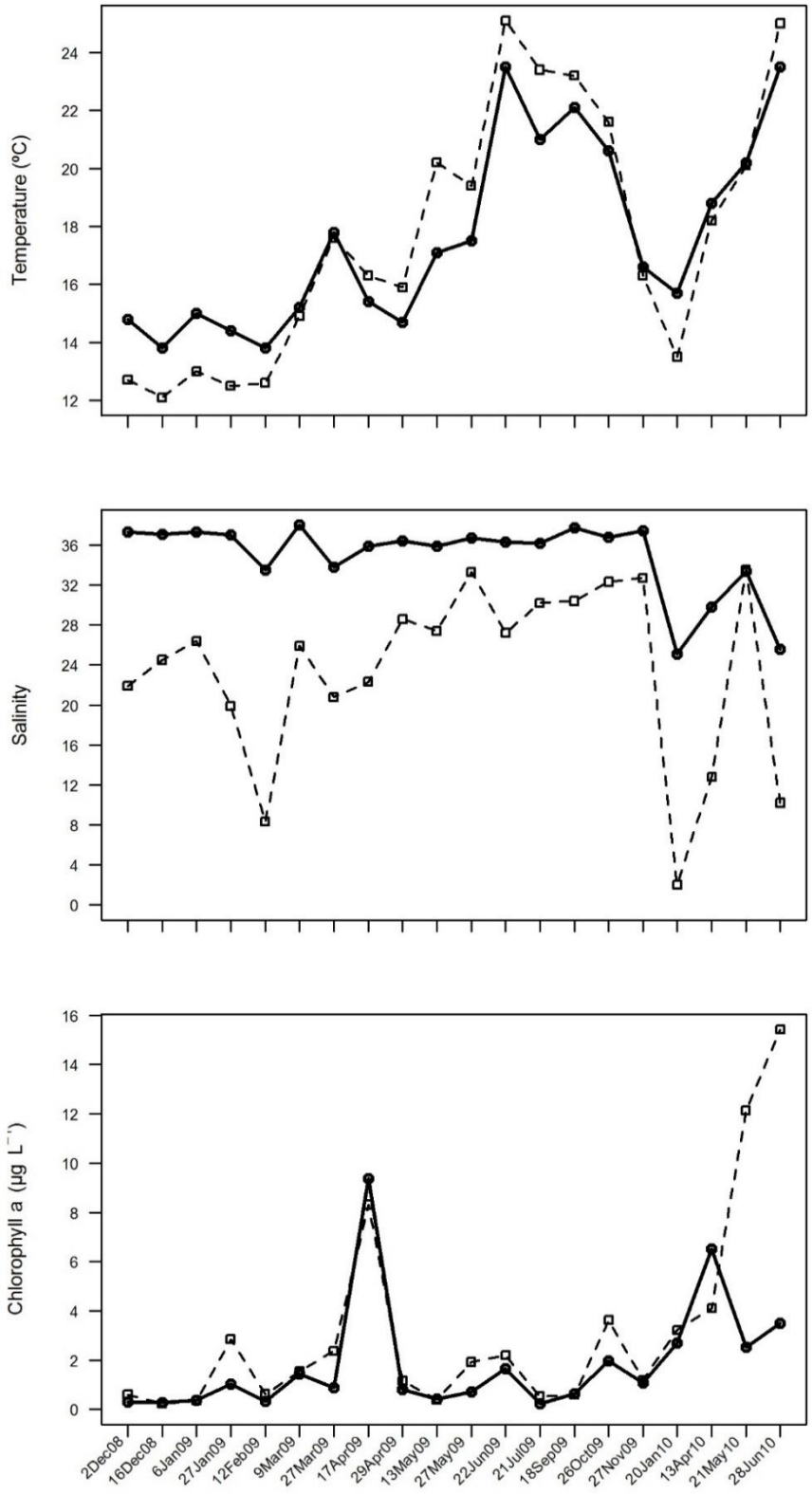

Fig. 4.2 - Seasonal variations in water temperature, salinity and chlorophyll *a* concentration in the two sampling stations; dashed line corresponds to inside Guadiana river estuary and solid line to adjacent coastal area.

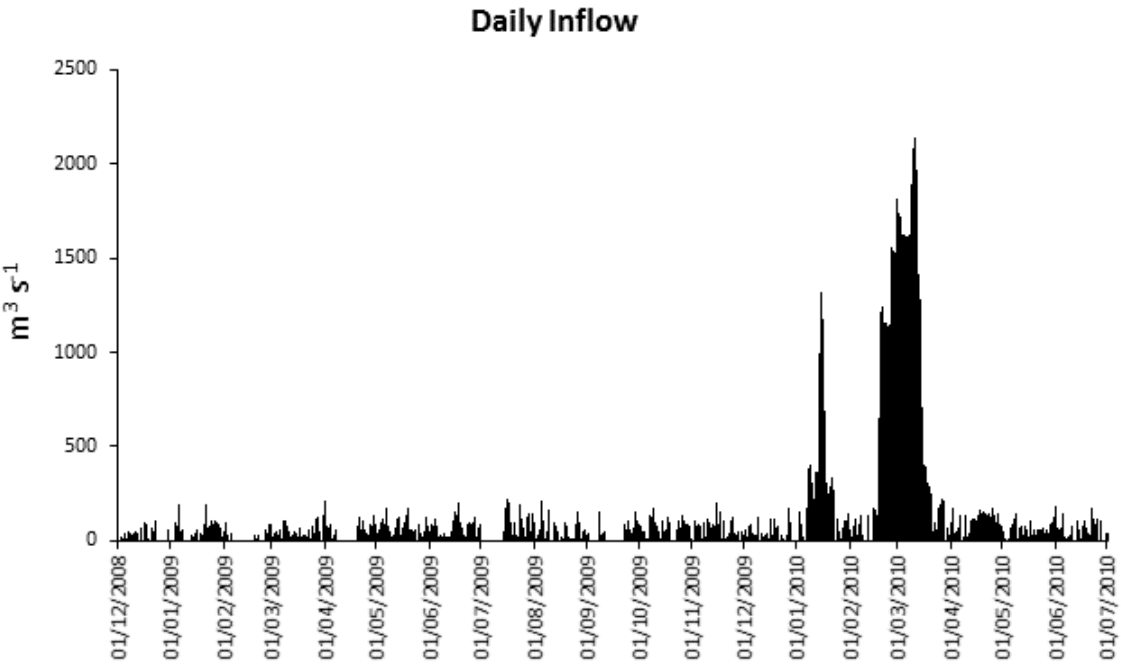

Fig. 4.3 – Daily freshwater discharge during the sampling period. Data was measured at the Pulo do Lobo hydrometric station and obtained from the Sistema Nacional Informação Recursos Hídricos (http://snirh.pt/)

4.3.2 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. 4.5). *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. 4.6). 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. 4.6). 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.

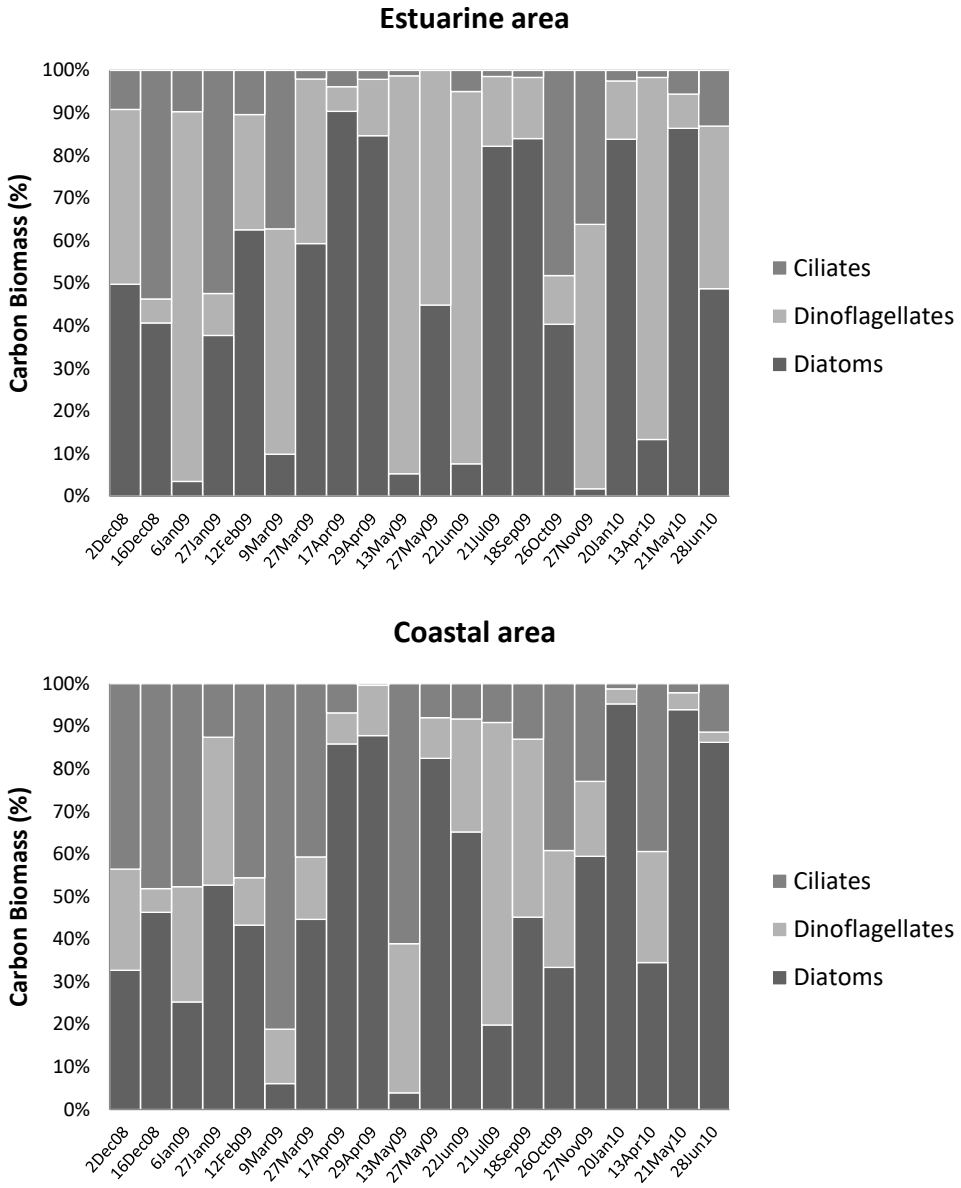

Fig. 4.4 - Carbon biomass of the evaluated microplankton groups at both stations. Data are represented as %.

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. 4.7).

The RNA:DNA ratio varied between 0.26-2.38 and 0.49-2.44 for *A. tonsa* and *A. clausi* females, respectively (Fig. 4.8). The highest value of the RNA:DNA ratio for both species occurred during May 2009.

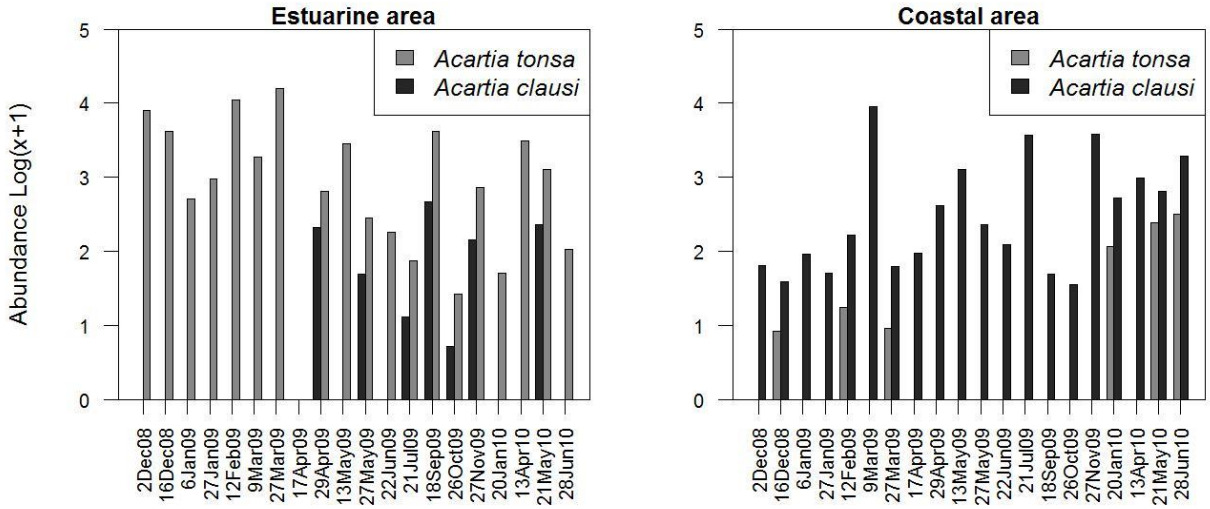

Fig. 4.5 – *Acartia tonsa* and *Acartia clausi* total abundance in the estuarine and coastal area stations; *A. tonsa* in grey bars and *A. clausi* in black bars; values are log transformed.

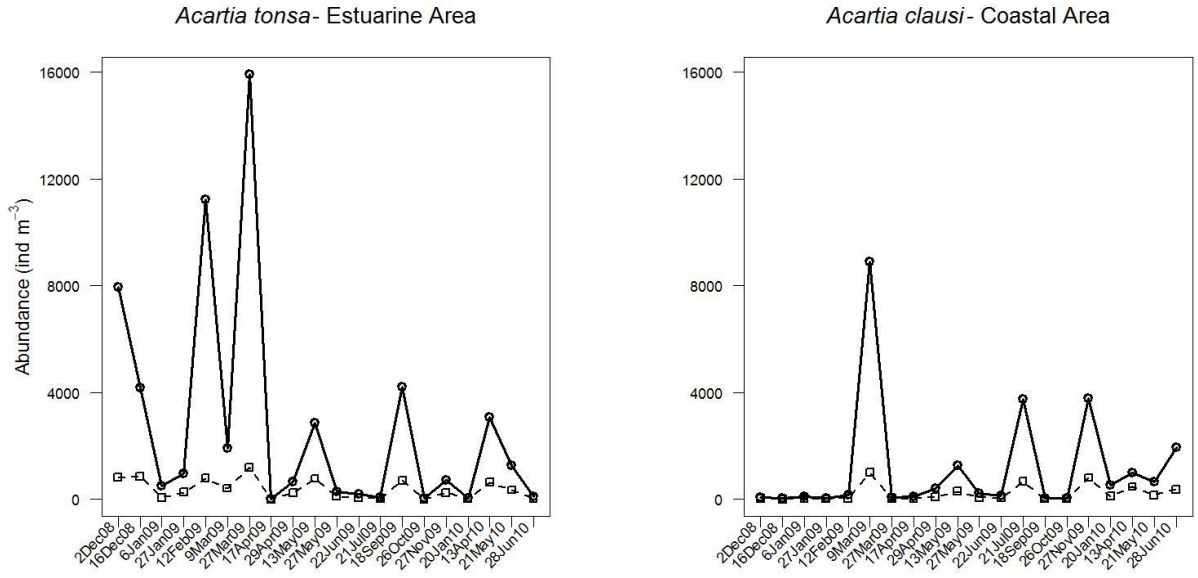

Fig. 4.6 – *Acartia tonsa* and *Acartia clausi* total and females' abundances in estuarine and coastal area stations; solid line is total abundance and dashed line is female abundance.

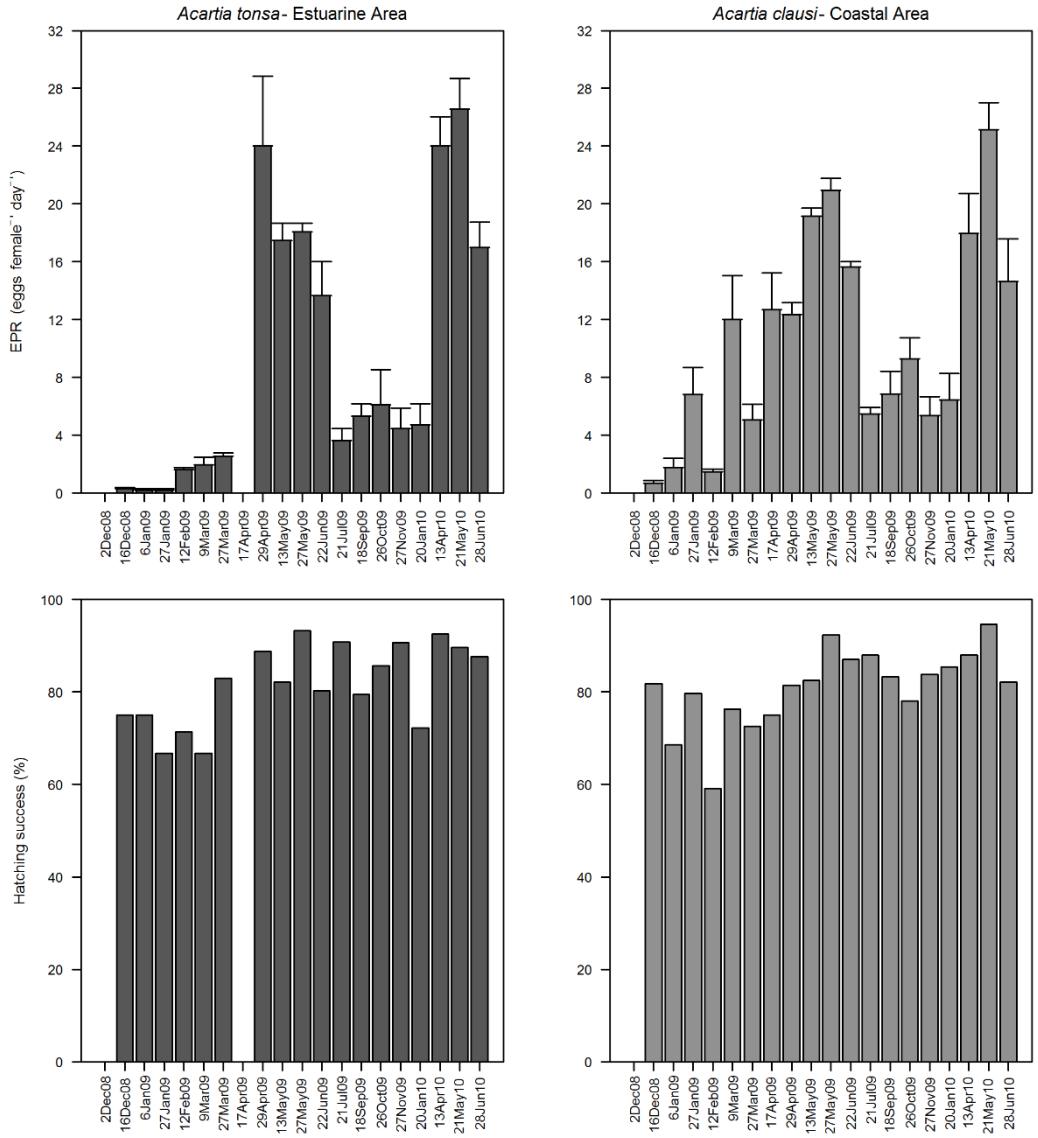

Fig. 4.7 – Reproductive traits of *Acartia tonsa* and *Acartia clausi*; egg production rates (EPR) as eggs female^{-1} day^{-1} and hatching success (%). Error bars indicate ± standard deviation.

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 4.1) 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 4.1).

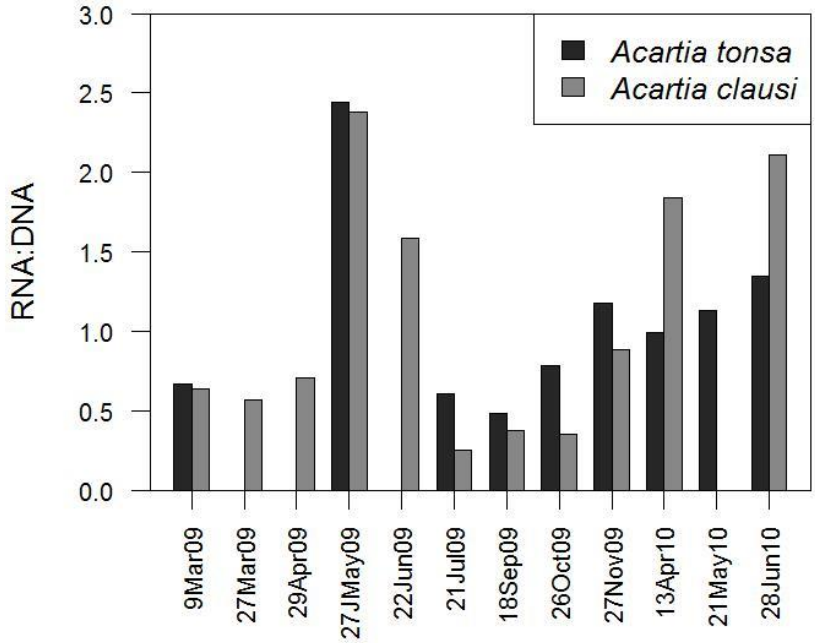

Fig. 4.8 – RNA:DNA ratios of *Acartia tonsa* and *Acartia clausi* females.

Table 4.1 - 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.

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

4.3.3 Females secondary production and recruitment

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 4.2). 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 4.2). Hatching success was very similar between seasons for both species, reaching the lowest values during winter (Table 4.2).

Table 4.2 – 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.

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

4.4 Discussion

4.4.1 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 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 like the present study. The temperature range found in the present study and used in the laboratory varied between 14.9 and 25ºC, 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 in 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. At the same time, 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.

4.4.2 Factors influencing reproduction

This study presents the first description of the reproduction dynamics of *Acartia clausi* and *Acartia tonsa* at southwestern Iberia, being one of the few studies analyzing copepod secondary production in the 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 in this region 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. Chícharo 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 availability of a more diverse prey assemblage. This is the result of the freshwater pulse leading to nutrient enrichment 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 neighbourhood coastal area is regulated by the tidal cycle and the amount of freshwater reaching the coastal area 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 (Paffenhₒfer 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 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 approximately 10-12, the egg production rates were high, because 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*, since the secondary production was kept high despite the salinity decrease, as nutrients and consequently primary producers increase.

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.

4.4.3 Females 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 4.3). 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 4.3, suggesting that the Guadiana estuary is a very productive system.

Table 4.3 - 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.

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

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). The early stages of small pelagic fish species, such as *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 of *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.

4.5 Conclusions

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 *Acartia clausi*, are influenced by different environmental variables. Egg production rate of *A. tonsa* was related to freshwater inflow, chlorophyll *a* and dinoflagellates biomass, while the egg production of *A. 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 small pelagic fish species inhabiting the Guadiana estuary, which means they can be used as indicators of food availability for their early development stages.

4.6 Acknowledgments

The authors wish to thank to Antero Fernandes, David Piló and Renata Gonçalves for their support during field work. J.C. was supported by Portuguese Foundation for Science and Technology (FCT) through a Doctoral Fellowship (SFRH/BD/28198/2006). MAT would like

to thanks also FCT through a sabbatical fellowship (SFRH/BSAB/113684/2015). The authors also acknowledge European Regional Development Fund (COMPETE program- Operational Competitiveness Programme), and by national funds from FTC through project UID/Multi/04326/2013. S.G. is supported by FCT through a Post-Doctoral Fellowship (SFRH/BPD/105419/2014) and by the Investigador FCT Program (IF/01546/2015).

4.7 References

Amaral, V., Penha-Lopes, G, Paula, J. (2009) RNA/DNA ratio of crabs as an indicator of mangrove habitat quality. *Aquatic Conservation: Marine and Freshwater Ecosystems*, 19: S56-S62.

Ambler, J.W. (1985) Seasonal factors affecting egg production and viability of eggs of *Acartia tonsa* Dana from East Lagoon, Galveston, Texas. *Estuarine Coastal and Shelf Science*, 20: 743-760.

Ara, K. (2001) Daily egg production rate of the planktonic calanoid copepod *Acartia lilljeborgi* Giesbrecht in the Cananeia Lagoon estuarine system, Sao Paulo, Brazil. *Hydrobiologia*, 445: 205-215.

Arendt, K.E., Jónasdóttir, S.H., Hansen, P.J., Gärtner, S. (2005) Effects of dietary fatty acids on the reproductive success of the calanoid copepod *Temora longicornis*. *Marine Biology*, 146: 513-530.

Ayukai, T (1987) Predation by *Acartia clausi* (Copepoda: Calanoida) on two species of tintinnids. *Marine Microbiology Food Webs*, 2: 45-52.

Azeiteiro, U.M., Marques, S.C., Vieira, L.M.R., Pastorinho, M.R.D., Ré, P.A.B., Pereira, M.J., Morgado, F.M.R. (2005) Dynamics of the *Acartia* genus (Calanoida: Copepoda) in a temperate shallow estuary (the Mondego estuary) on the western coast of Portugal. *Acta Adriatica*, 46: 7-20.

Bentle, L.A., Dutta, S., Metcoff, J. (1981) The sequential enzymatic determination of DNA and RNA. *Anal Biochemistry*, 116: 5-16.

Borme, D., Tirelli, V., Brandt, S.B., Fonda Umani, S., Arneri, E. (2009) Diet of *Engraulis*

encrasicolus in the northern Adriatic Sea (Mediterranean): ontogenetic changes and feeding selectivity. *Marine Ecology Progress Series*, 392: 193-200.

Boyer, S., Bouvy, M., Bonnet, D. (2013) What triggers *Acartia* species egg production in a Mediterranean lagoon? *Estuarine, Coastal and Shelf Science*, 117: 125-135.

Buckley, L.J., Caldarone, E.M., Clemmesen, C. (2008) Multi-species larval fish growth model based on temperature and fluorometrically derived RNA/DNA ratios: results from a meta-analysis. *Marine Ecology Progress Series*, 371: 221-232.

Buitenhuis, E., Le Quéré, C., Aumont, O., Beaugrand, G., Bunker, A., Hirst, A., Ikeda, T., O'Brien, T., Piontkovski, S., Straile, D. (2006). Biogeochemical fluxes through mesozooplankton. *Global Biogeochemical Cycles*, 20: GB2003.

Bulow, J.F. (1987) RNA-DNA ratios as indicators of growth rates in fish: A review. In The age and growth of fish; Summerfelt, R.C., Hall, G.E. Eds.; The Iowa State University Press: Ames, Iowa, pp. 45-64.

Caldarone, E.M., Clemmesen, M.C., Berdalet, E., Miller, T.J., Folkvord, A., Holt, G.J., Olivar, M.P., Suthers, I.M. (2006) Intercalibration of four spectrofluorometric protocols for measuring RNA:DNA ratios in larval and juvenile fish. *Limnology and Oceanography: Methods*, 4: 153-163.

Caldeira, C., Santos, A.M.P., Ré, P., Peck, M.A., Saiz, E., Garrido, S. (2014) Effects of prey concentration on ingestion rates of European sardine *Sardina pilchardus* larvae in the laboratory. *Marine Ecology Progress Series*, 517: 217-228.

Calliari, D., Andersen, C.M., Thor, P., Gorokhova, E., Tiselius, P. (2006) Salinity modulates the energy balance and reproduction success of co-occuring copepods *Acartia tonsa* and *A. clausi* in different ways. *Marine Ecology Progress Series*, 312: 177-188.

Castro-Longoria, E. (2003) Egg production and hatching success of four *Acartia* species under different temperature and salinity regimes. *Journal of Crustacean Biology*, 23: 289-299.

Chícharo, M.A., Chícharo, L. (2008) RNA:DNA ratio and other nucleic acid derived indices in marine ecology. *International Journal of Molecular Sciences*, 9: 1453-1471.

Chícharo, M.A., Esteves, E., Santos, A.M.P., dos Santos, A., Peliz, A., Ré, P. (2003) Are sardine larvae caught off northern Portugal in winter starving? An approach examining

nutritional conditions. *Marine Ecology Progress Series*, 257: 303-309.

Chícharo, M.A., Chícharo, L., Morais, P. (2006a). Inter-annual differences of ichthyofauna structure of the Guadiana estuary and adjacent coastal area (SE Portugal/SW Spain): Before and after Alqueva dam construction. *Estuarine, Coastal and Shelf Science*, 70: 39-51.

Chícharo, L., Chícharo, M.A., Ben-Hamadou, R. (2006b). Use of a hydrotechnical infrastructure (Alqueva Dam) to regulate planktonic assemblages in the Guadiana estuary: Basis for sustainable water and ecosystem services management. *Estuarine, Coastal and Shelf Science*, 70: 3-18.

Chinnery, F.E., Williams, J.A. (2004) The influence of temperature and salinity on *Acartia* (Copepoda: Calanoida) nauplii survival. *Marine Biology*, 145: 733-738.

Costalago, D., Palomera, I., Tirelli, V. (2014) Seasonal comparison of the diets of European anchovy *Engraulis encrasicolus* and sardine *Sardina pilchardus* juveniles in the Gulf of Lions. *Journal of Sea Research*, 89: 64-72.

Cushing, D.H. (1995) The long-term relationship between zooplankton and fish. *ICES Journal of Marine Science*, 52: 611-626.

Day, J. W., Hall, C. A. S., Kemp, W. M., Yáñez-Arancibia, A. (1989) Estuarine Ecology. John Wiley & Sons, Inc., (eds). New York, Chichester, Brisbane, Toronto, Singapore, 558 pp.

Dias, J.M.A., Gonzalez, R., Ferreira, Ó. (2004) Natural versus anthropic causes in variations of sand export from river basins: an example from the Guadiana River mouth (Southwestern Iberia). *Polish Geological Institute Special Papers*, 11: 95-102.

Domingues, R.B., Barbosa, A.B., Sommer, U., Galvão, H.M. (2012) Phytoplankton composition, growth and production in the Guadiana estuary (SW Iberia): Unraveling changes induced after dam construction. *Science of Total Environment*, 416: 300-313.

Domingues, R.B., Barbosa, A.B., Galvão, H.M. (2014) River damming leads to decreased phytoplankton biomass and disappearance of cyanobacteria blooms. *Estuarine, Coastal and Shelf Science*, 136: 129-138.

Dutz, J., Peters, J. (2008) Importance and nutritional value of large ciliates for the reproduction of *Acartia clausi* during the post spring-bloom period in the North Sea. *Aquatic*

Microbial Ecology, 50: 261-277.

Elser, J.J., Sterner, R.W., Gorokhova, E., Fagan, W.F., Markow, T.A., Cotner, J.B., Harrison, J.F., Hobbie, S.E., Odell, G.M., Weider, L.W. (2000) Biological stoichiometry from genes to ecosystems. *Ecology Letters*, 3: 540-550.

Escaravage, V., Soetaert, K. (1995) Secondary production of the brackish copepod communities and their contribution to the carbon fluxes in the Westerschelde estuary (The Netherlands). *Hydrobiologia*, 311: 103-114.

Evjemo, J.O., Tokle, N., Vadstein, O., Olsen, Y. (2008) Effect of essential dietary fatty acids on egg production and hatching success of the marine copepod *Temora longicornis*. *Journal of Experimental Marine Biology and Ecology*, 365: 31-37.

Faria, A., Morais, P., Chícharo, M.A. (2006) Ichthyoplankton dynamics in the Guadiana estuary and adjacent coastal area, South-East Portugal. *Estuarine, Coastal and Shelf Science*, 70: 85-97.

Fileman, E., Petropavlovsky, A., Harris, R. (2010) Grazing by the copepods *Calanus helgolandicus* and *Acartia clausi* on the protozooplankton community at station L4 in the Western English Channel. *Journal of Plankton Research*, 32: 709-724.

Fiúza, A.F.G. (1983) Upwelling patterns off Portugal. In: Suess, E. and Thiede, J. (eds) Coastal Upwelling: its sediment record. Plenum Press, pp 85-98.

Garel, E., Ferreira, Ó. (2015) Multi-year high-frequency physical and environmental observations at the Guadiana Estuary. *Earth System Science Data*, 7: 299-309.

Garrido, S., van der Lingen, C. (2014) Chapter 4 - Feeding biology and ecology. In: Ganias, K. (Ed.), Book: Biology and Ecology of Sardines and Anchovies. CRC Press/Taylor & Francis e Science Publishers.

Garrido, S., Ben-Hamadou, R., Oliveira, P.B., Cunha, M.E., Chícharo, M.A., van der Lingen, C.D. (2008) Diet and feeding intensity of sardine *Sardina pilchardus*: correlation with satellite-derived chlorophyll data. *Marine Ecology Progress Series*, 354: 245-256.

Garrido, S., Silva, A., Pastor, J., Dominguez, R., Silva, A.V., Santos, A.M. (2015) Trophic ecology of pelagic fish species off the Iberia: diet overlap, cannibalism and intraguild predation. *Marine Ecology Progress Series*, 539: 271-285.

Gómez-Gutiérrez, J., Palomares-Garcia, R., De Silva-Dávila, R. (1999) Copepod daily egg production and growth in Bahia Magdalena, México. *Journal of Plankton Research*, 21: 2227-2244.

Gonçalves, R., Correia, A.D., Atanasova, N., Teodósio, M.A., Ben-Hamadou, R., Chícharo, L. (2015) Environmental factors affecting larval fish community in the salt marsh area of Guadiana estuary (Algarve, Portugal). *Scientia Marina*, 79(1): 25-34.

Gorokhova, E. (2003) Relationships between nucleic acid levels and egg production rates in *Acartia bifilosa*: implications for growth assessment of copepods in situ. *Marine Ecology Progress Series*, 262: 163-172.

Gorokhova, E., Kyle, M. (2002) Analysis of nucleic acids in *Daphnia*: development of methods and ontogenetic variations in RNA–DNA content. *Journal of Plankton Research*, 24: 511-522.

Gusmão, L.F.M., McKinnon, A.D. (2011) Nucleic acid indices of egg production in the tropical copepod *Acartia sinjiensis*. *Journal of Experimental Marine Biology and Ecology*, 396: 122-137.

Hasle, G.R. (1978) The inverted-microscope method. In: Phytoplankton Manual, Ed.: A. Sournia, Monographs on Oceanographic Methodology, UNESCO, 88-98 pp.

Hay, S. (1995). Egg production and secondary production of common North Sea copepods: field estimates with regional and seasonal comparisons. *ICES Journal of Marine Sciences*, 52: 315-327.

Hérnandez-Léon, S., Ikeda, T. (2005) A global assessment of mesozooplankton respiration in the ocean. *Journal of Plankton Research*, 27: 153-158.

Hirst, A.G., Bunker, A.J. (2003) Growth of marine planktonic copepods: Global rates and patterns in relation to chlorophyll *a*, temperature, and body weight. *Limnology and Oceanography*, 48:1988–2010.

Hogfors, H., Holmborn, T., Hajdu, S., Gorokhova, E. (2011) Does female RNA content reflect viable egg production in copepods? A test with the Baltic copepod *Acartia tonsa*. *Journal of Plankton Research*, 33: 1460-1463.

Holste, L., Peck, M.A. (2006) The effects of temperature and salinity on egg production and

hatching success of Baltic *Acartia tonsa* (Copepoda: Calanoida): A laboratory investigation. *Marine Biology*, 148: 1061-1070.

Huntley, M.E., Lopez, M.D.G. (1992) Temperature-dependent production of marine copepods: a global synthesis. *The American Naturalist*, 140: 201-242.

Ikeda, T., Motoda, S. (1978) Estimated zooplankton production and their ammonia excretion in the Kuroshio and adjacent seas. *Fishery Bulletin US*, 76: 357–367.

Ikeda, T., San, F.; Yamaguchi, A., Matsuishi, T. (2007) RNA:DNA ratios of calanoid copepods from the epipelagic through abyssopelagic zones of the North Pacific Ocean. *Aquatic Biology*, 1: 99-108.

Jónasdóttir, S.H., Kiørboe, T. (1996) Copepod recruitment and food composition: Do diatoms affect hatching success? *Marine Biology*, 125: 743-750.

Jung, Y., Kang, H-K., Kang, Y.J. (2004) In situ egg production rate of the planktonic copepod *Acartia steueri* in Ilkwang Bay, southeastern coast of Korea. *Journal of Plankton Research*, 26: 1547-1553.

Kimmerer, W.J., Ferm, N., Nicolini, M.H., Peñalva, C. (2005) Chronic food limitation of egg production in populations of copepods of the genus *Acartia* in the San Francisco estuary. *Estuaries*, 28: 541-550.

Kiørboe, T., Sabatini, M. (1995) Scaling of fecundity, growth and development in marine planktonic copepods. *Marine Ecology Progress Series*, 120: 285-298.

Kiørboe, T., Møhlenberg, F., Hamburger, K. (1985) Bioenergetics of the planktonic copepod *Acartia tonsa*: relation between feeding, egg production and respiration, and composition of specific dynamic action. *Marine Ecology Progress Series,* 26: 85-97.

Kleppel, G.S. (1992) Environmental regulation of feeding and egg production by *Acartia tonsa* off southern California. *Marine Biology*, 112: 57-65.

Kleppel, G.S., Hazzard, S.E. (2000) Diet and egg production of the copepod *Acartia tonsa* in Florida Bay. II. Role of the nutritional environment. *Marine Biology*, 137: 111-121.

Kleppel, G.S., Burkart, C.A., Houchin, L. (1998) Nutrition and the regulation of egg production in the calanoid copepod *Acartia tonsa*. *Limnology and Oceanography*, 43: 1000-1007.

Kobari, T., Mori, H., Tokushige, H. (2013) Nucleic acids and protein content in ontogenetically migrating copepods in the Oyashio region as influenced by development stage and depth distribution. *Journal of Plankton Research*, 35: 97-104.

Leandro, S. M., Morgado, F., Pereira, F., Queiroga, H. (2007) Temporal changes of abundance, biomass and production of copepod community in a shallow temperate estuary (Ria de Aveiro, Portugal). *Estuarine, Coastal and Shelf Science*, 74: 215-222.

Leandro, S.M., Tiselius, P., Marques, S.M., Avelelas, F., Correia, C., Sá, P., Queiroga, H. (2014) Copepod production estimated by combining in situ data and specific temperature-dependent somatic growth models. *Hydrobiologia*, 741: 139-152.

Mauchline, J. (1998) The biology of calanoid copepods. *Advances in Marine Biology*, 33: 1-710.

Morais, P., Chícharo, M.A., Chícharo, L. (2009) Changes in a temperate estuary during the filling of the biggest European dam. *Science of the Total Environment*, 407: 2245-2259.

Morote, E., Olivar, M.P., Villate, F., Uriarte, I. (2010) A comparison of anchovy (*Engraulis encrasicolus*) and sardine (*Sardina pilchardus*) larvae feeding in the Northwest Mediterranean: influence of prey availability and ontogeny. *ICES Journal of Marine Science*, 67: 897-908.

Muxagata, E., Amaral, W.J.A., Barbosa, C.N. (2012) *Acartia tonsa* production in the Patos Lagoon estuary, Brazil. *ICES Journal of Marine Science*, 69: 475-482.

Nakata, K., Nakano, H., Kikuchi, H. (1994) Relationship between egg productivity and RNA/DNA ratio in *Paracalanus* sp. In the frontal waters of the Kurshio. *Marine Biology*, 119: 591-596.

Ning, J., Li, C., Yang, G., Wan, A., Sun, S. (2013) Use of RNA:DNA ratios to evaluate the condition and growth of the copepod *Calanus sinicus* in the Southern Yellow Sea. *Deep-Sea Research II*, 97: 109-116.

Nybakken, J.W. (2001). Marine biology: An ecological approach. San Francisco: Benjamin Cummings.

Olivar, M.P., Diaz, M.V., Chícharo, M.A. (2009) Tissue effect on RNA: DNA ratios of marine fish larvae. *Scientia Marina*, 73S1: 171-182.

Paffenhöfer, G. A., Stearns, D. (1988) Why is *Acartia tonsa* (Copepoda: Calanoida) restricted to nearshore environments? *Marine Ecology Progress Series*, 42: 33-38.

Pagano, M., Kouassi, E., Arfi, R., Bouvy, M., Saint-Jean, L. (2004) In situ spawning rate of the calanoid copepod *Acartia clausi* in a tropical lagoon (Ebrié, Côte d'lvoire): Diel variations and effects of environmental factors. *Zoological Studies*, 43: 244-254.

Pastorinho, R., Vieira, L., Ré, P., Pereira, M., Bacelar-Nicolau, P., Morgado, F., Marques, J.C., Azeiteiro, U. (2003) Distribution, production, histology and histochemistry in *Acartia tonsa* (Copepoda: Calanoida) as means for life history determination in a temperate estuary (Mondego estuary, Portugal). *Acta Oecologica*, 24: S259-S273.

Peck, M.A., Holste, L. (2006) Effects of salinity, photoperiod and adult stocking density on egg production and egg hatching success in *Acartia tonsa* (Calanoida: Copepoda): Optimizing intensive cultures. *Aquaculture*, 255: 341-350.

Peck, N., Peters, J., Diekmann, R., Laakmann, S., Renz, J. (2014) Interactive effects of temperature and salinity on population dynamics of the calanoid copepod *Acartia tonsa*. *Journal of Plankton Research*, 37: 197-210.

Poulet, S.A., Ianora, A., Laabir, M., Klein Bretler, W.C.M. (1995) Towards the measurement of secondary production and recruitment in copepods. *ICES Journal of Marine Science*, 52: 359-368.

Purcell, J.E. (1997) Pelagic cnidarians and ctenophores as predators: selective predation, feeding rates, and effects on prey populations. *Annales de l'Institut Océanographique*, 73: 125-137.

R Development Core Team (2013) R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. (available. from http://www.Rproject.org).

Reynolds, C.S. (1984) The Ecology of Freshwater Phytoplankton. Cambridge Univ. Press, Cambridge and New York, p. 384.

Rocha, C., Galvão, H., Barbosa, A. (2002) Role of transient silicon limitation in the development of cyanobacteria blooms in the Guadiana estuary, south-western Iberia. *Marine Ecology Progress Series*, 228: 35-45.

Rodriguez, V., Guerrero, F., Bautista, B. (1995) Egg production of individual copepods of

Acartia grani Sars from coastal waters: seasonal and die1 variability. *Journal of Plankton Research*, 17: 2233-2250.

Rollwagen-Bollens, G.C., Penry, D.L. (2003) Feeding dynamics of *Acartia* spp. copepods in a large, temperate estuary (San Francisco Bay, CA). *Marine Ecology Progress Series*, 257: 139-158.

Saiz, E., Calbet, A., Fara, A., Berdalet, E. (1998) RNA content of copepods as a tool for determining adult growth rates in the field. *Limnology and Oceanography*, 43: 465-470.

Sakamoto, Y., Ishiguro, M., Kitagawa, G. (1986) Akaike information criterion statistics. D. Reidel Publishing Company, Dordrecht, The Netherlands.

Sautour, B., Castel, J. (1995). Comparative spring distribution of zooplankton in three macrotidal European estuaries. *Hydrobiologia*, 311: 139-151.

Schmidt, K., Kähler, P., von Bodungen, B. (1998) Copepod egg production in the Pomeranian Bay (Southern Baltic Sea) as a function of phytoplankton abundance and taxonomic composition. *Marine Ecology Progress Series*, 174: 183-195.

Smayda (1978) From phytoplankters to biomass. In: Sournia A (ed) Phytoplankton manual. UNESCO, New York, p 273-279.

Speekmann, C.L., Hyatt, C.J., Buskey, E.J. (2006) Effects of *Karenia brevis* diet on RNA:DNA ratios and egg production of *Acartia tonsa*. *Harmful Algae*, 5: 693-704.

Sykes, A., Domingues, P., Andrade, J.P. (2004) Nucleic acid derived indices or instantaneous growth rate as tools to determine different nutritional condition in cuttlefish (*Sepia officinalis*, Linnaeus 1758) hatchlings. *Journal of Shellfish Research*, 23: 585-591.

Thompson, A.M., Durbin, E.G., ScDurbin, A.G. (1994) Seasonal changes in maximum ingestion rate of *Acartia tonsa* in Narragansett Bay, Rhode Island, USA. *Marine Ecology Progress Series*, 108: 91-105.

Uriarte, I., Cotano, U., Villate, F. (1998) Egg production of *Acartia bifilosa* in the small temperate estuary of Mundaka, Spain, in relation to environmental variables and population development. *Marine Ecology Progress Series*, 166: 197-205.

Uriarte, I., Cotano, U., Villate, F. (2005) Effects of estuarine conditions and organic enrichment on the fecundity and hatching success of *Acartia clausi* in contrasting systems. *Journal*

of Experimental Marine Biology and Ecology, 320: 105-122.

Üstün, F., Bat, L. (2014) The Egg Production Rate of *Acartia* (*Acartiura*) *clausi* Giesbrecht, 1889 (Copepoda) in Sinop Peninsula (Southern Black Sea). *Turkish Journal of Fisheries and Aquatic Sciences*, 14: 605-613.

Uye, S. (1981) Fecundity studies of neritic calanoid copepods *Acartia clausi* Giesbrecht and *A. Steueri* Smirnov: a simple empirical model of daily egg production. *Journal of Experimental Marine Biology and Ecology*, 50: 255-271.

Vehmaa, A., Larsson, P., Vidoudez, C., Pohnert, G., Reinikainen, M., Engström-Öst, J. (2011) How will increased dinoflagellate:diatom ratios affect copepod egg production? – A case study from the Baltic Sea. *Journal of Experimental Marine Biology and Ecology*, 401: 134-140.

Vehmaa, A., Kremp, A., Tamminen, T., Hogfors, H., Spilling, K., Engström-Öst, J. (2012) Copepod reproductive success in spring-bloom communities with modified diatom and dinoflagellate dominance. *ICES Journal of Marine Science*, 69: 351-357.

Venables, W.N., Ripley, B.D. (2002) Modern Applied Statistics with S, 4th ed. Springer-Verlag, New York.

Vieira, L., Morgado, F., Ré, P., Nogueira, A., Pastorinho, R., Pereira, M., Bacelar-Nicolau, P., Marques, J.C., Azeiteiro, U.M. (2003) Population dynamics of *Acartia clausi* from a temperate estuary (Mondego Estuary, Western Portugal). *Invertebrate Reproduction and Development*, 44: 9-15.

Wagner, M., Durbin, E., Buckley, L. (1998) RNA:DNA ratios as indicators of nutritional condition in the copepod *Calanus finmarchicus. Marine Ecology Progress Series*, 162: 173-181.

Wagner, M.M., Campbell, R.G., Boudreau, C.A., Durbin, E.G. (2001) Nucleic acids and growth of *Calanus finmarchicus* in the laboratory under different food and temperature conditions. *Marine Ecology Progress Series*, 221: 185-197.

White, J.R., Roman, M.R. (1992) Egg production by the calanoid copepod *Acartia tonsa* in the mesohaline Chesapeake Bay: the importance of food resources and temperature. *Marine Ecology Progress Series*, 86: 239-249.

Chapter 5

Plankton community and copepod production in a temperate coastal lagoon: what is changing in a short temporal scale?

Joana Cruz, M. Alexandra Teodósio, Susana Garrido, Radhouane Ben-Hamadou, Luís Chícharo, Pedro Ré and A. Miguel P. Santos

Journal of Sea Research (submitted May 2019)

Plankton community and copepod production in a temperate coastal lagoon: what is changing in a short temporal scale?

Abstract

Coastal lagoons are often exposed to intense short-term environmental changes and strong anthropogenic pressures influencing zooplanktonic communities and production. However, most works focus on long-term temporal scales using monthly or seasonal sampling strategies. The present study analysed the structure of the mesozooplanktonic assemblages, the production (egg production rates) and physiological condition (RNA:DNA ratio) of the copepod *Acartia clausi* in a temperate coastal lagoon (Ria Formosa) during the summer, using an intensive sampling approach. Salinity was the main factor affecting the short-term variability of mesozooplankton composition, followed by tidal phase (ebb tides) and semilunar cycle (spring tides). There was a positive relationship between the abundance of Appendicularia and *Penilia avirostris* and the toxic dinoflagellate *Gymnodinium catenatum*, suggesting no deleterious effects. The egg production rate of *A. clausi* was influenced by salinity and ammonia concentration, with a positive correlation between the egg productivity and the toxic macronutrient, showing a possible adaptation of this calanoid species. The RNA:DNA index was positively related to egg production rate, suggesting that it is a good proxy for the reproductive output of copepods, even in short-term periods. This study shows that different timescales need to be included in regular monitoring of planktonic assemblages in coastal lagoons in order to understand the influence of environmental and anthropogenic variables on marine organisms.

Keywords: *Acartia clausi*, egg production rate, Ria Formosa coastal lagoon, RNA:DNA ratio.

5.1 Introduction

Coastal lagoons are shallow nutrient-rich ecosystems, with a typically unstable environment threatened by climate changes and usually under intense anthropogenic pressures (Barbosa and Chícharo, 2011). Planktonic organisms respond rapidly to modifications in the environment, therefore are considered good indicators of environmental change in the ecosystems. However, it is difficult to infer the specific links between environmental variability and plankton dynamics in coastal lagoons. Tidal dynamics is usually strong and the major mechanical energy input in coastal lagoons, forcing water circulation through turbulent mixing and driving the physical, chemical and biological interactions inside these ecosystems (Schelske and Odum, 1962). Besides changes in tides, daily variability in wind stress on the water surface and freshwater input generally provide part of the mechanical energy necessary for the structuring of coastal lagoons (Barbosa, 2010).

The dynamics of planktonic communities in transitional systems highlights the unsteadiness of their spatial and temporal features. Most studies in these areas are developed under a monthly sampling strategy (e.g. Primo et al., 2009; Vieira et al., 2015), missing the short-term changes in the structure and production in those ecosystems, related to circatidal, circadian and circalunidian periodicities (Last et al., 2009). Although there is a significant number of studies of zooplankton communities in coastal lagoons worldwide, the patterns of planktonic production and the trophic interactions of their assemblages are poorly known (e.g. Repelin, 1985; Heerkloss et al., 1991; Sprung, 1994; Marques, 2005).

Planktonic communities can be quite distinct between coastal lagoons located in the same geographic area, depending on their interactions with the sea, local hydrodynamics and the influence of other physical parameters. These communities have a relevant role in sustaining the functioning and productivity of these areas, serving as a breeding and feeding ground for many species of fish and birds, and supporting a wide range of human activities, such as fisheries, shellfish farming and tourism (Barbosa, 2010). There are several examples of planktivorous fishes using these coastal lagoons as nursery areas (e.g. anchovies, sardines and some sparids), or living here as residents (e.g. atherinids) (Chícharo et al., 2012). The planktonic production of these areas also supports the important production of bivalves (Chícharo and Chícharo, 2001). At the same time, these planktonic assemblages can also be a threat to the ecosystem services of coastal lagoons, when noxious blooms occur, including toxic microalgae

that threaten higher trophic levels, zooplankton and bivalves (Cerejo and Dias, 2007), or harmful jellies that quickly consume early phases of fishes in nursery areas (Pereira et al., 2014). Therefore, the planktonic dynamics and production of these ecosystems need to be investigated in a significantly higher temporal resolution than the one usually used to study them.

Copepoda is the dominant group of mesozooplanktonic communities (Mauchline, 1998) and represents the major link between microbial food webs and higher trophic levels (Kiørboe, 1997). Among Copepoda, the genus *Acartia* is one of the most ubiquitous and abundant inhabiting coastal lagoons and estuarine systems (Azeiteiro et al., 2005; Leandro et al., 2007), thus, it is extremely important to estimate its secondary production. The most common technique used to estimate secondary production in laboratory and field studies is the egg production rate method (EPR) (Runge and Roff, 2000), that indicates the current nutritional status of wild caught copepods as well as their adaptations to environmental variability over a short period of time (~24 h). However, in the last decades, biochemical approaches such as the determination of RNA:DNA ratio have been developed to assess the physiological condition of copepods (Chícharo and Chícharo, 2008), as an indicator of growth (Elser et al., 2000; Wagner et al., 2001), nutritional condition (Wagner et al., 1998; Vehmaa et al., 2012), dormant condition (Kobari et al., 2013) and egg viability (Hogfors et al., 2011). Additionally, the RNA:DNA ratio has been shown to be correlated with female egg production (Nakata et al., 1994; Saiz et al., 1998; Gorokhova, 2003; Cruz et al., 2017), making it a potential alternative method for the estimation of production, with several advantages over the traditional techniques such as sensitivity, precision and repeatability.

In this study, we aim to analyze the short-term variability of the mesozooplanktonic assemblage structure in a temperate coastal lagoon (Ria Formosa) during the peak production period that occurs during summer, which is typical of the unimodal annual cycle of planktonic production that occurs in the temperate coastal lagoons (Barbosa and Chícharo, 2011). For that, we investigate the correlation between the mesozooplankton and the environmental conditions (abiotic and biotic parameters). Particularly, the production (EPR and females' secondary production) and physiological condition (RNA:DNA ratio) of *Acartia clausi* are assessed in relation to the main hydrological parameters and phytoplanktonic prey availability.

5.2 Materials and methods

5.2.1 Study area

Ria Formosa is a highly productive system of great ecological and economic importance recognized by the Ramsar Convention and Natura 2000, being a National Park since 1987. It is a mesotidal coastal lagoon system located in the south of Portugal with approximately 55 km length and a maximum 6 km width, with a total wetland of circa 110 km^2, five sandy barrier islands, being permanently connected with the Atlantic Ocean by six inlets (Fig. 5.1). The tidal range varies between 0.5 m at neap tides and 3.5 m during spring tides, causing semidiurnal and fortnightly tidal amplitudes variations (Falcão and Vale, 1990). It is a considerably shallow system with average depths of 2 m, reaching an average of 6 m at the main navigable channels (Newton and Mudge, 2003). The freshwater inputs are scarce except for few months in autumn and winter when rainfall occurs, leading to low run-offs. The salinity of the water ranges on average from 32 to 36.5 and the hydrodynamic circulation is dominated by the tidal cycle (Newton and Mudge, 2003). The climate is predominantly Mediterranean, characterized by hot dry summers and warm wet winters.

5.2.2 Sampling

Sampling was performed at a fixed station (37º00'16.91" N - 7º59'14.13"W) in the Ria Formosa lagoon system from July 28th to September 3rd, 2009 (Fig. 5.1). Mesozooplankton samples were collected with a WP2 conical net with 0.13 m^2 mouth opening and 200 μm mesh size, horizontally towed just below the sea surface, for 5 min at approximately 2 knots, equipped with a HydroBios flow meter. For 19 days, two hauls were taken in the morning around 11h00, and in the afternoon around 16h00. At the end of each haul, the cod end content was preserved in 4% buffered formalin for posterior determination of zooplankton composition and abundance. Environmental variables such as temperature, salinity, pH and dissolved oxygen were measured with a hand-held meter (VWR Symphony SP90M5). Precipitation data during the sampling period was obtained from the *Sistema Nacional Informação Recursos Hídricos* (http://snirh.pt/). Surface water samples were taken for the determination of nutrients

concentration (nitrates, nitrites, ammonia, phosphates, and silica) and for identification and quantification of the major microplankton taxa. The dissolved inorganic macronutrients concentrations were determined according to a spectrophotometric method using Spectroquant cell test photometric kits (Merck Millipore) and the Spectroquant Nova 60 photometer (Merck Millipore). Samples of microplankton were preserved with acid Lugol's solution and subsamples of 50 ml were concentrated by gravimetric sedimentation by the Utermöhl technique (Hasle, 1978) and observed using a Zeiss IM35 inverted microscope with phase contrast and bright field illumination. Zooplankton samples were fractioned with a Folsom plankton splitter, and at least 500 organisms were counted and identified under a Leica S8 APO stereoscopic microscope. The abundance of taxa was expressed as the number of individuals per cubic meter (ind m^{-3}).

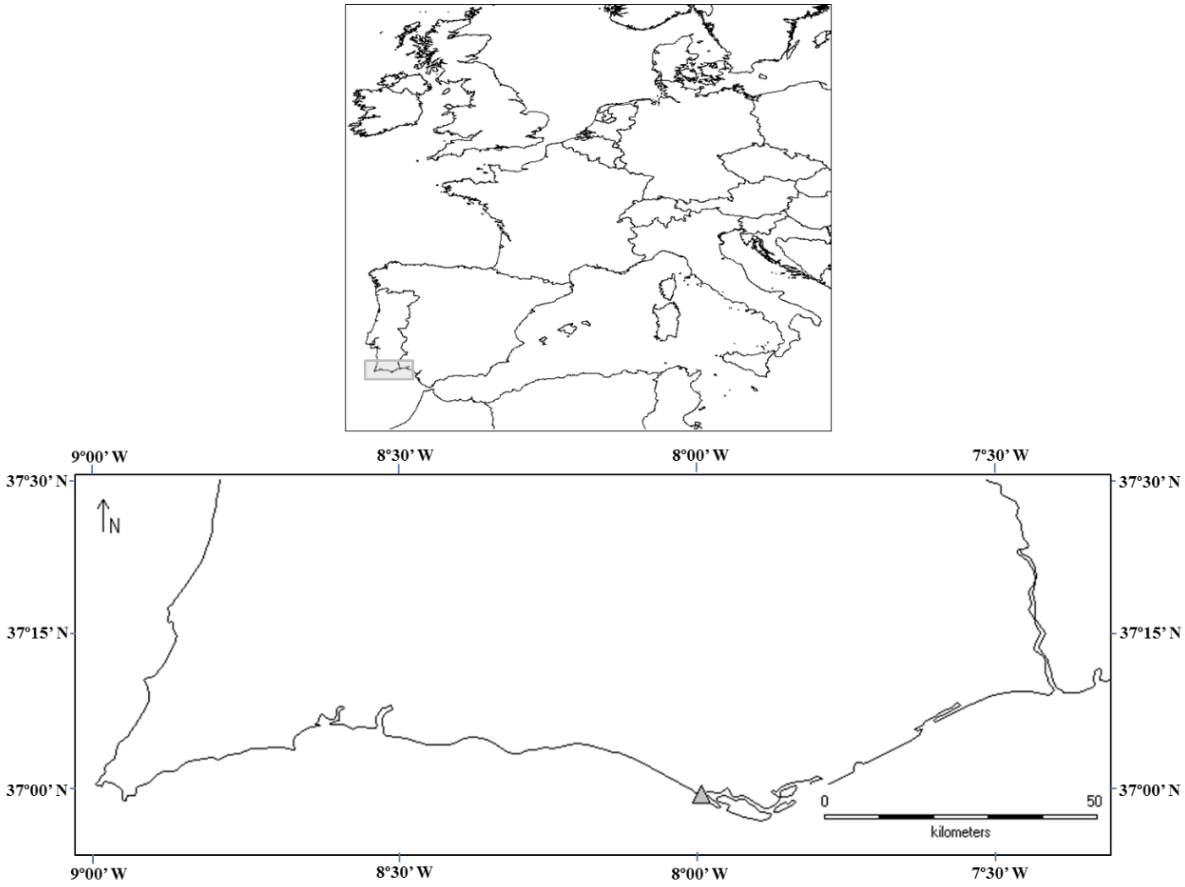

Fig. 5.1 – Map of the studied area with the fixed sampling station located in the western part of Ria Formosa Coastal lagoon (light grey triangle).

To obtain the total biomass, the displacement volume was calculated and converted to dry weight using the following equation (Wiebe, 1988):

$$Log_{10}(DV) = -1.842 + 0.865\, Log_{10}(DW)$$

where *DV* is the displacement volume (mL) and *DW* is dry weight (mg).

5.2.3 Egg production experiments

Egg production rates (EPR) experiments were performed during 12 days of the sampling period during high tide. To catch females of the free-spawning copepod *Acartia clausi* an additional tow was conducted with the same conical net, but with a lower velocity to avoid copepods disturbance. The cod-end content was gently transferred to a clean insulated container and diluted with surface seawater for transport to the laboratory. There, females were gently sorted with the help of a glass pipette and placed in 500 ml glass goblets (experimental units), already filled with natural water sieved through 50 μm mesh to remove any copepod eggs and metazoan zooplankton prior to the experiments. Six experimental units (500 ml goblets) were used at each experimental day, and 5 to 8 undamaged and actively swimming females were placed in each. The experiment lasted for 24 hours, after which all the females were removed by sieving the water through a 200 μm mesh and placed in liquid nitrogen for posterior RNA:DNA ratio analysis. Additionally, all the existing eggs and nauplii were counted to determine the egg production rate.

The Carbon-specific egg production rates (*SEP*) of *Acartia clausi* was calculated according to the equation:

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

where *EPR* 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 0.04 μg C egg^{-1} (Kiørboe and

Sabatini, 1995). Female carbon weights were estimated from prosome lengths (PL) using the equation $\log(Wf) = 3.005 \times \log(PL)^{-8.444}$ (Ayukai, 1987). The biomass was calculated by multiplying the female carbon weight by the abundance of females. Female copepod production was then calculated by multiplying the female biomass by SEP.

5.2.4 RNA:DNA ratio analysis

RNA and DNA of adult females of *Acartia clausi* used in the EPR experiments were estimated with the microplate fluorescent assay (MFA) of Ikeda et al. (2007). The MFA assay is a modification of the sequential fluorometric method of Bentle et al. (1981) in which DNA and RNA in a single sample are determined sequentially by the addition of DNase and RNase. The sequential fluorometric method was modified to the MFA with 96-well microtiter plates and the DNase step was eliminated using a sarcosyl extraction technique (Wagner et al., 1998).

The wet weight (WW) of a batch of 5 to 30 females was determined to obtain at least 0.5 mg of WW. The organisms were homogenized by sonication (3 pulses of 50 A during 1 min) with cold sarcosyl extraction buffer. The volume of extraction buffer was 100 µl (0.5%). Afterwards, all the samples were shaken for 30 minutes at room temperature on a vortex mixer equipped with a multiple-vial head. Next, they were centrifuged (12000 × g; 0-4ºC) for 15 min to separate insoluble copepods remains. The samples were subsequently 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 µg $µl^{-1}$ 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 96-well microplates (type nuclon black round bottom). The mean ratio of the slopes of the standard curves was 2.5 ± 0.2, which allows comparing the RNA/DNA ratio results determined by other protocols (Caldarone et al. 2006). 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). After the first reading, RNase solution (15 µl, 0.12µg ml^{-1}) was added to each well and incubated at 37ºC for 30 minutes. The concentration of DNA was calculated directly by the standard curve. The concentration of RNA was determined indirectly by subtraction of DNA fluorescence (second

scan) from total fluorescence (first scan).

5.2.5 Data analysis

The diversity of the mesozooplankton communities was determined as the number of species (species richness) and the Shannon-Wiener index, using PRIMER-6 software (Clarke and Gorley, 2006).

After the exploratory analysis of the mesozooplankton data using linear models, the data distributions were checked. Generalized Additive Mixed Models (GAMMs) were used to evaluate the potential contribution of selected independent environmental variables (water temperature, salinity, dissolved oxygen, food availability, nutrients, tidal phase and tidal coefficient) in explaining the variability of the dependent variables (abundance of the main mesozooplankton taxa). GAMMs are a flexible class of statistical predictive models which allow nonlinear relationships between a set of predictors and a dependent variable, using data not collected according to a balanced design and dealing with heterogeneity or temporal correlation in the counts. The models were fitted in the open source software R 2.15.3 (R Development Core Team, 2013), using the gamm function from the mgcv library (Wood, 2006), with all smoothness parameters estimated using restricted maximum likelihood (REML). Smoothing splines were used to represent the nonlinear effect of the predictors. The tidal phase was used as a factor, attributing it to each sampling at each specific time. The dependent variable was modelled using Gaussian distribution functions with a logarithmic link. The temporal auto-correlation of the data was treated with an autoregressive model of order one (AR-1) (from the nlme library for R). From the full set of calculated models (considering different explanatory variables and lognormal distribution functions), we selected the best models, and thereby the explanatory variables most likely responsible for the variability of mesozooplankton abundances, based on Akaike Information Criterion (AIC; Sakamoto et al., 1986). AIC gives information about the degree of fit of a model with the number of variables, to find the most parsimonious model. The statistical significance of the terms in the model (based on the approximate p-values produced by GAMM) was also considered. All the dependent variables were log transformed prior the analysis.

Generalized Linear Models (GLM; Venables and Ripley, 2002) were used to analyse the variability of EPR and RNA:DNA ratio of *Acartia clausi*. For both response variables, a Gaussian GLM was used with an identity link. The following predictor variables were considered for EPR: temperature, salinity, dissolved oxygen, food availability and nutrients. To analyse the RNA:DNA ratio variability and possible relation to egg production rates, the same independent variables were used adding the EPR. Model predictors were selected and removed by backward elimination based on the AIC. Only those predictors which contributed significantly to the model were kept. All the model analyses were also performed using the open source software R 2.15.3 (R Development Core Team 2013).

5.3 Results

5.3.1 Environmental conditions and food availability

Water temperature ranged from 20.8 and 27.8ºC and salinity from 34.3 to 37.6, the latter with lower values registered at the end of August and beginning of September (Fig. 5.2). Dissolved oxygen ranged from 4.2 to 8.7 mg L^{-1} and pH between 8 and 8.3 (Fig. 5.2). Ammonia showed a minimum value of 0.04 mg L^{-1} and a maximum of 0.68 mg L^{-1}, nitrites with a minimum of 0.002 mg L^{-1} and a maximum of 0.056 mg L^{-1}, nitrates with values ranging from 0.03 to 0.24 mg L^{-1}, phosphate concentrations from 0.04 to 0.21 mg L^{-1}, and silica concentrations between 0.06 and 0.85 mg L^{-1} (Fig. 5.2). All the macronutrients increased the concentrations with time, showing maximum values at the end of August. There was no precipitation during all the sampling period.

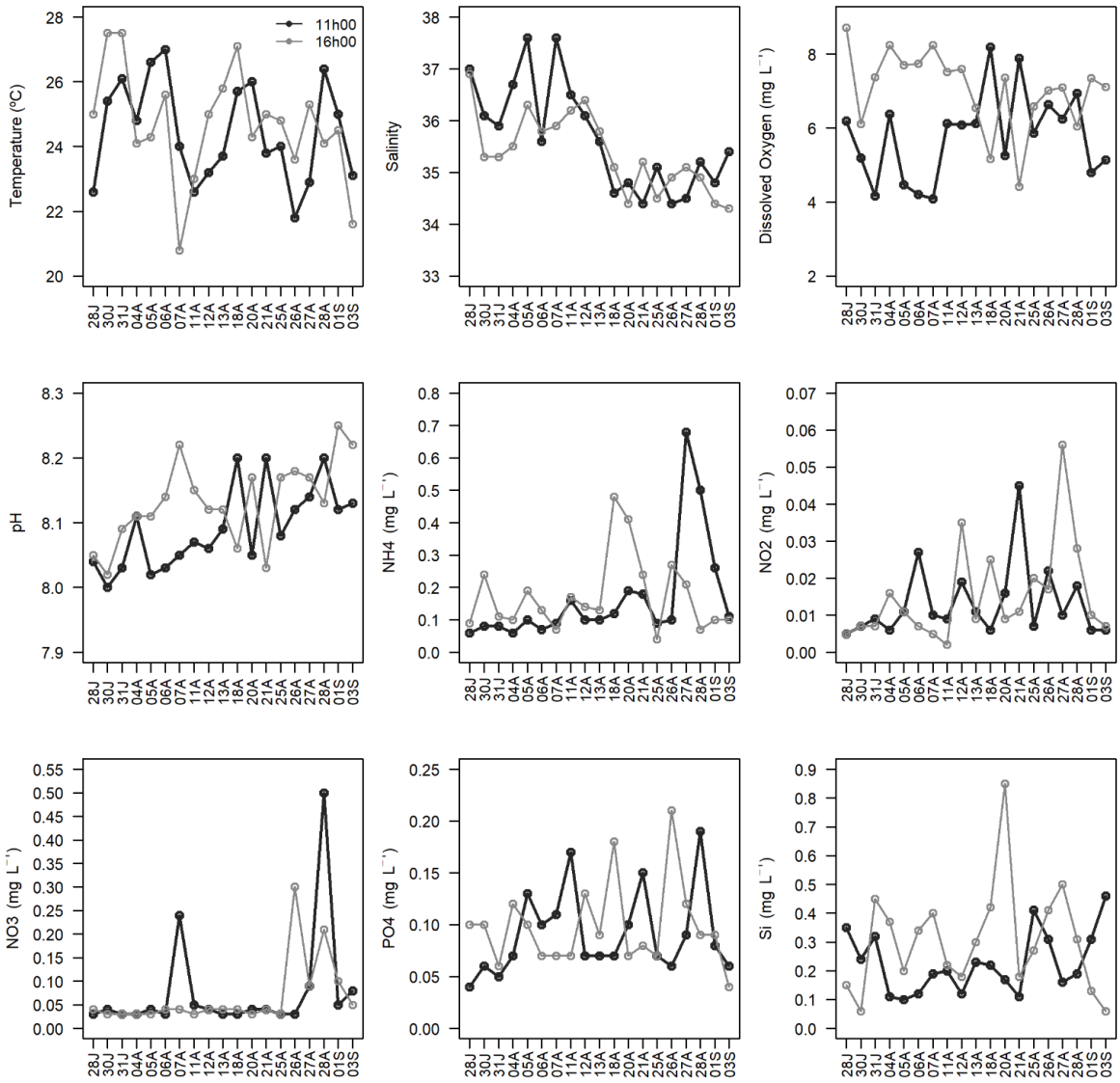

Fig. 5.2 - Environmental parameters measured in Ria Formosa lagoon during the sampling days from July 28th (28J) to September 3rd (03S), 2009, at 11h00 – black line, and 16h00 – grey line. (Temperature ºC, Salinity, Dissolved Oxygen mg L^{-1}, pH, NH4 – ammonia mg L^{-1}, NO2 – nitrites mg L^{-1}, NO3 –nitrates mg L^{-1}, PO4 – phosphorous mg L^{-1}, Si – silica mg L^{-1}).

Considering the carbon content of the main groups of microplankton (Fig. 5.3; Table 5.1), dinoflagellates were clearly dominant during this study, with densities ranging from 1.6 to 802.8 µg C L^{-1}, with higher values occurring at the end of the sampling period. The most abundant taxa were *Protoperidinium* spp., *Protoperidinium quinquecorne*, *Gymnodinium* spp., *Gymnodinium catenatum*, *Scrippsiella trochoidea* and *Prorocentrum* spp. (Fig 5.3; Table 5.1). The mean carbon content of ciliates oscillated throughout the sampling period, with a minimum value of 2.2 and a maximum of 72.4 µg C L^{-1}, while the diatoms were the least abundant group

(minimum: 0.3 μg C L^{-1} maximum: 16.4 μg C L^{-1}) (Fig. 5.3). The most representative taxa of diatoms were *Rhizosolenia* spp. and *Leptocylindrus* spp. (Fig. 5.3; Table 5.1).

Table 5.1 - Mean abundances (ind.m^{-3}) of mesozooplankton taxa and mean carbon content (μg C L^{-1}) of the main microplankton taxa identified in Ria Formosa lagoon from July 28th (28J) to September 3rd (03S), 2009.

Taxa	Mean ± SD	Taxa	Mean ± SD
Microplankton		*Temora longicornis*	2.0 ± 7.9
Diatoms	3.8 ± 3.1	*Temora stylifera*	1.9 ± 8.2
Rhizosolenia spp.	2.7 ± 2.4	*Centropages chierchiae*	737.9 ± 2320.4
Leptocilyndrus spp.	0.6 ± 2.5	Pontellidae	44.6 ± 116.7
Dinoflagellates	102.4 ± 157.6	*Oithona* spp.	634.8 ± 1630.4
Scrippsiella trochoidea	0.8 ± 1.4	*Oncaea* spp.	28.8 ± 86.7
Protoperidinium spp.	36.4 ± 134.7	*Corycaeus* spp.	5.1 ± 22.0
Protoperidinium quinquecorne	38.0 ± 73.1	Harpacticoida	1103.8 ± 1614.1
Gymnodinium spp.	8.5 ± 17.3	*Euterpina acutifrons*	158.6 ± 359.7
Gymnodinium catenatum	1.8 ± 4.3	*Clytemnestra* spp.	5.6 ± 19.9
Prorocentrum spp.	1.0 ± 1.4	*Microsetella* spp.	0.1 ± 0.6
Ciliates	30.6 ± 18.8	**Cirripedia**	131.0 ± 187.1
Mesozooplankton		Nauplii	105.9 ± 169.7
Hydromedusae	2.0 ± 5.9	Cyprids	25.1 ± 38.5
Siphonophora	0.3 ± 1.8	**Ostracoda**	0.8 ± 3.4
Mollusca	1144.3 ± 2930.8	**Cumacea**	0.2 ± 1.0
Gastropoda larvae	1131.7 ± 2934.5	**Decapoda**	36.8 ± 65.6
Bivalvia larvae	12.6 ± 29.3	Caridea	8.2 ± 33.2
Polychaeta larvae	6.3 ± 14.1	Thalassinidae	0.04 ± 0.2
Amphipoda	104.8 ± 139.2	Anomura	17.8 ± 45.1
Isopoda	4.0 ± 9.8	Brachyura	10.7 ± 16.6
Cladocera	1242.4 ± 2824.4	**Chaetognatha**	11.2 ± 26.0
Podon spp.	392.7 ± 971.5	**Doliolida**	9.1 ± 48.9
Evadne spp.	52.7 ± 119.1	**Appendicularia**	
Penilia avirostris	797.1 ± 2122.1	*Oikopleura* spp.	51.1 ± 154.8
Copepoda	3453.7 ± 4592.0	**Ascidiacea**	22.5 ± 32.3
Nauplii	125.3 ± 203.5	**Ichthyoplankton**	0.5 ± 0.9
Paracalanus spp.	204.1 ± 354.3	Eggs	0.1 ± 0.3
Clausocalanus spp.	0.6 ± 3.1	Larvae	0.4 ± 0.9
Acartia clausi	394.0 ± 821.4	**Total Zooplankton**	6220.9 ± 9225.8
Paracartia grani	6.6 ± 39.7		

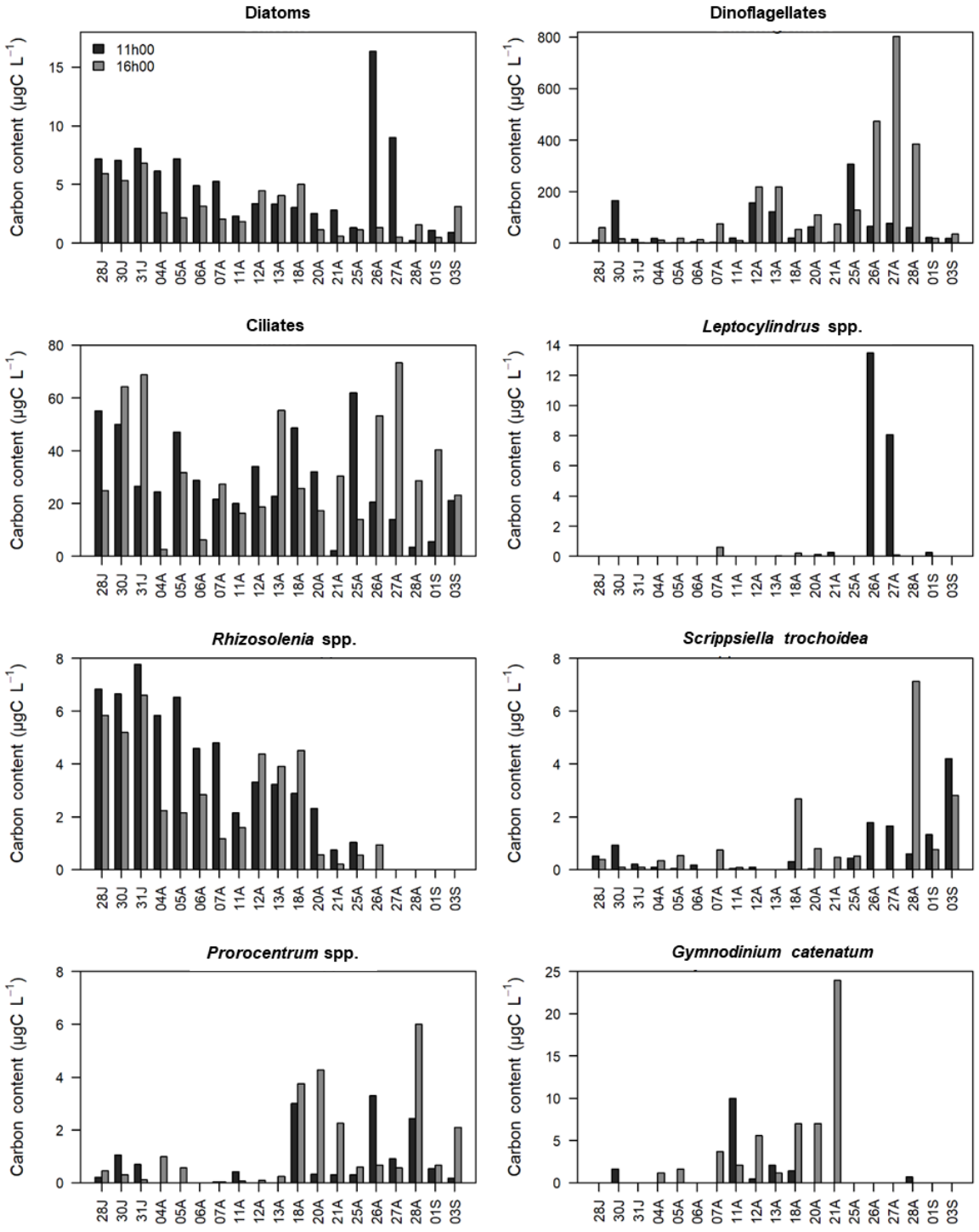

Fig. 5.3 - Carbon content (µg C L^{-1}) of the main microplankton groups and dinoflagellates species identified in Ria Formosa from July 28th to September 3rd, 2009, at 11h00 – black bar and 16h00 – grey bar. Note the change of scale in the y-axis.

5.3.2 Mesozooplankton biomass, diversity and abundance: environmental conditions influence

The biomass of mesozooplankton did not show any clear pattern with time and ranged from 3.3 to 208.8 mg m^{-3}. The Shannon-Wiener diversity index presented values between 1.1 and 2.3, while species richness varied between a minimum of 11 to a maximum of 24 taxa (Fig. 5.4), and these indices did not show any clear pattern with time, as well. Total mesozooplankton abundance was on average 6220.9 ± 9225.8 ind m^{-3} ranging between 206.4 and 38970.0 ind m^{-3}, and higher values were registered at the end of August and beginning of September (Fig. 5.5; Table 5.1).

A total of 41 taxa of mesozooplankton were identified during the sampling period (Table 5.1). Copepoda was the most abundant group with an average of 3453.7 ± 4592.0 ind m^{-3}, followed by Cladocera (1242.4 ± 2824.4 ind m^{-3}), Mollusca larvae (144.3 ± 2930.8 ind m^{-3}) and Cirripedia larvae (131.0 ± 187.1 ind m^{-3}). Copepoda abundance showed higher peaks in the second half of the sampling period, very similar to the total zooplankton abundances pattern, and the dominant species were the Calanoida *Paracalanus* spp., *Acartia clausi*, *Centropages chierchiae*, the Cyclopoida *Oithona* spp. and the Harpacticoida *Euterpina acutifrons* (Fig. 5.6; Table 5.1).

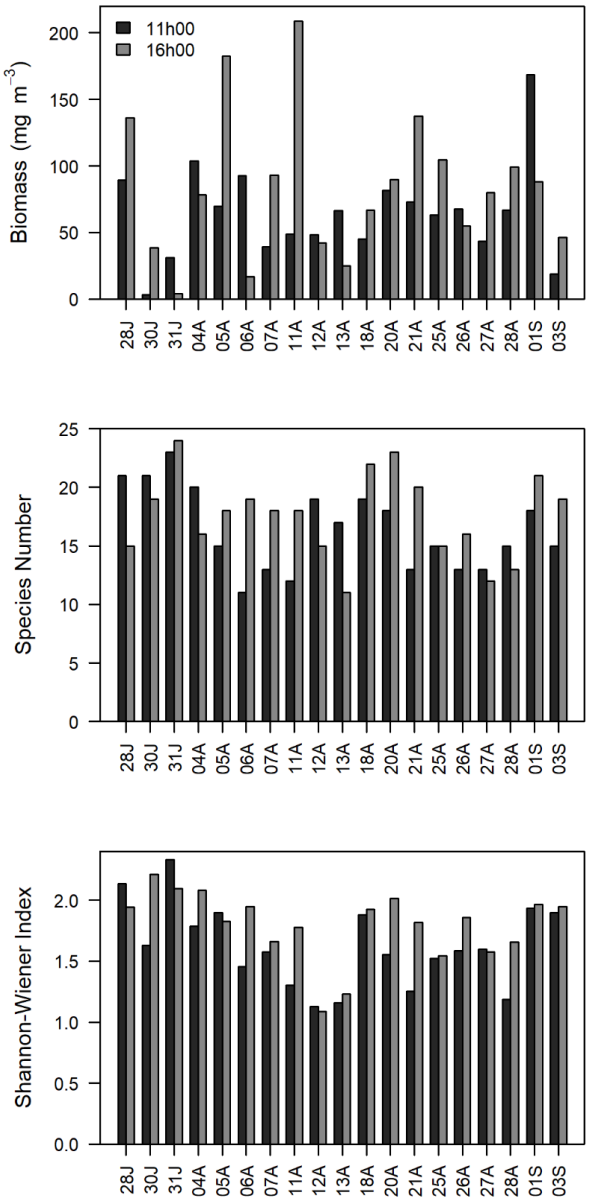

Fig. 5.4 – Mesozooplankton biomass (mg m^{-3}), species richness and Shannon-Wiener diversity index in Ria Formosa lagoon from July 28th (28J) to September 3rd (03S), 2009, at 11h00 – black bar and 16h00 – grey bar.

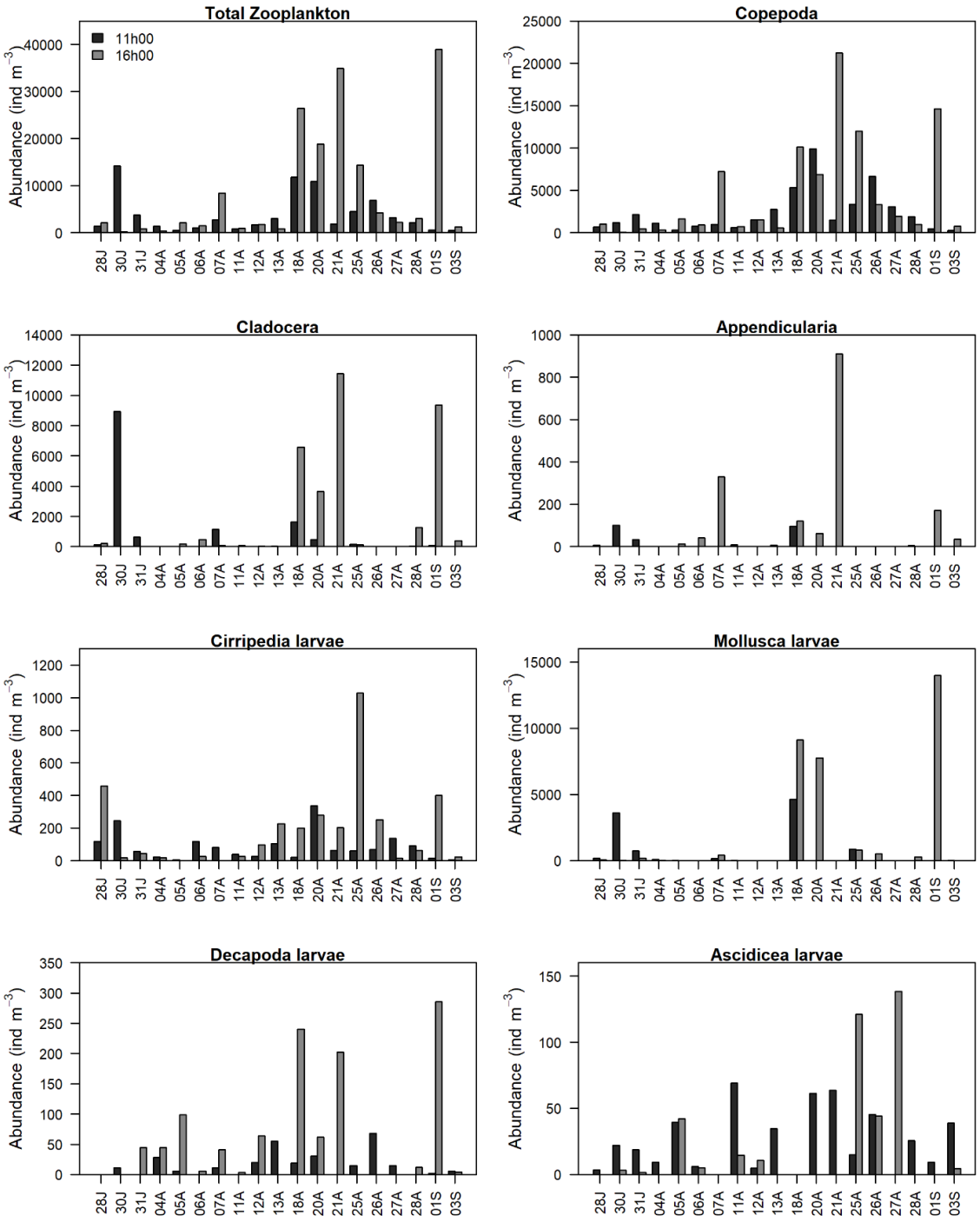

Fig. 5.5 - Abundances (ind.m^{-3}) of the main mesozooplankton taxa in Ria Formosa lagoon from July 28th (28J) to September 3rd (03S), 2009, at 11h00 – black bar and 16h00 – grey bar. Note the change of scale in the y-axis.

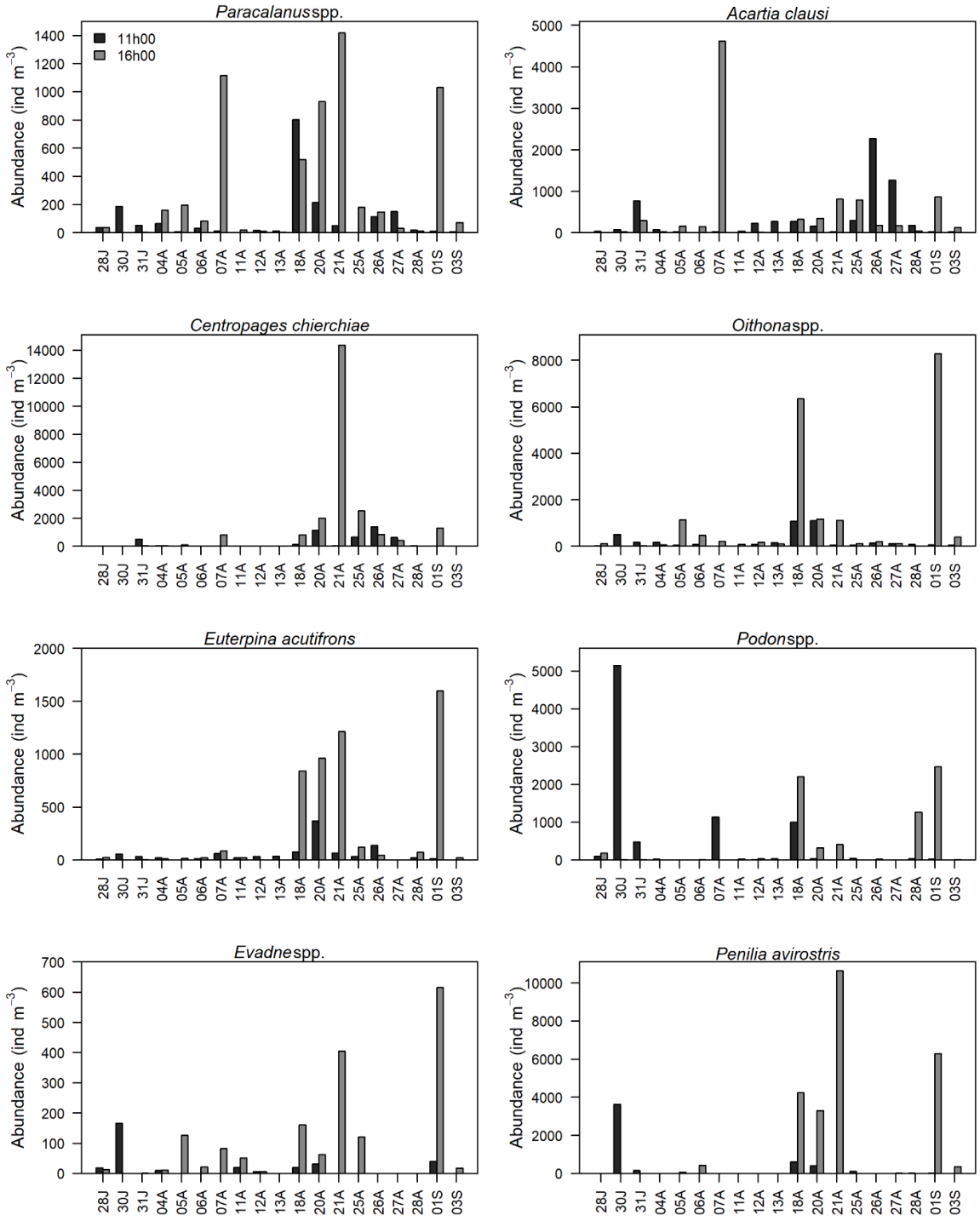

Fig. 5.6 - Abundances (ind.m^{-3}) of the main mesozooplankton species in Ria Formosa lagoon from July 28th (28J) to September 3rd (03S), 2009, at 11h00 – black bar and 16h00 – grey bar. Note the change of scale in the y-axis.

Salinity was the most important environmental factor that explains the distribution of most mesozooplankton taxa (Table 5.2). This parameter influenced the abundance of Copepoda, as well as of particular species within this group, such as *Paracalanus* spp., *Acartia clausi*, *Centropages chierchiae*, *Oithona* spp. and *Euterpina acutrifons*, but also influenced Cirripedia larvae. The abundance of all taxa seemed to respond to an optimal salinity around 34.5-35 and then decreased towards higher salinities. The tidal phase explained also the abundances of Copepoda (*Paracalanus* spp., *A. clausi*, *C. chierchiae* and *Oithona* spp.), Decapoda larvae and the species richness being positively related to ebb tides, negatively related to low tides and in some cases also to flood tides (*A. clausi*, Decapoda larvae and species richness) (Table 5.2). Tidal coefficient significantly influenced Copepoda (*Oithona* spp. and *E. acutifrons*) and Decapoda larvae, the three showing higher abundances when the tidal range increased, i.e. during spring tides (Table 5.2). Appendicularia registered higher abundances around 27ºC and was the only taxa influenced by the increase in temperature (Table 5.2). The abundance of the toxic dinoflagellate *Gymnodinium catenatum* was positively related to the abundance of Appendicularia and *Penilia avirostris* (Table 5.2), while two other dinoflagellates, *Scrippsiella trochoidea* and *Prorocentrum* spp., were significantly related to the variability in the abundance of the cladocerans *Evadne* spp. and *P. avirostris*, respectively, presenting higher abundances when the carbon content of the two dinoflagellate genus increased (Table 5.2). Higher biomass of total Diatoms influenced *Evadne* spp. abundance.

Table 5.2 - Results of Generalized Additive Mixed Models (GAMMs), species richness (SR) and abundance of mesozooplankton taxa in ind. m^{-3} (Copep – Copepoda; *Parac.* – *Paracalanus* spp.; *Acart.* – *Acartia clausi*; *Centrop.* – *Centropages chierchiae*; *Oith* – *Oithona* spp.; *Euterp.* – *Euterpina acutifrons*; *Evad.* – *Evadne* spp.; *Penil*. – *Penilia avirostris*; Append. – Appendicularia; Decap. – Decapoda larvae; Cirrip. – Cirripedia larvae) with indication of significant explanatory variables: tidal phase as a factorial parameter (F – Flood; H – High; L – Low) and smooth terms (T – temperature ºC; S – salinity; TC – tidal coefficient m; Diat – diatoms µg C L^{-1}; Scripp – *Scrippsiella trochoidea* µg C L^{-1}; Proroc - *Prorocentrum* spp. µg C L^{-1}; Gymnc– *Gymnodinium catenatum* µg C L^{-1}. AIC is the Akaike Information Criterion. (Signif. codes '***' p < 0.001 '**' p < 0.01 '*' p < 0.05).

Taxa	**SR**	**Copep**	***Parac***	***Acart***	***Centrop***	***Oith***	***Euterp***	***Evad***	***Penil***	**Append**	**Decap**	**Cirrip**
Parametric												
Tide E	18.7***	28.5***	9.0 ***	13.6***	6.8***	12.9***					6.9***	
Tide F	-3.5**	-1.4	-0.8	-3.7***	-1.4	-1.4					-3.1**	
Tide H	-0.2	-2.0	0.4	-1.4	0.1	0.2					-1.5	
Tide L	-2.7*	-3.0**	-3.1**	-5.0***	-2.4*	-5.7***					-3.5**	
Smooth terms												
s(T)										6.1**		
s(S)		7.7***	3.7*	5.2**	2.9*	5.0*	3.6*					5.3*
s(TC)		16.1***				14.6***	12.6**				6.6*	
s(Diat)								5.2**				
s(Scripp)								8.0***				
s(Proroc)									3.1*			
s(Gymnc)									4.4*	15.9***		
AIC		142.9	147.5	142.9	172.0	140.7	160.3	164.0	192.7	154.7	150.9	139.8

5.3.3 *Acartia clausi* EPR, RNA:DNA ratio and secondary production

The abundance of *Acartia clausi* females varied throughout the studied period, with maximum values of 662.5 ind. m^{-3}, a minimum of 7.3 ind. m^{-3}, and an average of 128.6 ± 178.4 ind. m^{-3} (Fig. 5.7a). The higher abundances were found at the end of August and beginning of September. EPR of *Acartia clausi* varied between 2 ± 0.6 and 12.5 ± 2.2 eggs $female^{-1}$ day^{-1}, with an average of 7.5 ± 2.9 eggs $female^{-1}$ day^{-1} (Fig. 5.7b). RNA:DNA ratio ranged from 0.1 and 3.6, with an average of 1.8 ± 1.3 and higher values registered during the second half of August and beginning of September (Fig. 5.7c). The secondary production of females reflected their biomass and varied between 1.73 and 207.8 µg C m^{-3} day^{-1} and an average summer

production of 42.6 ± 59 µg C m^{-3} day^{-1}. Higher values were found at the end of the sampling period (Fig. 5.7d).

Total EPR of *Acartia clausi* was statistically significant and positively related to salinity and ammonium (Table 5.3). The RNA:DNA ratio was significantly and positively related to EPR, and negatively related to temperature (Table 5.3).

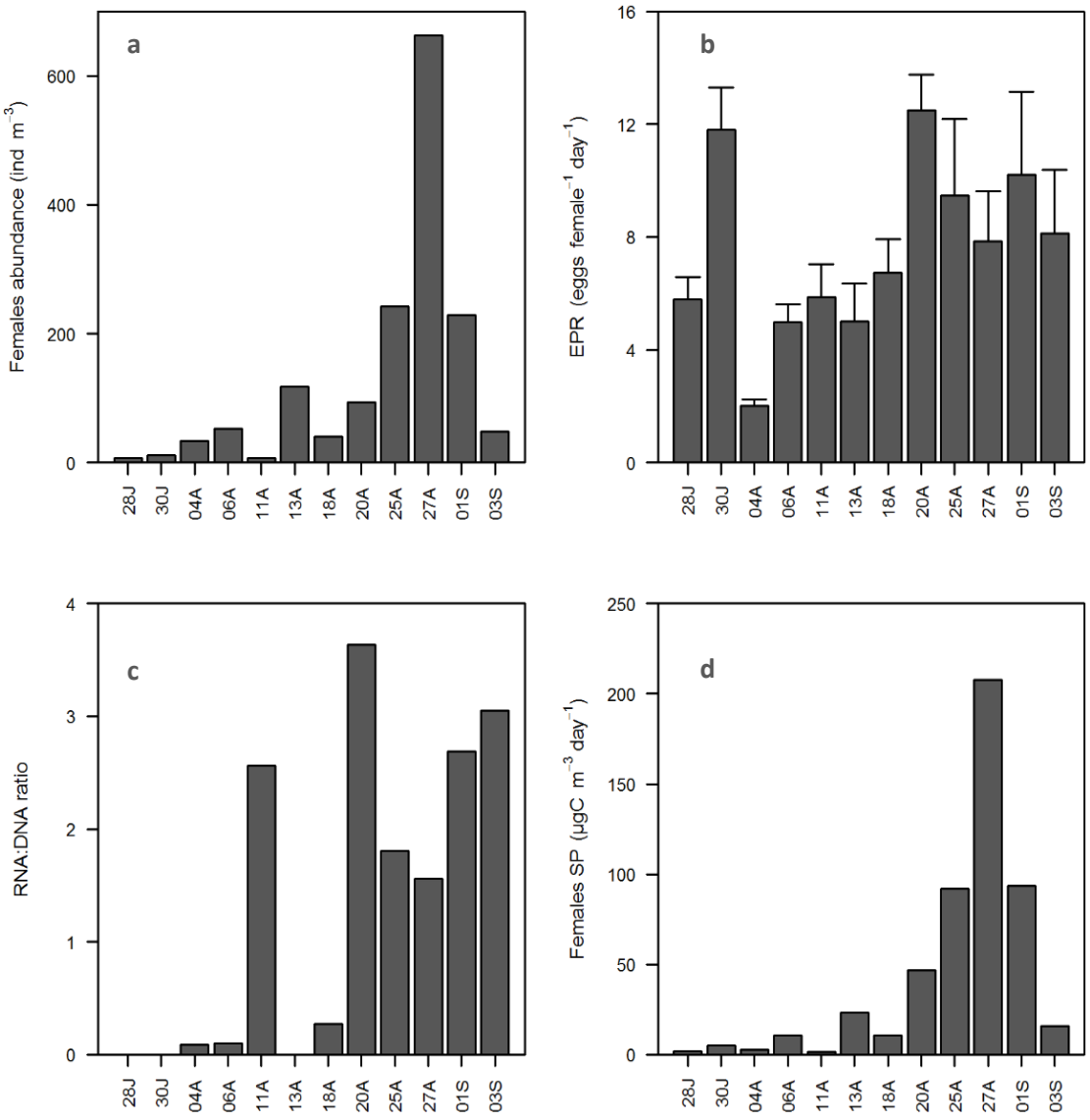

Fig. 5.7 – *Acartia clausi* females abundance (ind.m^{-3}) (a), egg production rates (eggs $female^{-1}$ day^{-1}) (b), RNA:DNA ratio (c) and females secondary production (µg C m^{-3} day^{-1}) (d) in Ria Formosa lagoon during twelve sampling days from 28th July (28J) to 3rd September (03S), 2009.

Table 5.3 - Coefficients and significance (p-value) of each of the explanatory variables of the Generalized Linear Models (GLMs - Gaussian) describing the variation of the egg production rates (EPR) and the RNA:DNA ratios of *Acartia clausi*. NH_4 represents ammonia. 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 is the degrees of freedom.

Coefficients	**Independent variables**	**EPR**	**RNA:DNA**
Gaussian	EPR	-	0.35**
(identity)	Temperature	n.i.	-0.54*
	Salinity	-2.07*	n.i.
	NH_4	19.40*	-
	Diatoms	n.i.	n.i.
	Dinoflagellates	n.i.	n.i.
	Ciliates	n.i.	n.i.
	AIC	54.69	19.7
	LogLik	-23.35	9.27
	DF	4	4

5.4 Discussion

5.4.1 Mesozooplankton community: influence of environmental conditions

Salinity has been considered an important factor influencing zooplankton community structure, especially in estuarine and coastal environments that are subject of constant changes (Gunter, 1961; Day et al., 1989; Greenwald and Hurlbert, 1993). In the present study, salinity was one of the main factors affecting the abundances and variability of several taxa, including Copepoda (*Paracalanus* spp., *Acartia clausi*, *Centropages chierchiae*, *Oithona* spp. and *Euterpina acutifrons*) and Cirripedia larvae. The optimal salinity that favoured higher abundances of all the referred taxa was around 34.5-35, above which the abundances started to decrease. This is in accordance to a previous study in the northern Adriatic Sea with salinities ranging up to ~38, where a negative correlation was found between the abundance of calanoid species and salinity, with species such as *Acartia clausi* and *Centropages kroyeri* preferring

lower salinities (Bojanić Varezić et al., 2015). Regarding the Cirripedia, several studies have shown that salinity is also one of the main factors influencing their early life stages (Anil and Kurian, 1996; Nasrohali et al., 2016). In fact, the lower values of the copepod species and Cirripedia larvae abundances observed at the beginning of the present study indicate that the salinity values (>36) inside the Ria Formosa lagoon were above their optimal metabolic conditions. There was no precipitation during the sampling period and the scarce and small rivers that provide freshwater inputs in this area are usually dry during summer months (Newton and Mudge, 2003). Therefore, salinity was considerably high during most of the sampling consequence of shallow depth and high temperatures leading to increase evaporation, as well as by the inflow of seawater through the tidal cycle. During the last days of the sampling period, salinity values were slightly lower (~34 – 35), which can be explained by the Submarine Groundwater Discharge (SGD). This discharge can be defined as any and all flow of water on continental margins from the seabed to the coastal ocean, regardless of the fluid composition or driving force (Burnett et al., 2003) and usually leads to an augment of nutrients concentrations in the area, that may increase the occurrence of algal blooms (Baptista, 1993). In fact, there was an increase of the majority of nutrients at the end of August and beginning of September, and the occurrence of harmful algal blooms, such as *Dynophysis acuta* and *Gymnodinium catenatum*, during August 2009 (Brito et al., 2012), supporting the idea of an SGD. The occurrence of SGD in the studied area was also reported by Leote et al. (2008) and Ibánhez et al. (2013), suggesting it as an important nutrient source to the Ria Formosa.

As expected, the tidal cycle influenced the abundance of several zooplanktonic taxa in the lagoon. Neritic copepod species such as *Paracaluns parvus*, *Acartia clausi*, *Centropages chierchiae* and *Oithona* spp., as well as species richness showed a high correlation with the ebb tidal phase. A previous work has shown a tendency of higher copepod abundances towards the outer lagoon, particularly of neritic calanoids and cyclopoids (Marques, 2005). The lower abundances at the inner lagoon can be explained by the fact that they may be avoiding a deeper transport into it, moving to lower depths during flood tide and ascending to upper water column on ebb flows. In fact, the present sampling station is in an intermediate place between the inlet and the inner lagoon, and these copepods can use this strategy to prevent from penetrating into the lagoon and rapidly return to the open sea. Ebb currents are generally faster than flood ones in the main inner channels (Pacheco et al., 2010), favouring this behaviour. Decapod larvae abundance was also correlated to the ebb flow, which is in accordance with previous works showing that these early life stages selectively use tidal currents to disperse into the open sea

and return to the lagoon or estuary during later larval stages to settle (Brookins and Epifanio, 1985; Morgan and Christy, 1997). The tidal coefficient also explained the variability of Copepoda (*Oithona* spp. and *Euterpina acutifrons*) and Decapoda larvae. The abundances of these taxa were higher when there was an increase of the tidal coefficients (spring tides). During spring tides, the studied area is mainly dominated by ebb tides presenting higher mean velocities when compared to flood tides (Pacheco et al., 2010), which can be related to higher copepod abundances occurring during these periods. Also, many decapods release their larvae to the environment on a semilunar cycle frequency when the tidal coefficient is higher (spring tides) (Forward, 1987; Flores et al., 2007), the same pattern showed by decapods during the present study.

During the summer period of the studied area, temperature affected the abundance of Appendicularia (*Oikopleura* spp.) species. Previous studies on appendicularians showed that temperature is one of the main factors influencing population dynamics (Troedsson et al., 2002, Troedsson et al., 2013). Short generation times and high maximum rate of lifetime reproductive fitness influenced by the increase of temperature and food concentrations together with a low number of predators would induce a higher abundance of Appendicularia (Deibel and Lowen, 2012). However, during the studied period, the presence of potential predators such as copepods (López-Urrutia et al., 2004) in high densities must have limited Appendicularia abundance, despite the increase when the temperature raised. Additionally, there was a positive correlation between the abundances of *Oikopleura* spp. and *Gymnodinium catenatum*, despite the toxicity produced by the latter. This result is in accordance with the study conducted by Badylak and Phlips (2008) during a harmful algal bloom event where *Oikopleura dioica* abundances mimicked the toxic dinoflagellate *Pyrodinium bahamense* abundances pattern, while two copepod species abundances, *Oithona colcarva* and *Acartia tonsa* declined. The cladoceran *Penilia avirostris* also showed a positive correlation with *G. catenatum*, revealing that this species is not affected by the presence of the toxic dinoflagellate. In fact, other planktonic organisms such as *Acartia clausi* can ingest the toxic dinoflagellate *G. catenatum* with no apparent adverse effects in the feeding and egg production rates (Palomares-García et al., 2006). Observations by Schultz and Kiørboe (2009) suggested that both *Pseudocalanus elongatus* and *Temora longicornis* could feed at high rates on *Karenia mikimotoi* for 24 h without any apparent deleterious effects. *A. clausi* was also shown to ingest more toxic cells of *Alexandrium minutum* as its concentration increased, although with this diet hatching success and nauplii production decreased (Frangópulos et al., 2000). In summary, present results add more evidence to the fact

that some organisms are not affected by the presence of toxic microalgae and must have developed defences against their toxicity.

In the present study, *Penilia avirostris* and *Evadne* spp. were correlated with *Prorocentrum* spp. and *Scrippsiella trochoidea*, respectively. Atienza et al. (2006) have found that the cladoceran *Penilia avirostris* ingests a broad spectrum of prey types including dinoflagellates and shows a behaviourally driven plasticity in prey selection. The fact that dinoflagellates were the dominant group of microplankton during this study may explain the positive correlation between these species. Due to the morphological characteristics of *Evadne spinifera*, Nival and Ravera (1979) suggested that this species can feed on organisms between 20 and 170 μm of total length, and probably can catch and hold animal prey or large algae. Moreover, Katechakis and Stibor (2004) studying feeding selectivity of cladoceran species, showed higher grazing coefficients of the congeneric *Evadne nordmanni* for the size classes 125, 175 and 205 μm, corresponding to an active selection of large diatoms. This supports the correlation found in the present study between *Evadne* spp. and diatoms, suggesting that this phytoplankton group is an important prey influencing the population dynamics of this species.

5.4.2 *Acartia clausi* production and RNA:DNA ratio: influence of environmental conditions

The main factors shaping the variability of egg production rates of *Acartia clausi* in a short temporal scale are salinity and ammonium. Previous studies reported that the main factors influencing *Acartia* reproduction are food availability (e.g. Uye, 1981, Kimmerer et al., 2005) and temperature (e.g. Uye, 1981, Castro-Longoria, 2003). Although it is well known that food is an important factor for the reproduction of *A. clausi* (Uye, 1981, Pagano et al., 2004), it was not the case in the present study as this factor was not selected by the model that explain the EPR variability (Table 5.3). This can be attributed to the fact that food was not a limiting factor during this season, when a relatively high abundance of dinoflagellates occurred, which are an essential food source for the reproduction of *A. clausi* (Band-Schmidt et al., 2008). Furthermore, most of the previous studies have been conducted in a higher temporal scale than the present one or in laboratory experimental conditions, therefore lacking a higher temporal resolution. As stated before, there was a salinity decrease (34-35) and macronutrients increased at the end of August and beginning of September, which corresponded to higher productivity of this calanoid

species. Castro-Longoria (2003) recorded the highest *A. clausi* EPR at a salinity of 35, in an experiment using a salinity range between 15 and 35. Moreover, *A. clausi* egg production in a Northern African lagoon was negatively correlated to salinity, where it decreased with values over 35 occurring during the summer (Annabi-Trabelsi et al., 2012). In fact, the metabolism of this species in terms of respiration rates was lowest at a salinity of 35, meaning that it is well adapted to these conditions (Gaudy et al., 2000). The decrease of reproduction in salinities > 35 mean that salinity could be a limiting factor for *A. clausi* even when it encounters suitable food conditions. The other factor influencing the reproduction was ammonia, showing an increment in egg production rates when this nutrient increased. Only a few previous studies have observed the same pattern despite the toxicity of this nutrient. Moraitou-Apostolopoulou and Verriopoulos (1981) have found that *A. clausi* inhabiting polluted areas always laid a high number of eggs. Additionally, Buttino (1994) showed that chronic exposure to ammonia produced a significant increment in egg production rates of the same species during the experimental period of exposure. It seems that *A. clausi* when exposed to stressful environments may have a reproductive strategy of increasing the egg production or even that the population has developed an adaptation to higher levels of pollutants.

The secondary production of *A. clausi* females was also higher at the end of the sampling period, peaking in the last days of August, and reflected mostly the curve of females' biomass rather than the egg production rates, which presented a lower variability throughout the sampling period. Although the egg production rate method includes only the adult fraction, underestimating the total secondary production, it has several advantages such as shorter periods of incubation, replicability and accuracy of biomass and fecundity measurements, and simplification in the identification (Poulet et al., 1995). Leandro et al. (2014) estimated the secondary production of *A. clausi* juvenile fraction in the coastal lagoon of Ria de Aveiro, NW Portugal, in a monthly basis, but using the growth rate approach with a temperature dependent growth model. They obtained higher values during September (23.9 µg C m^{-3} day^{-1}) which are comparable to what was found in the present study during the same season (average: 42.6 ± 59 µg C m^{-3} day^{-1}). Moreover, the summer production of *A. clausi* females in the adjacent coastal waters of Guadiana river estuary was higher than in Ria Formosa but also comparable to what was found in the present study (average values of 98.8 ± 84.3 µg C m^{-3} day^{-1}; Cruz et al., 2017). These results suggest that there is a potential considerable flow of energy and matter through the planktonic food web, especially to the early stages of the important fisheries resources (eg.

Sardina pilchardus, *Engraulis encrasicolus*, Sparidae) (Chícharo et al., 2012) that use Ria Formosa lagoon system as a nursery habitat.

RNA:DNA index of *A. clausi* adult females was positively related to the EPR and negatively to temperature, during the short sampling period. Previous studies, using different approaches such as laboratory experiments (*Acartia grani*: Saiz et al., 1998; *Acartia bifilosa*: Gorokhova, 2003) or *in situ* experiments (*Paracalanus* sp.: Nakata et al., 1994; *Calanus sinicus*: Ning et al., 2013; *Acartia* spp.: Cruz et al., 2017) have already shown this significant relationship between the two growth indices. This result is adding more evidence for the replacement of a more laborious and time-consuming method such as the egg production rate determination with the RNA:DNA ratio methodology.

Temperature has a strong effect on biochemical reactions such as the protein synthesis, i.e. higher temperatures will increase the metabolic rates without increasing the RNA concentration (Chícharo and Chícharo, 2008). Nakata et al. (1994) have found significant differences between the RNA:DNA ratio between sampling stations with different sea surface temperatures, and higher values of the index occurred when the temperature was lower. Saiz et al. (1998) also found a temperature dependency relationship between the EPR of *A. grani* and the RNA:DNA index. On the other hand, some studies did not find any correlation between temperature and the ecophysiological index (Gorockova, 2003; Ning et al. 2013; Cruz et al., 2017). The fact that there was no food limitation during the sampling period probably induced a more evident influence of temperature on the biochemical ratio. Also, even though the determination of RNA:DNA ratio occurred over a short temporal period, there was a high range of temperatures tested (between 23.1 and 27ºC). Previous studies indicate that with a broader temperature range (>2°C) there is a need to include both temperature and RNA:DNA ratio terms when modelling fish larvae growth rates (Buckley et al., 1999). Regarding the copepods, particularly the genus *Acartia*, there are still some incongruences among studies in relation to the best predictors to achieve a good model of EPR estimation. Therefore, further *in situ* and laboratory experiments should be accomplished using different time scales, relating the EPR, RNA:DNA ratio, temperature and food availability.

5.5 Conclusions

In the present study, salinity (particularly lower values ~34.5-35) was the main factor inducing higher abundances of several zooplanktonic taxa and production of the calanoid copepod *Acartia clausi*. The expected occurrence of warmer and drier summers in the future due to climate change may promote higher salinity values in Ria Formosa lagoon system (Williams, 2001) affecting the mesozooplankton abundance and production, which will probably disturb all the food web. Episodic events such as Submarine Groundwater Discharges will certainly be helpful to reverse a high salinity scenario.

Neritic copepods and decapod larvae seem to be favoured by ebb flows, using these currents to be transported to the open sea. Copepods seem to avoid the inner parts of the lagoon, while decapods use them to complete their larval development with the dispersion of newly hatched larvae into the ocean. To a lesser extent, semilunar cycle (tidal coefficient), temperature and food availability were also important in shaping zooplanktonic communities. The blooms of toxic dinoflagellates such as *Gymnodinium catenatum*, that are common in Ria Formosa lagoon during summer, do not have any deleterious effects in some taxa abundance (e.g. Apendicualaria and *Penilia avirostris*).

Besides salinity, the toxic pollutant ammonia present in low concentrations in the water, which are generally recorded in many eutrophic areas, and induced higher fecundity of *A. clausi* females, suggesting that this copepod is well adapted to these conditions. RNA:DNA ratio was proved to be a good proxy of egg production rate. However, further experiments should be conducted, in different time-scales, to understand how different variables influence these biological indexes and to achieve a good model to simplify the estimation of secondary production.

Ria Formosa coastal lagoon system presented a high variability in the plankton community (microplankton and mesozooplankton), as well as in the environmental factors that are constantly shaping species abundances, composition or production. It is unquestionable that long-term temporal scales studies using monthly or seasonal sampling strategies are important to continue monitoring plankton communities. However, the use of a more intensive sampling during the most productive seasons will be helpful to understand how sudden environmental changes can influence the community structure and production of these organisms, that are reflected in the interannual variability often difficult to explain.

5.6 Acknowledgements

The authors wish to thank David Piló, Renata Gonçalves and Eloise de Sá for their support during fieldwork and Centro de Ciência Viva do Algarve for the physical-chemical data. J.C. was supported by FCT through a Doctoral Fellowship (SFRH/BD/28198/2006). S.G. was supported by the Investigador FCT Program (IF/01546/2015). This study was supported by the European Regional Development Fund (COMPETE program- Operational Competitiveness Programme), and by Portuguese national funds from FTC through projects MODELA (PTDC/MAR/098643/2008), UID/Multi/04326/2016, UID/Multi/04326/2019 and UID/MAR/04292/2013.

5.7 References

Anil, A.C., Kurian, J. (1996) Influence of food concentration, temperature and salinity on the larval development of *Balanus amphitrite*. *Marine Biology*, 127: 115-124.

Annabi-Trabelsi, N., Daly-Yahia, M.N., Belmonte, G., Aleya, L., Ayadi, H. (2012) Impacts of very warm temperature on egg production rates of three Acartiidae (Crustacea, Copepoda) in a Northern African lagoon. *Journal of Thermal Biology*, 37: 445-453.

Atienza, D., Calbet, A., Saiz, E., Alcaraz, M., Trepat, I. (2006) Feeding ecology of the marine cladoceran *Penilia avirostris*: natural diet, prey selectivity and daily ration. *Marine Ecology Progress Series*, 315: 211-220.

Ayukai, T. (1987) Predation by *Acartia clausi* (Copepoda: Calanoida) on two species of tintinnids. *Marine Microbial Food Webs*, 2: 45-52.

Azeiteiro, U.M., Marques, S.C., Vieira, L.M.R., Pastorinho, M.R.D., Ré, P.A.B., Pereira, M.J., Morgado, F.M.R. (2005) Dynamics of the *Acartia* genus (Calanoida: Copepoda) in a temperate shallow estuary (the Mondego estuary) on the western coast of Portugal. *Acta Adriatica*, 46: 7-20.

Badylak, S., Phlips, E.J. (2008) Spatial and temporal distributions of zooplankton in Tampa Bay, Florida, including observations during a HAB event. *Journal of Plankton Research*, 30: 449-465.

Band-Schmidt, C.J., Pacheco-Chávez, R., Hernández-Trujillo, S. (2008) Influence of phytoplankton diets on the ingestion rate and egg production of *Acartia clausi* and *A. lilljeborgii* (Copepoda: Calanoida) from Bahía de La Paz, Gulf of California. *Hidrobiológica*, 18: 133-140.

Baptista, T.M.C. (1993) Microbiological observations in the Ria Formosa. M.Phil. Thesis, University of Wales, UK (unpublished)

Barbosa, AB (2010) Seasonal and interannual variability of planktonic microbes in a mesotidal coastal lagoon (Ria Formosa, SE Portugal): impact of climatic changes and local-human influences. In: Coastal Lagoons: critical habitats of environmental change, Eds.: H. Paerl and M. Kennish, CRC Press, Taylor & Francis Group, Marine Science Book Series, Boca Raton, 335-366 pp. ISBN: 978-1-4200-8830-4.

Barbosa, A.B., Chícharo, M.A. (2011) Hydrology and Biota Interactions as Driving Forces for Ecosystem Functioning. In: Treatise on Estuarine and Coastal Science, E. Wolanski and D.S. McLusky (Eds.) Waltham. Academic Press, 10, pp. 7–47.

Bentle, L.A., Dutta, S., Metcoff, J. (1981) The sequential enzymatic determination of DNA and RNA. *Analytical Biochemistry*, 116: 5-16.

Bojanić Varezić, D., Vidjak, O., Kraus, R., Precali, R. (2015) Regulating mechanisms of calanoid copepods variability in the northern Adriatic Sea: Testing the roles of west-east salinity and phytoplankton gradients. *Estuarine, Coastal and Shelf Science*, 164: 288-300.

Brito, A.C., Quental, T., Coutinho, T.P., Branco, M.A.C., Falcão, M., Newton, A., Icely, J., Moita, T. (2012) Phytoplankton dynamics in southern Portuguese coastal lagoons during a discontinuous period of 40 years: An overview. *Estuarine, Coastal and Shelf Science*, 110: 147-156.

Brookins, K.G., Epifanio, C.E. (1985) Abundance of brachyuran larvae in a small coastal inlet over six consecutive tidal cycles. *Estuaries*, 8: 60-67.

Buckley, L.J., Caldarone, E., Ong, T.-L. (1999) RNA–DNA ratio and other nucleic acid based indicators for growth and condition of marine fishes. *Hydrobiologia*, 401: 265-277.

Burnett, W.C., Bokuniewicz, H., Huettle, M., Moore, W.S., Taniguchi, M. (2003) Groundwater and pore water inputs to the coastal zone. *Biogeochemistry*, 66: 3-33.

Buttino, I. (1994) The effect of low concentrations of phenol and ammonia on egg-production rates, fecal pellet production and egg viability of the calanoid copepod *Acartia clausi*. *Marine Biology*, 119: 629-634.

Caldarone, E.M., Clemmesen, M.C., Berdalet, E., Miller, T.J., Folkvord, A., Holt, G.J., Olivar, M.P., Suthers, I.M. (2006) Intercalibration of four spectrofluorometric protocols for measuring RNA:DNA ratios in larval and juvenile fish. *Limnology and Oceanography: Methods*, 4: 153-163.

Castro-Longoria, E. (2003) Egg production and hatching success of four *Acartia* species under different temperature and salinity regimes. *Journal of Crustacean Biology*, 23: 289-299.

Cerejo, M., Dias, J.M. (2007) Tidal transport and dispersal of marine toxic microalgae in a shallow, temperate coastal lagoon. *Marine Environmental Research*, 63: 313-340.

Chícharo, L., Chícharo, M.A. (2001) Effects of environmental conditions on planktonic abundances, benthic recruitment and growth rates of *Ruditapes decussatus* in a coastal lagoon. *Fisheries Research*, 53: 235-250.

Chícharo, M.A., Chícharo, L. (2008) RNA:DNA ratio and other nucleic acid derived indices in marine ecology. *International Journal of Molecular Sciences*, 9: 1453-1471.

Chícharo, M.A., Amaral, A., Faria, A., Morais, P., Mendes, C., Piló, D., Ben-Hamadou, R., Chícharo, L. (2012) Are tidal lagoons ecologically relevant to larval recruitment of small pelagic fish? An approach using nutritional condition and growth rate. *Estuarine, Coastal and Shelf Science*, 112: 265-279.

Clarke, K.R., Gorley, R.N. (2006) Primer v6: user manual/tutorial. PRIMER-E, Plymouth.

Cruz, J., Teodósio, M.A., Ben-Hamadou, R., Chícharo, L., Garrido, S., Ré, P., Santos, A.M.P. (2017) RNA:DNA ratios as a proxy of egg production rates of *Acartia*. *Estuarine, Coastal and Shelf Science*, 187: 96-109.

Day, J.W.Jr., Hall, C.A.S., Kemp, W.M., Yáñez Arancibia, A. (1989) Estuarine ecology. Wiley-Interscience, New York, New York, USA, 558 pp.

Deibel, D., Lowen, B. (2012) A review of the life cycles and life-history adaptations of pelagic tunicates to environmental conditions. *ICES Journal of Marine Science*, 69: 358-369.

Elser, J.J., Sterner, R.W., Gorokhova, E., Fagan, W.F., Markow, T.A., Cotner, J.B., Harrison, J.F., Hobbie, S.E., Odell, G.M., Weider, L.W. (2000) Biological stoichiometry from genes to ecosystems. *Ecology Letters*, 3: 540-550.

Falcão, M.M., Vale, C. (1990) Study of the Ria Formosa ecosystem: benthic nutrient remineralization and tidal variability of nutrients in the water. *Hydrobiologia*, 207: 137-146.

Flores, A.A.V., Mazzuco, A.C.A., Bueno, M. (2007) A field study to describe diel, tidal and semilunar rhythms of larval release in an assemblage of tropical rocky shore crabs. *Marine Biology*, 151: 1989-2002.

Forward Jr, R.B. (1987) Larval release rhythms of decapod crustaceans: an overview. *Bulletin of Marine Science*, 41: 165-176.

Frangópulos, M., Guisande, C., Maneiro, I., Riveiro, I., Franco, J. (2000) Short-term and long-term effects of the toxic dinoflagellate *Alexandrium minutum* on the copepod *Acartia clausi*. *Marine Ecology Progress Series*, 203: 161-169.

Gaudy, R., Cervetto, G., Pagano, M. (2000) Comparison of the metabolism of *Acartia clausi* and *A. tonsa*: influence of temperature and salinity. *Journal of Experimental Marine Biology and Ecology*, 247: 51-65.

Gorokhova, E. (2003) Relationships between nucleic acid levels and egg production rates in *Acartia bifilosa*: implications for growth assessment of copepods in situ. *Marine Ecology Progress Series*, 262: 163-172.

Greenwald, G.M., Hurlbert, S.H. (1993) Microcosm analysis of salinity effects on coastal lagoon plankton assemblages. *Hydrobiologia*, 267: 307-335.

Gunter, G. (1961) Some relations of estuarine organisms to salinity. *Limnology and Oceanography*, 6: 182-190.

Hasle, G.R. (1978) The inverted-microscope method. In: Phytoplankton Manual, Ed.: A. Sournia, Monographs on Oceanographic Methodology, UNESCO, 88-98 pp.

Heerkloss, R., Schnese, W., Adamkiewicz-Chojnacka, B. (1991) Influence of eutrophication on seasonal variations of zooplankton biomass in shallow coastal lagoons of the Southern Baltic. *Acta Ichthyologica et Piscatoria*, 21: 67-76.

Hogfors, H., Holmborn, T., Hajdu, S., Gorokhova, E. (2011) Does female RNA content reflect viable egg production in copepods? A test with the Baltic copepod *Acartia tonsa*. *Journal of Plankton Research*, 33: 1460-1463.

Ibánhez, J.S.P., Leote, C., Rocha, C. (2013) Seasonal enhancement of submarine groundwater discharge (SGD)-derived nitrate loading into the Ria Formosa coastal lagoon assessed by 1-D modeling of benthic NO_3^- profiles. *Estuarine, Coastal and Shelf Science*, 132: 56-64.

Ikeda, T., San, F.; Yamaguchi, A., Matsuishi, T. (2007) RNA:DNA ratios of calanoid copepods from the epipelagic through abyssopelagic zones of the North Pacific Ocean. *Aquatic Biology*, 1: 99-108.

Katechakis, A., Stibor, H. (2004) Feeding selectivities of the marine cladocerans *Penilia avirostris*, *Podon intermedius* and *Evadne nordmanni*. *Marine Biology*, 145: 529-539.

Kimmerer, W.J., Ferm, N., Nicolini, M.H., Peñalva, C. (2005) Chronic food limitation of egg production in populations of copepods of the genus *Acartia* in the San Francisco estuary. *Estuaries*, 28: 541-550.

Kiørboe, T. (1997) Small-scale turbulence, marine snow formation, and planktivorous feeding. In: Lecture Notes on Turbulence and Plankton. *Scientia Marina*, 61(supl. 1): 141-158.

Kiørboe, T., Sabatini, M. (1995) Scaling of fecundity, growth and development in marine planktonic copepods. *Marine Ecology Progress Series*, 120: 285-298.

Kobari, T., Mori, H., Tokushige, H. (2013) Nucleic acids and protein content in ontogenetically migrating copepods in the Oyashio region as influenced by development stage and depth distribution. *Journal of Plankton Research*, 35: 97-104.

Last, K.S., Bailhache, T., Kramer, C., Kyriacou, C.P., Rosato, E., Olive, P.J. (2009) Tidal, daily, and lunar-day activity cycles in the marine polychaete *Nereis virens*. *Chronobiology International*, 26: 167-83.

Leandro, S.M., Morgado, F., Pereira, F., Queiroga, H. (2007) Temporal changes of abundance, biomass and production of copepod community in a shallow temperate estuary (Ria de Aveiro, Portugal). *Estuarine, Coastal and Shelf Science*, 74: 215-222.

Leandro, S.M., Tiselius, P., Marques, S.C, Avelelas, F., Correia, C., Sá, P., Queiroga, H. (2014) Copepod production estimated by combining in situ data and specific temperature-dependent somatic growth models. *Hydrobiologia*, 741: 139-152.

Leote, C., Ibánhez, J.S.P., Rocha, C. (2008) Submarine Groundwater Discharge as a nitrogen source to the Ria Formosa studied with seepage meters. *Biogeochemistry*, 88: 185-194.

López-Urrutia, A., Harris, R.P., Smith, T. (2004) Predation by calanoid copepods on the appendicularian *Oikopleura dioica*. *Limnology and Oceanography*, 49: 303-307.

Marques, A. (2005) Distribution of the Plankton Community in the Ria Formosa, a Coastal Lagoon in South Eastern Portugal. Philosophiae Doctor Thesis. University of Wales. 238 pp.

Mauchline, J. (1998) The biology of calanoid copepods. *Advances in Marine Biology*, 33: 1-710.

Moraitou-Apostolopoulou, M., Verriopoulos, G. (1981) Egg viability in two populations of *Acartia clausi* exposed to different degrees of pollution. *Vie et Milieu*, 31: 65-69.

Morgan, S.G., Christy, J.H. (1997) Planktivorous fishes as selective agents for reproductive synchrony. *Journal of Experimental Marine Biology and Ecology*, 209: 89-101.

Nakata, K., Nakano, H., Kikuchi, H. (1994) Relationship between egg productivity and RNA/DNA ratio in *Paracalanus* sp. In the frontal waters of the Kurshio. *Marine Biology*, 119: 591-596.

Nasrolahi, A., Havenhand, J., Wrange, A.-L., Pansch, C. (2016) Population and life-stage specific sensitivities to temperature and salinity stress in barnacles. *Scientific Reports*, 6: 32263.

Newton, A., Mudge, S.M. (2003) Temperature and salinity regimes in a shallow, mesotidal lagoon, the Ria Formosa, Portugal. *Estuarine, Coastal and Shelf Science*, 57: 73-85.

Ning, J., Li, C., Yang, G., Wan, A., Sun, S. (2013) Use of RNA:DNA ratios to evaluate the condition and growth of the copepod *Calanus sinicus* in the Southern Yellow Sea. *Deep-Sea Research Part II*, 97: 109-116.

Nival, S., Ravera, S. (1979) Morphological studies of the appendages of the marine cladoceran *Evadne spinifera* Müller by means of the scanning electron microscope. *Journal of Plankton Research*, 1: 207-213.

Pacheco, A., Ferreira, Ó., Williams, J.J., Garel, E., Dias, J.A. (2010) Hydrodynamics and equilibrium of a multiple-inlet system. *Marine Geology*, 274: 32-42.

Pagano, M., Kouassi, E., Arfi, R., Bouvy, M., Saint-Jean, L. (2004) In situ spawning rate of the calanoid copepod *Acartia clausi* in a tropical lagoon (Ebrié, Côte d'lvoire): Diel variations and effects of environmental factors. *Zoological Studies*, 43: 244-254.

Palomares-García, R., Bustillos-Guzmán, J., Band-Schmidt, C.J., López-Cortés, D., Luckas, B. (2006) Effect of the toxic dinoflagellate *Gymnodinium catenatum* on the grazing, egg production, and hatching success of the copepod *Acartia clausi*. *Ciencias Marinas*, 32: 111-119.

Pereira, R, Teodósio, M.A., Garrido, S. (2014) An experimental study of *Aurelia aurita* feeding behaviour: inference of the potential predation impact on a temperate estuarine nursery area. *Estuarine, Coastal and Shelf Science*, 146: 102-110

Poulet, S.A., Ianora, A., Laabir, M., Klein Bretler, W.C.M. (1995) Towards the measurement of secondary production and recruitment in copepods. *ICES Journal of Marine Science*, 52: 359-368.

Primo, A.L., Azeiteiro, U.M., Marques, S.C., Martinho, F., Pardal, M.A. (2009) Changes in zooplankton diversity and distribution pattern under varying precipitation regimes in a southern temperate estuary. *Estuarine, Coastal and Shelf Science*, 82: 341-347.

R Development Core Team (2013) R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. (available. from http://www.Rproject.org).

Repelin, R. (1985) Le zooplancton dans le système lagunaire Ivorien. Variations saisonnières et cycles nychtéméraux en Lagune Ebrié. *Documents Scientifiques Centre de Recherches Océanographiques Abidjan*, 16: 1-43.

Runge, J.A., Roff, J.C. (2000) The measurement of growth and reproductive rates. In Harris, R., Wiebe, P., Lenz, J., Skjoldal, H.R., Huntley, M. (eds), ICES Zooplankton Methodology Manual. Academic Press, London, pp. 401–454.

Saiz, E., Calbet, A., Fara, A., Berdalet, E. (1998) RNA content of copepods as a tool for determining adult growth rates in the field. *Limnology and Oceanography*, 43: 465-470.

Sakamoto, Y., Ishiguro, M., Kitagawa, G. (1986) Akaike information criterion statistics. D. Reidel Publishing Company, Dordrecht, The Netherlands.

Schelske, C.L., Odum, E.P. (1962) Mechanisms maintaining high productivity in Georgia estuaries. *Proceedings of the Gulf and Caribbean Fisheries Institute*, 14: 75-80.

Schultz, M., Kiørboe, T. (2009) Active prey selection in two pelagic copepods feeding on potentially toxic and non-toxic dinoflagellates. *Journal of Plankton Research*, 31: 553-561.

Sprung, M. (1994) High larval abundances in the Ria Formosa (Southern Portugal) – methodological or local effect? *Journal of Plankton Research*, 16: 151-160.

Troedsson, C., Bouquet, J.-M., Aksnes, D.L., Thompson, E.M. (2002) Resource allocation between somatic growth and reproductive output in the pelagic chordate *Oikopleura dioica* allows opportunistic response to nutritional variation. *Marine Ecology Progress Series*, 243: 83-91.

Troedsson, C., Bouquet, J.-M., Lobon, C.M., Novac, A., Nejstgaard, J.C., Dupont, S., Bosak, S., Jakobsen, H.H., Romanova, N., Pankoke, L.M., Isla, A., Dutz, J., Sazhin, A.F., Thompson, E.M. (2013) Effects of ocean acidification, temperature and nutrient regimes on the appendicularian *Oikopleura dioica*: a mesocosm study. *Marine Biology*, 160: 2175-2187.

Uye, S. (1981) Fecundity studies of neritic calanoid copepods *Acartia clausi* Giesbrecht and *A. Steueri* Smirnov: a simple empirical model of daily egg production. *Journal of Experimental Marine Biology and Ecology*, 50: 255-271.

Vehmaa, A., Kremp, A., Tamminen, T., Hogfors, H., Spilling, K., Engström-Öst, J. (2012) Copepod reproductive success in spring-bloom communities with modified diatom and dinoflagellate dominance. *ICES Journal of Marine Science*, 69: 351-357.

Venables, W.N., Ripley, B.D. (2002) Modern Applied Statistics with S, 4th ed. Springer-Verlag, New York.

Vieira, L.R., Guilhermino, L., Morgado, F. (2015) Zooplankton structure and dynamics in two estuaries from the Atlantic coast in relation to multi-stressors exposure. *Estuarine, Coastal and Shelf Science*, 167: 347-367.

Wagner, M., Durbin, E., Buckley, L. (1998) RNA:DNA ratios as indicators of nutritional condition in the copepod *Calanus finmarchicus*. *Marine Ecology Progress Series*, 162: 173-181.

Wagner, M.M., Campbell, R.G., Boudreau, C.A., Durbin, E.G. (2001) Nucleic acids and growth of *Calanus finmarchicus* in the laboratory under different food and temperature conditions. *Marine Ecology Progress Series*, 221: 185-197.

Wiebe, P.H. (1988) Functional regression equations for zooplankton displacement volume, wet weight, dry weight, and carbon: A correction. *Fisheries Bulletin*, 86: 833-835.

Williams, W.D. (2001) Anthropogenic salinization of inland waters. *Hydrobiologia*, 466: 329-337.

Wood, S.N. (2006) Generalized Additive Models: An Introduction with R. Boca Raton: Chapman and Hall–CRC, London, 391 p.

Chapter 6

General Conclusions

6.1 General conclusions

The general aim of this thesis was to improve the knowledge of copepods reproduction, more precisely of production estimations using simpler methods such as the egg production rate and the biochemical index RNA:DNA, relating them with environmental conditions. For this purpose, different copepod species were used in the field and laboratorial experimental studies.

In Chapters 2 and 3, the target species was the calanoid *Centropages chierchiae*, a climate change indicator, common in Portuguese coastal waters, that has been migrating towards northern latitudes possibly associated with the increase of sea surface temperature. With this experimental approach the feeding (Chapter 2), reproductive and respiration rates (Chapter 3) were determined and related to temperature (8, 13, 19 and 24ºC) and food (quantity and quality). As expected, temperature had a great influence in all physiological rates. Feeding rates were temperature dependent, showing a dome-shaped response with an optimal temperature at 19ºC, value that usually occur off western Portuguese coast when *C. chierchiae* presents its highest abundances (Sobrinho-Gonçalves et al., 2013). Similarly, egg production rates (EPR) increased when females were exposed to 19ºC, while respiration rates were temperature dependent increasing from 8 until 24ºC. Hatching success (HS) was significantly lower at cooler temperatures (13ºC) than with warmer ones (19 and 24ºC). Regarding food concentration and type, this copepod species presented high feeding rates for both diatoms and dinoflagellates, and its selective-feeding behaviour was dependent on food concentration, with larger prey (diatom *Ditylum brightwellii*) being selected only at low concentrations. Reproduction rates were higher when a diatom species (*Phaeodactylum tricornutum*) was given to *C. chierciae* females, particularly in high concentrations. On the other hand, an opposite result was verified for the hatching success, with higher values when the copepods fed on dinoflagellates (*Gymnodinium* sp.), which can indicate different nutritional needs for each reproductive rate.

Integrating the results of both chapters regarding the daily rations, i.e. the percentage of body carbon ingested per day, it was possible to compare the energy requirements obtained when a natural range of food concentration was given to the copepods (Chapter 2) with the 10-folded minimal requirements to maintain the basal metabolic rate (Chapter 3), that corresponds to the total carbon demands needed to assure growth, molting or reproduction. For most temperatures, the daily rations obtained with natural food concentrations were higher than the

minimum needed to assure the basic physiological conditions, except at 24ºC where daily ration do not seem to fulfil the energetic requirements. All these results indicate that *C. chierchiae* physiology is highly temperature dependent, meaning that its poleward migration is mostly linked to the ocean warming that has been occurring in the northern European seas for the past decades (MacKenzie and Shiedek, 2007). In a future warming scenario, that will correspond to the highest temperature tested in these studies (24ºC), this species will probably suffer a temporal shift in its production that may lead to a mismatch between *C. chierchiae* and their predators or prey. Furthermore, at such high temperature this species will need to consume more food per unit body size, and feeding may be critical since this genus shows high metabolic rates and low levels of metabolic reserves (Dagg, 1977). Although *C. chierchiae* presented low fecundity rates in the present study at 13ºC, the fact that a population of this species is established in northern latitudes, indicates that it probably has adapted to colder temperatures, involving different life history traits and thermal stress tolerance. Further studies should be considered to compare both *C. chierchiae* populations from the Iberian Peninsula and northern areas, in terms of their respiration physiology, reproductive biology and feeding ecology, in order to understand the phenotypic acclimation processes of this copepod species.

In situ field studies conducted with the ubiquitous genus *Acartia* in different ecosystems and time scales revealed that reproduction is affected by distinct environmental factors (Chapters 4 and 5). During the interannual study in the Guadiana estuary (Chapter 4), EPR of the calanoid *Acartia tonsa*, found predominantly in the lower estuary, was influenced by freshwater inflow, chlorophyll *a* and dinoflagellates biomass, while *Acartia clausi* that occurred mainly in the adjacent coastal waters was only related to dinoflagellates. On the other hand, in the intensive sampling in Ria Formosa during the summer (Chapter 5), *A. clausi* EPR was related with salinity and ammonia concentration. The main factors that are usually thought to have more influence on the copepod reproduction are temperature and food, although in some cases this is not so evident. In the present studies, temperature did not seem to be limiting this physiological rate, despite the broad range found in both sampling strategies, while food was an important factor for both *Acartia* species in the Guadiana estuary and adjacent coastal area. Dinoflagellates have already been considered a good food source, containing the essential nutrients to sustain the reproductive rates of copepods. However, during the experiments conducted with the calanoid *C. chierchiae* (Chapter 3), females attained a higher EPR when fed with a diatom species in detriment of a dinoflagellate species. This reveals that different prey species act differently on the reproduction of the copepod species tested, depending also on

whether laboratory experiments or field studies were conducted. The use of a more specific prey species instead of a higher taxonomical group to infer the influence of food on this physiological rate, will also promote different results. As stated before, temperature had a great influence in the reproductive rate of *C. chierchiae* during the experiments. Once more, results were different from the field studies, probably due to the use of a controlled environment under a limited number of factors (temperature and food), instead of using copepods directly caught from the natural environment with several biotic and abiotic factors simultaneously influencing them.

The freshwater discharges induced higher productivity downstream with nutrient enrichment, which led to a positive influence on the amount of eggs laid by *A. tonsa* in the lower estuary of Guadiana river. However, since 2002, with the construction of the Alqueva dam, changes in the inflow regime had an impact on phytoplankton communities that consequently may have altered copepods reproduction. Salinity is negatively correlated with the freshwater inflow, however it does not seem to be the factor affecting the EPR of *A. tonsa*, since low salinity usually induce lower fecundity rates. On the other hand, in the intensive study conducted in Ria Formosa lagoon, salinity (~34.5-35) was the main factor inducing a higher production of *A. clausi*. Besides salinity, the toxic pollutant ammonia induced a higher fecundity of *A. clausi* females. The nutrient was present in low concentrations in the water, which is generally recorded in many eutrophic areas, suggesting that this copepod is well adapted to these conditions.

Hatching success was constantly high during the sampled period for both *Acartia* species and was not related to any environmental variable, probably due to a lack of any limiting factor (Chapter 4). In contrast, hatching success in the *C. chierchiae* experiments (Chapter 3) was influenced by food, with higher percentages of nauplii hatched when fed with a dinoflagellate, and was lower at 13ºC than with higher temperatures (19 and 24ºC). This effect was probably more evident due to a controlled environment using only two variable factors (temperature and food).

In the Guadiana estuary, female's 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 this ecosystem, which means they can be used as indicators of food availability for their early developmental stages. In both studies (Chapters 4 and 5), the females' secondary production presented similar values to the ones estimated for the

total production in other environments (Pastorinho et al., 2003; Vieira et al., 2003). If total production would have been estimated in the present studies, most probably it would have attained higher or equal values, suggesting that these ecosystems may be considered very productive. Moreover, these results show that a simpler method such as the egg production rate (Poulet et al., 1995) to estimate copepod female's secondary production, even though it is an underestimation of the total production, should be considered as a more practical and easier method to be used in the future, until new more accurate methodologies are developed.

There was a positive relationship between RNA:DNA ratio and egg production rate of *Acartia* species, in both studies (Chapters 4 and 5), 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. Previous studies have already presented similar results regarding this correlation using laboratory experiments (Saiz et al., 1998; Gorokhova, 2003) or in situ experiments (Nakata et al., 1994; Ning et al., 2013). The only difference between both studies (Guadiana estuary and Ria Formosa lagoon system) was that temperature also entered in the model for the Ria Formosa lagoon system. Temperature has a strong influence in biochemical reactions such as protein biosysnthesis, suggesting a temperature dependency of any index containing RNA. However, different results have been obtained in previous studies, with some presenting a relationship between temperature and RNA:DNA index (Nakata et al, 1994; Saiz et al., 1998; Wagner et al., 2001), while others lacked this correlation (Gorockova, 2003; Ning et al., 2013). The fact that there was no food limitation during the summer study in Ria Formosa, may have evidenced the influence of temperature on the biochemical ratio. However, further experiments should be conducted, in different time-scales, to understand how different variables influence these biological indexes and to achieve a good model to simplify the estimation of secondary production in the future.

Regarding the mesozooplankton community of Ria Formosa during the most productive season, several environmental factors influenced different taxa. Neritic copepods and decapod larvae seem to be favoured by ebb flows, using these currents to be transported to the open sea. Copepods seem to avoid the inner parts of the lagoon, while decapods use them to complete their larval development with the dispersion of newly hatched larvae into the ocean. Semilunar cycle (tidal coefficient), temperature and food availability were also important in shaping zooplanktonic communities. The blooms of toxic dinoflagellates such as *Gymnodinium*

catenatum, that are common in Ria Formosa lagoon during summer, do not have any deleterious effects in some taxa abundance (Apendicularia and *Penilia avirostris*).

The expected occurrence of warmer and drier summers in the future due to climate change may promote higher salinity values in Ria Formosa lagoon system (Williams, 2001) affecting the mesozooplankton abundance and production, which will probably disturb the entire food web. Episodic events such as Submarine Groundwater Discharges will certainly be helpful to reverse a high salinity scenario. Ria Formosa coastal lagoon system presented a high variability in the plankton community (microplankton and mesozooplankton), as well as in the environmental factors that are constantly shaping species abundances, composition or production. It is unquestionable that long-term temporal scales studies using monthly (Chapter 4) or seasonal sampling strategies are important to continue monitoring plankton communities. However, the use of a more intensive sampling during the most productive seasons (Chapter 5) will be helpful to understand how sudden environmental changes can influence the community structure and production of these organisms that are reflected in the interannual variability often difficult to explain.

In conclusion, there is still much work to do related to the study of copepods physiology, secondary production estimations, zooplankton communities together with all the environmental factors that may influence them. In a constant changing world, with several anthropogenic and climate changes, further understanding how zooplankton will respond to them, that goes through species until community level, is an extremely important issue that needs an emergent attention. Hopefully, the present thesis will be helpful to fill some gaps regarding a more precise methodology of copepods secondary production estimations, and also to improve the knowledge on zooplanktonic species and communities off Portuguese coastal waters. Furthermore, future studies should rely on the development of models to estimate secondary production (Prestidge et al., 1995), along Portuguese coastal waters, using remote sensing in the same way it is used to estimate primary production (Joint and Groom, 2000). For this purpose, satellite retrieved sea surface temperature and chlorophyll *a* together with present results of copepods egg production rates and abundances used to validate the model, would be helpful to infer copepods secondary production, supporting fisheries resources management and the monitoring of climate changes.

6.2 References

Dagg, M. (1977) Some effects of patchy food environments on copepods. *Limnology and Oceanography*, 22: 99-107.

Gorokhova, E. (2003) Relationships between nucleic acid levels and egg production rates in *Acartia bifilosa*: implications for growth assessment of copepods in situ. *Marine Ecology Progress Series*, 262: 163-172.

Joint, I., Groom, S.B. (2000) Estimation of phytoplankton production from space: current status and future potential of satellite remote sensing. *Journal of Experimental Marine Biology and Ecology*, 250: 233-255.

MacKenzie, B.R., Schiedek, D. (2007) Daily ocean monitoring since the 1860s shows record warming of northern European seas. *Global Change Biology*, 13: 1335-1347.

Nakata, K., Nakano, H., Kikuchi, H. (1994) Relationship between egg productivity and RNA/DNA ratio in *Paracalanus* sp. In the frontal waters of the Kurshio. *Marine Biology*, 119: 591-596.

Ning, J., Li, C., Yang, G., Wan, A., Sun, S. (2013) Use of RNA:DNA ratios to evaluate the condition and growth of the copepod *Calanus sinicus* in the Southern Yellow Sea. *Deep-Sea Research Part II*, 97: 109-116.

Pastorinho, R., Vieira, L., Ré, P., Pereira, M., Bacelar-Nicolau, P., Morgado, F., Marques, J.C., Azeiteiro, U. (2003) Distribution, production, histology and histochemistry in *Acartia tonsa* (Copepoda: Calanoida) as means for life history determination in a temperate estuary (Mondego estuary, Portugal). *Acta Oecologica*, 24: S259-S273.

Prestidge, M.C., Harris, R.P., Taylor A.H. (1995) A modelling investigation of copepod egg production in the Irish Sea. *ICES Journal of Marine Science*, 52: 693-703.

Saiz, E., Calbet, A., Fara, A., Berdalet, E. (1998) RNA content of copepods as a tool for determining adult growth rates in the field. *Limnology and Oceanography*, 43: 465-470.

Sobrinho-Gonçalves, L., Moita, M.T., Garrido, S., Cunha, M.E. (2013) Environmental forcing on the interactions of plankton communities across a continental shelf in the Eastern Atlantic upwelling system. *Hydrobiologia*, 713: 167-182.

Vieira, L., Morgado, F., Ré, P., Nogueira, A., Pastorinho, R., Pereira, M., Bacelar-Nicolau, P., Marques, J.C., Azeiteiro, U.M. (2003) Population dynamics of *Acartia clausi* from a temperate estuary (Mondego Estuary, Western Portugal). *Invertebrate Reproduction and Development*, 44: 9-15.

Wagner, M.M., Campbell, R.G., Boudreau, C.A., Durbin, E.G. (2001) Nucleic acids and growth of *Calanus finmarchicus* in the laboratory under different food and temperature conditions. *Marine Ecology Progress Series*, 221: 185-197.

Williams, W.D. (2001) Anthropogenic salinization of inland waters. *Hydrobiologia*, 466: 329-337.