POCTI
Programa Operacional
Ciência Tecnologia Inovação

FCT
Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA EDUCAÇÃO E CIÊNCIA

UAlg FCT
UNIVERSIDADE DO ALGARVE
FACULDADE DE CIÊNCIAS E TECNOLOGIA

UNIVERSIDADE DO ALGARVE

Faculdade de Ciências e Tecnologia

The importance of the pelagic larval phase of the wedge shell *Donax trunculus* (L.): implications for the management of the fishery

Alexandre Miguel Moura Pereira

Doutoramento em Ciências do Mar

Especialidade em Biodiversidade Marinha

Trabalho realizado sob a orientação de:

Doutor Miguel Baptista Gaspar

Professora Doutora Josefina Méndez Felpeto

Professor Doutor Luís Manuel Zambujal Chícharo

2013

UNIVERSIDADE DO ALGARVE

Faculdade de Ciências e Tecnologia

The importance of the pelagic larval phase of the wedge shell *Donax trunculus* (L.): implications for the management of the fishery

Alexandre Miguel Moura Pereira

Doutoramento em Ciências do Mar

Especialidade em Biodiversidade Marinha

Trabalho realizado sob a orientação de:

Doutor Miguel Baptista Gaspar

Professora Doutora Josefina Méndez Felpeto

Professor Doutor Luís Manuel Zambujal Chícharo

2013

A realização desta tese foi possível graças ao financiamento da Fundação para a Ciência e Tecnologia, com co-financiamento do Fundo Social Europeu, através da bolsa de doutoramento SFRH/BD/40430/2007.

Declaração de autoria de trabalho:

Declaro ser a autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Alexandre Miguel Moura Pereira

Acknowledgements/Agradecimentos

O presente trabalho foi desenvolvido no Centro Regional de Investigação Pesqueira do Sul – CRIPSul, na estação experimental de moluscicultura de Tavira, ambos parte do Instituto Português do Mar e da Atmosfera – IPMA, e nos laboratórios do grupo XENOMAR do departamento de biologia celular e molecular da faculdade de ciências da universidade da Corunha.

Este trabalho foi financiado por uma bolsa de doutoramento atribuida pela FCT – Fundação para a Ciência e Tecnologia - SFRH/BD/40430/2007.

O desenvolvimento deste trabalho em todos os seus momentos dependeu da contribuição de várias pessoas e instituições a quem eu desejo expressar os meus sinceros agradecimentos:

Em primeiro lugar ao meu orientador Doutor Miguel Baptista Gaspar por acreditar neste projecto de doutoramento desde o início e por ter aceite supervisionar todo o meu trabalho. Agradeço-lhe todo o conhecimento que me tem transmitido ao longo destes anos em que trabalhamos juntos, por toda a dedicação e suporte durante a realização deste trabalho. Agradeço também por me ter deixado cometer os meus erros aprendendo com eles.

Ao Doutor Carlos Costa Monteiro (antigo Director do IPIMAR) por ter aceite, encorajado e providenciado todo o suporte e condições necessárias para o desenvolvimento deste estudo.

À Professora Doutora Josefina Méndez Felpeto coordenadora do grupo XENOMAR da Universidade da Corunha por ter aceite orientar este trabalho numa vertente que me era estranha até ao início desta tese. Agradeço-lhe todo o suporte e disponibilidade durante as minhas estadias na Corunha.

Ao Professor Doutor Luís Zambujal Chícharo por ter aceite em ser meu orientador na Universidade do Algarve e por toda a ajuda facultada e apoio na supervisão desta tese.

Ao Doutor Juan Fernandez-Tajes do grupo XENOMAR pelo apoio incansável no desenvolvimento da componente de biologia molecular desta tese. Por me ter dado todo o apoio nas minhas estadias e ter tido a paciência de me iniciar quase do zero no mundo da biologia molecular.

À Dr. Sandra Joaquim da estação experimental de moluscicultura de Tavira por toda a ajuda incansável na componente de produção de larvas de conquilha desde a implementação do sistema de desova, colheita e crescimento de ovos e larvas, recolha e demais processo de análises bioquímcas. Também pelas suas críticas ao longo da realização dos manuscritos.

À Dr. Paula Moura que partilha o gabinete comigo, por toda a amizade e ajuda nas imensas amostragens na praia da Lota.

Ao Dr. José Garcia Gil do grupo XENOMAR por me acompanhar no laboratório ensinando-me incansavelmente as regras e procedimentos.

À Eng. Margarete Matias pelo auxílio no cultivo larvar e manutenção dos sistemas de produção de larvas.

Ao Sr. Maurício Teixeira pelo auxílio na preparação dos tanques e de toda a ajuda na manutenção dos sistemas de produção.

À Doutora Ruth Freire Alvarez do grupo XENOMAR por toda a ajuda e ensinamentos facultados.

Ao Jorge Ramos, Paulo Vasconcelos, Pedro Lino, Susana Carvalho, João Cúrdia, Fábio Pereira, David Piló, Domitília Matias, Alexandra Leitão por toda a ajuda e amizade ao longo destes anos em que trabalhámos na mesma casa.

Ao Pedro Range pela ajuda nas amostragens na praia da Lota, pelo trabalho desenvolvido na estação de Molluscicultura e na realização do manuscripto.

À Milu, Luz Abreu, Lina Oliveira, Tibério Simões, José Luís Ova, Fernando Catarino por toda a ajuda em diferentes fases do desenvolvimento desta tese.

A todo o restante pessoal do CRIPSul por toda a amizade durante os anos que partilhei esse espaço convosco.

A todos os meus amigos que me têm acompanhado ao longo dos anos

A ti Cláudia por partilhares a tua vida comigo e por todo apoio que me tens dado ao longo destes anos que estamos juntos por vezes em momentos nem sempre fáceis. Pela tua ajuda nas amostragens, na revisão do inglês e criticismo.

Aos meus filhos Pedro Miguel e João Afonso por todos os momentos incríveis que me têm proporcionado desde que entraram na minha vida. Por melhorarem os meus dias com os vossos sorrisos e gargalhadas.

E por fim às minhas irmãs e em particular aos meus pais Alda e Joaquim por todo o apoio que me deram ao longo da minha vida, dos meus estudos, sempre apoiando as minhas escolhas e sem os quais a conclusão deste trabalho não seria possível.

A todos o meu enorme agradecimento.

“*We can judge our progress by the courage of our questions and the depth of our answers, our willingness to embrace what is true rather than what feels good.*”

Carl Sagan

Abstract

The wedge shell, *Donax trunculus*, inhabits high energy environment of exposed sandy beaches from the Atlantic coast of France to Senegal. Like all Donacidae, it is relative small, flat-shaped with elongated solid shells. It is a highly demanded and valuable species mainly in Algarve, with the dredge fleet increasing the pressure on species stocks. In 1986 the Portuguese Institute for the Ocean and Atmosphere (IPMA) initiated a bivalve survey program to evaluate the stock status of species with economical valuable but always on the adult population. Since then, several managing measures were implemented to guarantee bivalves' sustainable exploitation. Despite the available information on the abundance and distribution of the wedge shell along the Algarve coast, no information on the larval planktonic phase is available. To fill in this gap, the present study aimed at obtaining, for the first time information on the broodstock condition and on the pelagic phase of the wedge shell in the Algarve coast.

Through the determination of broodstock proteins, glycogen, total lipids and condition index (CI) as well as environmental factors such as sea surface temperature (SST), chlorophyll-a and phycotoxins, data obtained suggests that odd events (high SST and phycotoxins) during the spawning season were reflected in the CI of the wedge shell showing that this index could be a valuable indicator for fisheries management. In larvae, from the parameters analyzed, total lipids and the energy available were found to play an important role in the hatching success and development into D-larvae. More, the Total Lipid plus Carbohydrate to Protein ratio can be used to predict the hatching success in *D. trunculus*. Identification and discrimination of the wedge shell from the other three *Donax* species was successful achieved with a specific primer design on 5S rDNA. Larvae quantification in samples was possible using a specific primer amplifying the ITS1 region with real-time PCR. Since the Intergovernmental Panel on Climate Change (IPCC) predicts the increase of ocean acidification in the next centuries, the effect of high pCO_2 on larvae hatching rate and development was also considered and found to affect negatively the hatching rate success. It also seems to delay hatching. Results obtained suggest that high acidified environment also produce more abnormal larvae than the less acidified ones.

The results obtained here demonstrate that larval information is relevant and should be taken into consideration to improve managing measures and to timely detect possible recruitment failures.

Keywords: Bivalve, *Donax trunculus*, Broodstock, Larvae, Biochemistry, Condition index, Real-time PCR, Ocean acidification, Fisheries, Management

Resumo

A conquilha, *Donax trunculus*, habita em sedimentos arenosos de praias com algum hidrodinamismo desde a costa atlântica de França até ao Senegal. Tal como outros Donacidae, é relativamente pequena, achatada com uma concha sólida e alongada. É uma espécie com elevado valor económico e muito procurada especialmente no Algarve, levando a que exista um aumento da pressão por parte da frota de arrasto de ganchorra sobre a população. Em 1986 o Instituto Português do Mar e da Atmosfera (IPMA) iniciou um programa de monitorização dos recursos bivalves de modo a avaliar o estado dos estoques populacionais das espécies com valor económico, embora sempre sobre a população adulta. Desde esse período, várias medidas de gestão têm sido implementadas garantido a sustentabilidade da pesca. Apesar de existir variada informação acerca da população adulta ao longo da costa algarvia, não existe qualquer informação sobre a fase planctónica desta espécie. Com o intuito de colmatar esta falha, o presente estudo teve como objectivo obter pela primeira vez informação acerca da condição dos progenitores e da fase planctónica da conquilha na costa Algarvia.

Através da determinação nos progenitores das proteinas, glicogénio, lípidos totais e índice de condição (CI), assim como de factores ambientais como a temperatura superficial do mar (SST), a clorofila-a e as ficotoxinas, os dados obtidos durante a época de desova sugerem que eventos incomuns (elevada SST e ficotoxinas) reflectem-se no CI da conquilha mostrando que este índice pode ser um indicador valioso para a gestão da pescaria. Da análise dos lípidos totais e da energia disponível nas larvas, descobriu-se que estes desempenham um papel importante no sucesso da eclosão e posterior desenvolvimento em larva-D. Também o rácio entre Lípidos Totais mais Carboidratos e as Proteínas pode ser usado para prever o sucesso da eclosão na espécie *D. trunculus*. A identificação e discriminação da conquilha das outras três espécies de *Donax* existentes foi bem-sucedida com recurso ao desenvolvimento de um primer específico na região 5S rDNA. A quantificação de larvas em amostras foi possível através de Real-time PCR, utilizando um primer específico que amplifica na região ITS1. Uma vez que o Painel Intergovernamental para as Alterações Climáticas (IPCC) prevê o aumento da acidificação dos oceanos nos próximos séculos, o efeito do aumento de pCO_2 na eclosão das larvas e desenvolvimento foi avaliado, tendo sido encontrado um efeito negativo na taxa de eclosão e também um atraso nessa mesma eclosão. Os resultados obtidos também demonstram que ambientes acidificados aumentam o número de larvas com malformações do que em ambientes menos acidificados.

Os resultados aqui obtidos demonstram que a informação sobre as larvas é relevante e deve ser considerada no melhoramento das medidas de gestão de modo a antecipar a possível detecção de falhas de recrutamento.

Palavras chave: Bivalves, *Donax trunculus*, Progenitores, Larvas, Bioquímica, Índice de condição, Real-time PCR, Acidificação do oceano, Pescas, Gestão

Table of contents

List of tables xiii

List of figures xiv

Aims and Chapters 1

Chapter I. General Introduction 5

- Study area characterization 11
- Species characterization - Geographical and spatial distribution 12
- Taxonomy and species description 14
- Growth 15
- Reproduction and settlement 16
- Fishery and management 16

Chapter II. Biochemistry of *Donax trunculus* 20

- Is condition index a valuable indicator for the management of the *Donax trunculus* (Linnaeus, 1758) fishery in the Algarve coast? 21
  - Abstract 22
  - Introduction 23
  - Material and methods 25
  - Results 28
  - Discussion 32
  - Acknowledgments 37
- Is the biochemistry of *Donax trunculus* (Linnaeus 1758) egg and larvae suitable for managing purposes? 39
  - Abstract 40
  - Introduction 41
  - Materials and methods 43
  - Results 45
  - Discussion 51
  - Acknowledgments 55

Chapter III. Identification and quantification of *Donax trunculus* larvae. 58

- Identification of the wedge clam *Donax trunculus* by a simple PCR technique. 59
  - Abstract 60
  - Introduction 61
  - Material and methods 63
  - Results 65
  - Discussion 66

Acknowledgments 67

Quantification of wedge shell, *Donax trunculus* (L.), larvae by Real Time PCR. 69

Abstract 70

Introduction 71

Material and methods 74

Results 78

Discussion 81

Acknowledgements 83

Chapter IV. Ocean acidification effect on *Donax trunculus* larvae 86

Larval hatching and development of *Donax trunculus* (L.) under increased CO_2 and reduced pH scenarios. 87

Abstract 88

Introduction 89

Material and methods 91

Results 93

Discussion 98

Acknowledgements 101

Chapter V: General conclusions 104

General references 109

List of tables

Table 2.1. Data mean values for all biochemical parameters determined along the three years of study ($x \pm s.d.$). 31

Table 2.2. Results of Pearson correlation between studied parameters. SST- Sea Surface Temperature; r - Pearson correlation coefficient; P - P value; n.c. - no correlation was found. 32

Table 2.3. Biochemical components expressed as organic weight percentage, composition of lipid class and energetic value in *Donax trunculus* eggs during the spawning season in 2009, 2010 and 2011. 47

Table 2.4. Biochemical components expressed as organic weight percentage, composition of lipid classes and energetic value in *Donax trunculus* D-larvae during the spawning season in 2009, 2010 and 2011. 49

Table 2.5. Results of Spearman correlation between studied parameters. SST – Seawater Surface Temperature; (TL+C/P) – Total Lipids + Carbohydrates to Protein ratio; *r* – Spearman correlation coefficient; *p* – *p* value; n.c. – no correlation was found. 51

Table 3.1. Sequence of primers used initially to amplify ITS1 and COI regions and specific primers design for *Donax trunculus* in qPCR-RT analysis. 75

Table 3.2. Larvae templates used in ITS1 and COI linear functions and the number of replicates used. CV – coefficient of variation within samples with same number of larvae. 77

Table 3.3. Measures of DNA concentrations (ng.μl^{-1}) in two sets of samples with different amount of larvae. 78

Table 3.4. Ranges of Ct-values obtained by real-time PCR, used for relative quantification of *D. trunculus* larvae on plankton samples.. 80

Table 4.1. Seawater carbonate chemistry variables (Mean ± SE); Measurements of pH (total scale), Temperature (T), Salinity (S) and Total Alkalinity (TA); Dissolved inorganic carbon (DIC); partial pressure of $CO2$in seawater ($pCO2$) and saturation state for calcite (Ωcal) and aragonite (Ωara) were calculated from in situ T and S, corrected pH and TA.. 94

List of figures

Figure 1.1. *Donax trunculus* shell exterior and interior (Illustration by A.M. Arias, ICTIOTERM).. 15

Figure 2.1. Sampling location of *Donax trunculus* and monthly average values of sea surface temperature (SST) and chlorophyll-a (mg/m^3) at sampling site during the years 2009, 2010 and 2011. 25

Figure 2.2. Condition index, protein, glycogen and total lipids mean values (µg mg^{-1} DW) for a standard 26mm specimen. 29

Figure 2.3. Sampling location of *Donax trunculus* progenitors and monthly average values of sea surface temperature (SST) at sampling site during the years 2009, 2010 and 2011.. 45

Figure 2.4. *Donax trunculus* eggs hatching rate (HR), and Total lipid plus Carbohydrates to Protein ratio (TL+C to P) during 2009, 2010 and 2011 spawning seasons. 50

Figure 3.1. Image of 3% agarose gel electrophoresis of amplicons from different *Donax* species after staining with 0.5µg/ml ethidium bromide. XIII – step ladder 50-750 bp.. 65

Figure 3.2. Plot of the relation between larval number and estimated Ct values using ITS1. (♦). White circles represent blind test samples of: 0.1; 1; 5; 10; 50; 100; 250 and 500 larvae. Linear regression equation and r^2 displayed. 79

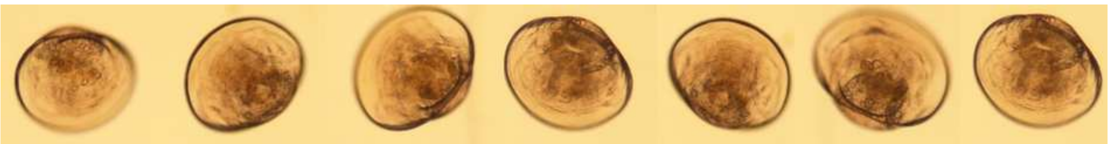
Figure 3.3. Plot of the relation between larval number and estimated Ct values using COI (♦). Linear regression equation and r^2 displayed. 80

Figure 4.1. Hatching rate percentage for Donax trunculus, 48 h after spawning, in the control ΔpH=0.0 (CT) in ΔpH=-0.3 (medium) and ΔpH=-0.6 (extreme) treatments (mean±sd)... 94

Figure 4.2. Abundance of *Donax trunculus* veligers (normal and abnormal), trochophore larvae and total survivors (trochophore plus veligers) during the 9 days of experiment, for the three treatments (ΔpH=0.0; ΔpH=-0.3; ΔpH=-0.6), (standard error bars displayed). 95

Figure 4.3. Larvae of *Donax trunculus* (a – trochophore; b – normal D-larvae; c – abnormal D-larvae with unequal valves; d – abnormal D-larvae with convex hinge; e – abnormal D-larvae with shell protuberances; f – abnormal D-larvae with mantle protuberant) (bars displayed = 10μm length)... 96

Figure 4.4. Diameter (μm) and Area (μm^2) of *Donax trunculus* veligers (normal and abnormal), trochophore larvae and total survivors (trochophore plus veligers) measured on days 5 and day 9 of the experiment in the three treatments (ΔpH=0.0; ΔpH=-0.3; ΔpH=-0.6), (standard error bars displayed). 97



Aims and Chapters

The objective of the present study was to improve knowledge on the pelagic larval phase of the wedge shell, *Donax trunculus*, and to discuss how the information gathered can be useful in the management of the fishery. This knowledge may help to timely detect possible recruitment failures anticipating, if necessary, the early implementation of proper managing measures. The present study fills in the lack of information on *D. trunculus* larval phase in Portugal and in particular in the Algarve coast. There were five main aims:

- To understand how biochemical quality of *D. trunculus* broodstock is affected by environmental factors;
- To find out if there is a relationship between *D. trunculus* larval quality and hatching rate.
- To develop a simple and easy methodology to successfully discriminate *D. trunculus* from the other three *Donax* species available in the Algarve coast.
- To develop methodologies to discriminate and quantify larvae of *D. trunculus* from seawater samples;
- To infer how the ocean acidification affect shell development and survival of early life stages of *D. trunculus*.

The present thesis is organized into five chapters containing the compilation of articles submitted or published in the scope of this study.

Chapter I provides a general introduction to the bivalves in the Algarve coast, the bivalves species with commercial interest and the information on stock population that has been gathered by the Portuguese Institute for the Ocean and Atmosphere (IPMA). A species characterizations, habitat and description of sudy area is provided, as well as a discussion on the importance of information on the planktonic stage of the wedge shell and the limitations that studies on this subject must deal with. Information on the taxonomic identification and quantification of larvae are presented. Information on the bivalve dredge fleet and on managing measures is described and a compilation of different studies on this species is also provided.

The Chapter II focus on the biochemistry of *Donax trunculus* in two particular stages of their life cycle. First, the broodstock condition is evaluated along three consecutive spawning seasons. The proteins, glycogen, total lipids and condition index were determined and the variation of these components with the surface seawater temperature, chlorophyll a and phycotoxins presence were analysed. Second, the Proteins, Total lipids, Carbohydrates and energy content of both eggs and D-larvae were determined in order to assess the influence of these components on hatching rate.

Chapter III deals with a problem that has hindered studies on bivalve larvae dynamic and dispersal: the identification and quantification time needed and difficulties in processing these type of samples using traditional methodologies. Successfully distinction of *Donax trunculus* from the other 3 *Donax* species available at the Algarve coast was achieved and identification and quantification of the amount of *Donax trunculus* larvae by real time PCR was used as an indicator of how many larvae are available to settle and later to recruit to the population.

In Chapter IV, the effect of ocean acidification on survival and abnormal shell formation in the larvae of *D. trunculus* after spawning was assessed. This is not information affecting directly the annual recruitment, but rather the resource itself in a long term scenario. In this chapter two scenarios were tested, one more mild and plausible, considered by the Intergovernmental Panel on Climate Change (IPCC) as an actual threat to occur in the next 100 years and one more extreme scenario, where the total carbon dioxide resulting from the depletion of all fossil fuels reserves would be released.

Finally, Chapter V provides the main conclusions of this work and highlights the main contributions and future works.

Chapter I

General introduction

Chapter I. General Introduction

The Algarve coast is the habitat for more than 70 bivalve species distributed from 0 to more than 50 meters depth, occurring in different granulometry types of sediments, such as sand, mud, sandy/muddy and coarse sediments, ranging from very fine to very coarse sand (Gaspar 1996; Freitas *et al.* 2011). Of these, only four have commercial valuable: the wedge shell *Donax trunculus*; the surf clam *Spisula solida*; the striped venus *chamelea gallina* and the razor clam *Ensis siliqua*. These four species are found along the coast up 15 meters depth, inhabiting the very fine sand usually found from 0 up to 7-10 meters and part of the coarser sand found beyond this depth (Freitas *et al.* 2011).

Although there are four species with economical interest, the dredge fishing fleet operating in the Algarve coast target mainly the surf clam (*S. solida*), the striped venus (*C. gallina*) and the wedge shell (*D. trunculus*). Of these, the wedge shell is one of the most valuable and is extremely appreciated gastronomically. The high demand of this species during summer, leads the dredge fleet to direct fishing effort mainly to this particular species, increasing the pressure on the available stocks. Apart from the boats that operate along the coast, licensed hand dredgers targeting the wedge clam also operate in several places along the shore. In hand dredging, while walking backwards, the fisherman pushes and pulls the handle repeatedly to facilitate towing the gear over the sediment surface (Gaspar *et al.* 2013) One problem observed and never properly addressed is the after catch procedure implemented by the users of hand dredges. A sieve is used to separate smaller wedge shells (less than 25 mm length) from the ones with the legal length, but unfortunately, usually the smaller ones are left on the beach sand in areas that are hardly affected by tides. This causes the remaining smaller specimens as well as other bivalve species and other invertebrates to be left to dehydration eventually ending up dying. The extent of this effect is not studied, but it is a very common practice among the hand dredge fishermen.

Due to the easy access to this species, and the low hydrodynamics of the beaches in the Algarve during the summer, vacationers also harvest specimens of *Donax trunculus* for consumption. This practice takes place during low tide and the depth segregation observed on this species, with smaller individuals in shallower waters and the larger ones deeper (Gaspar *et al.* 2002a), leads the vacationers to harvest mostly juveniles. This touristic activity is not controlled and there is no information available on total catches occurring annually. All this pressure on the wedge shell population occurs mainly during the summer, coincident in part with the spawning season of this species (late March until the end of July, early August).

The Portuguese Institute for the Ocean and Atmosphere (IPMA) has addressed over the years several studies regarding the bivalve species available along the Portuguese coast with emphasis on the Algarve coast. These studies have been performed since 1986 with usually two bivalve surveys held each year and with a primary focus on gathering information on the economically important species exploited by the dredge fleet. These bivalve surveys are usually made in April and September with two bivalve dredges mounted on board of a research vessel. The dredges used in those surveys respect the legal standards dredge grid size and mouth tooth size used by commercial fishing vessel operating in these fishing areas. The starboard dredge used is specifically designed to capture mainly clams and the port dredge especially designs to capture razor clams. The main difference found in the dredges used are related to the length of teeth (10cm length in the starboard dredge and 35mm in the port dredge) and is justified by the maximum burrowing depth of the target species (Gaspar 1996). These annual surveys comprehends 108 transects perpendicular to the coastline along the Algarve coast separated each other by one and a half nautical mile. At each transect three stations at different depths are towed and the specimens collected. For more information on the survey program held by IPMA see Rufino *et al* (2010). Although all bivalve species captured are sampled (measured and weighed), naturally these campaigns focus on the four commercially valuable and exploited species, from which additional information has been collected over the years.

Since dredges specifications are built according legal standards, part of the smaller individuals (<25 mm shell length for clams and <100mm for razor clams) pass through the meshes of the net bag of the dredge and are usually not taken on board neither to the laboratory for further analysis. Thus the information obtained from the specimens captured refers mostly to the adult fraction of the population, reflecting the status of the adult population only. The information obtained throughout the years, has been useful as a tool to design and implement managing measures to protect the resources from overfishing. Several managing measures have been implemented in order to guarantee the sustainable exploitation of the commercial bivalve populations, such as: dredge and hand-dredges specifications; limit of the number of fishing licenses; limit of the fishing time per day; daily quotas per species and boat or harvester; minimum landing size of the species; and a closed season from May 1st to June 15th. For more detailed information on these general managing measures see Pereira *et al.* (2007). It is important to underline that hand dredgers are allowed to operate during the closed season. Nevertheless, during this period the total daily catches are reduced from 30 kg to 5 kg of bivalves *per* fisherman.

Despite the quantitative information available on the abundance and distribution of the wedge shell population along the Algarve coast, no information on the larval stage is available. All managing measures mentioned above can only be applied or adjusted after the annual survey has been carried out and all the data has been processed. Moreover, the results obtained reflect mainly the status of the adult population due to the selective properties of the dredges used. As a result, the sampling design adopted in the IPMA surveys although effective to analyze the adult population status is not a totally effective method to prevent possible recruitment failures during critical moments in the life cycles of marine bivalves such as spawning and settlement. Aside from the quantity information on the larvae, also quality information on bivalve larvae in the Algarve coast is still scarce or even inexistent, being the study made by Joaquim *et al.* (2008a) on *Spisula solida* an exception.

It is then essential to collect new and more complete information on the wedge shell stock. The data gathered during the monitoring surveys must be complemented with information on the quality and condition of the broodstock, allowing us to understand how energy reserves accumulation affect the reproductive success and how individual organisms respond to environmental stress such as atypical changes of temperature and chlorophyll-a. Also the larvae biochemistry must be considered since the physiological condition of the embryos and larvae has been related successfully to larvae settlement and recruitment (Ojea *et al.* 2008; Marshall, McKinley, & Pearce 2010). This information is of utmost relevance to the resource management, since it will allow the timely adjustment of the fishing effort if a recruitment failure is detected.

Species specific information on larval distribution can be critical to the management of commercial species as it allows the understanding of recruitment variation and success and helps predicting population demographic patterns (Livi *et al.* 2006). Understanding the larval dispersal dynamic of bivalves is of utmost importance in order to interpret stock structure, population dynamics and estimating the abundance of larvae within plankton communities to properly manage the exploited species with emphasis on those in decline (Morgan & Rogers 2001; Vadopalas *et al.* 2006). This requires knowledge on the planktonic stage of the species, when high dispersal is achieved allowing long movement and colonization of new habitats (Livi *et al.* 2006). The studies of these stages, in particular the spatial-temporal patterns of the planktonic larval supply of recruitment, is extremely difficult to measure despite their extreme importance in population, community and ecosystem ecology (Morgan & Rogers 2001; Garland & Zimmer 2002).

Obtaining samples of bivalve larvae may be fairly easy if we consider a few samples, but it rapidly becomes a cumbersome task if hundreds of samples are needed. Moreover it is laborious and difficult to process a great amount of samples in a reasonable time. Those difficulties have hindered studies in the open waters at a large scale. Another problem is the accurate taxonomic identification which is vital for the correct interpretation of ecological data sets as well as establish links between conspecific adults and larvae so that a population can be tracked in space and time from one generation to the next (Morgan & Rogers 2001). The taxonomic identification is a difficult process when one tries to differentiate species from the same genus or even from different genera. Some authors have addressed this problem in their works finding different solutions to deal with this identification difficulty. Morphology based methods and molecular based methods have been used to identify bivalve larvae (Lutz *et al.* 1982; Hare, Palumbi, & Butman 2000; Le Goff-Vitry, Chipman, & Comtet 2007).

Morphological based methods include the traditional microscopic examination of larvae, but this is subjective, time consuming and even a trained person would have serious difficulties in differentiating larval species from the same genus and is, therefore extremely dependent on the expertise of the person identifying de larvae. Moreover, there are more than 70 species in the Algarve coast only, with four species belonging to the genus *Donax*. The morphological based identification in larva requires comparison among morphological traits for a definitive identification with the existence of published larval descriptions. However, these are not available or exist only for a particular species (Garland & Zimmer 2002). Moreover, the majority of bivalve larva is so similar in morphology that some taxonomic groups are morphologically indistinguishable even at later stages. Molecular methods potentially decreases the subjectivity inherent to the morphological based methods and are much accurate and precise in identification (Garland & Zimmer 2002). Molecular techniques are diverse and include immunological, isoenzymes, DNA-based such as hybridization, PCR-RFLP, PCR-RAPD, microsatellites, multiplex PCR and PCR.

The identification of bivalve larva is fairly simple when we are dealing with one specimen only or if we only want to detect the presence of the target species in a given sample, e.g. we may only want to detect the presence of an invasive species in a certain place, or in a survey along a shore we may only want information about the presence/absence of a certain species along the shore. But the majority of biological studies need quantitative information, which bring us to a different problem: how to easily quantify larva in the environment? Quantification of larvae has two main problems associated. First, counting larva manually is a cumbersome and extremely time consuming task when there are hundreds or thousands of larvae per sample. Studies dealing with hundreds of samples, each one with hundreds or thousands larvae are not realistically feasible. This lead us to the second problem, different species of bivalve and other meroplanktonic organisms may be present in each sample, and as mentioned above discriminating them visually is difficult. Some interesting work has been done in visually discriminating zebra mussel larva from other organisms with polarized light, although no real discrimination was actually made between this species and other bivalve species present in the samples (Johnson 1995). Machine learning and image analysis have been used (Tiwari & Gallager 2003; Thompson, Hare, & Gallager 2012) but although with some degree of correctness discriminating species using multiscale texture and color invariants in prepared sample test, when applied to samples collected from the environment the results are still far from the expected. Molecular methods have been applied to a diverse number of species with planktonic larva: abalone (*Haliotis kamtschatkana*) larvae quantified using mitochondrial DNA marker cytochrome oxidase I via quantitative PCR (Vadopalas *et al.* 2006), manila clam (*Ruditapes philipinarum*), harbour crab

(*Liocarcinus depurator*), velvet swimming crab (*Necora puber*), green crab (*Carcinus maenas*), edible crab (*Cancer pagurus*), the king crab (*Paralithodes camtschaticus*) and several barnacle species from the Japanese coast with real time PCR (Pan *et al.* 2008; Endo *et al.* 2010; Quinteiro *et al.* 2011; Jensen *et al.* 2012). Polychaets from genus *Osedax* and *Ophelia* and barnacles from the genus *Balanus* has been tested with rRNA targeted sandwich hybridization assay (SHA) using oligonucleotides probes to detect and enumerate the larva (Jones *et al.* 2008) and fluorescence in situ hybridization using species specific DNA probes was also used in marine bivalves (Heaney, Maloy, & Slater 2011).

Since the beginning of the industrial revolution the carbon dioxide released to the atmosphere have changed the carbonate chemistry of seawater (Meehl et al., 2007) (Orr *et al.* 2005; Meehl *et al.* 2007). This Carbonate chemistry is extremely important to the mineral formation of calcium carbonate ($CaCO_3$) a structural element of bivalve shells (Fabry *et al.* 2008). Since the shell formation in bivalves occurs at the beginning of the planktonic stage still as a trocophore larvae (Weiss *et al.* 2002), any detrimental effect during this period may jeopardize the survival capability and/or later development. The biological and economical implications that may arise during the next century are not fully understand but must be addressed as sooner as possible. At the present there is no information about the OA effect on *D. trunculus*, although some negative effects have been found to occur in other species: *Ruditapes decussatus* (Range *et al.* 2011; Fernández-Reiriz *et al.* 2011); *Crassostrea virginica* and *Mercenaria mercenaria* (Matoo *et al.* 2013; Ivanina *et al.* 2013); *Arctica islandica* (Hiebenthal *et al.* 2012); *Macoma balthica* (Jansson, Norkko, & Norkko 2013) and in the genus Mytilus (Gazeau *et al.* 2007; Rodolfo-Metalpa *et al.* 2011; Fernández-Reiriz *et al.* 2012; Range *et al.* 2012; Hiebenthal *et al.* 2012).

The majority of studies dealing with different aspects of *Donax trunculus*, refer to post-settled individuals and covers distribution, diet and biochemical composition (Özden, Erkan, & Deval 2009; Boussoufa *et al.* 2011; Martínez-Pita *et al.* 2012), identification (Pereira *et al.* 2012), pollution effect (Bresler & Fishelson 1994; Beldi & Gimbert 2006; Altug, Cardak, & Ciftci 2008; Özden, Erkan, & Deval 2009; Yawetz *et al.* 2010; Tlili *et al.* 2011), biological indicator of grain size (Huz, Lastra, & López 2002; La Valle *et al.* 2011), eco-toxicology (Mansour *et al.* 1998; Fishelson, Bresler, & Manelis 1999; Neuberger-Cywiak, Achituv, & Garcia 2003, 2007;

Moukrim *et al.* 2004; Tlili *et al.* 2010), ecology (Romeo & Gnassiabarelli 1988; Neuberger-Cywiak, Achituv, & Mizrahi 1990; Rufino *et al.* 2010), growth and reproduction (Bayed 1990; Ramón, Abelló, & Richardson 1995; Tirado & Salas 1998; Gaspar, Ferreira, & Monteiro 1999; Delgado & Defeo 2007; Deval 2009), cytogenetic (Cornet & Soulard 1990; Gonzalez-Tizon *et al.* 1998; Martínez *et al.* 2002; Petrović *et al.* 2009), genetics and molecular biology (Plohl & Cornudella 1996, 1997; Petrović & Plohl 2005; Petrović *et al.* 2007; Theologidis *et al.* 2008), population dynamics (Bayed & Guillou 1985; Guillou & Bayed 1991; Gaspar *et al.* 2002a; Zeichen *et al.* 2002), parasitology (Ramon 1999) and pathology (Sousa & Oliveira 1994).

To the author best knowledge, there are only a three studies dealing with wedge shell larvae, namely larvae rearing conditions (Ruiz-Azcona, Rodriguez-Sierra, & Martin 1996), larvae detection in planktonic samples (Livi *et al.* 2006) and settlement (Voliani *et al.* 1997). Ruiz-Azcona, Rodriguez-Sierra, & Martin (1996) showed that high temperatures (20°C) in rearing conditions were better to *D. trunculus* larvae culture than lower temperatures and that multispecies diets including Chaetoceros gracilis improved growth when compared to monospecies diets. Livi *et al* (2006) aiming at identifing the species that were presented in planktonic samples (including *D. trunculus*) developed a primer set by amplifying a region from the 18s rDNA using PCR-SSCP. This method allowed a fast sample processing and to detect if a species is absent or present in a sample. Finally, Voliani *et al* (1997) in their work quantified the amount of juveniles of *D. trunculus* that settled for square meter during the annual cycle in Toscan coast.

Study area characterization

The Algarve coast is characterized by a wind forced near surface offshore transport, coastal upwelling of cold subsurface waters and generation of alongshore currents to depths of 30 meters or more (Relvas & Barton 2005). The wave pattern is dominated by W-SW waves that represent about 71% of waves, followed by short-period SE waves generated by regional winds (“Levante”) that represent 23% of wave types (Costa, Silva, & Vitorino 2001). Wave energy is moderate with an annual average of offshore wave height of 1.0 meter and average peak period of 8.2 seconds (Costa, Silva, & Vitorino 2001). Along the year this region is affected by storm events, although these only account for 1% of the offshore wave climate (Costa, 1994). A large range of sediment grains can be found along the coast, with more muddy areas and finer sediment grains near the outflow of the Guadiana River and coarse sand found on the western coast (Rufino *et al.* 2010; Freitas *et al.* 2011). Moreover as we approach the Guadiana river, the grains size decreases and the beaches tend to be more dissipative, although more hydrodynamic and consequently less sediment stable due to exposure, having an higher turnover of nutrients favorable to suspension feeders like *Donax trunculus* (Rufino *et al.* 2010).

The study are, Lota beach (7°30′32.21″W, 37°9′56.33″N) in eastern Algarve, was chosen for its' easy access and low hydrodynamics during Spring and Summer, allowing the use of a hand dredge. It is classified as possessing clean medium sand (Freitas *et al.* 2011), where *Donax trunculus* occurs in quantity throughout the years.

Species characterization - Geographical and spatial distribution

The wedge shell, *Donax trunculus*, is an Atlantic-Mediterranean warm-temperate species, extending from the Atlantic coast of France (Ansell & Lagardère 1980) to Senegal (Tebble 1976). It inhabits the high energy environment of exposed sandy beaches with tidal rhythm, intense wave action and sediment instability (Ansell & Lagardère 1980), where it forms extensive dense beds (Gaspar, Ferreira, & Monteiro 1999) becoming the dominant macrobenthic organism in communities characterized by a low specific diversity (Donn *et al.* 1986; Ramón, Abelló, & Richardson 1995).
Along the Portuguese coast, this species is one of the four belonging to the genus *Donax*. Apart from *D. trunculus* also *D. semistriatus* (Poli 1795), *D. vittatus* (da Costa 1778) and *D. variegatus* (Gmelin 1791) inhabit the soft sediments along the shore. Although also present in the Northwest coast and Southwest coast of Portugal, it is in the South coast that the wedge shell presence is more expressive. In the Algarve, *D. trunculus* occurs along the entire coast with emphasis on the East part Quarteira to Vila Real de Santo António.

The wedge shell is the most inshore surf clam species in Portuguese waters, occurring up to 6 m depth, with higher densities between 0 and 3 m depth (Gaspar, Ferreira, &

Monteiro 1999). Several authors have referred a depth segregation phenomenon defining this species vertical distribution, with juveniles located in shallower depths at mid tide level, while adults are located deeper near the species bathymetrical limit (Ansell & Lagardère 1980; Guillou & Bayed 1991; Gaspar *et al.* 2002a; Zeichen *et al.* 2002). A study on depth segregation on *Donax serra* has demonstrated that there is an intimate correlation between temperature and depth, with juveniles positioning themselves in shallower waters where the higher temperature enhance the burrowing speed and avoid dislodgement and predation (Stenton-Dozey & Brown 1994). An inverse pattern distribution has been observed to occur in the Moroccan coast (Bayed & Guillou 1985) where juveniles were situated at greater depths than adults, since juveniles have lower tolerance and resistance to the high temperatures of surface sediments (30°- 40°). It was described that this phenomenon of depth segregation observed in Algarve coast may result from larval fixation at shallower depths, that undergo a depth migration as they grow allowing space to be available for further larva fixation (Gaspar *et al.* 2002a).

This species lives close to the sediment surface, a compromise between the possibility to be flushed away or exposed to predation and the more availability of food (Rufino *et al.* 2010). As all Donacidae, *Donax trunculus* is a suspension-feeder on phytoplankton and suspended particulate matter, which determines its vertical distribution. The wedge shell inhabits wash zones with a certain degree of hydrodynamism, favoring the presence of particulate matter and impeding its rapid sedimentation (Zeichen *et al.* 2002). It is a rapid and efficient burrower, with a shell shape designed to allow easy penetration into the sediment (McLachlan *et al.* 1995) and is considered a species sensitive to substrate grain size variations in particular during early growth stages (Huz, Lastra, & López 2002). Coarse sediment could limit the presence of *Donax trunculus* on exposed sandy beaches since individual may be carried away by waves and currents reducing the effectiveness of their burrowing (Huz, Lastra, & López 2002).

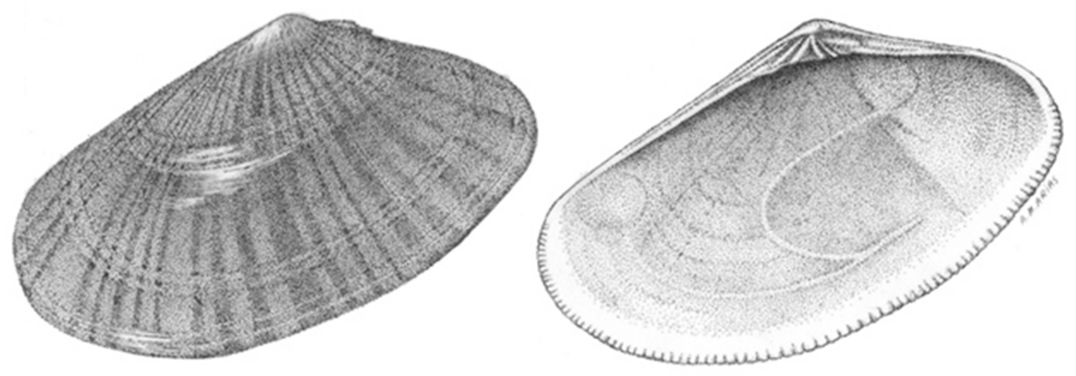
Taxonomy and species description

The wedge shell was first described by Linnaeus in 1758 in his "*Systema Naturae per regna tria naturae*" where it was referred as possessing a smooth shell, internally violet, with crenulate margins. Presently, its taxonomical classification is as follows:

Kingdom **Animalia** (Linnaeus, 1758)
Phylum **Mollusca** (Limmaeus, 1758)
Class **Bivalvia** (Linnaeus, 1758)
Subclass **Heterodonta** (Neumayr, 1884)
Order **Veneroida** (Gray, 1854)
Superfamily **Tellinoidea** (Blainville, 1814)
Family **Donacidae** (Fleming, 1828)
Genus ***Donax*** (Linnaeus, 1758)
Species ***Donax trunculus*** (Linnaeus, 1758)

Like all Donacidae, *Donax trunculus* is relative small flat-shaped with elongated shells. The shell is solid with a wedge shape (hence its common name "wedge shell"), equivalve, inequilateral and triangular in outline with margins straight. Beaks are positioned in the posterior half inwards and backwards with ligament as a small brown arched band. Almost invisible radial lines can be observed in the shell. Shell color white-yellowish, yellow, brown, purple or various shades of these colors. Sometimes with rays of white or purple radiating from the umbone and often with bands of color concentrated about the growth lines. The periostracum is glossy, thin, greenish, yellow or dark yellow to brown. Sculpture of very fine evenly spaced incised radiating lines over the greater surface area are visible as well as the growth stages. Right valve with two cardinal teeth (the posterior is stout and bifid and the anterior short and ridge like), two posterior and one lateral; left valve with two thin cardinal teeth (the posterior bifid and one weak anterior) and one posterior lateral. Interior of the shell color may vary, being white or purple, sometimes tinted yellow or orange. Muscle scars well marked as well as the pallial sinus, deep, extending half-way inside the shell with its lower border partially confluent with the pallial line.

Margin crenulated inside from the anterior border to the behind the ligament and rough to the touch (Nobre 1932; Tebble 1976; Macedo, Macedo, & Borges 1999).



Left valve, exterior

Right valve, interior

Figure 1.1. *Donax trunculus* exterior and interior (Illustration by A.M. Arias, ICTIOTERM).

Growth

Growth in *Donax trunculus* is controlled by the interaction of ecological factors such as temperature, density and food availability (Ansell, Frenkiel, & Moueza 1980; Bodoy 1982; Gaspar, Ferreira, & Monteiro 1999). The growth in this species shows a point of inflection beyond average sizes of 25-30 mm and growth rates also decrease at lower latitudes (Bayed & Guillou 1985). The analysis of the microstructure of the shell of the wedge clam populations that occur along the Algarve, allowed to evidence a seasonal discrimination of growth, with a rapid shell growth occurring in spring and summer when wide micro-growth increments were deposited in the shell, and a slow growth season occurring in late summer and early autumn with the deposition of narrow growth increments and the formation of a well defined annual growth ring (Gaspar, Ferreira, & Monteiro 1999). These authors reported a rapid growth during the first two years of life, reaching the minimum legal size of fishing (25 mm) in about one year.

Reproduction and settlement

In southern Portugal, the gametogenic cycle of *D. trunculus* usually begin in late November and the spawning occurs from March to late July. It is a species with a continuous spawning activity during all the spawning season, and the gamete presence at all stages of maturity reveal an individually successive spawning activity (Gaspar, Ferreira, & Monteiro 1999). After the end of the spawning season the majority of adults are in a spent state with gonads completely empty and contracted making the macroscopically sexual differentiation difficult (Zeichen *et al.* 2002). It has a life expectancy of 3 to 4 years, reaches maturity during the first year of life and maturity is a function of age and not size (Gaspar 1996; Gaspar, Ferreira, & Monteiro 1999). The wedge shell in the Algarve coast present a sex ratio that has been found to be slightly in favor of males (Gaspar, Ferreira, & Monteiro 1999) although other studies in different geographical areas have found no such difference (Deval 2009).

The increase of seawater temperature in March triggers the mass gamete release initiating the spawning season, a synchronism observed by Moueza & Frenkiel (1973); Neuberger-Cywiak, Achituv, & Mizrahi (1990); Gaspar, Ferreira, & Monteiro (1999). After spawning, the larvae planktonic period can last up to 2 months (Neuberger-Cywiak, Achituv, & Mizrahi 1990) although settlements of 4-6 months after the end of gonad activity have been reported by Moueza & Frenkiel (1973). Within 24 hours after spawning, the eggs develop into D-larvae and later develop the velum, a feeding structure that allows larvae to feed.

Fishery and management

The bivalve fishery is one of the most important artisanal fisheries in the Portuguese coast, being one of the most profitable. Along the South coast, *Donax trunculus* is an important commercial species, being exploited by an artisanal fleet comprising 57 boats divided between local and coastal boats (Oliveira, Camanho, & Gaspar 2013). Local boats operate near the homeport or adjacent ports and have an overall length smaller than 9 m, a gross tonnage (GT) between 1.18 and 9.41 tons and up to 75kW engines. Coastal boats can operate within all the fishing area they are registered for, their overall length is above 9m, ranging between 3.19 and 23.64 GT and an up to 130kW engine power.

The exploitation of bivalves along the Portuguese coast started in late 1960s (Gaspar *et al.* 2003). Until 1986, minimum landing sizes were the only management measure available, but due the increase in landings, fishing power and resource conservation concerns, the IPMA (ex-IPIMAR) initiated a bivalve survey program to evaluate the stock status of the bivalve species with economical valuable. The results obtained led to the implementation of new managing measures that were altered that year. Apart technical regulations such as gear restrictions and fishing seasons, other measures were implemented to control fishing effort namely maximum engine power and limitation to the number of fishing licenses. A season fishing ban period was introduced in 1987 and since then annual surveys were held allowing the suggestion of new managing measures to the competent authorities in order to improve the management of this fishery. The technical characteristics of the dredges were later changed in order to meet biological and ecological characteristics of the target species. These modifications included minimum mesh size, dredge mouth width, tooth length and spacing. In addition a seasonal closure was set between May 1st and 15th June (Gaspar & Chícharo 2007). In 1997 some signs of overexploitation of the bivalve stocks led to the implementation of the maximum number of six fishing days per week and maximum daily fishing quotas adjusting catches to the status of the resource (Gaspar & Chícharo 2007). Later research culminated in the introduction, in the year 2000, of a new dredge design which is more selective and efficient than the traditional one (Gaspar, Castro, & Monteiro 1999; Gaspar *et al.* 2002b, 2003). Since then the managing measures that regulate the bivalve fishery remained unchanged. However daily quotas are reviewed annually in order to adjust the catches to the conservation status of the fishing resources.

Chapter II

Biochemistry of *Donax trunculus*

Chapter II. Biochemistry of *Donax trunculus*

Is condition index a valuable indicator for the management of the *Donax trunculus* (Linnaeus, 1758) fishery in the Algarve coast?

Pereira, AM; Joaquim, S; Chícharo, L; Gaspar, MB. Is condition index a valuable indicator for the management of the *Donax trunculus* (Linnaeus, 1758) fishery in the Algarve coast? (Accepted for publication in Fisheries Research).

Abstract

Donax trunculus is one of the most important commercial species that occurs along the southern Portuguese coast. A high demand for this species occurs during spawning season. In order to prevent overexploitation, several management measures, based only on quantitative data have been applied. However, quality information is important to understand how the nutritional status of the broodstock population may affect reproduction and consequently recruitment success. The variation in the condition index reflects the individual reproductive activity and is influenced by variations in biochemical composition, namely proteins, glycogen and total lipids. We also tested the influence of sea surface temperature and chlorophyll-a on the condition index and biochemical contents. Our data suggests that during spawning season, wedge shell is accumulating and simultaneously converting glycogen into total lipids indicating an opportunistic reproductive strategy. Our data suggests that both CI and biochemical components storage and use are affected by the presence of phycotoxins. This affected the wedge shell feeding response and energy intake, leading to the use of proteins reserves. It was demonstrated that odd events such as unusual high sea surface temperatures and abnormal presence of phycotoxins during the spawning season was reflected in the condition index of the wedge shell showing that the variation of the CI could be a valuable indicator for fisheries management.

Introduction

According to Rees (2010), the bivalve production industry in Europe covers mainly oysters and mussels, since these are highly demanded species in several countries (e.g. United kingdom, France, Denmark, Ireland, Netherlands and Spain). Nevertheless, as well as the oyster or the carpet shell, the less valuable wedge shell (*Donax trunculus*) has become increasingly important in Portugal and other southern European countries such as Italy and Spain (Ruiz-Azcona, Rodriguez-Sierra, & Martin 1996). Although captured all year round, it is during the summer season that the demand for this species increases dramatically, mainly due to the gastronomical tourism offered in the South of the Iberian Peninsula. It is during this season that may become apparent an overfishing of this and other commercial bivalve species (Rufino *et al.* 2010). The fishing effort set on this species and other bivalves in the South of the Iberian Peninsula and in particular in the Algarve coast (South Portugal) has an important socio-economical role due to the size of the fishing fleet and the number of people directly and indirectly dependent upon this activity (Gaspar *et al.* 2002a), which is unlikely to decline in the near future. Furthermore, alongside with the fishing fleet, hand dredgers also operate in shallow waters capturing *D. trunculus*: the wedge shell is well distributed across a narrow sandy zone of the South coast of Portugal between 0 to 5 meters depth. Hand operated dredges have easy access almost all year round, except winter, when some hydrodynamics are observed (Costa, Silva, & Vitorino 2001). Moreover, vacationers tend to catch *D. trunculus* by hand. However, due to the depth segregation observed in this species almost only juvenile specimens are captured, because smaller individuals are usually found near the shore (Gaspar *et al.* 2002a). Unfortunately all this fishing activity occurs exactly during the spawning period of *D. trunculus*, that usually begins with the rise of temperature in late March–early April (Gaspar, Ferreira, & Monteiro 1999), and therefore some managing measures have been implemented to protect the available stocks from over-exploitation. The fishery is managed by a closure season (from May 1st to Jun15th), maximum daily catch per boat and harvester and a minimum shell length of 25 mm. Daily quotas may change annually, depending on the results obtained from the annual bivalve surveys held by the Portuguese Institute for the Ocean and Atmosphere (IPMA) along the Algarve coast. Unfortunately, the information obtained refers only to quantitative information without reference to the quality status of the broodstock.

This quality or nutritional status information is of fundamental importance, since energy reserve accumulation and the importance of each gross biochemical component has in the reproductive process, play a role in the adaptive strategies of a species to the habitat (Goodman 1979; Crosby & Gale 1990; Dahlhoff 2004). One way to follow these nutrient reserve changes during critical stages of their life cycle, is using condition indices (Crosby & Gale 1990). The most used CI is that proposed by Walne & Mann (1975), since it reflects the individuals reproductive activity (Massapina *et al.* 1999; Mladineo *et al.* 2007; Joaquim *et al.* 2008a; Boussoufa *et al.* 2011). More important, CI may reveal how individual organisms respond to rapid environment changes and how the involved factors interact at larger temporal and spatial scales (Dahlhoff 2004). The condition index is influenced by variations in biochemical composition, that occur due to energy trades during spawning, as well as food availability (Matozzo, Tomei, & Marin 2005). Indeed, CI is related to the organic matter (proteins, glycogen and total lipids) found dispersed within in the organic tissue.

In bivalves, proteins play a structural role, that are only used as a last resource of energy reserve in cases where the organisms suffer from extreme stress or when no other source of energy is available (González *et al.* 2001; Gopalakrishnan & Vijayavel 2009). Glycogen is the major energy source in bivalves, converted into lipids during gamete production that are later lost during the spawning process (Liu *et al.* 2008). Total lipids, with its high caloric content act as an important immediate energy reserve, being used by most marine bivalve species as a basic energetic reserve for sustaining embryonic and larval development (Fraser 1989).

To the authors best knowledge there are only four studies addressing the biochemical composition of *D. trunculus*. Three in the Mediterranean Sea, focusing on the effect of chemical pollutants to the energy reserves such as glycogen and lipids (Tlili *et al.* 2011) and on the seasonal variation in the condition and fatty acids composition (Boussoufa *et al.* 2011; Hamdani & Soltani-Mazouni 2011). The fourth study, outside the Mediterranean Sea, is related to the effect of a controlled diet in total lipids and fatty acids composition, although information on CI and total lipids of the wild broodstock is also presented (Martínez-Pita *et al.* 2012).

We believe that is still lacking some information related to Atlantic populations of *D. trunculus* in particular related to the broodstock condition during the critical spawning period. This particular type of information should be monitored yearly to complement the quantitative data already obtained. With this work we aimed at studing the variation of the Condition Index, proteins, glycogen and total lipids in the broodstock population of *D. trunculus* during the spawning season in order to understand the reproductive strategy adopted by *Donax trunculus* and to find out if the CI is sensitive to odd natural events and can be an useful indicator for fishing management.

Material and methods

Sample collection

A total of 1100 individuals of *D. trunculus* were collected in years 2009, 2010 and 2011 during the spawning season between March and July at Lota beach (7°30′32.21″W, 37°9′56.33″N) located in the southeastern Algarve coast, (Figure 2.1).

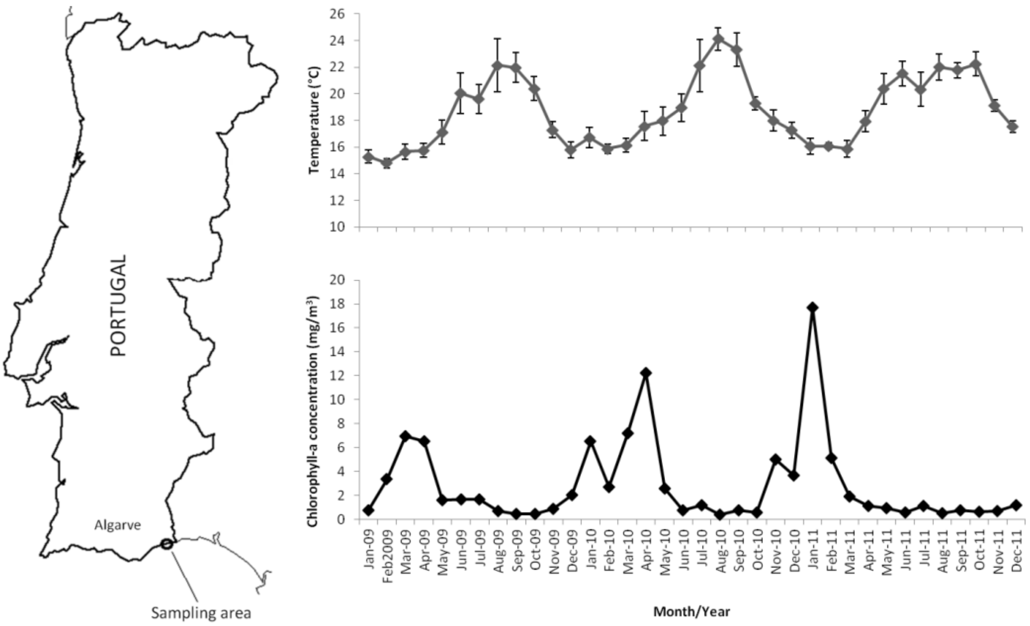


Figure 2.1. Sampling location of *Donax trunculus* and monthly average values of sea surface temperature (SST) and chlorophyll-a (mg/m^3) at sampling site during the years 2009, 2010 and 2011.

Spawning season has previously been used by other authors to assess broodstock quality in species such as the sea star *Asterias vulgaris* (Raymond, Himmelman, & Guderley 2007) and the swimming crab *Portunus trituberculatus* (Wu *et al.* 2010). During this five month of spawning season, the good hydrodynamic conditions facilitate easy access to the broodstock, a key factor to annually implement a sample survey.

Individuals were caught during low tide using a bivalve hand dredge, similar to those used by local harvesters. After been captured, the specimens were taken to the laboratory and placed for a period of 24 hours in a closed water system at lower seawater temperature (±15°C) without being fed, to avoid spawning and to clean the digestive tract. After this period, the closed water system was verified to ascertain that no spawning occurred, and specimens were packed up and frozen at - 20°C for posterior determination of CI and biochemical contents (protein, glycogen and total lipids).

Sea Surface Temperature (SST) and chlorophyll-a

Data on SST and food availability was obtained in order to evaluate their influence in both CI and biochemical content of *D. trunculus*. SST was provided by the Hydrographical Institute (IH). The chlorophyll-a concentration, which can be estimated remotely using ocean color satellites, is the most widely used and remains the best proxy of phytoplankton biomass for studies of primary production (Hout *et al.* 2007). Therefore, chlorophyll-a concentrations were derived from satellite remote sensing data, collected from the Giovanni online data system (MODIS-Aqua 4 km, monthly processed data, available at http://disc.sci.gsfc.nasa.gov/giovanni) developed and maintained by the NASA GES DISC (Acker & Leptoukh 2007).

Condition index determination

For the determination of the condition index 30 specimens per sample were used. Shell length was measured to the nearest mm using a digital caliper before removing the edible part. Both, dry soft tissues and shell weight were determined after oven drying at 80°C for 24 h. The dry meat was then converted to ash in a muffle furnace for 24 h at 450ºC. Afterwards it was once again weighed (ash weight). All weights were determined in an electronic scale (Mettler AJ100) with a precision of 0.0001g. The CI was calculated according to Walne and Mann (1975) using the following formula:

$$CI = \frac{dry\ meat\ weight\ (mg) - ash\ (mg)}{dry\ shell\ weight\ (mg)} \times 100$$

This index was determined for a standard animal of 26 mm SL, in order to suppress the results of growth, so that accumulation or loss of organic matter associated with reproduction can be shown (Gaspar, Ferreira, & Monteiro 1999).

Biochemical content

Biochemical content was determined separately for males and females. The specimens were sexed according to the color of the gonad, i.e., violet in females, yellowish-orange in males. For each sampling period, the soft tissues of five males and five females ranging from 26 mm to 30 mm SL were removed from the shell and homogenized individually with a blender (Ultra-Turrax^{TM}). For each individual it was determined: (1) protein content using the modified Lowry method (Shakir *et al.* 1994), (2) glycogen from dried homogenate (80 °C for 24h) using the anthrone reagent (Viles & Silverman 1949) and (3) total lipids were extracted from fresh homogenized material in chloroform/methanol (Folch, Lees, & Sloane-Stanley 1957) and estimated spectrophotometrically after charring with concentrated sulfuric acid (Marsh & Weinstein 1966). Duplicate determinations were performed in all cases and values expressed in µg mg^{-1} of dry weight (DW).

Statistical analysis

One Way Analysis of Variance (ANOVA) was performed to test differences in sea surface temperature, chlorophyll-a, condition index and biochemical contents across a time-series, whereas a t-test was used in order to investigate significant differences within years between males and females. Whenever the assumptions of ANOVA or the t-test were not met, the nonparametric Kruskal Wallis One Way Analysis of Variance on Ranks test or the Mann-Whitney Rank Sum test was performed, respectively. Each time ANOVA or Kruskal-Wallis tests detected significant differences among groups; pairwise multiple comparisons were made using Tukey or Dunn *post hoc* tests. Finally, the Pearson's correlation was used to seek the possible correlations between SST, chlorophyll-a, condition index, proteins, glycogen and total lipids. Whenever the normality assumption was not verified, the Spearmen's correlation coefficient was used. All statistical tests were performed using the Sigma Stat 2.0 statistical package.

Results

The evolution of monthly SST along the three years of study is shown in figure 2.1. The lower SST was registered in February 2009 with 14.81°C and the highest SST during August 2010 with 24.14 °C. Although the trend in year 2011 was similar to the observed in previous years, temperature rose earlier and stayed above 20°C for six months. These differences were detected by ANOVA, that revealed significant differences between SST observed during the spawning season of 2011 and both 2009 (Tukey test q=5.51, p<0.05) and 2010 (Tukey test q=4.07, p<0.05).

The maximum values of chlorophyll-a in 2009 and 2010 were observed in March (6.96 mg/m^3) and in April (12.21 mg/m^3) respectively. By opposition, the minima were observed in September (0.47 mg/m^3) and in August (0.37 mg/m^3) respectively (Figure 1). In 2011 the maximum value was observed earlier than in previous years, in January (17.72 mg/m^3), and the minimum was registered in August (0.50 mg/m^3) (Figure 1). Notwithstanding, the ANOVA results showed no significant differences between years for chlorophyll-a (K-W, *d.f.*=2, H=1.578 p=0.454).

The CI values during the spawning season followed a descent pattern (Figure 2.2). The highest value was registered in 2009 both for males (11.95±1.72) and females (11.46±1.31) whilst the lowest was registered in 2011 both for males (4.99±0.88) and females (4.72±0.93). A significant difference among the years was observed both for males (K-W, H=94.4, $d.f.$= 2, p<0.001) and females (K-W, H=73.2, $d.f.$= 2, p<0.001), with 2011 values differing from the other two years studied both for males (Dunn test: ${}_{2009\mathrm{vs}2011}q=9.44$ $p<0.05$; ${}_{2010\mathrm{vs}2011}q=7.61$, $p<0.05$) and females (${}_{2009\mathrm{vs}2011}q=8.41$ $p<0.05$; ${}_{2010\mathrm{vs}2011}q=6.32$, $p<0.05$). Significant differences in CI were also observed between 2011 and the other years values when data from males and females were pooled (${}_{2009\mathrm{vs}2011}q=12.56$ $p<0.05$; ${}_{2010\mathrm{vs}2011}q=9.94$, $p<0.05$).

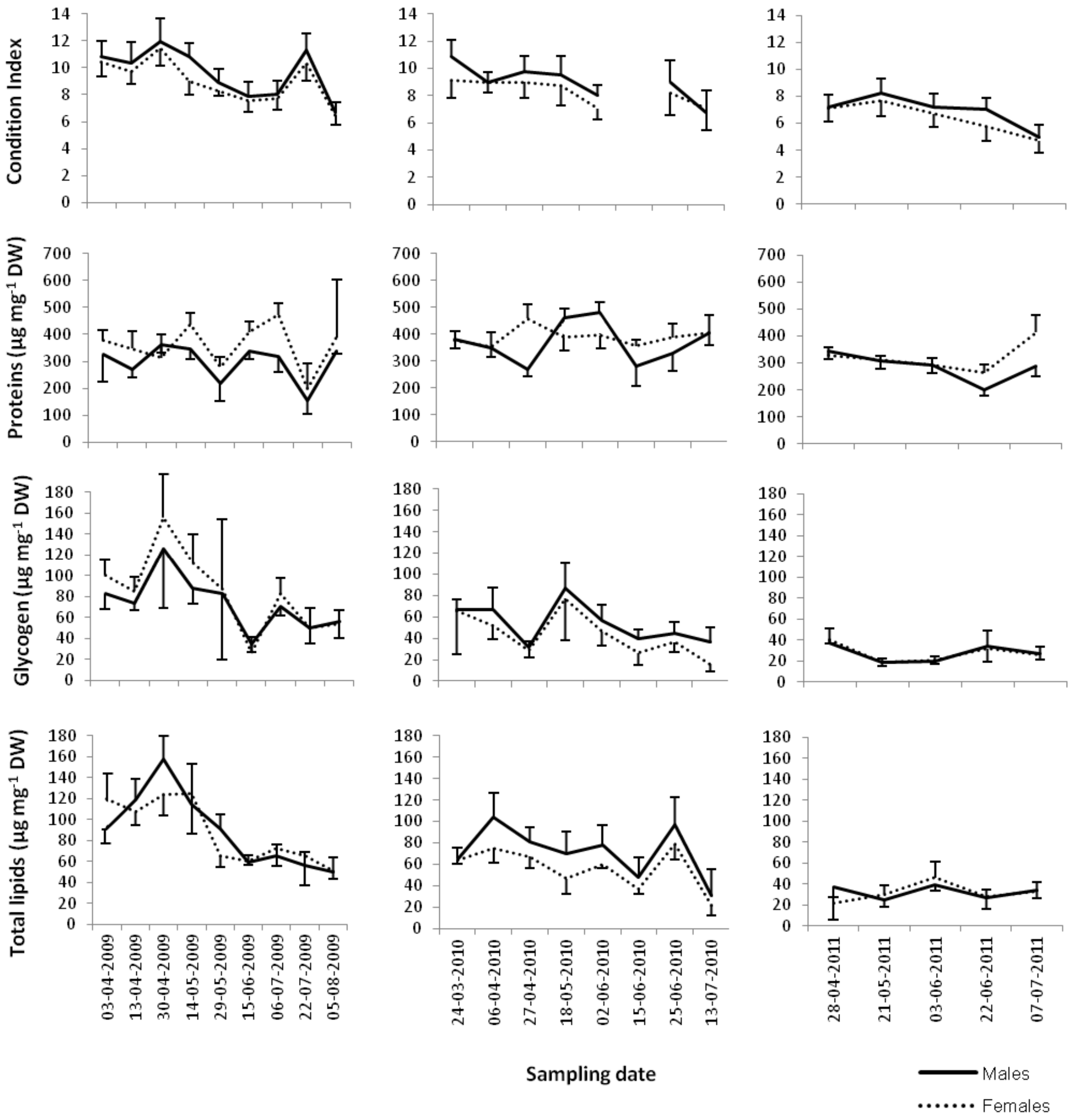


Figure 2.2. Condition index, protein, glycogen and total lipids mean values (µg mg^{-1} DW) for a standard 26mm specimen.

The proteins were the predominant component in every year during the spawning season representing more than 66%, 77% and 83% in 2009, 2010 and 2011, respectively (Figure 2.2). As observed in Table 2.1, the highest value of protein content was recorded for males in June 2010 (481.10±58.62 µg mg^{-1} DW) and for females in July 2009 (472.15±42.45 µg mg^{-1} DW) whereas the lowest values were observed in July 2009 both for males and females (154.29±51.06 µg mg^{-1} DW and 198.53±95.00 µg mg^{-1} DW, respectively). During the spawning season the mean protein values determined in males differed significantly only between 2010 and the other two years (Dunn $test_{2010vs2009}$: q=4.77, $p<0.05$; Dunn $test_{2010vs2011}$: q=5.27, $p<0.05$). In females, significant differences were observed for protein between 2011 and the previous years (Dunn $test_{2009vs2011}$: q=4.00, $p<0.05$; Dunn $test_{2010vs2011}$: q=5.87, $p<0.05$). When data was grouped, males plus females, there was differences among the three years (Dunn $test_{2009vs2011}$: q=3.54, $p<0.05$; Dunn $test_{2010vs2009}$: q=5.37, $p<0.05$; Dunn $test_{2010vs2011}$: q=7.98, $p<0.05$).

Regarding glycogen, the highest values were observed both in males and females in April 2009 (126.05±57.29 µg mg^{-1} DW and 155.49±62.91 µg mg^{-1} DW, respectively), whilst the lowest values were observed in May 2011 for males (18.52±3.69 µg mg^{-1} DW) and in July 2010 for females (16.67±6.88 µg mg^{-1} DW) (Table 2.1). Glycogen content generally decreased from the beginning to the end of the spawning season in years 2009 and 2010, but not in 2011 (Figure 2.2). Significant differences were observed between the three years in analysis for both males and females ($K\text{-}W_{males}$: H=78.59, $p<0.001$; $K\text{-}W_{females}$: H=72.57, $p<0.001$). Those differences were verified along the spawning season of all three years (Dunn test: Males ${}_{2009vs2011}q$=8.85, $p<0.05$; ${}_{2009vs2010}q$=3.14, $p<0.05$; ${}_{2010vs2011}q$=5.97, $p<0.05$; Females ${}_{2009vs2011}q$=8.08, $p<0.05$; ${}_{2009vs2010}q$=5.76, $p<0.05$; ${}_{2010vs2011}q$=3.05, $p<0.05$). The same differences among the years were also observed when data from males and females were pooled (Dunn test: ${}_{2009vs2011}q$=12.04, $p<0.05$; ${}_{2009vs2010}q$=6.41, $p<0.05$; ${}_{2010vs2011}q$=6.31, $p<0.05$).

Table 2.1. Data mean values for all biochemical parameters determined along the three years of study ($\bar{x}\ \pm s.d.$).

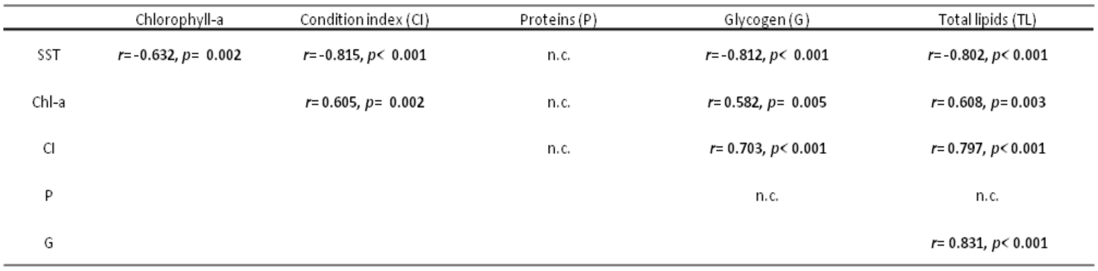
	Proteins (µg mg^{-1} DW)			Glycogen (µg mg^{-1} DW)			Total Lipids (µg mg^{-1} DW)		
Date	Males	Females	Total	Males	Females	Total	Males	Females	Total
03-04-2009	325.41 ± 99.46	379.10 ± 39.78	352.25 ± 78.70	82.72 ± 15.03	100.36 ± 15.00	91.54 ± 17.18	91.08 ± 15.26	119.41 ± 28.95	105.24 ± 26.80
13-04-2009	269.91 ± 30.67	347.35 ± 64.13	308.63 ± 63.02	73.17 ± 6.54	85.15 ± 13.22	79.16 ± 11.87	118.79 ± 19.73	107.17 ± 12.13	112.98 ± 17.02
30-04-2009	360.42 ± 30.35	313.75 ± 86.38	337.08 ± 67.41	126.05 ± 57.29	155.49 ± 62.91	140.77 ± 60.48	157.88 ± 24.23	123.57 ± 19.81	140.72 ± 27.82
14-05-2009	347.19 ± 38.40	437.23 ± 42.69	392.21 ± 60.79	87.87 ± 14.68	112.51 ± 26.93	100.19 ± 24.60	114.70 ± 38.64	125.06 ± 38.94	119.88 ± 38.13
29-05-2009	216.39 ± 62.93	278.81 ± 37.45	247.6 ± 59.71	82.81 ± 63.00	87.08 ± 67.48	84.95 ± 63.57	91.12 ± 14.27	65.26 ± 10.70	78.19 ± 18.08
15-06-2009	339.26 ± 29.54	409.94 ± 36.69	374.6 ± 48.64	34.19 ± 7.32	26.20 ± 14.87	29.99 ± 12.29	58.50 ± 6.45	60.23 ± 4.01	59.37 ± 5.30
06-07-2009	316.11 ± 56.29	472.15 ± 42.45	394.13 ± 93.60	70.81 ± 9.35	82.73 ± 14.70	76.77 ± 13.46	65.70 ± 10.47	72.86 ± 16.80	69.28 ± 14.11
22-07-2009	154.29 ± 51.06	198.53 ± 95.00	176.41 ± 77.62	49.63 ± 14.26	50.28 ± 18.26	49.95 ± 15.95	55.68 ± 13.54	65.17 ± 27.97	60.42 ± 21.93
05-08-2009	339.23 ± 112.87	389.69 ± 214,12	364.46 ± 168.59	55.70 ± 15.43	53.99 ± 12.60	54.85 ± 13.74	49.68 ± 14.24	51.23 ± 7.78	50.46 ± 11.20
24-03-2010	381.60 ± 34.20	378.00 ± 34.73	379.80 ± 33.60	67.99 ± 9.58	65.41 ± 40.45	66.70 ± 9.84	65.85 ± 10.28	64.71 ± 3.80	65.34 ± 7.89
06-04-2010	349.82 ± 34.23	352.77 ± 53.67	351.30 ± 43.84	66.41 ± 20.97	51.27 ± 12.59	58.84 ± 18.54	104.52 ± 22.02	75.03 ± 14.11	89.78 ± 23.51
27-04-2010	268.59 ± 26.09	458.02 ± 55.08	363.31 ± 105.84	31.41 ± 6.21	29.08 ± 6.57	30.24 ± 6.34	80.59 ± 13.66	67.18 ± 10.99	73.89 ± 13.89
18-05-2010	460.71 ± 28.56	390.72 ± 40.16	425.72 ± 49.39	96.59 ± 24.01	77.09 ± 38.60	86.84 ± 32.76	69.69 ± 20.53	46.65 ± 14.40	58.17 ± 20.92
02-06-2010	481.10 ± 58.62	398.38 ± 44.23	444.33 ± 66.44	57.37 ± 14.41	45.07 ± 13.92	51.28 ± 15.16	78.29 ± 18.49	59.74 ± 3.29	69.02 ± 16.05
15-06-2010	280.45 ± 73.73	355.76 ± 22.19	318.11 ± 65.58	39.46 ± 8.51	26.40 ± 11.52	32.93 ± 11.92	47.85 ± 18.39	37.01 ± 4.54	42.43 ± 14.17
25-06-2010	331.61 ± 67.31	389.48 ± 50.68	360.55 ± 65.14	44.28 ± 10.83	33.47 ± 9.78	38.87 ± 11.47	96.77 ± 25.39	78.67 ± 13.84	86.71 ± 21.27
13-07-2010	405.22 ± 46.97	405.24 ± 66.70	405.23 ± 56.26	37.08 ± 13.54	16.67 ± 6.88	26.87 ± 14.80	30.56 ± 24.80	21.55 ± 8.93	25.55 ± 17.79
28-04-2011	343.21 ± 27.58	328.42 ± 31.69	335.81 ± 29.89	36.68 ± 14.59	40.27 ± 3.03	38.38 ± 10.67	37.35 ± 31.03	21.77 ± 5.19	29.56 ± 23.08
21-05-2011	308.73 ± 28.56	310.76 ± 16.12	309.75 ± 22.60	18.52 ± 3.69	18.32 ± 2.84	18.42 ± 3.21	25.27 ± 7.55	30.24 ± 8.42	27.76 ± 8.19
03-06-2011	293.65 ± 29.90	293.12 ± 24.83	293.39 ± 26.75	19.20 ± 4.96	21.02 ± 4.19	20.11 ± 4.56	39.33 ± 5.7	46.01 ± 15.59	42.67 ± 11.93
22-06-2011	199.59 ± 19.34	262.93 ± 32.73	231.26 ± 41.72	34.54 ± 14.49	32.55 ± 12.43	33.54 ± 13.18	26.72 ± 10.21	28.31 ± 6.12	27.52 ± 8.23
07-07-2011	289.55 ± 39.07	412.79 ± 63.97	335.77 ± 77.96	27.36 ± 6.70	25.56 ± 4.43	26.46 ± 5.60	33.81 ± 7.82	33.00 ± 9.26	33.40 ± 8.35

In what concerns total lipids, the highest value was observed in April 2009 for males (157.88±24.23 µg mg^{-1} DW) and May 2009 for females (125.06±38.94 µg mg^{-1} DW), whereas the lowest values were observed in May 2011 for males (25.27±7.55 µg mg^{-1} DW) and in July 2010 for females (21.55±8.93 µg mg^{-1} DW) (Table 2.1). As observed previously for glycogen, total lipids generally decreased along the spawning season and decreased from 2009 until 2011 (Figure 2.2). Significant differences were observed between the three years, both for males and females (K-W: Males H=84.45, p<0.001; Females H=100.66, p<0.001). For males, statistical differences were found between 2009 and 2011 (Dunn test: q=9.02, p<0.05) and between 2010 and 2011 (Dunn test: q=6.98, p<0.05). In the case of females, significant differences were observed between three years (Dunn test: $_{\mathrm{2009vs2011}}q$=9.97, p<0.05; $_{\mathrm{2009vs2010}}q$=5.10, p<0.05; $_{\mathrm{2010vs2011}}q$=5.36, p<0.05). Similar differences among the three years were observed when data from males and females were grouped (Dunn test: $_{\mathrm{2009vs2011}}q$=13.49, p<0.05; $_{\mathrm{2009vs2010}}q$=5.12, p<0.05; $_{\mathrm{2010vs2011}}q$=8.73, p<0.05).

From the three year time-series analysis, same correlations were observed for males and females separately; henceforward the correlations are shown regardless of sex. During the spawning period, the sea surface temperature (SST) was correlated negatively with all the parameters studied: chlorophyll-a (r=-0.632, p=0.002), CI (r=-0.815, p<0.001), glycogen (r=-0.812, p<0.001) and total lipids (r=-0.802, p<0.001)

(Table 2.2). Proteins were the exception with no correlation observed. This biochemical component does not correlated with any other parameter analyzed during the studied period. Chlorophyll-a correlated positively with CI (r=0.605, p=0.002), glycogen (r=0.582, p=0.005) and total lipids (r=0.605, p=0.002). Condition Index also correlated positively with glycogen (r=0.703, p<0.001) and total lipids (r=0.797, p<0.001). Finally, glycogen and total lipids also correlated positively (r=0.831, p<0.001) during the spawning period (Table 2.2).

Table 2.2 Results of Pearson correlation between studied parameters. SST- Sea Surface Temperature; r - Pearson correlation coefficient; P - P value; n.c. - no correlation was found.



	Chlorophyll-a	Condition index (CI)	Proteins (P)	Glycogen (G)	Total lipids (TL)
SST	**r=-0.632, p= 0.002**	**r=-0.815, p< 0.001**	n.c.	**r=-0.812, p< 0.001**	**r=-0.802, p< 0.001**
Chl-a		**r=0.605, p= 0.002**	n.c.	**r=0.582, p= 0.005**	**r=0.608, p=0.003**
CI			n.c.	**r= 0.703, p< 0.001**	**r=0.797, p< 0.001**
P				n.c.	n.c.
G					**r= 0.831, p< 0.001**

Discussion

In the present study, the condition index was used to evaluate the broodstock condition of the wedge shell during the spawning season. The CI is likely to reflect variations in the environmental conditions as well as the occurrence of odd events during this critical phase of the wedge shell life cycle.

The CI values obtained during 2009 and 2010 spawning season are in accordance to the ones observed by Boussoufa *et al* (2011) and by Tlili *et al* (2011) in the Gulf of Tunis. In the same period glycogen values are similar to ones obtained by Tlili *et al* (2011) and total lipids values observed are similar to the ones observed by Boussoufa *et al* (2011) and lower to the ones registered by Tlili *et al* (2011) both in the Gulf of Tunis. The CI, glycogen and total lipids values registered during 2011 spawning season were much lower than the ones observed by the above cited authors.

The SST values followed the tendency observed for this region in previous years. The temperature starts to rise during Spring until reach its peak in August during the summer, decreasing afterwards to minimum levels between December and February during Winter. This pattern is normal for the studied area and similar to that observed in previous studies (Gaspar *et al.* 2004; Joaquim *et al.* 2008b).

The observed negative correlation between SST and the chlorophyll-a was expected since during winter, when SST is lower, an upwelling event occurs in southern Portugal (Soares 2004). This event is responsible for the peak in chlorophyll-a concentration in later winter and early spring (Falcão *et al.* 2007). The concentration of chlorophyll-a decreases afterwards as SST rises.

A negative correlation was also observed between SST and CI, glycogen and total lipids. Correlations between SST and CI and biochemical components present in tissues occur in many bivalve species since SST is associated to the reproductive development, controlling the onset of gametogenesis in many bivalve species and it is also the key factor triggering the spawning as reported by several authors (Bayne 1976; Ansell & Lagardère 1980; Dridi, Romdhane, & Elcafsi 2007; Liu *et al.* 2008; Joaquim *et al.* 2008b; Cerón-Ortiz *et al.* 2009; Fearman & Moltschaniwskyj 2010; Ezgeta-Balić *et al.* 2011; Matias *et al.* 2013). The above correlation was observed not only in *D. trunculus* for other areas (Boussoufa *et al.* 2011; Martínez-Pita *et al.* 2012), but also in other bivalve species that occur in the Portuguese coast, such as: *Ensis siliqua*, *Venus striatula*/*Chamelea gallina* (Gaspar & Monteiro 1998), *Spisula solida* (Gaspar & Monteiro 1999) and *Callista chione* (Moura, Gaspar, & Monteiro 2008).

In our study, the negative correlation found between SST and CI, suggests that glycogen is being used and transformed in total lipids for gamete production. Indeed, SST is also correlated negatively with glycogen and total lipids, as expected, since CI is a reflection of the biochemical variation of these two components during spawning (Beninger & Lucas 1984; Mouneyrac *et al.* 2008). Interestingly, CI correlates positively with both glycogen and total lipids during the studied period, which seems to indicate that while transforming glycogen into total lipids during gamete production, the species continues to accumulate and store energy (Vite-García & Saucedo 2008; Gómez-Robles & Saucedo 2009) which explains the positive correlation between glycogen and total lipids. This result indicates that *D. trunculus* in southern Algarve has an opportunistic reproductive strategy allowing investment of any surplus energy into gamete production. Surplus energy is probably ensured by a high pumping rate to succeed in periods of low food concentration (Pouvreau *et al.* 2000).

The above cited hypothesis contrasts to the conservative strategy used by species such as *Argopecten irradians concentricus* in Florida (Barber & Blake 1981) and *Ruditapes decussatus* in Galicia (Ojea *et al.* 2004) that stores energy previously the gametogenesis, when plenty food is available; until the relative short spawning period occur during which the energy reserves become depleted (Barber & Blake 1985).

The opportunistic reproductive strategy adopted by the wedge shell was also observed in other species such as *Ostrea edulis* (Bayne 1976), *Mytella charruana* and *M. guyanensis* (Cardenas & Aranda 2000). This strategy in wedge shell is well adapted to its long spawning season, allowing the species not to concentrate all the reproductive effort in a short temporal period, allocating surplus energy to reproduction under environmental favorable conditions.

Our results revealed that during 2011 something must have occurred, since the CI values of this particular year were statistically different from those observed in 2009 and 2010. The values of proteins and in particular the values of glycogen and total lipids were also lower than the ones observed in the previous two years. The SST in 2011 was statistically different from 2010 and 2009, with a faster increase of the SST in March/April and with mean values reaching values clearly above 20°C during 6 months whereas in 2009 and 2010 values of SST above 20º were only observed in four and three months respectively. Although no differences were found, it was observed that chlorophyll-a peaked two to three months earlier than usual during 2011. We believe that the unusual high SST values during this season were responsible for the abnormal occurrence of phycotoxins registered in the spawning season of 2011. Usually in this area, phycotoxins occur every year with the rise of SST and after a week are no longer detected in the water, reappearing again once or twice during the summer. Although the SST pattern in 2009 and 2010 were similar, in 2009 phycotoxins were detected in one day only (April 15th), while in 2010 phycotoxins were recorded during a longer period (June 25th to July 23th). In 2011, phycotoxins appeared when the SST rose in March and remained above legally established limits from March 25th to April 28th and from July 6 to August 11th, leading to prolonged fishing bans due to public health hazard.

Although there are no studies published about the direct effect of phycotoxins on biochemical composition of marine bivalves and reproductive success, some effects of phycotoxins on bivalves have been reported by several authors. Haberkorn *et al* (2010) has demonstrated that in *Crassostrea gigas*, the PSP (paralytic shellfish poisoning) phycotoxin had an effect on the spermatozoa quality and motility. Medhioub *et al* (2012) also reported an adverse effect of PSP phycotoxins in *C. gigas* digestive gland tubules, affecting the digestive and absorption capacity of the specimens exposed to this phycotoxin. In addition, the recovering process of digestive gland tubules after the exposure revealed to be energy demanding. PSP phycotoxin may also affects the clearance rate of some bivalve species such as *Paphies donacina*, *Dosinia anus* and *Pecten novaezelandiae* as observed by Contreras, Marsden, & Munro (2012), while in other species such as *Spisula solidissima* the clearance rate is not affected at all (Shumway *et al.* 1994). Bricelj & Shumway (1998) reported that phycotoxins may affect the burrowing capability and the feeding response of bivalves, an effect that probably is reflected in the energy intake during the phycotoxin blooms as observed in *Ruditapes philippinarum* and *Perna viridis* where the growth rate and the scope for growth were affected when phycotoxins were present (Li, Wang, & Hsieh 2002). Thus, if the presence of phycotoxins affects the feeding response and consequently the energy intake by bivalves, then the unusual phycotoxin presence during the spawning season in 2011 may explain the difference in the CI observed during 2011 as well as the lower values of proteins, glycogen and total lipids registered. In fact, although no correlation has been found between proteins and the other parameters during the spawning season, protein values differed among the three years, with lower values occurring in 2011. It seems that during the beginning of the 2011 spawning season, *D. trunculus* was unable to recover glycogen reserves due the phycotoxins presence in the water and therefore used proteins as a last energy resource to suppress energetic needs (Gopalakrishnan & Vijayavel 2009) although for a short period, since a recover of proteins was observed later in the season.

In the first two years of this study, the SST as well as the chlorophyll-a values were similar. However, glycogen and total lipids differed among years, with a slightly decrease observed from 2009 to 2010 spawning season. In this same period, an increase in phycotoxins presence from one day in 2009 to almost one month in 2010 was registered. This phycotoxin increase may be related to the decrease observed in glycogen and total lipids in 2010 spawning season.

The chlorophyll-a peak observed in January 2011 may also have affected the CI values in the beginning of the spawning and may have compromised the energy intake prior to the beginning of the spawning season, although we have no data related to the CI outside this season.

It was observed by the authors *in situ* during sampling, that when phycotoxins were present in the water, the mantle and foot of the wedge shell wrinkled indicating that phycotoxins affects somehow this species. Moreover, during laboratory experiments, when spawning was induced none of the individuals collected during 2011 responded to thermal shock, which contrasted with the previous years, where induced spawning was successfully achieved (unpublished data).

The spawning season is a critical period of the life cycle of *D. trunculus* and, as our data shows, unusual events such as the high SST observed and the phycotoxins presence seems to be reflected in both CI and the biochemical composition of the broodstock population. Additional information on larva quality may help understanding the implications of odd events on the non exploitable phase of the resource (planktonic larval stage and settlement) and posterior recruitment success. This knowledge would allow a precautionary implementation of protective measures, if needed, *i.e.*, a low number of larvae is detected, settlement fail or/and low recruitment occur.

In conclusion, like other bivalve mollusks, *D. trunculus* is highly dependent of temperature which is responsible for triggering the spawning event. Our results indicate that this species has adopted an opportunistic reproductive strategy accumulating energy in the form of glycogen while being converted into total lipids used in gamete production. It seems to be sensitive to the presence of phycotoxin in the water, although more studies on this subject must be undertaken. Our data showed that the occurrence of odd events during the spawning season is reflected in the CI, making this index a valuable parameter for the management of the fishery.

Acknowledgments

The authors would like to thank Dr. Alexandra Leitão, the coordinator of IPMA Molluscan Aquaculture Experimental Station, to Eng. Margarete Matias for her help in the water maintenance system and aid during biochemical analysis and to Dr. Jorge Ramos for his help editing the English language of the manuscript. This work was supported by the grant SFRH/BD/40430/2007 financed by Fundação para a Ciência e Tecnologia.

Is the biochemistry of *Donax trunculus* (Linnaeus 1758) egg and larvae suitable for managing purposes?

Pereira, A.M., Joaquim, S., Chicharo, L., Gaspar, M.B. Is the biochemistry of *Donax trunculus* (Linnaeus 1758) egg and larvae suitable for managing purposes? (Submitted to Scientia Marina).

Abstract

Knowledge on the energy reserves of the wedge shell *Donax trunculus* egg and larvae is important to help predicting recruitment, allowing in case of recruitment failure to timely adjust the fishing effort. We aimed at evaluating wedge shell egg and larvae quality, in order to improve development and implementation of future managing measures. Egg and D- larvae total lipids, proteins and carbohydrates were determined and energy was calculated using the energy conversions factors used for lipid, carbohydrate and protein (35.24, 17.16 and 18.00 kJ g^{-1} respectively). A total lipid + carbohydrates to protein ratio was determined for eggs. Total lipids and proteins were the main components along the study period with carbohydrates representing only a small percentage. Total lipids play an important role in the hatching success of the species as well as in the amount of energy available to ensure the successful development into D-larvae. Despite the differences in total lipids registered, neutral lipid showed no differences indicating that a threshold may exist. The total lipid plus carbohydrate to protein results may be used to predict hatching success in *D. trunculus* and we set 0.82±0.09 as the value above which recruitment occurs successfully. This information has not been used in bivalve stock management, being this a first step into an earlier detection of recruitment failure.

Introduction

Studies on bivalve larvae quality, address almost exclusively, hatchery conditioning techniques based on diet effect on growth and survival rate of larvae and in most cases with a restocking perspective in mind (e.g. Powell *et al.* 2002; Hendriks, van Duren, & Herman 2003; Leverone *et al.* 2006; Matias *et al.* 2010; Pettersen *et al.* 2010; Przeslawski, Perino, & Padilla 2012; Gonzalez Araya *et al.* 2012). Other few cases focus on particular topics such as the differences in larval quality from different latitude broodstocks populations (Lagos 2012), the effect of antibiotics on the dissoconch development and posterior metamorphosis success (Pernet, Bricelj, & Cartier 2006), the effect of harmful algae on lipid storage (Przeslawski *et al.* 2008), the food limitation in larvae development (Bos *et al.* 2006) or egg and larvae quality in growth and survival success (Videla *et al.* 1998; Burke, Bataller, & Miron 2008). No studies have addressed larvae quality in wild populations with a managing perspective in mind.

The wedge shell *Donax trunculus* is a widely distributed species along the Algarve coast and although captured all year round, it is during the summer season that the demand for this species greatly increases. This demand is mainly due to tourism and occurs during part of the spawning period of *D. trunculus* that begins with the rise of temperature in late March early April and ends in late July (Gaspar, Ferreira, & Monteiro 1999). After spawning the eggs released into the water dictate the beginning of the meroplancktonic stage of the wedge shell that lasts until settlement takes place (Savina & Ménesguen 2008).

During the first 24 hours, the wedge shell eggs, as in other bivalve species, must rely solely on the endogenous energy reserves provided by the progenitors during the gametogenesis (Holland & Hannant 1973; Holland & Spencer 1973; Gallager & Mann 1986; Whyte, Bourne, & Ginther 1990; Massapina *et al.* 1999; Matias *et al.* 2013) This is a stressful and crucial period with tissue reorganization (Pernet, Bricelj, & Cartier 2006) that, if successful, culminates in the hatching of a D-shape larvae ready to grow. To accomplish successfully this transformation, embryos need to use their primary nutritional reserves that consist mainly in eggs lipids particularly on the neutral lipid fraction (Holland & Hannant 1974; Ferreiro, Pérez-Camacho, & Labarta 1990). The neutral lipids are also fundamental for larval growth until they become competent pediveligers (e.g. Holland & Spencer 1973; Ferreiro, Pérez-Camacho, & Labarta 1990; Videla *et al.* 1998; Przeslawski *et al.* 2008; Gonzalez Araya *et al.* 2012).

Along with total lipids, proteins are also an abundant component in tissues during the embryonic development, essential to ensure larva body growth and maintenance beyond the embryonic phase until metamorphosis occurs (Gopalakrishnan & Vijayavel 2009; Matias *et al.* 2010). According to da Costa *et al* (2011) protein may also be used as an energy source, and has been proven by Uriarte *et al* (2004) that in *Crassostrea gigas* and *Argopecten purpuratus* proteins are likely to improve larval survival as settlement approaches. Carbohydrates are other component available in the eggs and larvae of bivalves, and although it has been suggested that carbohydrates do not play an important role during larval development (Holland & Spencer 1973; Gallager, Mann, & Sasaki 1986), according to Haws, DiMichele, & Hand (1993) they may be used to optimize the utilization of other reserves available in tissues.

In the majority of bivalves the physiological condition of the embryos and larvae, as well as their capacity to grow and survive under various environmental conditions during the species life cycle, has been related successfully to larvae settlement and recruitment (Phillips 2002; Racotta 2003; Ojea *et al.* 2008; Marshall, McKinley, & Pearce 2010). This success depends on the acquisition by the eggs, and later on by the larvae, of energy reserves that suffice the metamorphosis energetic demands (Pernet, Bricelj, & Cartier 2006). The knowledge on the egg and larvae quality of the *D. trunculus* is therefore of utmost importance since it may be used to predict recruitment. From the management point of view this knowledge is fundamental since in case of recruitment failure it allows the timely adjustment of fishing effort contributing, this way, to the sustainability of the fishery.

This work aims at evaluating egg and larva quality on the wedge shell *D. trunculus*, as well as the implications of seawater temperature in the energetic reserves in eggs and D-larvae quality. We believe this will provide new insights during such a critical period of the life cycle of the wedge shell (post spawning and hatching period), improving the development and implementation of future managing measures.

Materials and methods

Adult collection and spawning

A total of 1100 individuals of *D. trunculus* were collected in years 2009, 2010 and 2011 between Mars and July at Lota beach (7°30′32.21″W, 37°9′56.33″N) in the Algarve coast, western Portugal (Figure 1). The captures were made at low tide using a bivalve hand dredge similar to the ones used by local harvesters. For this location, data on seawater surface temperature (SST) was obtained from the Hydrographical Institute (IH). After captured, the specimens were taken to the laboratory and placed in three previously prepared spawning tanks. Filtered and aerated seawater was used in these tanks and food (*Isochrysis galbana* and *Chaetoceros calcitrans*) was provided *ad libitum*. Adults were placed in the tanks and left overnight to spawn. After spawning, eggs were collected at the exit end of each tank using a 40 µm sieve. Whenever spawning did not occur naturally, a thermal shock of water system temperature plus 5°C was applied for one hour, after which, the adults were retrieved to the original tanks. This procedure was repeated two to three times.

Egg and larva collection

After spawning, eggs were removed from the sieve, counted and 150,000 were rinsed with isosmotic ammonium formate (3%) to remove salt, and frozen at -20°C. The remaining eggs were placed in an aerated tank with food available during 24 hours until they hatch in D-shape larvae. At this stage samples of 50,000 D-shape larvae were collected and preserved, following the same procedures used with eggs. Hatching rate was determined by counting the number of 1-day hatched larvae relative to the total number of incubated eggs.

Sample fractioning and biochemical content

A micro-analytical fractionated extraction scheme developed by Holland & Gabbott (1971) and Holland & Hannant (1973) was followed for the determination of the contents of biochemical components. Lyophilized samples were homogenized in

500µl distilled water and sonicated in an ice water bath for three intervals of 10 s at 20 W each to obtain a thoroughly homogenized sample. Two samples of 200 µl each were taken from the initial homogenate, of which one was used to determine total lipids and neutral lipids content whereas the other was used to determine proteins, total carbohydrates and free reducing sugars. Total lipid content was extracted by the method of Bligh & Dyer (1959) and taken up in 500 µl chloroform. Total lipids were determined by the methods of Marsh & Weinstein (1966) using tripalmitin as a standard, and the absorbance was determined at 375 nm. Neutral lipids were determined in the same way as total lipids; 200 µl samples of neutral lipids in chloroform were dried for 20 min at 100°C and used for determinations. Phospholipids were determined as the difference between total and neutral lipids. Proteins were precipitated by cold 5% trichloroacetic acid (TCA), and the precipitate was washed in warm 1.0 N NaOH. Protein concentration was assayed by the method of Lowry *et al* (1951), modified by Bensadoun & Weinstein (1976) and Hess, Lees, & Derr (1978), at 750 nm using serum albumin as a standard. Hydrolyzed and unhydrolyzed samples of TCA supernatant were used for the determination of total carbohydrates and free reducing sugars by a modification of the method of Folin and Malmros (1929). The components were quantified with a ferricyanate reduction reaction at 420 nm using glucose as a standard. Duplicate determinations were performed in all cases and values expressed as organic weight percentages. Energy conversion factors used for lipid, carbohydrate and protein were 35.24, 17.16 and 18.00 kJ g^{-1}, respectively (Beukema & De Bruin 1979). The Total lipid + Carbohydrates to Protein ratio was determined for eggs (Hofmann *et al.* 2004).

Statistical analysis

Statistical analyses were computed using Sigma-Stat for Windows. Differences between years in each component were assessed by analysis of variance (ANOVA) and when assumptions of normality and homoscedasticity were not fulfilled the nonparametric Kruskal–Wallis test was used. When results suggested significant differences, pairwise multiple comparisons were made using Tukey or Dunn’s tests, respectively (Zar 1984). Prior to any analysis data was transformed to arcsine square root values. Results were regarded as significant for $p<0.05$. Correlations between the components were assessed by the Spearman Rank Order Correlation.

Results

Seawater Surface Temperature (SST)

The evolution of monthly SST along the three years of study is shown in Figure 2.3. Lowest SST value was registered in February 2009 with 14.81°C and the highest SST value during August 2010 with 24.14 °C. Although the trend in year 2011 was similar to the observed previously in 2009 and 2010, temperature rose earlier and stayed above 20°C for a period of six months. These differences were detected by ANOVA, that revealed significant differences between SST observed in 2011 and both 2009 (Tukey test q=5.51, p<0.05) and 2010 (Tukey test q=4.07, p<0.05).

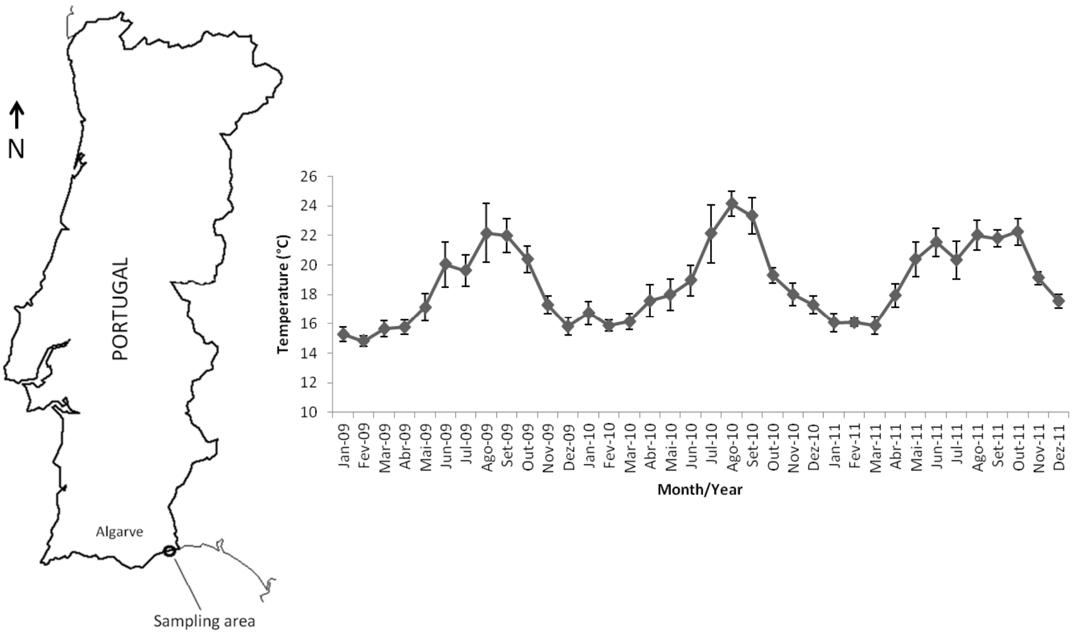


Figure 2.3. Sampling location of *Donax trunculus* progenitors and monthly average values of sea surface temperature (SST) at sampling site during the years 2009, 2010 and 2011.

Biochemical components

The percentage of proteins, carbohydrates and total lipids in the organic weight and the composition of total lipids and carbohydrates classes in percentage are shown in tables 2.3 and 2.4 for eggs and D-larvae, respectively.

Eggs

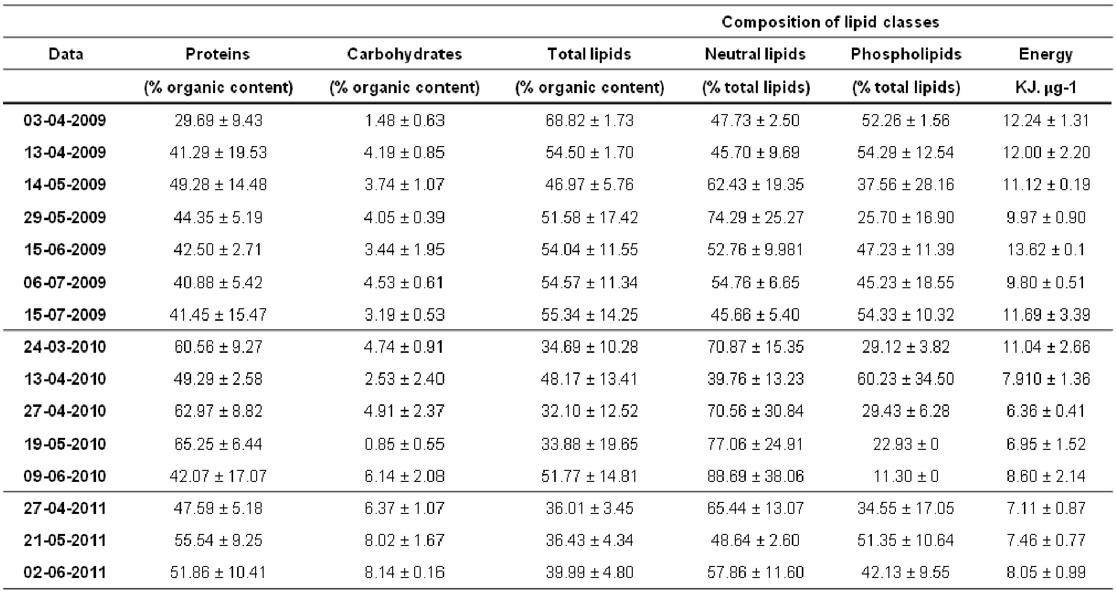
Total lipids were the predominant constituent in the eggs in year 2009 with a percentage between 46.97±5.76% and 68.82±1.73% whereas in 2010 and 2011 total lipids were the second predominant constituent ranging between 32.10±15.52% and 51.77±14.81% and between 36.01±3.45% and 39.99±4.80% of organic weight, respectively (Table 2.3). The percentage of total lipids in the total organic weight decreased in 2010 and 2011 compared to 2009 values. Indeed, total lipids content in 2009 were statistically different from both 2010 (Dunn´s test q=3.27 p<0.05) and 2011 (Dunn´s test q=3.94 p<0.05). Total lipids were represented by neutral lipids and phospholipids. Both neutral lipids and phospholipids presented similar percentages, oscillating along each year with some samples with higher percentage of neutral lipids and other with phospholipids more represented. However, in 2010 this oscillation was not observed. During this year, the percentage of neutral lipids was always higher that 70% with the exception of the sample obtained in April where the proportion of phospholipids was higher than neutral lipids (Table 2.3).

Proteins were the second predominant constituent in eggs in the year 2009 with only the first sample (in April 3rd 2009) registering a percentage bellow 40% (29.69±9.43%). In 2010 and 2011 proteins were generally more represented than total lipids, ranging between 42.07±17.07% and 60.56±9.27% in 2010 and between 47.59±5.18% and 55.54±9.25% in 2011 (Table 2.3). Although the mean percentage of proteins in the total organic weight registered in 2009 were lower than in the following years, no statistical differences were detected among years (ANOVA: H=4.9 *d.f*=2 P>0.05).

Carbohydrates were the less represented fraction in the total organic weight in all three years of study ranging in 2009 from 1.48±0.63% to 4.53±0.61%, in 2010 from 0.85±0.55% to 6.14±2.08% and from 6.37±1.07% to 8.14±0.16% in 2011 (Table 2.3).

Significant differences in the percentage of carbohydrates in the total organic weight were observed between 2011 and both 2009 (Dunn´s Test, q=4.7 p<0.05) and 2010 (Dunn´s Test, q=4.6 p<0.05).

Table 2.3. Biochemical components expressed as organic weight percentage, composition of lipid class and energetic value in *Donax trunculus* eggs during the spawning season in 2009, 2010 and 2011.



				Composition of lipid classes		
Data	**Proteins**	**Carbohydrates**	**Total lipids**	**Neutral lipids**	**Phospholipids**	**Energy**
	(% organic content)	**(% organic content)**	**(% organic content)**	**(% total lipids)**	**(% total lipids)**	**KJ. µg-1**
03-04-2009	29.69 ± 9.43	1.48 ± 0.63	68.82 ± 1.73	47.73 ± 2.50	52.26 ± 1.56	12.24 ± 1.31
13-04-2009	41.29 ± 19.53	4.19 ± 0.85	54.50 ± 1.70	45.70 ± 9.69	54.29 ± 12.54	12.00 ± 2.20
14-05-2009	49.28 ± 14.48	3.74 ± 1.07	46.97 ± 5.76	62.43 ± 19.35	37.56 ± 28.16	11.12 ± 0.19
29-05-2009	44.35 ± 5.19	4.05 ± 0.39	51.58 ± 17.42	74.29 ± 25.27	25.70 ± 16.90	9.97 ± 0.90
15-06-2009	42.50 ± 2.71	3.44 ± 1.95	54.04 ± 11.55	52.76 ± 9.981	47.23 ± 11.39	13.62 ± 0.1
06-07-2009	40.88 ± 5.42	4.53 ± 0.61	54.57 ± 11.34	54.76 ± 6.65	45.23 ± 18.55	9.80 ± 0.51
15-07-2009	41.45 ± 15.47	3.19 ± 0.53	55.34 ± 14.25	45.66 ± 5.40	54.33 ± 10.32	11.69 ± 3.39
24-03-2010	60.56 ± 9.27	4.74 ± 0.91	34.69 ± 10.28	70.87 ± 15.35	29.12 ± 3.82	11.04 ± 2.66
13-04-2010	49.29 ± 2.58	2.53 ± 2.40	48.17 ± 13.41	39.76 ± 13.23	60.23 ± 34.50	7.910 ± 1.36
27-04-2010	62.97 ± 8.82	4.91 ± 2.37	32.10 ± 12.52	70.56 ± 30.84	29.43 ± 6.28	6.36 ± 0.41
19-05-2010	65.25 ± 6.44	0.85 ± 0.55	33.88 ± 19.65	77.06 ± 24.91	22.93 ± 0	6.95 ± 1.52
09-06-2010	42.07 ± 17.07	6.14 ± 2.08	51.77 ± 14.81	88.69 ± 38.06	11.30 ± 0	8.60 ± 2.14
27-04-2011	47.59 ± 5.18	6.37 ± 1.07	36.01 ± 3.45	65.44 ± 13.07	34.55 ± 17.05	7.11 ± 0.87
21-05-2011	55.54 ± 9.25	8.02 ± 1.67	36.43 ± 4.34	48.64 ± 2.60	51.35 ± 10.64	7.46 ± 0.77
02-06-2011	51.86 ± 10.41	8.14 ± 0.16	39.99 ± 4.80	57.86 ± 11.60	42.13 ± 9.55	8.05 ± 0.99

The highest energy value obtained was 13.62±0.1 KJ. μg^{-1} in 15th June 2009 and the lowest registered was 6.36±0.4 KJ. μg^{-1} in 27th of April 2010. A significant decrease in the energy values was observed between 2009 and both 2010 and 2011 (Dunn´s test q=3.53, p<0.05; q=3.85, p<0.05, respectively) (Table 2.3).

The Total lipid + Carbohydrates to Protein ratio data followed the tendency of the HR decreasing from 2009 to 2011 spawning season (Figure 2.4). This ratio in 2009 differed significantly from 2010 and 2011 ratios (M-W t=19, p=0.03 and M-W t=6, p=0.017 respectively).

D-Larvae

The percentage of total lipid in organic weight of larvae was similar along the three years of study with values varying from 50.00±4.00% to 83.87±35.43% in 2009, from 34.90±33.71% to 59.76±14.42% in 2010 and from 46.63±9.90% to 53.36±2.94% in

2011 (Table 2.4). Despite the two highest percentages registered in 2009 (73.74±28.81% - 30th May and 83.87±35.43% 16th July) no statistical differences were observed among years in the amount of total lipids. However, in terms of the percentage of total lipids in the total organic weight it was observed a significant decrease between 2009 and both 2010 and 2011 (Dunn´s test q=3.27 $p<0.05$; q=3.94 $p<0.05$, respectively). The total lipid class was also represented by both neutral lipids and phospholipids. In 2009 and 2011, both neutral lipids and phospholipids presented similar percentages, oscillating along the spawning season with some samples showing a higher percentage of neutral lipids and others with phospholipids more represented. In 2010 this pattern was not observed. With the exception of the sample collected late April, the percentage of neutral lipids was always higher that 70%.

In larvae, proteins were the second predominant constituent in 2009 with a percentage ranging between 13.43±1.36% and 42.55±13.88%. In 2010 and 2011 protein percentage was similar to the total lipid ones. In 2010 it ranged from 36.83±3.46% to 53.81±6.34% of total organic weight and in 2011 it was fairly constant ranging from 44.65±1.27% to 49.63±9.42% of total organic weight (Table 2.4). The percentages of proteins observed in 2009 differed statistically from the values of 2010 (Dunn´s test q=6.62, $p<0.05$) and 2011 (Dunn´s test q=5.0, $p<0.05$).

Carbohydrates were the less represented fraction of organic weight in all three years of study ranging in 2009 from 2.68±0.83% to 9.45±2.43%, in 2010 from 3.20±1.15% to 11.28±5.65% and in 2011 from 1.97±0.1% to 3.72±0.77% (Table 2.4). The only statistical difference observed among years was between 2010 and 2011 (Dunn´s test q=3.00, $p<0.05$).

Energy in the larvae was lower than the observed in the eggs with the highest value observed in 25th March 2010 (5.74±0.46 KJ. μg^{-1}) and the lowest observed in 7th July 2009 (1.15±0.24 KJ. μg^{-1}) (Table 2.4). Values from 2009 were slightly lower than the observed in the other years, however the only statistically significant difference found was between 2009 and 2010 (Tukey test q=5.6 p=0.002).

Table 2.4. Biochemical components expressed as organic weight percentage, composition of lipid classes and energetic value in *Donax trunculus* D-larvae during the spawning season in 2009, 2010 and 2011.

				Composition of lipid classes		
Data	**Proteins**	**Carbohydrates**	**Total lipids**	**Neutral lipids**	**Phospholipids**	**Energy**
	(% organic content)	**(% organic content)**	**(% organic content)**	**(% total lipids)**	**(% total lipids)**	**KJ. µg-1**
04-04-2009	33.80 ± 15.28	7.98 ± 3.32	58.21 ± 6.18	54.58 ± 13.37	45.41 ± 22.46	2.77 ± 0.54
14-04-2009	33.29 ± 0.72	5.60 ± 4.47	61.09 ± 5.77	64.49 ± 5.12	35.50 ± 14.56	3.17 ± 0.10
15-05-2009	42.55 ± 13.88	7.44 ± 1.28	50.00 ± 4.00	48.73 ± 30.47	51.26 ± 38.44	2.32 ± 0.19
30-05-2009	19.40 ± 8.25	6.85 ± 5.36	73.74 ± 28.81	69.10 ± 20.79	30.89 ± 24.19	3.25 ± 1.24
16-06-2009	20.65 ± 5.04	9.45 ± 2.43	69.89 ± 20.07	53.37 ± 3.55	46.62 ± 25.16	3.41 ± 0.73
07-07-2009	35.49 ± 11.27	8.25 ± 1.64	56.25 ± 8.75	32.62 ± 3.22	67.37 ± 29.59	1.15 ± 0.24
16-07-2009	13.43 ± 1.36	2.68 ± 0.83	83.87 ± 35.43	58.77 ± 20.79	41.22 ± 21.54	3.05 ± 0.10
25-03-2010	53.74 ± 3.65	6.67 ± 0.78	39.57 ± 13.35	40.54 ± 14.15	59.45 ± 12.49	5.74 ± 0.46
14-04-2010	36.83 ± 3.46	3.40 ± 0.23	59.76 ± 14.42	58.33 ± 30.88	41.66 ± 21.17	5.47 ± 0.69
28-04-2010	53.81 ± 6.34	11.28 ± 5.65	34.90 ± 33.71	67.05 ± 6.41	32.94 ± 21.47	2.56 ± 0.82
20-05-2010	46.62 ± 5.38	3.29 ± 0.22	50.08 ± 3.44	54.29 ± 12.54	45.70 ± 15.16	3.26 ± 0.12
10-06-2010	43.19 ± 14.41	3.20 ± 1.15	53.60 ± 5.26	59.31 ± 3.19	40.68 ± 8.35	4.09 ± 0.05
28-04-2011	44.65 ± 1.27	1.97 ± 0.1	53.36 ± 2.94	30.63 ± 4.47	69.36 ± 9.15	3.08 ± 0.10
22-05-2011	49.63 ± 9.42	3.72 ± 0.77	46.63 ± 9.90	37.24 ± 3.98	62.75 ± 17.90	4.03 ± 0.40
03-06-2011	45.45 ± 5.82	2.19 ± 0.78	52.34 ± 4.93	26.30 ± 2.40	73.69 ± 11.23	4.03 ± 0.21

Hatching rate (HR)

In the three years, the highest hatching rate observed occurred in the 13th April 2009 sample, with an 84.9% of hatching success into D-larvae, whilst the lowest was observed in the sample from 24th April 2010 with only a success of 25.9%. Despite the slight decrease observed in HR values from the year 2009 to 2011 (Figure 2.4), no statistical differences were observed among the three years.

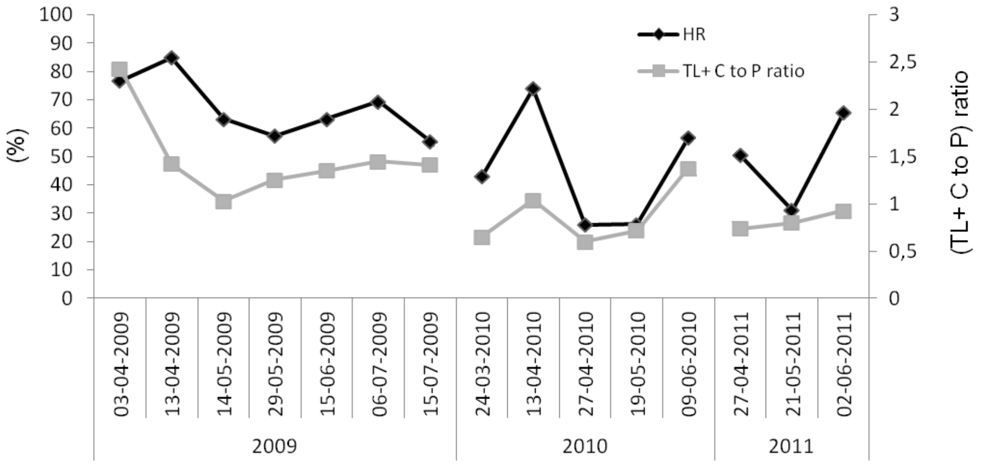
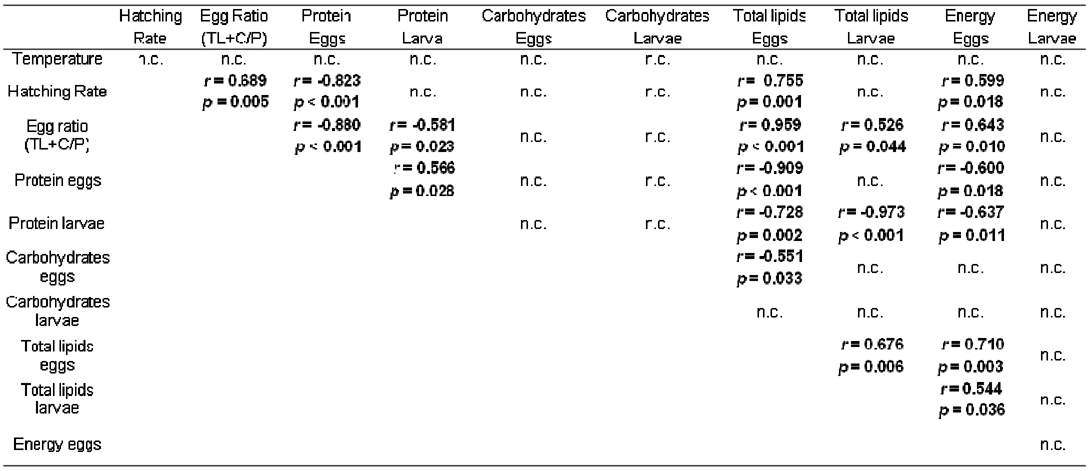


Figure 2.4. *Donax trunculus* eggs hatching rate (HR), and Total lipid plus Carbohydrates to Protein ratio (TL+C to P) during 2009, 2010 and 2011 spawning seasons.

Correlations

The results of the Spearman Rank Correlation test are shown in Table 2.5. From its analysis it can be concluded that SST did not correlate with any of the parameters analyzed. The hatching rate correlated negatively with eggs proteins and positively with eggs total lipids and energy. The TL+C to P ratio were found to correlate negatively with proteins and positively with total lipids both in eggs and larvae. A positive correlation was also found between this ratio and the energetic value in the eggs. Eggs proteins correlated positively with larvae proteins and negatively with both eggs total lipids and energy. D-larvae proteins also correlated negatively with eggs energy and with total lipids in eggs and in larvae. While eggs carbohydrates correlated negatively with eggs total lipids no correlation was found between D-larvae carbohydrates and any other component. Eggs total lipids correlated positively with its energy and with total lipids in larvae. Finally, total lipids in larvae correlated positively with eggs energy.

Table 2.5. Results of Spearman correlation between studied parameters. SST – Seawater Surface Temperature; (TL+C/P) – Total Lipids + Carbohydrates to Protein ratio; *r* – Spearman correlation coefficient; *p* – *p* value; n.c. – no correlation was found.



	Hatching Rate	Egg Ratio (TL+C/P)	Protein Eggs	Protein Larva	Carbohydrates Eggs	Carbohydrates Larvae	Total lipids Eggs	Total lipids Larvae	Energy Eggs	Energy Larvae
Temperature	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
Hatching Rate		**$r = 0.689$** **$p = 0.005$**	**$r = -0.823$** **$p < 0.001$**	n.c.	n.c.	n.c.	**$r = 0.755$** **$p = 0.001$**	n.c.	**$r = 0.599$** **$p = 0.018$**	n.c.
Egg ratio (TL+C/P)			**$r = -0.880$** **$p < 0.001$**	**$r = -0.581$** **$p = 0.023$**	n.c.	n.c.	**$r = 0.959$** **$p < 0.001$**	**$r = 0.526$** **$p = 0.044$**	**$r = 0.643$** **$p = 0.010$**	n.c.
Protein eggs				**$r = 0.566$** **$p = 0.028$**	n.c.	n.c.	**$r = -0.909$** **$p < 0.001$**	n.c.	**$r = -0.600$** **$p = 0.018$**	n.c.
Protein larvae					n.c.	n.c.	**$r = -0.728$** **$p = 0.002$**	**$r = -0.973$** **$p < 0.001$**	**$r = -0.637$** **$p = 0.011$**	n.c.
Carbohydrates eggs							**$r = -0.551$** **$p = 0.033$**	n.c.	n.c.	n.c.
Carbohydrates larvae							n.c.	n.c.	n.c.	n.c.
Total lipids eggs								**$r = 0.676$** **$p = 0.006$**	**$r = 0.710$** **$p = 0.003$**	n.c.
Total lipids larvae									**$r = 0.544$** **$p = 0.036$**	n.c.
Energy eggs										n.c.

Discussion

Marine bivalves have their life cycle strongly related to environmental factors such as the sea surface temperature (SST) or the type and availability of food (Chícharo & Chícharo 2001; Burke, Bataller, & Miron 2008; Marshall, McKinley, & Pearce 2010). These create a broodstock-recruitment relation that is reflected in the annual variability observed in shellfish recruitment (Hoffmann, Pfaff, & Branch 2012). During our study, the SST trend in 2011 was similar to the two previous years; nevertheless it rose earlier, staying above the 20°C for a six month period, a longer period than observed in 2009 and 2010. The 2011 SST values were statistically different from the ones in 2009 and 2010. The influence of SST in the wedge shell life cycle in the Algarve has already been demonstrated by Gaspar, Ferreira, & Monteiro (1999), with the rise of the SST in March/April triggering the spawning event of the species. Although no information is available on the effect of the SST on eggs and larvae of the wedge shell in the wild, Ruiz-Azcona, Rodriguez-Sierra, & Martin (1996) demonstrated under controlled conditions that the percentage of surviving larvae of this species was greater at 20ºC than at 17ºC. In our study we found no correlation between SST and any other parameter analyzed, although the long period with high SST registered in 2011 has been previously related to the high number of days with phycotoxins registered in that particular year in the Algarve coast (Pereira *et al*. submitted). The effect of these phycotoxins on adult bivalves have been studied by several authors (Shumway *et al.* 1994; Bricelj & Shumway 1998; Li, Wang, & Hsieh 2002; Haberkorn *et al.* 2010; Contreras, Marsden, & Munro 2012; Medhioub *et al.* 2012) but still lack information in both eggs and larvae. Only Yan *et al* (2001) referred in scallop *Chlamys farreri* an inhibition on eggs hatching into D-larvae due to the presence of phycotoxins. We found no such effect on our data, with no differences in the hatching rate (HR) among the three years of study observed, despite the slightly decrease from 2009 to 2011 (Figure 2.3). Nevertheless, since phycotoxins could be a factor of stress, and since it is known that stressful environmental conditions affect the survival of larvae with poor quality (Phillips 2002) further studies should be undertaken to assess the direct effect of phycotoxins on egg and larvae, exploring possible relations. It worth noting, however, that we did register difficulties in inducing spawning during 2011 especially from June to August, when all attempts for inducing spawning failed. This result seems to suggest that although not directly related to HR, the phycotoxins presence may have affected the spawning event by delaying it, probably until phycotoxins were no longer present.

When spawning occurs, the released eggs must rely solely on their endogenous reserves to develop successfully into D-larvae (Ojea *et al.* 2008). As in other bivalve species, fertilized eggs of the wedge shell develop into veliconcha larvae within 24 hours and the positive correlation found between energy reserves in the eggs and hatching rate indicates that this period of major transformations requires that the progenitors provided the eggs with sufficient energy to successfully achieve D-larva. The effect of eggs energetic reserves on the hatching success and posterior larval survival has been demonstrated by other authors (Gallager & Mann 1986; Whyte, Bourne, & Ginther 1990; Burke, Bataller, & Miron 2008). In our study, the energetic levels in 2009 were significantly higher than the ones observed in 2010 and 2011. Since the energy in eggs must suffice the metamorphosis energetic needs and, later on, the energetic needs of growing larvae until feeding structures are completely formed, it would be expected that the D-larvae hatched in 2009 would have higher energy values to continue to grow than in the other years. Nonetheless our data showed exactly the opposite, with the energetic values in D-larva in 2009 being lower than the ones determined in 2010 and 2011 (Tables 2.3 and 2.4). When we analyzed the percentage reduction in the amount of energy between eggs and larvae, this reduction was higher and statistically different in 2009 with a loss in energy levels above 67.4% reaching 88.27%, while in 2010 and 2011 it ranged between 30.85%-59.75% and 45.98%-56.68%, respectively. It seems that despite the higher amount of energy available in 2009 eggs, more energy was used to achieve the D-larva stage when compared to the other years. It would be interesting to evaluate in further studies if a higher consumption of energy is reflected later in the survival rate of larvae, since individuals that complete larval development with minimal energetic reserves are known to fare poorly (Phillips 2002). The relation between high energetic values and successful metamorphosis and settlement was previously reported for *Crassadoma gigantea* (Whyte, Bourne, & Ginther 1990). These authors observed that larvae with an energy value around 6.1-6.3 kJ g^{-1} were better equipped to successfully metamorphose into competent larvae being 4.5-5.0 kJ g^{-1} considered the minimum energy to achieve success.

Energy comes mainly from total lipids, proteins and to a lesser extent, from carbohydrates: the three biochemical components available in the eggs and larvae tissues (Ferreiro, Pérez-Camacho, & Labarta 1990). Nevertheless the total lipids in the eggs are the ones considered responsible for the hatching success in bivalves (Gallager & Mann 1986; Utting & Millican 1997; Massapina *et al.* 1999; Matias *et al.* 2013). The positive correlation found for the wedge shell, indicates that total lipids also play an important role in hatching success of this species.

Total lipids available in the eggs are in part used/loss during egg development and hatching. This total lipid loss (from eggs to D-larvae) was higher in 2009 (52% to 89%), a value in accordance to the 70% lipid loss registered during the development of the *Mercenaria mercenaria* and *Crassostrea virginica* eggs by Gallager & Mann (1986) and contrasting with the lower total lipid loss observed in 2010 (19.5% to 53.2%) more similar to the 19% total lipid loss observed in *Crassostrea gigas* by Waldock & Nascimento (1979). In 2011 the loss in total lipid ranged widely from 38.5% to 79.1% positioning between the other years results. Interestingly, Burke et al. (2008a) registered in *Ostrea edulis* an increase in 15% of the total lipid content from the eggs to the D-larvae which did not occur in any moment during our observations.

The total lipids class comprises the neutral lipids and the phospholipids. Neutral lipids are considered an important energy source in pelagic veligers, especially in periods of low food availability or during starvation (Gallager, Mann, & Sasaki 1986; Kheder *et al.* 2010) while phospholipids play a more structural function in cell membranes (Chícharo *et al.* 2003). In our data the percentage of neutral lipids in the eggs did not show any differences among years despite the difference between total lipids percentage in 2009 and the remaining years. Although a higher total lipid loss was observed in 2009 as mention above, neutral lipids loss from egg to larvae did not show any differences between years. This result may indicate that despite the oscillation in total lipids percentage in eggs, a threshold in neutral lipids may exist, assuring that the embryogenesis is completed successfully as suggested by Utting & Millican (1997). Lipid content in the eggs of *Argopecten purpuratus* and *Ostrea edulis* affect their hatching success (Helm *et al.* 1991; Navarro *et al.* 2000) although we did have in *D. trunculus* a significant higher percentage of total lipids in eggs during 2009 when compared to 2010 and 2011 no significant differences among years were observed in the hatching rate.

The majority of studies on biochemical content in bivalve larvae focus only in the lipid class content, mainly due to proteins being considered as having a structural importance and used for somatic growth only. Hofmann *et al* (2004) considered, in their model, that egg rich in protein grow rapidly, but store insufficient lipid to sustain a successful egg and larvae development. In our study the proteins in eggs correlated negatively both with the HR and total lipid content which is in accordance with this author’s assumption that protein is not metabolized into other biochemical constituents, affecting the egg growth. Our data suggests that when more total lipids are available more energy is available to ensure the successful development into D-larvae.

The third and less represented component both in eggs and larvae of *D. trunculus*, were the carbohydrates and although it has been suggested that they do not play an important role during larval development (Holland & Spencer 1973; Gallager, Mann, & Sasaki 1986), carbohydrates may optimize the utilization of other reserves available in tissues (Haws, DiMichele, & Hand 1993). According to our results, the Total Lipids plus Carbohydrates to Protein Ratio trend was similar to the HR along the three years of study. The correlation between these two parameters was positive suggesting that this ratio may be used to predict hatching success in *D. trunculus*. Since *D. trunculus* landings between 2010 and 2012 did not vary (DGRM landings database) it can be assumed that recruitment was successful during the study period than this indicates that both mean ratio and mean hatching rate observed can be used to predict recruitment success. Therefore, based in our results, it can be assumed that a mean ratio higher than 0.82±0.09 (the lowest mean value observed during the present study) may lead to a good recruitment. Below this value managers should be aware that recruitment could fail. This type of information has not been used in bivalve stock management, being a first step into an earlier detection of recruitment failure, complementing stock assessment. Notwithstanding, further studies on larval survival should be conducted to understand how larvae quality is reflected in the survival rate when they become competent.

Acknowledgments

The authors would like to thank Dr. Alexandra Leitão, the coordinator of IPMA Mollusk Aquaculture Experimental Station and to Eng. Margarete Matias for her help in the water maintenance system and aid during biochemical analysis. This work was supported by the grant SFRH/BD/40430/2007 financed by Fundação para a Ciência e Tecnologia.

Chapter III

Identification and quantification of *Donax trunculus* larvae.

Chapter III. Identification and quantification of *Donax trunculus* larvae.

Identification of the wedge clam *Donax trunculus* by a simple PCR technique.

Pereira, AM; Fernández-Tajes, J; Gaspar, MB; Méndez, J (2012) Identification of the wedge clam *Donax trunculus* by a simple PCR technique. Food Control, 23:268-270.

Abstract

The wedge clam *Donax trunculus* is an important bivalve commercial species in Portugal which can be easily mistaken with other three morphologically similar species (*D. semistriatus*, *D. vittatus* and *D. variegatus*) that have a lower market price. This may lead fish sellers to make false claims about the authenticity of their products in order to get higher profits. To overcome this problem it is important to develop analytical techniques that can be used to test the authenticity of the species that is being sold. In this study we present two DNA extraction methodologies and a simple PCR method for the accurate identification of *D. trunculus* based on the amplification of the nuclear marker 5S rDNA. The PCR amplification results showed that this method is reliable to differentiate *D. trunculus* and *D. variegatus* from the remaining *Donax* species, since fragments of *D. trunculus* were about 275-300bp while *D. variegatus* were about ~450bp a little lower molecular weight than DNA fragments of the other two species (~500bp).

Introduction

The demand of bivalves in the Algarve (southern Portugal) highly increases during the summer, mainly due to tourism and the large quantities of shellfish festivals held during this season. This high demand of bivalve products sometimes offered to the consumer shelled may lead to mislabeling, either intentionally or not.

In the Algarve region the wedge clam *Donax trunculus* is one of the most economically important bivalve species. In Portugal this species occurs along the south and southwest Portuguese coast in very shallow waters of sandy beaches at a depth ranging from 0 to 6 meters (Gaspar, Castro, & Monteiro 1999) where is subject to an intensive fishery carried out by both dredger boats and hand-dredgers. Concurrently with *D. trunculus*, other *Donax* species such as *Donax semistriatus*, *Donax vittatus* and *Donax variegatus* can also be caught in the same fishing grounds. Although *Donax* species can be distinguished by their morphological traits, this is not a straightforward task for the consumer since the shell of these species but *D. variegatus* are very similar in size, shape and colour. Therefore, it is likely to find in the fish market other *Donax* species being sold as *D. trunculus*.

In order to protect the consumer, European Union introduced the Council Regulation EC 104/2000. This regulation state at Chapter 2, Article 4, that fishery product may not be offered for retail sale to the final consumer unless appropriate marking and labeling indicates commercial designation of the species, the production method and the catch area. Moreover this regulation enforced all member states to publish a list reporting the scientific name and the corresponding commercial name(s) for each commercial species. Despite this regulation, when only edible parts of bivalves are available to retail, the uncertainty that the product correspond to the species claimed in the label may arise, since it is impossible to the consumer to differentiate between species. It is therefore important the setting up of fast, reliable, reproducible and low cost techniques to be applied to test the authenticity of the bivalve species that are being used as raw material in order to avoid economic fraud. To achieve this goal, several techniques based on protein analysis (electrophoretic, chromatographic and immunological methods) have been developed for fish species identification (Carrera *et al.* 1997; Mackie *et al.* 2000). However according to Lockley & Bardsley (2000) those methodologies have some limitations especially in processed products, where processing techniques may easily result in protein denaturation.

Contrary to immunologic techniques, the DNA-based techniques have proven to be reliable, sensitive and fast for fish species authentication (Asensio *et al.* 2009). Actually, DNA molecule offers several advantages over proteins, especially when extracted from processed products, since it is present in all tissues, contains more information than proteins and is more stable towards processing procedures (Lin & Hwang 2008). DNA techniques have been used by several authors to assess their effectiveness in the certification of species present in commercial food, in detection of food mislabeling and in detection of fraudulent species substitution (Russell *et al.* 2000; Machado-Schiaffino, Martinez, & Garcia-Vazquez 2008; Sánchez *et al.* 2009).

Most of the DNA based methods used to verify fish and bivalve species identity are based on polymerase chain reaction amplification of polymorphic portion of genomic DNA due to its simplicity, specificity and sensitivity (Asensio *et al.* 2009). Some examples of PCR based techniques are: sequencing of PCR products based on DNA, restriction digestion of PCR products, species-specific PCR primers, single strand conformational polymorphism analysis and random amplified polymorphic DNA (RAPD) analysis. A more detailed description of these and other techniques can be found elsewhere (e.g. Lockley & Bardsley 2000; Asensio Gil 2007). Both nuclear and mitochondrial DNA are available in cells. Mitochondrial DNA has several advantages over nuclear DNA, mainly due to its higher abundance, mutation rate and number of copies inside the cell (Mackie *et al.* 2000). Nevertheless Ferguson *et al* (1995) stated that nuclear markers may be useful for species discrimination because of the existence of introns of different sizes which allow the amplification of species-specific DNA fragments. The 5S rDNA forms a multigene family of tandem arrays with a highly conserved region of 120 bp and a non-transcribed spacer (NTS) that evolves more rapidly, this makes it an interesting and widely used marker to discriminate several commercial fish/shellfish species (Fernandez-Tajes & Méndez 2007).

The present article deals with a reliable and fast protocol based on the comparison of two DNA extraction methodologies, and a simple PCR amplification method in order to properly detect or confirm the identity of *D. trunculus* in raw products. Actually the setting up of reproducible, fast, reliable and low cost techniques to identify shellfish species used both as raw and processed matter in industry is an important goal for food safety and consumer rights protection.

Material and methods

Bivalve sampling

The adult specimens (20 individuals of each species) were captured off Lota beach (N37º9'57.92'', W7º30'36.79'') South Portuguese coast, with a manual dredge in the case of *Donax trunculus* and by a bivalve dredge mounted onboard the research vessel NI Diplodus in the case of *D. semistriatus*, *D. variegatus* and *D. vittatus*. All specimens were taken alive to the laboratory and were identified by their morphological characteristics according to Macedo, Macedo, & Borges (1999) and Tebble (1966). For each species, the foot and adductor muscle were removed and preserved in 95% ethanol for posterior DNA extraction.

DNA extraction

Two DNA extraction methodologies were used on non processed tissue in order to compare extraction times, costs and feasibility and to establish the most appropriate method to use.

Method 1 - bivalve specific extraction method

A piece of adductor muscle of each sample (20 mg) was incubated with 400µl of lysis buffer (Fernandez-Tajes & Méndez 2007) and 2 µl of proteinase K at 65℃ for 2 hours. Then 100µl of precipitation buffer were added and the suspension was vortexed vigorously for 30 seconds and centrifuged at 13000g for 7 minutes. Supernatant was dropped in an autoclaved 1.5µl tube and 500µl of isopropanol were added and centrifuged once more at 13000g for 7 minutes. After isopropanol removal, 1ml of 70% ETOH was added and the suspension was centrifuged at 13000g for 7 minutes. After ETOH removal, the content of tubes was dried at room temperature and resuspended.

Method 2 – Chelex 100 ™ (Bio-Rad- Barcelona - Spain) resin based method

To each sample (2mm^3 of adductor muscle), 100 µl of a previous prepared Chelex 100™ solution (1g Chelex 100™ for 10 ml milliq water) was added, followed by a vigorous stirring, after which the sample was placed into a thermalcycler for 20 minutes at 100ºC. After being centrifuged for 1 minute, the supernatant was transferred to an autoclaved tube.

Primer design and PCR amplification

In this study a set of primers designed by Fernandez-Tajes & Méndez (2007) for amplifying the 5S rDNA gene was used. These primers were successfully used by the same authors in differentiating *Ensis* species. The forward primer was the 5SF (5′ CGTCCGATCACCGAAGTTAA 3′) whilst the reverse was the 5SR (5′ ACCGGTGTTTTCAACGTCAT 3′). Primers specificity for DNA samples obtained by the two methodologies described above was assessed. PCR amplification was carried out in a final volume of 25µl containing 15 ng of genomic DNA, 0.24µM of each dNTP, 2mM of MgCl2+, 1µM of each primer, 0.625 U of Taq polymerase (Roche Applied Science, Barcelona, Spain) and the buffer recommended by the polymerase suppliers. The amplification process was performed using a Gene Cycler thermal cycler (BIO-RAD – Barcelona - Spain) and consisted in an initial denaturation step of 2 minutes at 95 ºC, 35 cycles with a denaturation step at 95ºc for 30 seconds, an annealing step at 55ºC for 30 seconds and an extension step for 1 minute at 72ºC, and a final extension step at 72ºC for 5 minutes. The length of fragments was determined by comparison with Step Ladder 50-750bp marker from Roche^{TM} after electrophoresis in a 3% agarose gel. Gel were stained by immersion in 0.5 µg/ml ethidium bromide solution for 30 minutes, visualized and recorded on a transilluminator (Gel Doc XR Systems, Bio-Rad, Barcelona, Spain).

Results

Extraction of DNA from the tissue samples was successfully achieved with both methodologies. In the case of the bivalve specific extraction method, six reagents and five major steps were used. This method took about two and a half hours to obtain DNA from the tissue (without the overnight resuspension step). The method based on Chelex 100™ resin used two reagents and consists of three major intermediary steps. With this method the extraction process was concluded in only half an hour and the samples were ready for PCR amplification. Even though we kept the DNA extractions in the second method at 4ºC, it was observed that after a month, successful PCR amplifications became more difficult or even impossible to obtain.

The amplification of the 5S rDNA was successfully obtained with both DNA extraction techniques and for the four *Donax* species (Figure 3.1). Length was about 275-300 bp for *D. trunculus* fragments, about 450bp for *D. variegatus* and around 500 bp for fragments obtained from the other two *Donax* species.

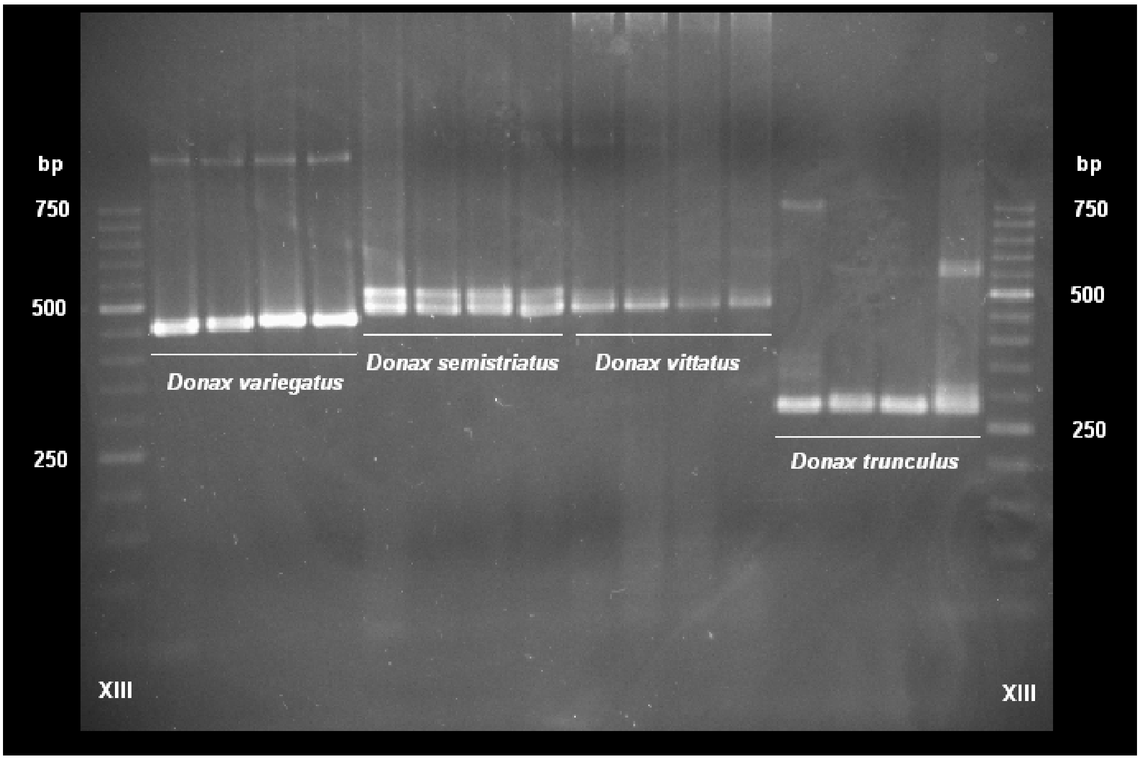


Figure 3.1. Image of 3% agarose gel electrophoresis of amplicons from different *Donax* species after staining with 0.5µg/ml ethidium bromide. XIII – step ladder 50-750 bp.

Discussion

Designing a proper method that could be easily implemented by state or private entities that deal with food safety and consumer rights are fundamental to dispel or confirm suspicions about mislabeled food and to ensure that consumers do not incur economic fraud. In the present study, two methodologies were used to extract DNA to be used to discriminate *D. trunculus* from other three *Dona*x species (with lower commercial value) and both proved to be effective. However, differences were observed in the time needed to perform the extraction. Indeed, the method based on Chelex 100™ resin is much faster than the bivalve specific extraction method in obtaining proper DNA to be used in an amplification process. This resin has been used successfully for DNA extraction in the pacific oyster (Launey & Hedgecock 2001; Aranishi & Okimoto 2006), arkshells and in pen shells (Steiner & Muller 1996). Furthermore, this method requires less reagents and intermediary steps making it also cheaper and easy to apply. This makes the difference when a large number of samples has to be analyzed rapidly and at a lower cost.

The oligonucleotides used were designed from 5S rDNA region because this ribosomal gene has two main advantages for species identification: a highly conserved region (even among different taxa) and a more variable spacer that allows species-specific differentiation (Freire, Insua, & Mendez 2005). Although primers 5SF and 5SR, which annealed in the coding region, are not specific to genus *Donax* these oligonucleotides are mollusc-specific and were previously used with success in the differentiation of species of the genus *Ensis* (Fernandez-Tajes & Méndez 2007). In the case of the genus *Donax*, despite successfully identifying *D. trunculus* and *D. variegatus*, this set of oligonucleotides were unsuitable for discriminating the other two *Donax* species, and therefore a set of specific oligonucleotides should be developed for this genus. Indeed, it was observed that 5S rDNA of *D. vittatus, D. semistriatus* presented a similar molecular weight (~500 bp) which hinders the authentication of these species.

One of the main drawbacks using 5S rDNA for species identification is the organization of ribosomal genes in multiple tandem arrays. The intraspecific variation that could exist in the different repeats at individual level could give rise to possible variations in array size. Nevertheless, concerned evolution, the main force that rules the evolution of tandem repeat arrays, tends to homogenize the repeat units present inside and among individuals of the species (Dover, Coen, & Strachan 1982).

Problems in using the above methodologies may arise if processed food is analyzed since heat and pressure processing may lead to DNA fragmentation reducing the sensitivity of the analysis. Hird *et al* (2006) subjected several samples of turkey meat to different processing conditions (baking, boiling, autoclaving and canning) and concluded that in general, when compared to DNA from raw samples, there was no differences in the amplification rates of DNA extracted from boiled or baked ones. Moreover these authors stated that amplification rates were not compromised for amplicons higher than 150 bp. Nevertheless, in the case of bivalves, this is not a problematic issue since they are usually sold alive or frozen.

Acknowledgments

The authors would like to thank the valuable comments of two anonymous referees. This work was supported by the grant SFRH/BD/40430/2007 financed by Fundação para a Ciência e Tecnologia.

Quantification of wedge shell, *Donax trunculus* (L.), larvae by Real Time PCR.

Pereira, A.M.; Fernandez-Tajes, J.; Gaspar, M.B.; Mendez, J. The wedge shell, *Donax trunculus* (L.), larvae quantification by Real Time PCR.

Abstract

Methodologies dealing with bivalve larvae identification and quantification are a key step to improve the management of bivalve fishery. Currently, in the Algarve, the managing measures applied on this fishery are based only on the adult population ignoring the larval stage. Knowledge on larvae quantification along the Algarve coast is important to better understand recruitment variations in economically valuable species such as the wedge shell, *Donax trunculus*. Larvae quantification of a single species is a cumbersome task to perform manually and if several species are present in seawater samples, it is practically impossible to accomplish successfully. To solve the discrimination and quantification problem in *D. trunculus* larvae, we used a real time PCR methodology to determine the amount of larvae in seawater samples. We developed two sets of primers on ITS1 and COI regions and tested them in both adults and larvae of the four *Donax* species occurring along the Algarve coast, in order to verify that amplification was species specific. After tested successfully for specificity, developed primers were used to obtain Ct-values from samples with different amount of larvae. Our results showed a high efficiency in both amplified regions (ITS1 and COI), however, only ITS1 registered a high r^2=0.9902 between Ct-values and the number of larvae, allowing us to predict the larvae abundance in water samples from the Ct-values obtained. During our experiment we found that high DNA concentration due to the large amount of larvae in samples as well as the phytoplankton presence in seawater samples have an inhibitory effect in Real-time PCR reactions and should be further studied in future works. The variability in DNA concentration revealed in samples with same amount of larvae and the difference in size of larvae within each sample led us to develop Ct-values intervals enabling us to assign the Ct-value obtained from blind-test samples to the respective larvae abundance interval, obtaining the general amount of larvae. We demonstrated that a Real-time PCR method may successfully quantify *Donax trunculus* larvae from water samples improving the information available for a better management of the resource.

Introduction

Some bivalve species, due to their economical importance are subjected to a high demand and tend to be overfished. This overexploitation usually leads to the implementation of management measures to protect the resource from depletion. These protective measures must be based on data frequently collected along the life cycle of the species. Presently at the Algarve coast, the only data available about bivalve stock populations, is obtained by the Portuguese Institute for the Ocean and Atmosphere through monitoring bivalve surveys that are carried in a yearly basis (for further information on these surveys see Rufino *et al.*, 2010). The results obtained through these surveys are extremely important since it allows to adjust daily quotas to the conservation status of the stocks. Currently, these measures are based on juveniles and mostly on adults, ignoring the larval stages and limiting the scientific and/or management questions that could be addressed if such data was available (Garland & Zimmer 2002). With data on larval distribution of a target species, it would be possible to understand recruitment variations (Mann 1988; Weinberg 1999; Garland & Zimmer 2002) and predict population demographic patterns of a species (Morgan & Rogers 2001; Livi *et al.* 2006), being therefore an important tool aiding the management of the resources (Morgan & Rogers 2001; Vadopalas *et al.* 2006).

Regardless the importance in understanding the causes affecting recruitment in bivalves, the problems in obtaining field data on a large scale usually leads to a scarcity or total absence of information (Hare, Palumbi, & Butman 2000). Usually studies on planktonic larval distribution are limited by the difficulty in obtaining large number of samples with adequate spatial and temporal coverage and their subsequent analysis in due time, because of the large amount of samples generated (Levin 1990; Garland & Zimmer 2002). The two main problems in this type of studies are the identification of the larvae species present in the samples and the quantification of the amount of larvae of the desired species present in those samples.

Classically, bivalve larvae identification has been made based on morphological characteristics (Larsen *et al.* 2005) despite the particular difficulty in identifying bivalve larvae trough their morphological traits using a light microscope (Hare, Palumbi, & Butman 2000). This can be rather laborious, time consuming and costly, since specialized personnel is needed and the identification of a single individual sometimes requires a great deal of training, experience and dedication and usually requires a long time to process (Pan *et al.* 2008). Independent of the degree of such expertise, the phenotypic variability caused by environmental factors also increases the uncertainty in the identification of many species (Pan *et al.* 2008) with most cases ending with larvae only identified to larger *taxa* and not to the species level (Bell & Grassle 1998). Many of the youngest larval stages in bivalves (frequently the most abundant invertebrate larvae in coastal samples) are so similar in appearance during their early development that they cannot be identified to the species by using gross morphological criteria alone (Loosanoff, Davis, & Chanley 1966; Garland & Zimmer 2002).

Also problematic is the quantification of the larva, with two main problems hindering studies on this subject. First, counting larvae manually is a cumbersome task when hundreds or thousands of larvae are present in each sample and therefore extremely time consuming. Studies dealing with dozens of samples, each one with dozens or hundreds of larvae are not realistically feasible. This leads us to the second problem, different species of bivalve and other meroplanktonic organisms may be present in each sample, and as mentioned above, discriminating them visually is unpractical. Some interesting work has been done in discriminating visually bivalve larvae from other organisms with polarized light such as the larvae of zebra mussel (Johnson 1995). Notwithstanding, no real discrimination was actually made between this species and other bivalve species presented in the samples but rather between bivalve larvae and debris. Also machine learning and image analysis have been used (Tiwari & Gallager 2003; Thompson, Hare, & Gallager 2012) with some degree of correctness discriminating species using multiscale texture and color invariants in prepared sample tests. Nevertheless when applied to samples collected from the environment the results are still poor.

To solve the discriminating problem in bivalve larvae, scientists have turned to several solutions in order to identify and quantify larval species in samples. The majority of those solutions use molecular methods for identifying bivalve larvae to species, thereby alleviating some of the ambiguity or subjectivity of traditional morphology-based taxonomy and eventually expediting sample-processing (Garland

& Zimmer 2002). Molecular methodologies have been used successfully to identify visually indistinguishable early stage in gadoid eggs (Taylor *et al.* 2002; Fox *et al.* 2005), to estimate the abundance of diatoms and dinoflagelates (Hosoi-Tanabe & Sako 2005; Godhe *et al.* 2008) and in the quantification and identification of algae in copepod stomachs content (Troedsson *et al.* 2008). Polymerase chain reaction (PCR) has been the most robust reliable and versatile methodology used, although numerous technical challenges, and development of these approaches require extensive optimization and empirical testing before they can be routinely applied (Larsen *et al.* 2005).

Several species of marine invertebrates with planktonic larvae have been tested, using real time PCR methodologies: the abalone (*Haliotis kamtschatkana*), in which larvae were quantified using mitochondrial DNA marker cytochrome oxidase I (Vadopalas *et al.* 2006), the manila clam (*Ruditapes philipinarum*) (Quinteiro *et al.* 2011), the crustaceans larvae from *Liocarcinus depurator, Necora puber*, *Carcinus maenas* and *Cancer pagurus* using taqman® probes designed in the cytochrome oxidase I (Pan *et al.* 2008), some barnacle species from the Japanese coast (Endo *et al.* 2010) and the King crab *Paralithodes camtschaticus* were quantified via primer development and dual labeled probe for use in real-time PCR assay (Jensen *et al.* 2012). The use of fluorescence in situ hybridization using species specific DNA probes in marine bivalves was also performed by Heaney *et al.* (2011). Polychaets from genus *Osedax* and *Ophelia* as well as barnacles from the genus *Balanus* have also been tested with rRNA targeted sandwich hybridization assay (SHA) using oligonucleotides probes to detect and enumerate the larva (Jones *et al.* 2008).

Although widely use, real time PCR may be problematic with larvae that largely increase cell numbers as they grow. Since the real time PCR essentially count genomes, the counts of these organisms will be biased as they grow, unless standard curves are derived from larvae containing a similar number of cells as the unknown samples or standard curves are derived for earlier and later stages in larval development (Vadopalas *et al.* 2006).

The wedge shell, *Donax trunculus* is an economical important species in the Algarve, and although knowledge on adult population is retrieved every year, there is a tremendous lack on information on larval abundance and distribution of this species in the Algarve coast. Aside from *D. trunculus*, only three more species of this genus are found along the Algarve coast (*D. vittatus*, *D. variegatus* and *D. semistriatus*). This species is perfect to test if a real time PCR methodology can be used to identify and quantify larvae since there is no information on its larval abundance and distributions and there is an easy access to the stock population. In our study we intended to develop a real-time PCR assay in order to routinely and accurately identify and quantify larvae from the wedge shell *Donax trunculus* from unsorted plankton samples. The success in discriminating and quantifying larvae in samples gathered from the Algarve coast is an important step to improve the management of this fishery. Moreover, this methodology could be easily transferred to other bivalve or invertebrate species with planktonic larval stages after species specific primers are developed and tested.

Material and methods

Larvae samples

Larvae of *D. trunculus* were obtained from adults previously captured at Lota beach (7°30′32.21″W, 37°9′56.33″N) in the Algarve coast, western Portugal. The captures were made at low tide, during the morning and using a bivalve hand dredge similar to the ones used by local harvesters. After captured, the specimens were taken to the laboratory facilities and placed in three previous prepared nurseries. Spawning occurred overnight and eggs were collected and placed for 24 hours in 50 l tanks until hatched into D-larvae. Larvae were collected and aggregated in groups (1, 10, 50, 100, 250, 500, 1000, 5000) and preserved in 96% ethanol. The remaining larvae were allowed to achieve umbunate state after which were collected, preserved in 96% ethanol and grouped (1, 10, 50, 100, 250, 500, 1000, 5000) for further use.

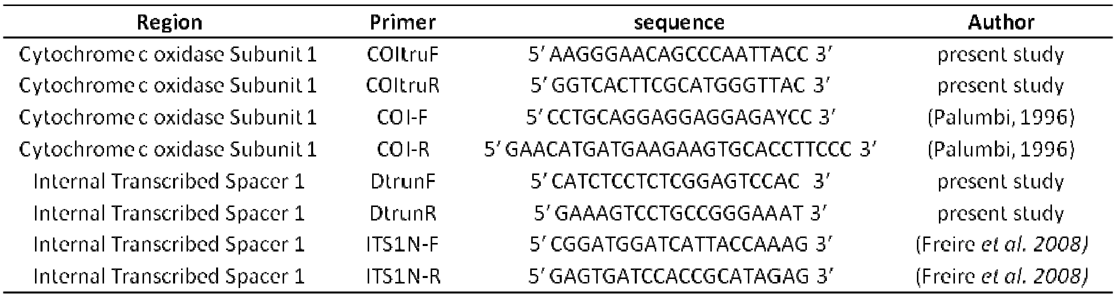
Extraction of template DNA from the larvae

Larvae DNA was extracted according the methodology used by Taris *et al.* (2005) with an additional first step, where samples were centrifuged at 12000 rpm, in order to break the shell and improve extraction quality. Ethanol from the larvae samples was carefully removed, and different lyses buffer (Roche™) volumes were added depending on the number of larvae (60µl to groups of 1-500 larvae and 100µl to groups above 1000 larvae). Samples were briefly vortexed and centrifuged for 2 minutes at 10000 rpm. Different volumes of proteinase k were added depending on larvae number (5µl to larvae groups from 1 to 500 and 7µl for groups above 1000 larvae) and heated at 55ºC for 1 hour, vortex briefly and heated at 100ºC for 20 minutes. Supernatant was used as DNA template.

Primer design

A piece of adductor muscle (20 mg) of each adult specimen from the 4 existing *Donax* species in the Algarve coast was used to extract DNA following the method described by Fernandez-Tajes and Méndez (2007). ITS1 region was amplified using the primers described in Freire *et al.* (2008) and COI region was obtained by means of amplification with the primers designed by Palumbi (1996) (Table 3.1). Fresh PCR products were ligated into the plasmid pCR®2.1-TOPO® using the TOPO-TA Cloning kit (Invitrogen) and transformed into TOP10F' cells. Plasmid DNA purification was carried out using QIAprep Miniprep Kit (Qiagen, Barcelona, Spain), which employs the modified alkaline method of Bimboim and Doly (1979). Sequencing of both strands of the insert of 4–6 clones/individual was performed with an automatic capillary DNA sequencer (CEQTM8000 Genetic Analysis System).

Table 3.1. Sequence of primers used initially to amplify ITS1 and COI regions and specific primers design for *Donax trunculus* in qPCR-RT analysis.



Region	Primer	sequence	Author
Cytochrome c oxidase Subunit 1	COItruF	5' AAGGGAACAGCCCAATTACC 3'	present study
Cytochrome c oxidase Subunit 1	COItruR	5' GGTCACTTCGCATGGGTTAC 3'	present study
Cytochrome c oxidase Subunit 1	COI-F	5' CCTGCAGGAGGAGGAGAYCC 3'	(Palumbi, 1996)
Cytochrome c oxidase Subunit 1	COI-R	5' GAACATGATGAAGAAGTGCACCTTCCC 3'	(Palumbi, 1996)
Internal Transcribed Spacer 1	DtrunF	5' CATCTCCTCTCGGAGTCCAC 3'	present study
Internal Transcribed Spacer 1	DtrunR	5' GAAAGTCCTGCCGGGAAAT 3'	present study
Internal Transcribed Spacer 1	ITS1N-F	5' CGGATGGATCATTACCAAAG 3'	(Freire *et al.* 2008)
Internal Transcribed Spacer 1	ITS1N-R	5' GAGTGATCCACCGCATAGAG 3'	(Freire *et al.* 2008)

The sequences obtained were used for designing primers species-specific suitable for qPCR-RT analysis (Table 3.1). Briefly, nucleotide sequences were aligned using ClustalX software (Thompson *et al.* 1997), and edited with GeneDoc software (Nicholas, Nicholas, & Deerfield 1997). Interspecific differences found at ITS1 and COI regions of *Donax* species were employed for designing reverse species-specific primers using the Primer3 software (Rozen & Skaletzky 2000). The obtained ITS1 and COI primers were tested in adults and larvae of the four *Donax* species to verify that amplification only occur in *D. trunculus*. Blastn searches for homology was also undertaken to test in-silico for possible cross amplifications.

PCR amplifications

Primers were used in PCR amplification both with a final volume of 25µl containing 15 ng of genomic DNA, 0.24µM of each dNTP, 2mM of MgCl2+, 1µM of each primer, 0.625 U of Taq polymerase (Roche Applied Science, Barcelona, Spain) and the buffer recommended by the polymerase suppliers. PCR fragment amplifications were performed using a Gene Cycler thermal cycler (BIO- RAD – Barcelona - Spain). For ITS1 it consisted of an initial denaturation step of 5 minutes at 95 ºC, 40 cycles with a denaturation step at 94ºc for 20 seconds, an annealing step at 56ºC for 20 seconds and an extension step for 1 minute at 72ºC, and a final extension step at 72ºC for 5 minutes. In the case of, COI PCR fragment amplifications consisted of an initial denaturation step of 3 minutes at 94 ºC, 34 cycles with a denaturation step at 94ºc for 45 seconds, an annealing step at 60ºC for 45 seconds and an extension step for 45 seconds at 72ºC, and a final extension step at 72ºC for 3 minutes. PCR products were migrated using a 2% agarose gel. Gels were stained by immersion in 0.5 µg/ml ethidium bromide solution for 30 minutes, visualized and recorded on a transilluminator (Gel Doc XR Systems, Bio-Rad, Barcelona, Spain).

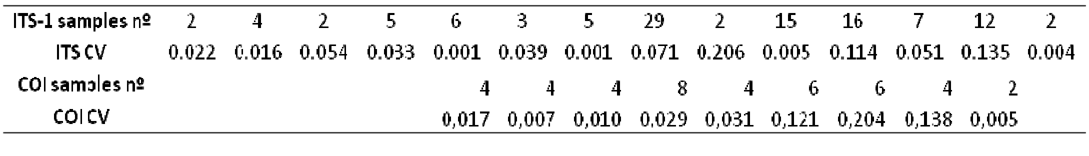
Real time PCR amplification

Oligonucleotide primers previously designed were used. Real Time PCR was run in triplicate using LightCycler® SYBR green I Master Kit (Roche) and the LightCycler® 480 Real-time PCR Detection System (Roche). The PCR conditions were 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 5 s, for 45 cycles and final extension of 5 min. A subsequent melting temperature curve of the amplicon was performed. Prior to running samples, efficiency of target amplification was optimized for each pair of primers by assaying four primer concentrations (200, 150, 100 and 50 nM). LightCycler software 1.5.0 (Roche) was used for computing the number of amplification steps required to reach the threshold cycle number (Ct). Constant Ct values were observed at a 100 nM final primer concentration for each of the primer pairs. Efficiency of PCR was calculated by means of the Ct-values estimation through a range of DNA concentrations: from 1.23 to 1.23×10^{-4} ng for ITS1 region and between 1.71 to $1.71\text{x}10^{-5}$ ng for COI region.

Ct- standard curve design

Linearity was evaluated by amplifying DNA isolated from a variable number of larvae and replicates as expressed in Table 3.2. The obtained Ct values enable the calculation of a coefficient of variation (CV) evaluating pipetting errors and possible variability and reproducibility in the DNA isolation procedure in samples with identical larvae number.

Table 3.2. Larvae templates used in ITS1 and COI linear functions and the number of replicates used. CV – coefficient of variation within samples with same number of larvae.



Larvae	0.01	0.02	0.05	0.1	1	2	5	10	20	50	100	250	500	5000
ITS-1 samples nº	2	4	2	5	6	3	5	29	2	15	16	7	12	2
ITS CV	0.022	0.016	0.054	0.033	0.001	0.039	0.001	0.071	0.206	0.005	0.114	0.051	0.135	0.004
COI samples nº					4	4	4	8	4	6	6	4	2	
COI CV					0,017	0,007	0,010	0,029	0,031	0,121	0,204	0,138	0,005	

Results

After extraction, DNA concentration measures using NanoDrop® revealed that as the number of larvae increased in samples also the DNA concentration increased, although some variation within samples with the same amount of larvae was registered (Table 3.3).

Table 3.3. Measures of DNA concentrations (ng.µl^{-1}) in two sets of samples with different amount of larvae.

number of larvae	DNA concentration (ng.µl^{-1})	
	sample set 1	sample set 2
100	2.12	4.12
250	8.12	12.97
500	16.91	31.35
1000	29.64	36.35

Prior to real-time PCR, primer specificity was tested in adult and larvae samples from *D. trunculus* and the other three *Donax* species that occur in the Algarve coast. Only success amplification was produced in *D. trunculus* samples corroborating the usefulness of our species-specific primers for distinguishing *Donax* species. After each real-time PCR run, Ct values were obtained from the different larvae concentrations and ranged in the ITS1 from a minimum of 16.5 registered in samples with 5000 larvae to a maximum of 35.34 in samples with 0.01 larvae In the COI the Ct-values ranged from 24.95 to 35.85 both registered in samples with 100 larvae. Two replicates for each larvae sample were introduced in order to assure the reliability of PCR reaction. Negative controls were added for ascertain that no contamination existed in the PCR Master-Mix and Positive controls were also incorporated for controlling the success of PCR amplification.

As an additional quality control all Ct-values above 36 were not considered due to lack of liability and the samples with Ct values ≥40 were excluded since no signal was observed in template DNA. Besides, replicates with a Ct-difference higher than 0.7 were not considered in the analysis.

The PCR slope in the SYBR Green assay was –5.558, r^2=0.986, p<0.001 (ITS1) and -1.295, r^2=0.985 p<0.001 (COI), suggesting a very high efficiency of the real-time PCR method in both cases. The plot of the ITS1 linearity between the Ct value and the abundance of larvae (ln scale) is presented in figure 3.2. The linear equation obtained showed a r^2= 0.9902 indicating almost 100% efficiency and allowing to predict larvae abundance from the Ct-value obtained in the real-time PCR reaction. The plot of the COI linearity between the Ct-value and the abundance of larvae (ln scale) is presented in Figure 3.3. On the contrary to the observed for the ITS1, the linear equation obtained from COI Ct-values did not allow to predict larvae abundance (r^2=0.0195).

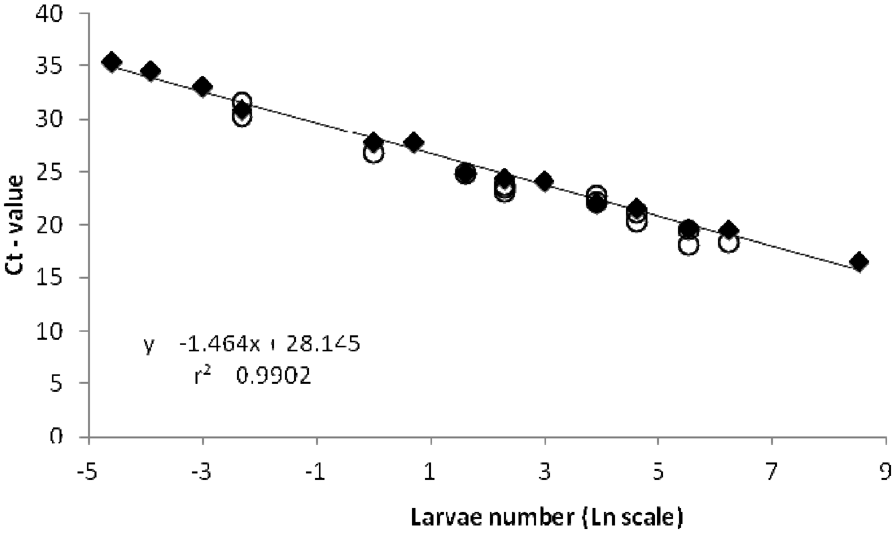


Figure 3.2 – Plot of the relation between larval number and estimated Ct-values using ITS1. (♦). White circles represent blind test samples of: 0.1; 1; 5; 10; 50; 100; 250 and 500 larvae. Linear regression equation and r^2 displayed.

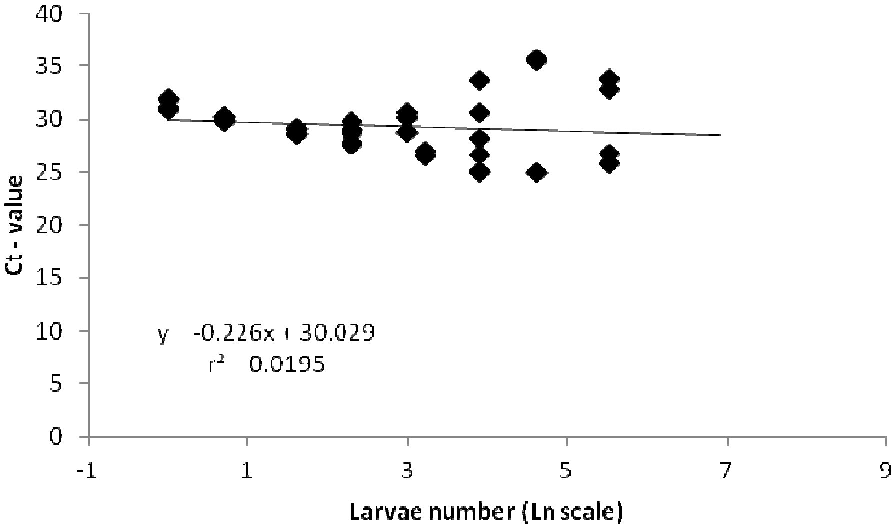


Figure 3.3. Plot of the relation between larval number and estimated Ct-values using COI (♦). Linear regression equation and r^2 displayed.

Although in ITS1 an r^2= 0.9902 was registered, differences in DNA extraction concentration and size differences among larvae within a sample leaded us to built Ct-values ranges (Table 3.4).

Table 3.4. Ranges of Ct-values obtained by real-time PCR, used for relative quantification of *D. trunculus* larvae on plankton samples.

Range of Ct-values	Larvae abundance
>100	<21.58
10-100	21.98-21.58
10-50	24.26-21.99
10-1	27.70-24.25
<1	>27.70

Test sample application

Since no linear relation between Ct-values and the amount of larvae was found using COI primers, blind-test samples were only applied in the ITS1 case. The samples used to test our real-time PCR approach were obtained as previous mentioned. Samples with 0.1; 1; 5; 10; 50; 100; 250 and 500 larvae were tested and Ct-values and larvae abundances fell successfully into the ranges described in Table 3.4.

Discussion

Methodologies dealing with larvae identification and quantification are most desired since monitoring the amount of larvae available and the determination of their available on the water is a key step to improve the management of the fisheries, Although some authors have used real-time PCR methods to accomplished quantification of larvae on marine invertebrate species such as *Haliotis kamtschatkana* (Vadopalas *et al.* 2006); *Liocarcinus depurator* (Pan *et al.* 2008); *Paralithodes camtschaticus* (Jensen *et al.* 2012) and several barnacles species (Endo *et al.* 2010), in bivalves the use of these methodologies is scarce (*Ruditapes philippinarum*, Quinteiro *et al.*, 2011).

In our study we used a real time PCR methodology based on SYBR green assay to determine the amount of larvae in seawater samples. The PCR methodologies developed for larval quantification have to overcome two critical elements: first, reactions that produce no species specific product because of an absence of target DNA must be distinguished from failure of the overall enzymatic reaction (Hare, Palumbi, & Butman 2000). We accomplished that by including positive control amplification in every reaction. Second, the method must be straightforward, relative simple and inexpensive to be applied in a large amount of samples even from preserved samples stored for several years in order to be considered for large scale use (Larsen *et al.* 2005).

We demonstrate that the development of such methodology may take some time in order to achieve the specificity required and to build the linear relation between the Ct-value and larvae abundance. Nevertheless, when is fully operational, its implementation is fairly easy and allow treating a high number of samples (ranging from 48 to 386 including replicates and controls) at the same time. Another important aspect of real-time PCR for larval quantification is to choose an appropriate DNA molecular marker. This marker has to be specific for the tag species and reliable. In our approach, a nuclear region (ITS1) and a mitochondrial one (COI) were selected.

By making use of interspecific variation at the ITS1 and COI loci we designed two sets of species-specific primers capable of distinguish the wedge shell species from the other three *Donax* species present in the Algarve coast. The next step was to establish the linear regression between the Ct-values and the amount of larvae for each region amplified. The Ct-value will depend on the amount of DNA available in the initial template and this is related to the initial amount of larvae in the samples. One problem is that while lecitotrophic larvae typically exhibit less daily growth than planktotrophic larvae and may have only a minor increase in cell number during their planktonic phase (Havenhand & Todd 1988) they also tend to have short planktonic phases such as in the case of *Haliotis kamtschatkana* with only one week as planktonic before become benthic (Sloan & Breen 1988). The opposite is observed in the wedge shell, a planktothrophic species with a one month planktonic phase that develop feeding structures allowing them to feed and grow larger.

Since the aim of a quantification methodology is their practicability and celerity, a previous size sorting would delay all the process and become unrealistic. To overcome this problem we use a mixture of D-shape and umbonated larvae. Our results showed a high efficiency in both amplified regions, However, only ITS1 registered a high r^2=0.9902 between Ct and number of larvae allowing us to predict their abundance in water samples from the Ct-values obtained. The poor correlation obtained for COI (r^2=0.0195) discarded the usefulness of this region for larvae quantification.

With the linearity determined, the blind tests were only undertaken using the ITS1 region. The variability in DNA concentration revealed in samples with same amount of larvae and the difference in size of larvae within each sample was reflected in Ct-value variability among replicas. This fact leads us to develop Ct-values intervals as presented in Table 3.4. By using these intervals we were able to assign the Ct-value from blind-test samples to the respective interval and know the general amount of larvae. Cycle threshold ranges was already used to quantify *Liocarcinus depurator* larvae in water samples by Pan et al. (2008) although with different Ct-values intervals than ours.

It should be mentioned that we did find that in samples with a number of larvae above 1000 Ct-values were extremely variable and most of the times a failure on the PCR reaction was observed. The inhibitory effect of a high DNA concentration seems to be the most plausible explanation for this observation. Similar results were also reported in samples with high number of manila clam larvae by Quinteiro *et al.* (2011). Another problem is related to the inhibitory effect that phytoplankton has when mixed with larvae samples prior DNA extraction. This inhibition may not only result in a reaction failure, but also in reaction delays that may give false or negative Ct-values estimates of initial template quantity (Vadopalas *et al.* 2006).

Overall, the Real-time PCR method base on SYBR green assay developed in this work revealed that it is possible to correctly quantify larvae of *Donax trunculus* in water samples using the nuclear region ITS1. The described procedure is relatively fast with no post-manipulation after the Ct-values have been obtained and does not require an exhaustive experience in bivalve identification. However, there are some issues that should be addressed in further studies in order to increase the methodology efficiency. The extraction protocol should be improved or replaced by other protocols more effective in reducing DNA concentration variability in samples. Since high concentrations of DNA may inhibit PCR reaction we recommend using dilutions, avoiding large larvae concentrations. Also the inhibitory effect that phytoplankton causes in the PCR reactions should be addressed and solved, either by dilution, by removal before DNA extraction or by using a reaction promoter such as UDG.

As a conclusion we can say that for the first time a PCR-based methodology that allows obtaining information on wedge shell larvae abundance along the Algarve coast is available and their use could allow using this information as an aid to the traditional stock assessment measures and other larvae dynamic studies.

Acknowledgements

We are grateful to Mr. Jose García Gil for his technical assistance. This work was supported by the grant SFRH/ BD/40430/2007 financed by Fundação para a Ciência e Tecnologia.

Chapter IV

Ocean acidification effect on *Donax trunculus* larvae

Chapter IV. Ocean acidification effect on *Donax trunculus* larvae

Larval hatching and development of *Donax trunculus* (L.) under increased CO_2 and reduced pH scenarios.

Pereira, A.M.; Range, P.; Campoy, A.; Oliveira, A.P.; Joaquim, S.; Matias, D.; Chícharo, L.; Gaspar, M.B. Larval hatching and development of *Donax trunculus* (L.) under increased CO_2 and reduced pH scenarios.

Abstract

Noticeable changes in global temperatures, climate and ocean carbon chemistry are the result of carbon dioxide increase in the atmosphere. This increase has been mitigated by the oceans capacity to absorb 25 to 26% of the carbon dioxide in the atmosphere although this CO_2 intake affects oceans carbonate chemistry. The detrimental effect of acidification in the development and shell formation in several bivalve species have been studied, although no information is available on the wedge shell *Donax trunculus,* a most gastronomically appreciated species and an important economical resource to the Algarve region. We evaluated the effect of $p\text{CO}_2$ increase on hatching and early life stages of *Donax trunculus* considering the two ocean acidification scenarios predicted by the IPCC. Our results showed that the hatching rate was negatively affected in the extreme scenario (ΔpH=-0.6) and a delay in hatching into D-larvae was registered with some trochophore larvae found in day 5 and day 9 of the experiment. Our results suggests that ΔpH=-0.6 acidified treatment produce more abnormal larvae than the ΔpH=-0.3 and ΔpH=-0.0 treatment. Also veligers under acidified treatments grow larger than veligers in ΔpH=-0.0 suggesting that wedge shell larvae under acidified conditions invested in growth, maybe to better resist the adverse environment since smaller larvae cannot feed as well as larger or due higher mortality occurring in the acidified treatments that may leave more food available to favoring growth. This was a first insight of the detrimental effects of pCO_2 increase in the wedge shell larvae according the predicted scenarios for the next centuries.

Introduction

With the beginning of the industrial revolution 250 years ago, human activity has been responsible for a concentration increase in atmosphere carbon dioxide (CO_2) (Meehl *et al.* 2007). This increase, according to Intergovernmental Panel on Climate Change (IPCC) is unequivocally accepted as causing global climate changes, affecting average air and ocean temperatures, causing not only a widespread melting of snow and ice and rising of average sea level but also noticeable changes in global temperatures, climate and ocean carbon chemistry (Raven *et al.* 2005).

Oceans have an important role mitigating the effects of carbon dioxide, since they are responsible for absorbing 25 to 26% of the total global emissions without which the values would be 55% higher than the presently observed (Sabine *et al.* 2004; Fabry *et al.* 2008; Feely, Doney, & Cooley 2009). Of course this oceanic uptake of CO_2also affect the oceans, changing the carbonate chemistry of seawater altered by the dissolution of anthropogenic CO_2, increasing the concentration of hydrogen (H^+) and bicarbonate (HCO_3^-) and reducing the carbonate ion (CO_3^{2-}) saturation, and pH (Feely *et al.* 2004; Orr *et al.* 2005). Estimations based on IPCC emissions scenarios indicate that pH will decline 0.3 to 0.5 units during the 21st century, and if there is an unrestricted burning of fossil fuels, an extreme scenario with a reduction of 0.7 units will occur until 24th century (Caldeira & Wickett 2003).

Carbonate chemistry in the seawater is extremely important to the mineral formation of calcium carbonate ($CaCO_3$), a structural element of shells and skeleton of marine calcifying organisms (Fabry *et al.* 2008). These organisms are seen as the most threatened since their ability to produce calcareous structures is expected to be directly affected by the seawater carbonate chemistry (Broecker & Takahashi 1966; Orr *et al.* 2005). Moreover, bivalves are most likely to be the organisms to have their calcification rates compromise over other organisms if IPCC predicted scenarios occur (Hendriks, Duarte, & Álvarez 2010). Indeed, of all marine calcifiers predicted to be affected by the ocean acidification (OA), bivalves are of most concern due to their important role in marine and coastal ecosystems, the contribution to the inorganic carbon cycle as carbonate producers and for being a link between the primary productivity and the upper trophic levels (Range *et al.* 2011; Barros *et al.* 2013). Moreover, in many coastal areas, bivalves are economical important resources for fisheries and aquaculture and the potential for significant economical and ecological impacts arising from the effects of OA on bivalves stocks could be of utmost importance, reason why the need of further experiments has been recognized (Cooley *et al.* 2012).

In bivalves, the process of shell segregation and mineralization initiates in the first hours after hatching, during the trocophora stage (Weiss *et al.* 2002) from the invagination of specialized ectotermic cell that form the shell gland and mantle and the remaining outer cells producing the outer layer of the shell, the periostracum (McConnaughey & Gillikin 2008). Unlike the shells of adult bivalves, that are built of calcite, aragonite or both, structured in several layers, the shells of all larvae contain aragonite (Weiss *et al.* 2002), the most soluble form of $CaCO_3$ (Barros *et al.* 2013). The detrimental effect of acidification in the development and shell formation in several species of bivalves have been studied, such as in the genus *Mytilus* (Gazeau *et al.* 2007; Rodolfo-Metalpa *et al.* 2011; Fernández-Reiriz *et al.* 2012; Range *et al.* 2012; Hiebenthal *et al.* 2012), *Ruditapes decussatus* (Range *et al.* 2011), *Crassostrea virginica* and *Mercenaria mercenaria* (Matoo *et al.* 2013; Ivanina *et al.* 2013), *Arctica islandica* (Hiebenthal *et al.* 2012) and *Macoma balthica* (Jansson, Norkko, & Norkko 2013).

The species targeted in the present study, the wedge shell *Donax trunculus*, has becoming increasingly important in Portugal and other southern European countries such as Italy and Spain (Ruiz-Azcona, Rodriguez-Sierra, & Martin 1996). It is most appreciated gastronomically and is a source of income not only to the fishermen involved in the bivalve fishery but also to a network of secondary activities that depend on this fishery. The disappearance of such economical resource would be tragic for the sector, as a result studying the effects of ocean acidification in early-life stages of the wedge shell is essential to predict not only future consequences on the ecosystem but also to inform the industry on the problems that may arise, alloying to timely adapt. The present study aims at evaluating, for the first time, the effect of $p\mathrm{CO}_2$ increase on hatching and early life stages of *Donax trunculus*.

Material and methods

Rearing system

The rearing system was installed in the experimental bivalve hatchery Portuguese Institute for the Ocean and Atmosphere (IPMA) in Tavira, Southern Portugal (37°7′17.73″N, 7°37′12.19″W). Seawater was pumped from the Ria Formosa lagoon, sand filtered and stored in a 200 000 l concrete reservoir. Before entering the rearing system, it was aerated during 2-3 days in a 2000 l fiber glass tank before being transferred to the 250 l header tanks. Each pH level had a separate header tank (250 l) and pump, connected to the experimental 20 l plastic tanks by a loop of PVC tube. A flow through system was used to minimize the interference from metabolic waste products of the organisms. Seawater flow in the tanks was regulated manually to 6 L h-1, allowing the excess water to overflow so that the total volume in each tank was totally exchanged every 3 hours. Cleaning of tanks and sieves (40 µm mesh) were done whenever the tanks were emptied for larvae counting and observations.

Experimental design

Three levels of pH were applied, each with 3 replicate tanks: the control pH (ΔpH=0.0) and two manipulated pH levels (ΔpH=-0.3 and ΔpH=-0.6). The pH of seawater in the acidified treatments was adjusted to the predetermined set-points by diffusing pure gaseous CO_2into closed reactors (Aqua Medic-reactor 1000), installed before the headertanks. The flux of gas from the CO_2 to the reactors was controlled using a pH-stat system (Aqua Medic AT control), by adjusting a solenoid valve when deviation from the pH predetermined setting points exceeded ± 0.005 pH units.

Determinations of variables in seawater

Temperature (T), salinity (S) and pH were monitored continuously in each of the nine tanks during the course of the experiment, using dedicated electrodes and the data logger function of the controller. The automatics readings were validated by weekly manual determinations with an YSI Pro Plus multiprobe, standardized against Tris seawater buffers (ionic strength of 0.7M). All pH readings are expressed in the total scale (pHT). Salinity readings were calibrated with an AutoSal salinometer using IAPSO standard seawater.

Bioassay

Eggs from wedge shell were obtained from broodstock taken at Lota Beach (7°30′32.21″W, 37°9′56.33″N; Southern Portugal) in 9/05/2012 and placed in a rearing system to spawn overnight. After collection, the fertilized eggs were counted and 1,000,000 eggs were transferred to each replicate tank filled with seawater at natural pH. The larvae were fed with *Isochrysis aff. galbana* (T-ISO) through a continuous-flow system at a concentration of 50 to 100 cells µL-1. During the subsequent hours seawater in the tanks was gradually replaced to achieve the planned pH levels. Every 48 hours all replicates tanks were emptied for counts of trochofore larvae, normal and abnormal veligers until no specimens were found alive in the treatments, ending the experiment.

At each sampling day, one hundred larvae from each replicate were conserved in a 4% formaldehyde solution to evaluate shell growth. Photomicrographs were taken to measure shell diameter and area and to evaluate the morphology and shell abnormalities. Abnormal shells were classified according three shell abnormalities: convex hinge, valve inequality and shell protuberances.

Image processing and analysis of the larvae

Image processing and analysis was done using the program ImageJ. The first step was converting all the images to an 8-bits TIFF format to reduce memory requirements and allow images to be opened in stacks, enabling to work with multiple images at the same time. These stacks were subsequently converted to 8-bits greyscale and a Despeckle filter applied to remove noise. This is a median filter that replaces each pixel with the median value in its 3x3 neighborhood. The stacks were converted to

Binary images with an automatic threshold operation and the spatial calibration (5.961 pixels.µm-1) was defined from a reference image of a cell counter grid. The Set Measurements command was used to define the variables to measure: area, shape descriptors, perimeter and Feret´s diameter. Finally, measurements were done using the Analyze particles command, with boundaries defined for particule size (500-8000 µm2) and circularity (0.2-1.0), to exclude particles other than bivalve larvae.

Statistical analysis

Differences in hatching rate, mortality and growth were tested with analysis of variance (ANOVA). For multiple pairwise comparisons among means the post-hoc SNK test was used. For all test, differences were considered significant at $p<0.05$. All statistical tests were performed on Sigmaplot© software.

Results

The experiment was terminated after day 9 due to the high mortality registered. The seawater carbonate chemistry variables registered for the three treatments are presented in Table 4.1.

The hatching rate success was higher in the control treatment (CT) with 34.1%±11.7 decreasing in ΔpH=-0.3 (19.9%±7.6) and even more in ΔpH=-0.6 (10.4%±5.5) (Figure 4.1). Statistical differences were found only between CT treatment and the most extreme treatment ΔpH=-0.6 (ANOVA, F=6.2, df=2, p=0.035; SNK, q=5.0, $p<0.05$). In day 2 of the experiment a large amount of larvae were still in trocophore stage in all treatments and therefore no statistical differences were found among them. In day 5, all the larvae in CT and ΔpH=-0.3 were in a D-shape stage whereas in ΔpH=-0.6 13.0%±3.8 of the larvae were still in the trocophore stage.

Table 4.1. Seawater carbonate chemistry variables (Mean ± SE); Measurements of pH (total scale), Temperature (T), Salinity (S) and Total Alkalinity (TA); Dissolved inorganic carbon (DIC); partial pressure of CO_2 in seawater (pCO_2) and saturation state for calcite (Ωcal) and aragonite (Ωara) were calculated from in situ T and S, corrected pH and TA.

ΔpH	T (ºC)	S	pH_T	TA (µmol Kg^{-1})	DIC (µmol Kg^{-1})	*p*CO2 (µatm)	$\Omega_{cal.}$	$\Omega_{ara.}$
Fist spawning (11/05/2012 - 19/05/2012)								
CT	22,08 ± 0,04	31,41 ± 0,01	7,93 ± 0,01	3506 ± 8	3250 ± 14	890 ± 35	5,44 ± 0,15	3,53 ± 0,10
-0,3	22,21 ± 0,10	31,40 ± 0,02	7,54 ± 0,02	3481 ± 4	3426 ± 11	2344 ± 179	2,46 ± 0,17	1,60 ± 0,11
-0,6	22,29 ± 0,16	31,40 ± 0,02	7,27 ± 0,02	3485 ± 6	3556 ± 14	4555 ± 167	1,34 ± 0,05	0,87 ± 0,03
Field samppling (24/05/2012)								
	20,20 ± 1,72	35,96 ± 0,19	8,01 ± 0,04	3454 ± 34	3061 ± 32	641 ± 62	7,54 ± 0,58	4,98 ± 0,39

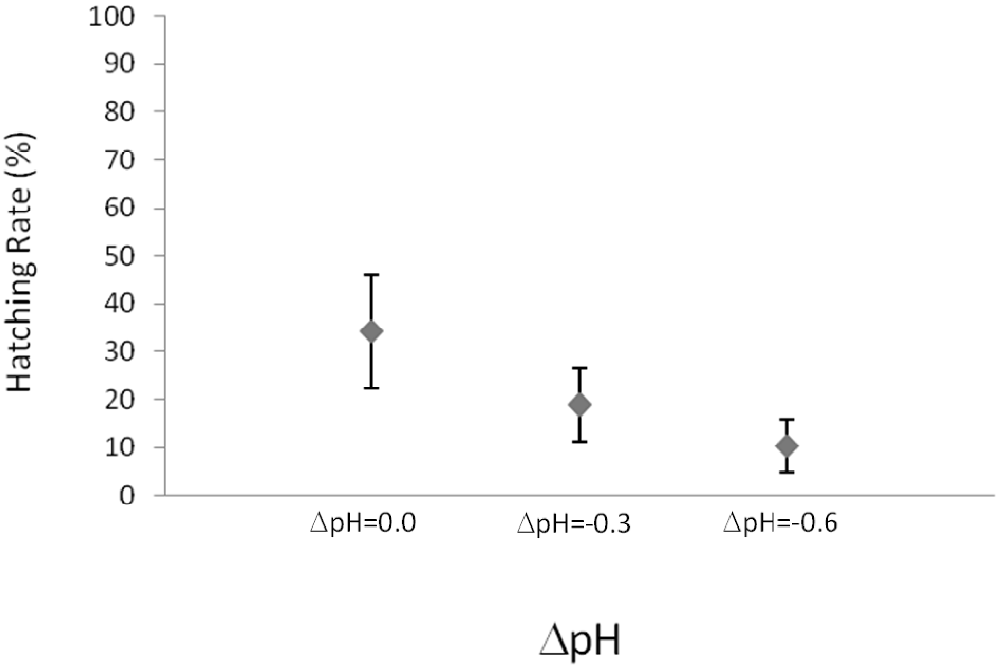


Figure 4.1. Hatching rate percentage for Donax trunculus, 48 h after spawning, in the control ΔpH=0.0 (CT) in ΔpH=-0.3 (medium) and ΔpH=-0.6 (extreme) treatments (mean±sd).

The number of larvae alive decreased in the three treatments during the time of experiment. Significant differences between treatments were only found in day 5 (ANOVA, F=6.0, df=2, p=0.037) and only between the CT and the ΔpH=-0.3 (SNK, q=4.83, p<0.05).

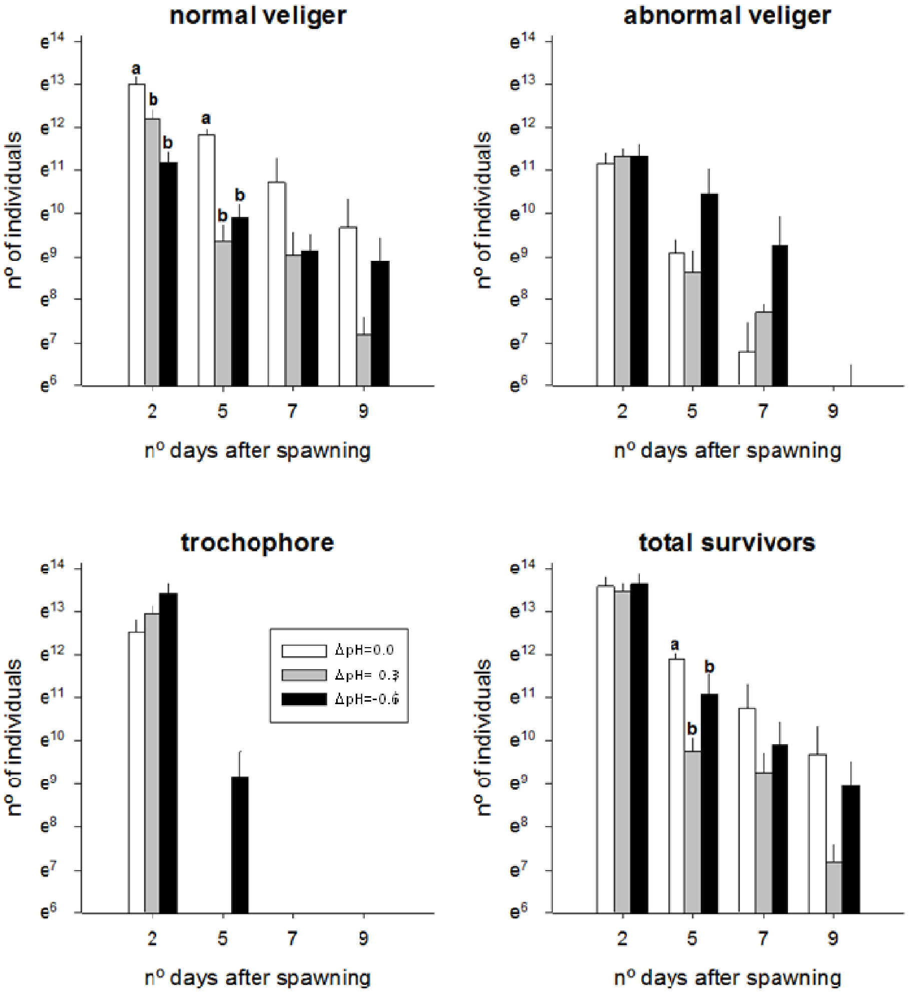


Figure 4.2. Abundance of *Donax trunculus* veligers (normal and abnormal), trochophore larvae and total survivors (trochophore plus veligers) during the 9 days of experiment, for the three treatments (ΔpH=0.0; ΔpH=-0.3; ΔpH=-0.6), (standard error bars displayed).

The amount of normal larvae in veliger stage decreased along the experiment, with ΔpH=-0.3 decreasing more than the ΔpH=-0.6 treatment. In day 2 differences between the CT treatment and the ΔpH=-0.3 and ΔpH=-0.6 were found (ANOVA, F=11.1, df=2, p=0.01; SNK, q=4.3, p<0.05 and SNK, q=6.6, p<0.05 respectively) and in day 5 differences between the CT and both ΔpH=-0.3 and ΔpH=-0.6 were registered (ANOVA, F=30.8, df=2, p<0.001; SNK, q=9.9, p<0.05 and, q=9.2, p<0.05 respectively). No differences were found among treatments in days 7 and 9 (Figure 4.2).

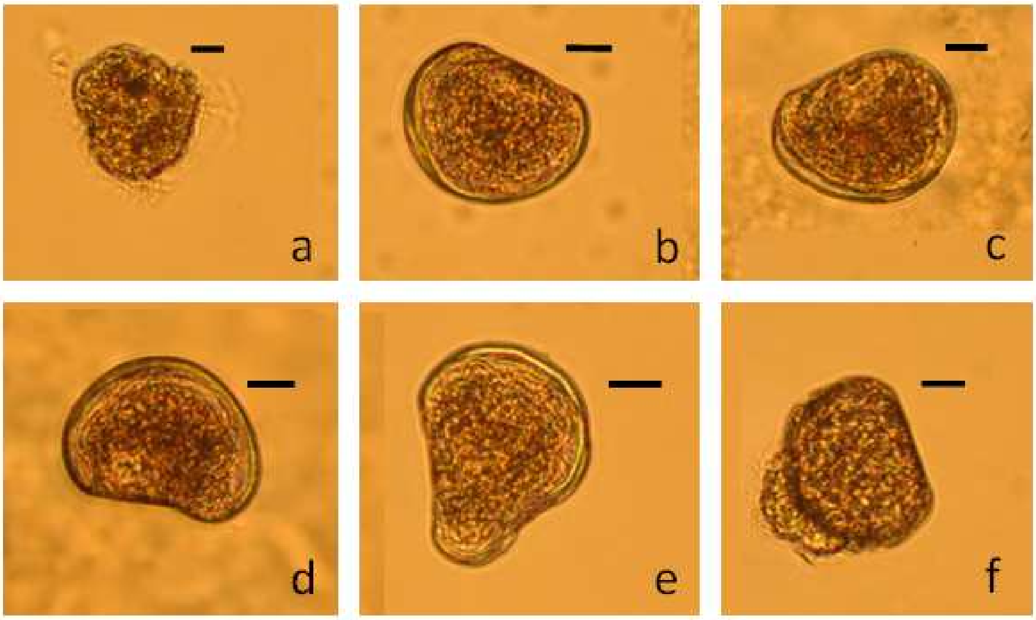


Figure 4.3 – Larvae of *Donax trunculus* (**a** – trochophore; **b** – normal D-larvae; **c** – abnormal D-larvae with unequal valves; **d** – abnormal D-larvae with convex hinge; **e** – abnormal D-larvae with shell protuberances; **f** – abnormal D-larvae with mantle protuberant) (bars displayed = 10µm length).

The amount of abnormal veligers (Figure 4.3) also decreased along the experiment but with a higher amount of abnormal veligers in the ΔpH=-0.3 treatment and even higher in the more acidified treatment ΔpH=-0.6. Notwithstanding, no statistical differences were found among treatments in any day of the experiment (Figure 4.2).

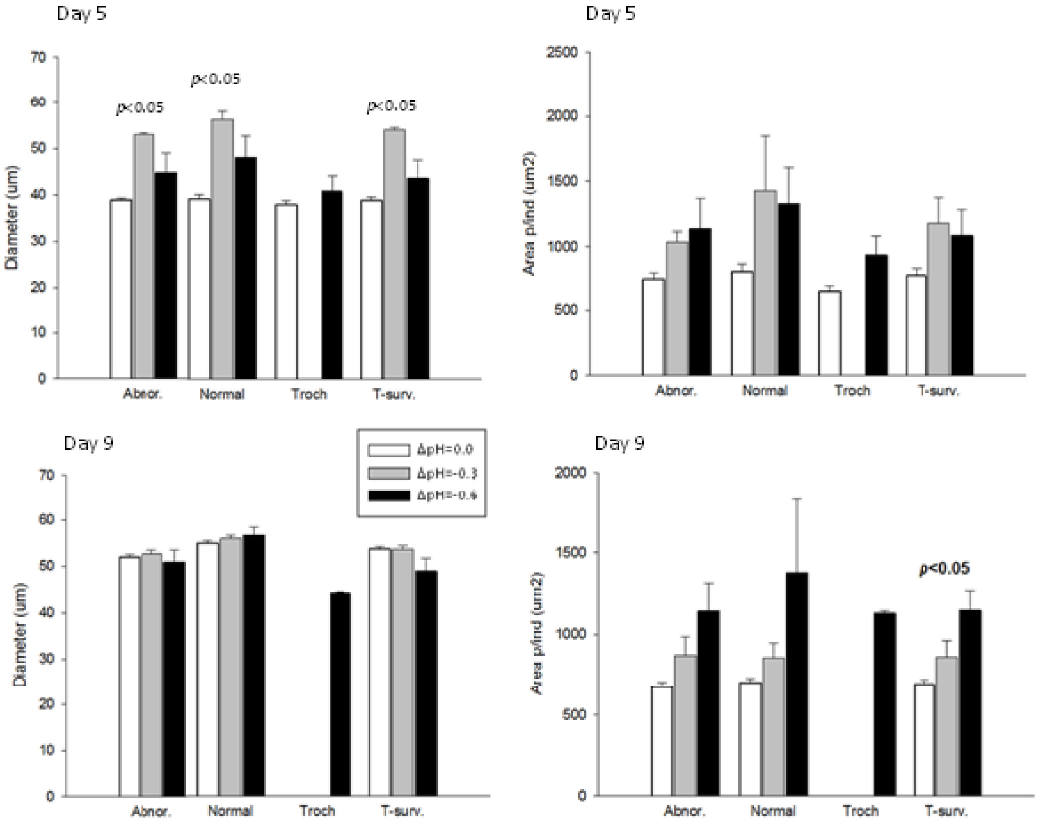


Figure 4.4. Diameter (µm) and Area (μm^2) of *Donax trunculus* veligers (normal and abnormal), trochophore larvae and total survivors (trochophore plus veligers) measured on days 5 and day 9 of the experiment in the three treatments (ΔpH=0.0; ΔpH=-0.3; ΔpH=-0.6), (standard error bars displayed).

The image analysis of larvae collected in days 5 and 9 of the experiment revealed that in day 5 the veligers in CT treatment were smaller than the ones in the acidified treatments (Figure 4.4). A statistical difference was found in normal veligers (ANOVA, F=8.25, df=2, $p<0.05$), abnormal veligers (ANOVA, F=8.16, df=2, $p<0.05$) and in total veligers alive (ANOVA, F=11.46, df=2, $p<0.001$). In day 9, the diameter of veligers in the CT increased and no differences to other treatments were found. The area of larvae determined with image analysis reveal that in day 5 the normal, abnormal veligers and trochophore larvae from the CT treatment were smaller than the ones in acidified treatments. This smaller area remained in day 9 of the experiment only with a significant difference registered in total survivors (ANOVA, F=6.74, df=2, $p<0.05$). The image processing revealed that in day 9, trochophore larvae still existed in the ΔpH=-0.6 treatment (Figure 4.4).

Discussion

The projections for global ocean acidification indicate that this process will continue in this century, affecting coastal ecosystems more vulnerable to CO_2 increase (Talmage & Gobler 2011), in particular bivalve larvae, which are more sensitive to low pH and future acidification than other organism groups (Fabry *et al.* 2008). The knowledge on the effects of ocean acidification in the development of bivalve larvae is still limited and the responses that each species may present in face of such effect are diverse.

In natural conditions, after spawning occurs, the wedge shell eggs develop into trochophore in few hours and after a 24 hours period hatch into D-larvae. In our data we found that 48 hours after the beginning of the experiment, not all larvae hatched into D-larvae and trochophore were still present in all treatments. At this time, the HR success was lower than registered in previous experiments by the authors, although HR may oscillate greatly during spawning season, being intimate related to the energetic reserves available in eggs (Pereira *et al. submited*). Although a decrease in HR was observed in ΔpH=-0.3 and in ΔpH=-0.6 (figure 1), the statistical difference between the CT and the ΔpH=-0.6 treatment indicate that in an extreme scenario, the hatching rate success is negatively affected. Similar effects were registered in *Crassostrea gigas* (Kurihara, Kato, & Ishimatsu 2007; Barros *et al.* 2013), *Mytillus galloprovincialis* (Kurihara *et al.* 2009), *Crassostrea virginica*, *Mercenaria mercenaria* (Calabrese & Davis 1966) and in *Macoma balthica* (Van Colen *et al.* 2012). Moreover, in this most extreme treatment, our data also shows a delay in hatching, since we still found trochophore larvae in day 5 and in day 9 (Figure 4.3). This delay implies that all structure development is also postponed, including the feeding structures that allow veligers to feed and get energy from the environment. Relying for a longer period on the reserves provided by genitors, may lead larvae to die if the energetic reserves do not suffice energetic needs. Since the main energetic source in the eggs of *D. trunculus* are total lipids, larvae need to be in high condition to survive such delay and successfully hatch.

There was a great decrease in veligers found alive during the 9 days of experiment. However, this decrease occurred more rapidly than we expected. In other species, a diverse total of experiment days were found, e.g. *Crassostrea gigas* – 6 days (Barros *et al.* 2013); *Ostrea edulis* – 18 days (Bamber 1990); *Macoma balthica* - 19 days (Van Colen *et al.* 2012). The present experiment was ended after day 9 due the 100% mortality observed in all treatments. We believe that this rapid mortality is mainly due to the species biology rather than the acidification process. The wedge shell has a planktonic stage for about a month after which it settles. In previous experiments conducted by the authors, high mortalities were registered and maintaining larvae after the umbonate stage was extremely difficult (unpublished data).

The differences registered in the amount of normal veligers in all treatments in days 2 and 5 reveal the negative effect of a higher pCO_2 in the first days of life. This general decrease observed in all treatments is normal in marine invertebrates, that have a natural high mortality in early life stages (Pedersen *et al.* 2008) although as suggested by Jansson, Norkko & Norkko (2013) even a slight change in survivorship may have long-lasting consequences.

The extreme acidified treatment seems to produce more abnormal larvae than the medium and CT treatment although we found no statistical difference. However the negative impact that pCO_2 increase have in calcifying stages in benthic mollusks was demonstrated by Fabry *et al* (2008) and found to produce shell abnormalities such as indented shell margins (*Crassostrea gigas*) (Kurihara, Kato, & Ishimatsu 2007) and convex hinge and protuberating mantle (*Macoma balthica*, *Saccostrea glomerata*, *Crassostrea gigas*) (Parker, Ross, & O’Connor 2010; Van Colen *et al.* 2012)

The image analyzes in day 5, revealed that the initial diameter in the acidified treatments was higher than the observed in CT, which was not expected to occur. Similar growth increments under ΔpH=-0.5 was reported in *Mytillus edulis*, although the author has attributed such increments to random variation (Berge *et al.* 2006). Nevertheless our data does not agree with the general pattern of inhibition/decrease in shell growth registered in larvae from a variety of species such as *Macoma balthica*, *Crassostrea gigas*, *Crassostrea virginica*, *Mytilus galloprovincialis*, *Mercenaria mercenaria* and *Argopecten irradians* when exposed to pH treatments between 7.4 and 7.8 (Kurihara *et al.* 2009; Talmage & Gobler 2011; Van Colen *et al.* 2012; Jansson, Norkko, & Norkko 2013; White *et al.* 2013; Barros *et al.* 2013). However in day 9 this difference cease to exist and the diameter among all treatments did not differ significantly. It seems that in first days under acidified treatment, the wedge shell larvae invested in growth, maybe to better resist the adverse environment since smaller larvae cannot feed as well as larger and therefore are more susceptible to starvation (Hart & Strathann 1995; Kurihara, Kato, & Ishimatsu 2007). Moreover, higher mortality occurring in the acidified treatments may have benefited the remaining individuals with more food availability, favoring growth. This food availability effect may outweighed pCO_2 effect as observed by Thomsen *et al* (2013) in *Mytilus edulis* larvae where growth was primarily dependent on food supply rather than pCO_2 increase. This increase in growth registered in the first days may potentiate survival in later post-larva stages (Bonsdorff, Norkko, & Boström 1995).

Our experiment is a first step to understand the effects of acidification in the wedge shell. It is important to notice that scenarios predicted by IPCC are long term scenarios that will occur in a century timeframe and some of these detrimental effects may be attenuated by the capacity of the organisms to acclimate or adapt to elevated pCO_2 over that period (Parker *et al.* 2012). The effect of ocean acidification should be considered as a serious threat to economically important resources for local economy, such as the wedge shell is. As demonstrated by our results, higher pCO_2 has a detrimental effect on wedge shell larvae survival in both acidified treatments and a negative impact in hatching success and hatching delay in the most acidified one.

The delay in the hatching could prolong the planktonic phase of later spawned larvae (late July) beyond September, when the hydrodynamics in the Algarve coast (south Portugal) increases washing larvae offshore from suitable sediment to settle. This may impact severely wedge shell populations since the population dynamic is largely dependent on settlement and recruitment success of post-larvae (Pedersen *et al.* 2008). This is a first insight on the effect of the pCO_2 increase in *Donax trunculus* early life stage, further studies should be also made on the effect of pCO_2 increase in shell formation and structure.

Acknowledgements

We are grateful to Sr. Mauricio Teixeira for system maintenance assistance. This work was supported by the grants SFRH/BD/40430/2007 and SFRH/BPD/69959/2010, awarded by Fundação para a Ciência e Tecnologia to AMP and PR, respectively.

Chapter V

General conclusions

Chapter V: General conclusions

The present work aimed at improving the knowledge on the planktonic phase of the wedge shell, filling the gap currently existing on the information about *Donax trunculus* larvae in the Algarve coast. The main goal was to develop tools and methodologies that could be used to help predict possible recruitment failures earlier than it is done presently. The tools and information here obtained are not meant to be used as standalone tools neither to replace traditional stock assessment methodologies, but as an important complement to the annual information on adult population presently obtained by IPMA, contributing to the understand of earlier life stages of the wedge shell.

This study focused on the spawning season, a critical period of the life cycle of the wedge shell. The information obtained and presented in chapter two is the result of data collection in three spawning season's (2009, 2010 and 2011). This period was chosen due to the necessity of collecting broodstock specimens ready to spawn as well as larvae from the broodstock and from the meroplankton.

The following up of the condition on broodstock prior spawning and later, the quality of the eggs and post hatched D-larvae was achieved. The condition index revealed to be an important indicator of the broodstock condition in this particular period, with odd events, such as the long periods of high SST registered in 2011, affecting the energetic reserves and therefore the condition of the broodstock. The wedge shell revealed to be sensitivity to the presence of phycotoxins that affect negatively the ready to spawn adults, delaying the spawning event. Despite the large spawning period that this species has, the delay in the spawning may reduce the availability of larvae in the water if it is to prolonged in time.

Monitoring the condition of the broodstock during the spawning season is an easy task with a low cost and effort, providing important information on the quality of the wedge shell during spawning. After spawning occurs, the fertilized eggs must rely on the energetic reserves provided by the progenitors to successfully hatch into D-larvae. Also in chapter two the quality of both eggs and larvae were studied and was demonstrated that eggs with less energetic reserves hatch less successfully into D-shape and, in eggs and larvae, the total lipids are the main energetic source that allow eggs to survive and hatch. Therefore it is important to monitor eggs quality to detect if hatching may be compromised affecting the amount of D-shape larvae availability. During this study it was found that the Total Lipids plus Carbohydrates to Protein Ratio may be used successfully to predict hatching success. This type of information is usually used in aquaculture to produce high value market bivalves but has not been previously used in bivalve stock management, being a first step into an earlier detection of future recruitment failure, complementing the presently used methodologies. Notwithstanding, further studies on larval survival should be conducted to understand how larvae quality is reflected in the survival rate through the entire planktonic phase until their metamorphose and settlement.

After hatching, successful veligers of *D. trunculus* larvae will disperse along the coast, but only some will find suitable sediment to settle, grow and recruit into adult population. At the present, no information neither methodologies exist to monitor the abundance of wedge shell larvae on the water. Monitoring the wedge shell larvae along the coast could help perceive larvae abundances variations and relate the quantity of larvae to recruitment in the following year, therefore in chapter III, we developed a methodology that allowed us to obtain information on the quantity of larvae present in the water by using Real-time PCR. The quantification of larvae from a single species in meroplanktonic samples would be a great improvement in larvae dynamic studies, especially on large sets of samples. Moreover, this quantification should be practical to obtain and could be implemented at a low cost.

Quantifying larva from meroplanktonic samples is only possible if the results obtained are promising and usable. The surveys currently held by IPMA are a great platform to collect data along the coast, with little increase in the time and effort of the survey. The results in chapter III showed that discriminating *D. trunculus* from the other *Donax* species present in the Algarve coast is possible and easy to achieve with an 5S primer developed and tested in the first part of this chapter. Nonetheless, it was necessary to obtain information on the quantity of larvae in the water column. New specific primers were design focusing on ITS-1 rDNA and COI mDNA regions and although discrimination was achieved successfully for these two regions, the standard curve linearity was only obtained with success for the ITS-1. Since we detected size variability in the larvae, we used Ct-values ranges, representing larvae abundances intervals, thus avoiding the need of a previous triage of larvae according to their size, a step that would nullify the main purpose of this method: fast and easy to apply to samples collected from the environment.

While larvae information is important to improve predictions on recruitment failure or success, and should be obtained annually with the information presently collected on adult population, some factors may slowly affect the development of larvae of the wedge shell. Such is the case of the ocean acidification; a slow process that according to IPCC predictions will not stop in the next decades. In chapter IV the two scenarios predicted by IPCC were simulated and their effect on larvae was assessed to understand how the pCO_2 increase in sea water may affect larvae development. It was evident that the most extreme scenario predicted (ΔpH=-0.6) was the one most affecting the wedge shell larvae. This increase in the pCO_2 not only reduced the hatching rate success but also delayed it, revealing that trochopore larvae may be found up to 9 days after fecundation. Along with the reduction in hatching success and survival, acidification increases the amount of abnormal larvae. Further experiments on the structure of the shell of larvae under acidified conditions should be made, growing larvae in these scenarios until settlement in order to understand how shell structure and resistance is affected. The effect of ocean acidification should be considered a serious threat to economically important resources, such as the wedge shell. This is of utmost importance in future studies, in order to understand if the sector must adapt their capture methodologies and gears to maintain shell intact upon capture.

The results obtained during this thesis revealed that it is possible and desirable to use information on larvae to improve the management and, in the future, timely predict possible recruitment failures of this important resource. The studies conducted allowed monitoring the condition of adults and larvae, their quantity and presence in the water column during the spawning season, easily and at low cost. The current surveys held by IPMA could be used as a platform to sample collection without extra cost providing access to the entire Algarve coast rapidly. It is important to group some information that is already available but disperse, such as the SST, phycotoxins and chlorophyll-a concentration. This thesis is a starting point to better understand how the planktonic phase may be reflected in the recruitment of the species and give the bivalve fishing sector and decisions makers' new tools to improve the management of this economical important resource. Further studies should be made on the later phase of the plankonic phase, in particular when metamorphose occurs and veligers became benthic pediveligers, initiating their life in the sediment. Also the quantification methodology should be improved to detect more accurately, the number of larvae. To determine how the results obtained impact the adult population over time, further studies linking larvae data to adult population data should be implemented prior to management measures design. Data presented in this work is easily obtained, increasing substantially the information available on this species and should be considered in future survey programs on *Donax trunculus*.

General references

Acker, J.G. & Leptoukh, G. (2007) Online analysis enhances use of NASA Earth science data. *Eos, Transactions American Geophysical Union*, **88**, 14–17.

Altug, G., Cardak, M. & Ciftci, P.S. (2008) Indicator and other bacteria in striped venus (*Chamelea gallina*, L.) and wedge clam (*Donax trunculus*) from the Northern coast of the Sea of Marmara, Turkey. *Journal of Shellfish Research*, **27**, 783–788.

Ansell, A.D., Frenkiel, L. & Moueza, M. (1980) Seasonal changes in tissue weight and biochemical composition for the bivalve *Donax trunculus* L on the Algerian coast. *Journal of Experimental Marine Biology and Ecology*, **45**, 105–116.

Ansell, A. & Lagardère, F. (1980) Observations on the biology of *Donax trunculus* and *D. vittatus* at Ile d'Oleron (French Atlantic Coast). *Marine biology*, **300**, 287–300.

Aranishi, F. & Okimoto, T. (2006) A simple and reliable method for DNA extraction from bivalve mantle. *Journal of applied genetics*, **47**, 251–4.

Asensio Gil, L. (2007) PCR-based methods for fish and fishery products authentication. *Trends in Food Science & Technology*, **18**, 558–566.

Asensio, L., González, I., Rojas, M., García, T. & Martín, R. (2009) PCR-based methodology for the authentication of grouper (*Epinephelus marginatus*) in commercial fish fillets. *Food Control*, **20**, 618–622.

Bamber, R. (1990) The effects of acidic seawater on three species of lamellibranch mollusc. *Journal of Experimental Marine Biology and Ecology*, **143**, 181–191.

Barber, B. & Blake, N. (1981) Energy storage and utilization in relation to gametogenesis in *Argopecten irradians concentricus* (say). *Journal of Experimental Marine Biology and Ecology*, **52**, 121–134.

Barber, B. & Blake, N. (1985) Intra-organ biochemical transformations associated with oogenesis in the bay scallop, *Argopecten irradians concentricus* (Say), as indicated by 14C incorporation. *The Biological Bulletin*, **168**, 39–49.

Barros, P., Sobral, P., Range, P., Chícharo, L. & Matias, D. (2013) Effects of sea-water acidification on fertilization and larval development of the oyster *Crassostrea gigas*. *Journal of Experimental Marine Biology and Ecology*, **440**, 200–206.

Bayed, A. (1990) Chromosome number and karyotype of *Donax trunculus* L (Mollusca, Bivalvia, Tellinacea). *Cahiers de Biologie Marine*, **31**, 159–169.

Bayed, A. & Guillou, J. (1985) A contribution to the study of populations of the *Donax* genus. The Population of *Donax trunculus* L (Mollusca, Bivalvia). *Annales de L'Institut Oceanographique*, **61**, 139–147.

Bayne, B.L. (1976) Aspects of reproduction in bivalve molluscs. *Estuarine processes*, 432–448.

Beldi, H. & Gimbert, F. (2006) Seasonal variations of Cd, Cu, Pb and Zn in the edible mollusc *Donax trunculus* (Mollusca, Bivalvia) from the gulf of Annaba, Algeria. *African Journal of Agricultural Research*, **1**, 85–90.

Bell, J. & Grassle, J. (1998) A DNA probe for identification of larvae of the commercial surfclam (*Spisula solidissima*). *Molecular marine biology and biotechnology*, **7**, 127–137.

Beninger, P.G. & Lucas, A. (1984) Seasonal variations in condition, reproductive activity, and gross biochemical composition of two species of adult clam reared in a common habitat: *Tapes decussatus* L. (Jeffreys) and *Tapes philippinarum*

(Adams & Reeve). *Journal of Experimental Marine Biology and Ecology*, **79**, 19–37.

Bensadoun, A. & Weinstein, D. (1976) Assay of proteins in the presence of interfering materials. *Analytical Biochemistry*, **70**, 241–250.

Berge, J.A., Bjerkeng, B., Pettersen, O., Schaanning, M.T. & Øxnevad, S. (2006) Effects of increased sea water concentrations of CO2 on growth of the bivalve *Mytilus edulis* L. *Chemosphere*, **62**, 681–7.

Beukema, J. & De Bruin, W. (1979) Calorific values of the soft parts of the tellinid bivalve *Macoma balthica* (L.) as determined by two methods. *Journal of Experimental Marine Biology and Ecology*, **37**, 19–30.

Bimboim, H.C. & Doly, J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* , **7** , 1513–1523.

Bligh, E. & Dyer, W. (1959) A rapid method for total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*, **37**, 911–917.

Bodoy, A. (1982) Seasonal growth of the bivalve *Donax trunculus* (L) in the northwestern mediterranean (France). *Malacologia*, **22**, 353–358.

Bonsdorff, E., Norkko, A. & Boström, C. (1995) Recruitment and population maintenance of the bivalve *Macoma balthica* (L.)—factors affecting settling success and early survival on shallow sandy bottoms. *Proceedings of the 28th European Marine Biological Symposium.* (eds A. Eleftheriou, A. Ansell & C. Smith), pp. 253– 260. Olsen and Olsen., Fredensborg.

Bos, O.G., Hendriks, I.E., Strasser, M., Dolmer, P. & Kamermans, P. (2006) Estimation of food limitation of bivalve larvae in coastal waters of north-western Europe. *Journal of Sea Research*, **55**, 191–206.

Boussoufa, D., Ghazali, N., Viciano, E., Navarro, J. & El Cafsi, M. (2011) Seasonal variation in condition and fatty acid composition of coquina clam, *Donax trunculus* (Linnaeus 1758)(Mollusca: Bivalvia) from the Tunisian coast. *Cahiers de biologie Maritime*, **52**, 47–56.

Bresler, V. & Fishelson, L. (1994) Microfluorometrical study of bioaccumulation of benzo(a)pyrene and marker xenobiotics in the bivalve *Donax trunculus* from clean and polluted sites along the Mediterranean shore of Israel. *Diseases of Aquatic Organisms*, **19**, 193–202.

Bricelj, V. & Shumway, S. (1998) Paralytic shellfish toxins in bivalve molluscs: occurrence, transfer kinetics, and biotransformation. *Reviews in Fisheries Science*, **6**, 315–383.

Broecker, W. & Takahashi, T. (1966) Calcium carbonate precipitation on the Bahama Banks. *Journal of Geophysical Research*, **71**, 1575.

Burke, K., Bataller, É. & Miron, G. (2008) Larval quality of a nonnative bivalve species (European oyster, *Ostrea edulis*) off the east Canadian coast. *Journal of Shellfish Research*, **27**, 701–710.

Calabrese, A. & Davis, H.C. (1966) The pH tolerance of embryos and larvae of *Mercenaria mercenaria* and *Crassostrea virginica*. *Biological Bulletin*, **131**, 427–436.

Caldeira, K. & Wickett, M. (2003) Anthropogenic carbon and ocean pH. *Nature,* **425**, 365–365.

Cardenas, E.B. & Aranda, D.A. (2000) A review of reproductive patterns of bivalve mollusks from Mexico. *Bulletin of Marine Science*, **66**, 13–27.

Carrera, E., García, T., Céspedes, A., González, I., Sanz, B., Hernández, P.E. & Martín, R. (1997) Immunostick Colorimetric ELISA Assay for the Identification of Smoked Salmon, Trout and Bream. *Journal of the Science of Food and Agriculture*, **74**, 547–550.

Cerón-Ortiz, A.N., Cordero, B., Arredondo-Vega, B.O. & Voltolina, D. (2009) Effect of algal diet and temperature on survival, growth and biochemical composition of spat of the lion's paw scallop *Nodipecten subnodosus*. *Aquaculture*, **298**, 64–69.

Chícharo, M., Amaral, A., Condinho, S. & Alves, F. (2003) Adenylic-derived indices and reburying time as indicators of the effects of dredging-induced stress on the clam Spisula solida. *Marine Biology*, **142**, 1113–1117.

Chícharo, L. & Chícharo, M. (2001) Effects of environmental conditions on planktonic abundances, benthic recruitment and growth rates of the bivalve mollusc *Ruditapes decussatus* in a Portuguese coastal lagoon. *Fisheries research*, **53**, 235–250.

Van Colen, C., Debusschere, E., Braeckman, U., Van Gansbeke, D. & Vincx, M. (2012) The early life history of the clam *Macoma balthica* in a high CO2 world. *PloS one*, **7**, e44655.

Contreras, A., Marsden, I. & Munro, M. (2012) Effects of short-term exposure to paralytic shellfish toxins on clearance rates and toxin uptake in five species of New Zealand bivalve. *Marine and Freshwater Research*, **63**, 166–174.

Cooley, S.R., Lucey, N., Kite-Powell, H. & Doney, S.C. (2012) Nutrition and income from molluscs today imply vulnerability to ocean acidification tomorrow. *Fish and Fisheries*, **13**, 182–215.

Cornet, M. & Soulard, C. (1990) Chromosome number and karyotype of *Donax trunculus* L (Mollusca, Bivalvia, Tellinacea). *Genetica*, **82**, 93–97.

Da Costa, F., Nóvoa, S., Ojea, J. & Martínez-Patiño, D. (2011) Changes in biochemical and fatty acid composition of the razor clam *Solen marginatus* (Solenidae: Bivalvia) during larval development. *Marine Biology*, **158**, 1829–1840.

Costa, M., Silva, R. & Vitorino, J. (2001) Contribuição para o estudo do clima de agitação marítima na costa portuguesa. *Proceedings das 2as Jornadas Portuguesas de Engenharia Costeira e Portuária*.

Crosby, M. & Gale, L. (1990) A review and evaluation of bivalve condition index methodologies with a suggested standard method. *Journal of Shellfish Research*, **9**.

Dahlhoff, E.P. (2004) Biochemical indicators of stress and metabolism: applications for marine ecological studies. *Annual review of physiology*, **66**, 183–207.

Delgado, E. & Defeo, O. (2007) Tisular and population level responses to habitat harshness in sandy beaches: the reproductive strategy of *Donax hanleyanus*. *Marine Biology*, **152**, 919–927.

Deval, M.C. (2009) Growth and reproduction of the wedge clam (*Donax trunculus*) in the Sea of Marmara, Turkey. *Journal of Applied Ichthyology*, **25**, 551–558.

Donn, T.E., Clarke, D.J., Mclachlan, A. & Dutoit, P. (1986) Distribution and abundance of *Donax serra* Roding (Bivalvia, Donacidae) as related to beach morphology. 1. semilunar migrations. *Journal of Experimental Marine Biology and Ecology*, **102**, 121–131.

Dover, G., Coen, E. & Strachan, T. (1982) The dynamics of sequence turnover and concerted evolution of families of DNA, rDNA, histone genes and non-coding DNA of Drosophila. *Heredity*, **49**, 141–141.

Dridi, S., Romdhane, M.S. & Elcafsi, M. (2007) Seasonal variation in weight and biochemical composition of the Pacific oyster, *Crassostrea gigas* in relation to the gametogenic cycle and environmental conditions of the Bizert lagoon, Tunisia. *Aquaculture*, **263**, 238–248.

Endo, N., Sato, K., Matsumura, K., Yoshimura, E., Odaka, Y. & Nogata, Y. (2010) Species-specific detection and quantification of common barnacle larvae from the Japanese coast using quantitative real-time PCR. *Biofouling*, **26**, 901–911.

Ezgeta-Balić, D., Rinaldi, A., Peharda, M., Prusina, I., Montalto, V., Niceta, N. & Sarà, G. (2011) An energy budget for the subtidal bivalve *Modiolus barbatus* (Mollusca) at different temperatures. *Marine environmental research*, **71**, 79–85.

Fabry, V., Seibel, B., Feely, R. & Orr, J. (2008) Impacts of ocean acidification on marine fauna and ecosystem processes. *ICES Journal of Marine Science*, **65**, 414–432.

Falcão, M., Santos, M.N., Vicente, M. & Monteiro, C.C. (2007) Biogeochemical processes and nutrient cycling within an artificial reef off Southern Portugal. *Marine environmental research*, **63**, 429–44.

Fearman, J. & Moltschaniwskyj, N.A. (2010) Warmer temperatures reduce rates of gametogenesis in temperate mussels, *Mytilus galloprovincialis*. *Aquaculture*, **305**, 20–25.

Feely, R., Doney, S. & Cooley, S. (2009) Ocean acidification: present conditions and future changes in a high-CO2 world. *Oceanography*, **22**, 36–47.

Feely, R., Sabine, C., Lee, K. & Berelson, W. (2004) Impact of anthropogenic CO2 on the CaCO3 system in the oceans. *Science*, **305**, 362–366.

Ferguson, A., Taggart, J., Prodöhl, P., McMeel, O., Thompson, C., Stone, C., McGinnity, P. & Hynes, R. (1995) The application of molecular markers to the study and conservation of fish populations, with special reference to *Salmo*. *Journal of Fish biology*, **47**, 103–126.

Fernández-Reiriz, M., Range, P., Álvarez-Salgado, X., Espinosa, J. & Labarta, U. (2012) Tolerance of juvenile *Mytilus galloprovincialis* to experimental seawater acidification. *Marine Ecology Progress Series*, **454**, 65–74.

Fernández-Reiriz, M., Range, P., Álvarez-Salgado, X. & Labarta, U. (2011) Physiological energetics of juvenile clams *Ruditapes decussatus* in a high CO2 coastal ocean. *Marine Ecology Progress Series*, **433**, 97–105.

Fernandez-Tajes, J. & Méndez, J. (2007) Identification of the razor clam species Ensis arcuatus, E. siliqua, E. directus, E. macha, and Solen marginatus using PCR-RFLP analysis of the 5S rDNA region. *Journal of agricultural and food chemistry*, **55**, 7278–82.

Ferreiro, M., Pérez-Camacho, A. & Labarta, U. (1990) Changes in the biochemical composition of *Ostrea edulis* larvae fed on different food regimes. *Marine Biology*, **106**, 395–401.

Fishelson, L., Bresler, V. & Manelis, R. (1999) Toxicological aspects associated with the ecology of *Donax trunculus* (Bivalvia, Mollusca) in a polluted environment. *The Science of the total environment*, **226**, 121–131.

Folch, J., Lees, M. & Sloane-Stanley, G. (1957) A simple method for the isolation and purification of total lipids from animal tissues. *Journal of biological chemistry*, 497–509.

Folin, O. & Malmros, H. (1929) Micro-method for glucose determination in 0.1 ml of blood. *Journal of Biological Chemistry*, **83**, 115.

Fox, C.J., Taylor, M.I., Pereyra, R., Villasana, M.I. & Rico, C. (2005) TaqMan DNA technology confirms likely overestimation of cod (*Gadus morhua* L.) egg abundance in the Irish Sea: implications for the assessment of the cod stock and mapping of spawning areas using egg-based methods. *Molecular ecology*, **14**, 879–84.

Fraser, A.J. (1989) Triacylglycerol Content as a Condition Index for Fish, Bivalve, and Crustacean Larvae. *Canadian Journal of Fisheries and Aquatic Sciences*, **46**, 1868–1873.

Freire, R., Fernández-Tajes, J. & Méndez, J. (2008) Identification of razor clams Ensis arcuatus and Ensis siliqua by PCR–RFLP analysis of ITS1 region. *Fisheries Science*, **74**, 511–515.

Freire, R., Insua, A. & Mendez, J. (2005) *Cerastoderma glaucum* 5S ribosomal DNA: characterization of the repeat unit, divergence with respect to *Cerastoderma edule*, and PCR-RFLPs for the identification of both cockles. *Genome*, **48**, 427–442.

Freitas, R., Ricardo, F., Pereira, F., Sampaio, L., Carvalho, S., Gaspar, M., Quintino, V. & Rodrigues, A.M. (2011) Benthic habitat mapping: Concerns using a combined approach (acoustic, sediment and biological data). *Estuarine, Coastal and Shelf Science*, **92**, 598–606.

Gallager, S. & Mann, R. (1986) Individual variability in lipid content of bivalve larvae quantified histochemically by absorption photometry. *Journal of plankton research*, **8**, 927–937.

Gallager, S.M., Mann, R. & Sasaki, G.C. (1986) Lipid as an index of growth and viability in three species of bivalve larvae. *Aquaculture*, **56**, 81–103.

Garland, E. & Zimmer, C. (2002) Techniques for the identification of bivalve larvae. *Marine Ecology Progress Series*, **225**, 299–310.

Gaspar, M.B. (1996) *Bivalves Do Litoral Oceânico Algarvio: Aspectos Da Biologia, Ecologia e Da Pescaria Dos Mananciais de Interesse Económico: Aplicação à Gestão Dos Recursos*. Universidade do Algarve.

Gaspar, M., Barracha, I., Carvalho, S. & Vasconcelos, P. (2013) Clam fisheries worldwide: main species, harvesting methods and fishing impacts. *Clam fisheries and aquaculture* (ed F. Costa), pp. 291–327. Nova Science Publishers Inc, New York.

Gaspar, M., Castro, M. & Monteiro, C. (1999) Effect of tooth spacing and mesh size on the catch of the Portuguese clam and razor clam dredge. *ICES Journal of Marine Science*, **56**, 103–110.

Gaspar, M. & Chícharo, L. (2007) Modifying dredges to reduce by-catch and impacts on the benthos. *By-catch Reduction in the World's Fisheries* (ed S.J. Kennelly), pp. 95–140. Springer, Dordrecht.

Gaspar, M., Chícharo, L., Vasconcelos, P., Garcia, A., Santos, A. & Monteiro, C. (2002a) Depth segregation phenomenon in *Donax trunculus* (Bivalvia: Donacidae) populations of the Algarve coast (southern Portugal). *Scientia Marina*, **66**, 111–121.

Gaspar, M., Ferreira, R. & Monteiro, C. (1999) Growth and reproductive cycle of *Donax trunculus* L., (Mollusca: Bivalvia) off Faro, southern Portugal. *Fisheries Research*, **41**, 309–316.

Gaspar, M., Leitão, F., Santos, M., Chícharo, M., Dias, M.D., Chícharo, A. & Monteiro, C.C. (2003) A comparison of direct macrofaunal mortality using three types of clam dredges. *ICES Journal of Marine Science*, **60**, 733–742.

Gaspar, M., Leitão, F., Santos, M., Sobral, M., Chícharo, L., Chícharo, A. & Monteiro, C. (2002b) Influence of mesh size and tooth spacing on the proportion of damaged organisms in the catches of the Portuguese clam dredge fishery. *ICES Journal of Marine Science*, **59**, 1228–1236.

Gaspar, M.B. & Monteiro, C.C. (1998) Reproductive cycle of the razor clam *Ensis siliqua* and the clam *Venus striatula* off Vilamoura, Southern Portugal. *Journal of the Marine Biological Association of the United Kingdom*, **78**, 1247–1258.

Gaspar, M.B. & Monteiro, C.C. (1999) Gametogenesis and spawning in the subtidal white clam *Spisula solida*, in relation to temperature. *Journal of the Marine Biological Association of the United Kingdom*, **79**, 753–755.

Gaspar, M., Pereira, A., Vasconcelos, P. & Monteiro, C. (2004) Age and growth of *Chamelea gallina* from the Algarve coast (southern Portugal): influence of seawater temperature and gametogenic cycle on growth rate. *Journal of Molluscan Studies*, **70**, 371–377.

Gazeau, F., Quiblier, C., Jansen, J.M., Gattuso, J.-P., Middelburg, J.J. & Heip, C.H.R. (2007) Impact of elevated CO 2 on shellfish calcification. *Geophysical Research Letters*, **34**, L07603.

Godhe, A., Asplund, M.E., Härnström, K., Saravanan, V., Tyagi, A. & Karunasagar, I. (2008) Quantification of diatom and dinoflagellate biomasses in coastal marine seawater samples by real-time PCR. *Applied and environmental microbiology*, **74**, 7174–82.

Le Goff-Vitry, M., Chipman, A. & Comtet, T. (2007) In situ hybridization on whole larvae: a novel method for monitoring bivalve larvae. *Marine Ecology Progress Series*, **343**, 161–172.

Gómez-Robles, E. & Saucedo, P. (2009) Evaluation of Quality Indices of the Gonad and Somatic Tissues Involved in Reproduction of the Pearl Oyster *Pinctada mazatlanica* with Histochemistry and Digital Image Analysis. *Journal of Shellfish Research*, **28**, 329–335.

Gonzalez Araya, R., Mingant, C., Petton, B. & Robert, R. (2012) Influence of diet assemblage on *Ostrea edulis* broodstock conditioning and subsequent larval development. *Aquaculture*, **364-365**, 272–280.

González, M., Caride, B., Lamas, A. & Taboada, C. (2001) Nutritive value of protein from sea urchin, and its effects on intestinal leucine aminopeptidase and intestinal and hepatic gamma-glutamyltranspeptidase. *International Journal of Food Sciences and Nutrition*, **52**, 219–24.

Gonzalez-Tizon, A., Martinez-Lage, A., Marinas, L., Freire, R., Cornudella, L. & Mendez, Y. (1998) Cytogenetic characterization of *Donax trunculus* (Mollusca, Bivalvia). *Cytogenetics and Cell Genetics*, **81**.

Goodman, D. (1979) Regulating reproductive effort in a changing environment. *American Naturalist*, **113**, 735–748.

Gopalakrishnan, S. & Vijayavel, K. (2009) Nutritional composition of three estuarine bivalve mussels, *Perna viridis*, *Donax cuneatus* and *Meretrix meretrix*. *International journal of food sciences and nutrition*, **60**, 458–63.

Guillou, J. & Bayed, A. (1991) Distributions of *Donax trunculus* L and *Donax venustus* Poli along the Moroccan Atlantic coast as related to environmental factors. *Oceanologica Acta*, **14**, 291–298.

Haberkorn, H., Lambert, C., Le Goïc, N., Moal, J., Suquet, M., Guéguen, M., Sunila, I. & Soudant, P. (2010) Effects of *Alexandrium minutum* exposure on nutrition-related processes and reproductive output in oysters *Crassostrea gigas*. *Harmful Algae*, **9**, 427–439.

Hamdani, A. & Soltani-Mazouni, N. (2011) Changes in biochemical composition of the gonads of *Donax trunculus* L.(Mollusca, Bivalvia) from the Gulf of Annaba (Algeria) in relation to reproductive events and Pollution. *Jordan Journal of Biological Sciences*, **4**, 149–156.

Hare, M., Palumbi, S. & Butman, C. (2000) Single-step species identification of bivalve larvae using multiplex polymerase chain reaction. *Marine Biology*, **137**, 953–961.

Hart, M. & Strathann, R. (1995) Mechanisms and rates of suspension feeding. *Ecology of Marine Invertebrate Larvae* (ed L. McEdwards), pp. 193–222. CRC Press, Boca Raton.

Havenhand, J. & Todd, C. (1988) Physiological ecology of *Adalaria proxima* (Alder et Hancock) and *Onchidoris muricata* (Müller)(Gastropoda: Nudibranchia). I. Freeding, growth and respiration. *Journal of Experimental Marine Biology and Ecology*, **118**, 151–172.

Haws, M., DiMichele, L. & Hand, S. (1993) Biochemical changes and mortality during metamorphosis of the Eastern Oyster, *Crassostrea virginica*, and the Pacific oyster, *Crassostrea gigas*. *Molecular Marine Biology and Biotechnology*, **2**, 207–217.

Heaney, S.A., Maloy, A.P. & Slater, J.W. (2011) Evaluation of fixatives and autofluorescence reduction treatments for marine bivalve larvae. *Journal of the Marine Biological Association of the United Kingdom*, **91**, 1567–1576.

Helm, M., Holland, D., Utting, S. & East, J. (1991) Fatty acid composition of early non-feeding larvae of the European flat oyster, *Ostrea edulis*. *Journal of the Marine Biological Association of the United Kingdom*, **71**, 691–705.

Hendriks, I.E., Duarte, C.M. & Álvarez, M. (2010) Vulnerability of marine biodiversity to ocean acidification: A meta-analysis. *Estuarine, Coastal and Shelf Science*, **86**, 157–164.

Hendriks, I.E., van Duren, L.A. & Herman, P.M. (2003) Effect of dietary polyunsaturated fatty acids on reproductive output and larval growth of bivalves. *Journal of Experimental Marine Biology and Ecology*, **296**, 199–213.

Hess, H., Lees, J. & Derr, J. (1978) A linear Lowry-Folin assay for both water soluble and sodium dodecyl sulfate solubilized proteins. *Analytical Biochemistry*, **85**, 259–300.

Hiebenthal, C., Philipp, E.E.R., Eisenhauer, A. & Wahl, M. (2012) Effects of seawater pCO2 and temperature on shell growth, shell stability, condition and cellular stress of Western Baltic Sea *Mytilus edulis* (L.) and *Arctica islandica* (L.). *Marine Biology*, **160**, 2073–2087.

Hird, H., Chisholm, J., Sanchez, A., Hernandez, M., Goodier, R., Schneed, K., Boltz, C. & Popping, B. (2006) Effect of heat and pressure processing on DNA fragmentation and implications for the detection of meat using a real-time polymerase chain reaction. *Food Additives and Contaminants*, **23**, 645–650.

Hoffmann, V., Pfaff, M. & Branch, G. (2012) Spatio-temporal patterns of larval supply and settlement of intertidal invertebrates reflect a combination of passive transport and larval behavior. *Journal of Experimental Marine Biology and Ecology*, **418-419**, 83–90.

Hofmann, E., Powell, E., Bochenek, E. & Klinck, J. (2004) A modelling study of the influence of environment and food supply on survival of *Crassostrea gigas* larvae. *ICES Journal of Marine Science*, **61**, 596–616.

Holland, D.L. & Gabbott, P.A. (1971) Micro-analytical scheme for determination of protein, carbohydrate, lipid and RNA levels in marine invertebrate larvae. *Marine and Freshwater Biology*, **51**, 659–668.

Holland, D.L. & Hannant, P.J. (1973) Addendum to a microanalytical scheme for the biochemical analysis of marine invertebrate larvae. *Journal of the Marine Biological Association of the United Kingdom*, **53**, 833–838.

Holland, D.L. & Hannant, P.J. (1974) Biochemical changes during growth of the spat of the oyster *Ostrea edulis*. *Journal of the Marine Biological Association of the United Kingdom*, **54**, 1007–1016.

Holland, D.L. & Spencer, B.E. (1973) Biochemical changes in fed and starved oysters, *Ostrea Edulis* L. during larval development, metamorphosis and early spat growth. *Journal of the Marine Biological Association of the United Kingdom*, **53**, 287–298.

Hosoi-Tanabe, S. & Sako, Y. (2005) Species-specific detection and quantification of toxic marine dinoflagellates *Alexandrium tamarense* and *A. catenella* by Real-time PCR assay. *Marine biotechnology (New York, N.Y.)*, **7**, 506–14.

Hout, B., Babin, M., Bruyant, F., Grob, C., Twardowski, M.S. & Claustre, H. (2007) Does chlorophyll a provide the best index of phytoplankton biomass for primary productivity studies? *Biogeosciences Discussions*, **4**, 707–745.

Huz, R.D. la, Lastra, M. & López, J. (2002) The influence of sediment grain size on burrowing, growth and metabolism of *Donax trunculus* L. (Bivalvia: Donacidae). *Journal of Sea Research*, **47**, 85–95.

Ivanina, A. V, Dickinson, G.H., Matoo, O.B., Bagwe, R., Dickinson, A., Beniash, E. & Sokolova, I.M. (2013) Interactive effects of elevated temperature and CO2 levels on energy metabolism and biomineralization of marine bivalves *Crassostrea virginica* and *Mercenaria mercenaria*. *Comparative biochemistry and physiology. Part A, Molecular & integrative physiology*, **166**, 101–11.

Jansson, A., Norkko, J. & Norkko, A. (2013) Effects of Reduced pH on *Macoma balthica* Larvae from a System with Naturally Fluctuating pH-Dynamics. *PloS one*, **8**.

Jensen, P.C., Purcell, M.K., Morado, J.F. & Eckert, G.L. (2012) Development of a Real-time PCR assay for detection of planktonic red king crab (*Paralithodes camtschaticus* (Tilesius 1815)) larvae. *Journal of Shellfish Research*, **31**, 917–924.

Joaquim, S., Gaspar, M., Matias, D., Ben-Hamadou, R. & Arnold, W.S. (2008a) Rebuilding viable spawner patches of the overfished *Spisula solida* (Mollusca: Bivalvia): a preliminary contribution to fishery sustainability. *ICES Journal of Marine Science*, **65**, 60–64.

Joaquim, S., Matias, D., Lopes, B., Arnold, W.S. & Gaspar, M.B. (2008b) The reproductive cycle of white clam *Spisula solida* (L.) (Mollusca: Bivalvia): Implications for aquaculture and wild stock management. *Aquaculture*, **281**, 43–48.

Johnson, L.E. (1995) Enhanced early detection and enumeration of zebra mussel (*Dreissena spp*) veligers using cross-polarized light microscopy. *Hydrobiologia*, **312**, 139–146.

Jones, W.J., Preston, C.M., Marin III, R., Scholin, C. a & Vrijenhoek, R.C. (2008) A robotic molecular method for in situ detection of marine invertebrate larvae. *Molecular ecology resources*, **8**, 540–50.

Kheder, R. Ben, Quéré, C., Moal, J. & Robert, R. (2010) Effect of nutrition on *Crassostrea gigas* larval development and the evolution of physiological indices. Part A: Quantitative and qualitative diet effects. *Aquaculture*, **305**, 165–173.

Kurihara, H., Asai, T., Kato, S. & Ishimatsu, A. (2009) Effects of elevated pCO2 on early development in the mussel *Mytilus galloprovincialis*. *Aquatic Biology*, **4**, 225–233.

Kurihara, H., Kato, S. & Ishimatsu, A. (2007) Effects of increased seawater pCO2 on early development of the oyster *Crassostrea gigas*. *Aquatic Biology*, **1**, 91–98.

Lagos, L. (2012) Effect of temperature on the culture of larvae of the bivalve *Mytilus chilensis* originated from broodstocks from different latitudes in a controlled environment. *Ciencias Marinas*, **38**, 543–550.

Larsen, J., Frischer, M., Rasmussen, L. & Hansen, B. (2005) Single-step nested multiplex PCR to differentiate between various bivalve larvae. *Marine Biology*, **146**, 1119–1129.

Launey, S. & Hedgecock, D. (2001) High genetic load in the Pacific oyster *Crassostrea gigas*. *Genetics*, **159**, 255–265.

Leverone, J.R., Blake, N.J., Pierce, R.H. & Shumway, S.E. (2006) Effects of the dinoflagellate *Karenia brevis* on larval development in three species of bivalve mollusc from Florida. *Toxicon : official journal of the International Society on Toxinology*, **48**, 75–84.

Levin, L.A. (1990) A review of methods for labeling and tracking marine invertebrate larvae. *Ophelia*, **32**, 115–144.

Li, S.-C., Wang, W.-X. & Hsieh, D.P.H. (2002) Effects of toxic dinoflagellate *Alexandrium tamarense* on the energy budgets and growth of two marine bivalves. *Marine environmental research*, **53**, 145–60.

Lin, W.-F. & Hwang, D.-F. (2008) A multiplex PCR assay for species identification of raw and cooked bonito. *Food Control*, **19**, 879–885.

Liu, W., Li, Q., Yuan, Y. & Zhang, S. (2008) Seasonal variations in reproductive activity and biochemical composition of the cockle *Fulvia mutica* (Reeve) from the eastern coast of China. *Journal of Shellfish Research*, **27**, 405–411.

Livi, S., Cordisco, C., Damiani, C., Romanelli, M. & Crosetti, D. (2006) Identification of bivalve species at an early developmental stage through PCR-SSCP and sequence analysis of partial 18S rDNA. *Marine Biology*, **149**, 1149–1161.

Lockley, a. . & Bardsley, R.. (2000) DNA-based methods for food authentication. *Trends in Food Science & Technology*, **11**, 67–77.

Loosanoff, V., Davis, H. & Chanley, P. (1966) Dimensions and shapes of larvae of some marine bivalve mollusks. *Malacologia*, **4**, 351–435.

Lowry, O., Rosebrough, N., Farr, A. & Randall, R. (1951) Protein measurement with the Folin phenol reagent. *Journal of biological chemistry*, **193**, 265–275.

Lutz, R., Goodsell, J., Castagna, M., Chapman, S., Newell, C., Hidu, H., Mann, R., Jablonski, D., Kennedy, V., Siddall, S., Goldberg, R., Beattie, H., Falmagne, C., Chestnut, A. & Partridge, A. (1982) Preliminary observations on the usefulness of hinge structures for identification of bivalve larvae. *Journal of Shellfish Research*, **2**, 65–70.

Macedo, M., Macedo, M. & Borges, J. (1999) *Conchas Marinhas de Portugal*, Editorial . Lisboa.

Machado-Schiaffino, G., Martinez, J.L. & Garcia-Vazquez, E. (2008) Detection of mislabeling in hake seafood employing mtSNPs-based methodology with identification of eleven hake species of the genus *Merluccius*. *Journal of agricultural and food chemistry*, **56**, 5091–5.

Mackie, I., Craig, A., Etienne, M., Jérôme, M., Fleurence, J., Jessen, F., Smelt, A., Kruijt, A., Yman, I.M., Ferm, M., Martinez, I., Pérez-Martín, R., Piñeiro, C., Rehbein, H. & Kündiger, R. (2000) Species identification of smoked and gravad fish products by sodium dodecylsulphate polyacrylamide gel electrophoresis, urea isoelectric focusing and native isoelectric focusing: a collaborative study. *Food Chemistry*, **71**, 1–7.

Mann, R. (1988) Field studies of bivalve larvae and their recruitment to the benthos: a commentary. *Journal of Shellfish Research*, **7**, 7–10.

Mansour, N., Yassien, N., Ibrahim, H.M. & Elmossalami, M.K. (1998) Microbial hazard of salted OM El-Kholoul (wedge shell *Donax trunculus*). *Zentralblatt für Veterinärmedizin. Reihe B. Journal of veterinary medicine. Series B*, **45**, 53–7.

Marsh, J.B. & Weinstein, D.B. (1966) Simple charring method for determination of lipids. *Journal of lipid research*, **7**, 574–6.

Marshall, R., McKinley, S. & Pearce, C.M. (2010) Effects of nutrition on larval growth and survival in bivalves. *Reviews in Aquaculture*, **2**, 33–55.

Martínez, A., Mariñas, A., Gozález-Tizón, A. & Méndez, J. (2002) Cytogenetic characterization of *Donax trunculus* (Bivalvia: Donacidae) by means of karyotyping, fluorochrome banding and fluorescent in situ hybridization. *Journal of Molluscan Studies*, **68**, 393–396.

Martínez-Pita, I., Hachero-Cruzado, I., Sánchez-Lazo, C. & Moreno, O. (2012) Effect of diet on the lipid composition of the commercial clam *Donax trunculus* (Mollusca: bivalvia): sex-related differences. *Aquaculture Research*, **43**, 1134–1144.

Massapina, C., Joaquim, S., Matias, D. & Devauchelle, N. (1999) Oocyte and embryo quality in *Crassostrea gigas* (Portuguese strain) during a spawning period in Algarve, South Portugal. *Aquatic Living Resources*, **12**, 327–333.

Matias, D., Joaquim, S., Matias, A.M., Moura, P., de Sousa, J.T., Sobral, P. & Leitão, A. (2013) The reproductive cycle of the European clam *Ruditapes decussatus* (L., 1758) in two Portuguese populations: Implications for management and aquaculture programs. *Aquaculture*.

Matias, D., Joaquim, S., Ramos, M., Sobral, P. & Leitão, A. (2010) Biochemical compounds' dynamics during larval development of the carpet-shell clam *Ruditapes decussatus* (Linnaeus, 1758): effects of mono-specific diets and starvation. *Helgoland Marine Research*, **65**, 369–379.

Matoo, O.B., Ivanina, A. V, Ullstad, C., Beniash, E. & Sokolova, I.M. (2013) Interactive effects of elevated temperature and CO(2) levels on metabolism and oxidative stress in two common marine bivalves (*Crassostrea virginica* and *Mercenaria mercenaria*). *Comparative biochemistry and physiology. Part A, Molecular & integrative physiology*, **164**, 545–53.

Matozzo, V., Tomei, A. & Marin, M.G. (2005) Acetylcholinesterase as a biomarker of exposure to neurotoxic compounds in the clam *Tapes philippinarum* from the Lagoon of Venice. *Marine pollution bulletin*, **50**, 1686–93.

McConnaughey, T. a. & Gillikin, D.P. (2008) Carbon isotopes in mollusk shell carbonates. *Geo-Marine Letters*, **28**, 287–299.

McLachlan, A., Jaramillo, E., Defeo, O., Dugan, J., de Ruyck, A. & Coetzee, P. (1995) Adaptations of bivalves to different beach types. *Journal of Experimental Marine Biology and Ecology*, **187**, 147–160.

Medhioub, W., Lassus, P., Truquet, P. & Bardouil, M. (2012) Spirolide uptake and detoxification by *Crassostrea gigas* exposed to the toxic dinoflagellate *Alexandrium ostenfeldii*. *Aquaculture*, 108–115.

Meehl, G., Stocker, T., Collins, W., Friedlingstein, P., Gaye, A., Kitoh, A., Knutti, R., Noda, A., Watterson, I. & Weaver, A. (2007) Global climate projections. *Climate change 2007: the physical science basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change* (eds S. Solomon, D. Qin, M. Manning, Z. Chen, M. Marquis, K.

Averyt, M. Tignor & H. Miller), pp. 747–846. Cambridge University Press, Cambridge.

Mladineo, I., Peharda, M., Orhanović, S., Bolotin, J., Pavela-Vrančić, M. & Treursić, B. (2007) The reproductive cycle, condition index and biochemical composition of the horse-bearded mussel *Modiolus barbatus*. *Helgoland Marine Research*, **61**, 183–192.

Morgan, T. & Rogers, A. (2001) Specificity and sensitivity of microsatellite markers for the identification of larvae. *Marine Biology,* **139**, 967–973.

Moueza, M. & Frenkiel, L. (1973) Study of biology of *Donax trunculus* L (Lamellibranch Mollusks) in Algeria - reproduction. *Cahieres de Biologie Marine*, **14**, 261–283.

Moukrim, A., El Hamidi, F., Lagbouri, A., Kaaya, A., Zekhnini, A., Bouhaimi, A. & Narbonne, J.F. (2004) Study of *Donax trunculus* as a sentinel species for environmental monitoring of sandy beaches on moroccan coasts. *Bulletin of Environmental Contamination and Toxicology*, **73**, 674–81.

Mouneyrac, C., Linot, S., Amiard, J.-C., Amiard-Triquet, C., Métais, I., Durou, C., Minier, C. & Pellerin, J. (2008) Biological indices, energy reserves, steroid hormones and sexual maturity in the infaunal bivalve *Scrobicularia plana* from three sites differing by their level of contamination. *General and Comparative Endocrinology*, **157**, 133–41.

Moura, P., Gaspar, M.B. & Monteiro, C.C. (2008) Gametogenic cycle of the smooth clam *Callista chione* on the south-western coast of Portugal. *Journal of the Marine Biological Association of the United Kingdom*, **88**, 161–167.

Navarro, J., Leiva, G., Martinez, G. & Aguilera, C. (2000) Interactive effects of diet and temperature on the scope for growth of the scallop *Argopecten purpuratus* during reproductive conditioning. *Journal of Experimental Marine Biology and Ecology*, **247**, 67–83.

Neuberger-Cywiak, L., Achituv, Y. & Garcia, E.M. (2003) Effects of zinc and cadmium on the burrowing behavior, LC(50), and LT(50) on *Donax trunculus* Linnaeus (Bivalvia-Donacidae). *Bulletin of Environmental Contamination and Toxicology*, **70**, 713–22.

Neuberger-Cywiak, L., Achituv, Y. & Garcia, E.M. (2007) Effects of sublethal Zn++ and Cd++ concentrations on filtration rate, absorption efficiency and scope for growth in *Donax trunculus* (Bivalvia; Donacidae). *Bulletin of environmental contamination and toxicology*, **79**, 622–627.

Neuberger-Cywiak, L., Achituv, Y. & Mizrahi, L. (1990) The ecology of *Donax trunculus* Linnaeus and *Donax semistriatus* Poli from the Mediterranean coast of Israel. *Journal of Experimental Marine Biology and Ecology*, **134**, 203–220.

Nicholas, K., Nicholas, H. & Deerfield, D. (1997) GeneDoc: analysis and visualization of genetic variation. *EMBNEWS news*, **4**, 14.

Nobre, A. (1932) *Molluscos Marinhos de Portugal*, Instituto . Porto.

Ojea, J., Pazos, A., Martínez, D., Novoa, S., Garcia-Martinez, P., Sánchez, J. & Abad, M. (2008) Effects of temperature regime on broodstock conditioning of *Ruditapes decussatus*. *Journal of Shellfish Research,* **27**, 1093–1100.

Ojea, J., Pazos, A.J., Martínez, D., Novoa, S., Sánchez, J.L. & Abad, M. (2004) Seasonal variation in weight and biochemical composition of the tissues of *Ruditapes decussatus* in relation to the gametogenic cycle. *Aquaculture,* **238**, 451–468.

Oliveira, M., Camanho, A. & Gaspar, M. (2013) The influence of catch quotas on the productivity of the Portuguese bivalve dredge fleet. *ICES Journal of Marine Science*.

Orr, J., Fabry, V., Aumont, O., Bopp, L., Doney, S., Feely, R., Gnanadesikan, A., Gruber, N., Ishida, A., Joos, F., Key, R., Lindsay, K., Maier-Reimer, E., Matear, R., Monfray, P., Mouchet, A., Najjar, R., Plattner, G., Rodgers, K., Sabine, C., Sarmiento, J., Schlitzer, R., Slater, R., Totterdell, I., Weirig, M., Yamanaka, Y. & Yool, A. (2005) Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. *Nature*, **437**, 681.

Özden, Ö., Erkan, N. & Deval, M.C. (2009) Trace mineral profiles of the bivalve species *Chamelea gallina* and *Donax trunculus*. *Food Chemistry*, **113**, 222–226.

Palumbi, S.R. (1996) What can molecular genetics contribute to marine biogeography? An urchin's tale. *Journal of Experimental Marine Biology and Ecology*, **203**, 75–92.

Pan, M., McBeath, A.J.A., Hay, S.J., Pierce, G.J. & Cunningham, C.O. (2008) Real-time PCR assay for detection and relative quantification of Liocarcinus depurator larvae from plankton samples. *Marine Biology*, **153**, 859–870.

Parker, L.M., Ross, P.M. & O'Connor, W. a. (2010) Comparing the effect of elevated pCO2 and temperature on the fertilization and early development of two species of oysters. *Marine Biology*, **157**, 2435–2452.

Parker, L., Ross, P., O'Connor, W., Borysko, L., Raftos, D. & Portner, H. (2012) Adult exposure influences offspring response to ocean acidification in oysters. *Global Change Biology*, **18**, 82–92.

Pedersen, T.M., Hansen, J.L.S., Josefson, A.B. & Hansen, B.W. (2008) Mortality through ontogeny of soft-bottom marine invertebrates with planktonic larvae. *Journal of Marine Systems*, **73**, 185–207.

Pereira, A.M., Fernández-Tajes, J., Gaspar, M.B. & Méndez, J. (2012) Identification of the wedge clam *Donax trunculus* by a simple PCR technique. *Food Control*, **23**, 268–270.

Pereira, A., Palanco, I., Rufino, M., Moreno, O. & Gaspar, M.. (2007) *La Pesca de Bivalvos En El Litoral Oceánico de La Costa Sur-Occidental de La Península Ibérica: Descripción de La Pesquería y Artes de Pesca, Medidas de Gestión, Biología de Las Especies Comerciales y Catálogode Especies Bivalvos.* (ed C Manzano). IFAPA, Sevilla.

Pernet, F., Bricelj, V.M. & Cartier, S. (2006) Lipid class dynamics during larval ontogeny of sea scallops, *Placopecten magellanicus*, in relation to metamorphic success and response to antibiotics. *Journal of Experimental Marine Biology and Ecology*, **329**, 265–280.

Petrović, V., Perez-Garcia, C., Pasantes, J.J., Prats, E. & Plohl, M. (2007) Characterization of a subtelomeric satellite DNA in the mollusc *Donax trunculus*. *FEBS Journal*, **274**, 344.

Petrović, V., Perez-Garcia, C., Pasantes, J.J., Satovic, E., Prats, E. & Plohl, M. (2009) A GC-rich satellite DNA and karyology of the bivalve mollusk *Donax trunculus*: a dominance of GC-rich heterochromatin. *Cytogenetic and Genome Research*, **124**, 63–71.

Petrović, V. & Plohl, M. (2005) Sequence divergence and conservation in organizationally distinct subfamilies of *Donax trunculus* satellite DNA. *Gene*, **362**, 37–43.

Pettersen, A.K., Turchini, G.M., Jahangard, S., Ingram, B.A. & Sherman, C.D.H. (2010) Effects of different dietary microalgae on survival, growth, settlement and fatty acid composition of blue mussel (*Mytilus galloprovincialis*) larvae. *Aquaculture*, **309**, 115–124.

Phillips, N. (2002) Effects of nutrition-mediated larval condition on juvenile performance in a marine mussel. *Ecology*, **83**, 2562–2574.

Plohl, M. & Cornudella, L. (1996) Characterization of a complex satellite DNA in the mollusc *Donax trunculus*: analysis of sequence variations and divergence. *Gene*, **169**, 157–64.

Plohl, M. & Cornudella, L. (1997) Characterization of interrelated sequence motifs in four satellite DNAs and their distribution in the genome of the mollusc *Donax trunculus*. *Journal of molecular evolution*, **44**, 189–98.

Pouvreau, S., Gangnery, A., Tiapari, J., Lagarde, F., Garnier, M. & Bodoy, A. (2000) Gametogenic cycle and reproductive effort of the tropical blacklip pearl oyster, *Pinctada margaritifera* (Bivalvia: Pteriidae), cultivated in Takapoto atoll (French Polynesia. *Aquatic Living Resources*, **13**, 37–48.

Powell, E.N., Bochenek, E. a, Klinck, J.M. & Hofmann, E.E. (2002) Influence of food quality and quantity on the growth and development of *Crassostrea gigas* larvae: a modeling approach. *Aquaculture*, **210**, 89–117.

Przeslawski, R., Bourdeau, P.E., Doall, M.H., Pan, J., Perino, L. & Padilla, D.K. (2008) The effects of a harmful alga on bivalve larval lipid stores. *Harmful Algae*, **7**, 802–807.

Przeslawski, R., Perino, L. & Padilla, D.K. (2012) Effects of larval density and food rations on the larval development of an ecologically important bivalve (*Mercenaria mercenaria*). *Molluscan Research*, **32**, 27–35.

Quinteiro, J., Perez-Dieguez, L., Sanchez, A., Perez-Martin, R.I., Sotelo, C.G. & Rey-Mendez, M. (2011) Quantification of Manila clam *Ruditapes philippinarum* (Adams & Reeve, 1850) larvae based on SYBR Green Real-Time Polymerase chain reaction. *Journal of Shellfish Research*, **30**, 791–796.

Racotta, I. (2003) Shrimp larval quality in relation to broodstock condition. *Aquaculture*, **227**, 107–130.

Ramon, M. (1999) *Bacciger bacciger* (Trematoda, Fellodistomidae) infection in commercial clams *Donax trunculus* (Bivalvia, Donacidae) from the sandy beaches of the Western Mediterranean. *Diseases of aquatic organisms*, **35**, 37–46.

Ramón, M., Abelló, P. & Richardson, C. (1995) Population structure and growth of *Donax trunculus* (Bivalvia: Donacidae) in the western Mediterranean. *Marine biology*, **121**, 665–671.

Range, P., Chícharo, M.A., Ben-Hamadou, R., Piló, D., Matias, D., Joaquim, S., Oliveira, A.P.P. & Chícharo, L. (2011) Calcification, growth and mortality of juvenile clams Ruditapes decussatus under increased pCO2 and reduced pH: Variable responses to ocean acidification at local scales? *Journal of Experimental Marine Biology and Ecology*, **396**, 177–184.

Range, P., Piló, D., Ben-Hamadou, R., Chícharo, M.A., Matias, D., Joaquim, S., Oliveira, A.P. & Chícharo, L. (2012) Seawater acidification by CO2 in a coastal lagoon environment: Effects on life history traits of juvenile mussels Mytilus galloprovincialis. *Journal of Experimental Marine Biology and Ecology*, **424-425**, 89–98.

Raven, J., Caldeira, K., Elderfield, H. & Hoegh-Guldberg, O. (2005) *Ocean Acidification Due to Increasing Atmospheric Carbon Dioxide*, The Royal. Clyvedon Press, Cardiff.

Raymond, J.F., Himmelman, J.H. & Guderley, H.E. (2007) Biochemical content, energy composition and reproductive effort in the broadcasting sea star *Asterias vulgaris* over the spawning period. *Journal of Experimental Marine Biology and Ecology*, **341**, 32–44.

Rees, G. (2010) *Safe Management of Shellfish and Harvest Waters* (eds G Rees, WH Organization, and IW Association). International Water Association Pub, London ; New York.

Relvas, P. & Barton, E.D. (2005) A separated jet and coastal counterflow during upwelling relaxation off Cape São Vicente (Iberian Peninsula). *Continental Shelf Research*, **25**, 29–49.

Rodolfo-Metalpa, R., Houlbrèque, F., Tambutté, É., Boisson, F., Baggini, C., Patti, F.P., Jeffree, R., Fine, M., Foggo, a., Gattuso, J.-P. & Hall-Spencer, J.M. (2011) Coral and mollusc resistance to ocean acidification adversely affected by warming. *Nature Climate Change*, **1**, 308–312.

Romeo, M. & Gnassiabarelli, M. (1988) The ecology of *Donax trunculus* Linnaeus and *Donax semistriatus* Poli from the Mediterranean coast of Israel. *Marine Biology*, **99**, 223–227.

Rozen, S. & Skaletzky, H. (2000) Primer3 on the WWW for General Users and for Biologist Programmers. *Bioinformatics Methods and Protocols: Methods in Molecular Biology.*, Humana Pre (eds S. Misener & S. Krawetz), pp. 365–386. Totowa.

Rufino, M.M., Gaspar, M.B., Pereira, A.M., Maynou, F. & Monteiro, C.C. (2010) Ecology of megabenthic bivalve communities from sandy beaches on the south coast of Portugal. *Scientia Marina*, **74**, 163–178.

Ruiz-Azcona, P., Rodriguez-Sierra, R. & Martin, J.B. (1996) Culture of coquina clam, *Donax trunculus*, larvae. *Aquaculture*, **139**, 151–155.

Russell, V.J., Hold, G.L., Pryde, S.E., Rehbein, H., Quinteiro, J., Rey-Mendez, M., Sotelo, C.G., Pérez-Martin, R.I., Santos, a T. & Rosa, C. (2000) Use of restriction fragment length polymorphism to distinguish between salmon species. *Journal of agricultural and food chemistry*, **48**, 2184–8.

Sabine, C.L., Feely, R. a, Gruber, N., Key, R.M., Lee, K., Bullister, J.L., Wanninkhof, R., Wong, C.S., Wallace, D.W.R., Tilbrook, B., Millero, F.J., Peng, T.-H., Kozyr, A., Ono, T. & Rios, A.F. (2004) The oceanic sink for anthropogenic CO2. *Science (New York, N.Y.)*, **305**, 367–71.

Sánchez, A., Quinteiro, J., Rey-Mendez, M., Perez-Martín, R.I. & Sotelo, C.G. (2009) Identification of European Hake species (*Merluccius merluccius*) using real-time PCR. *Journal of agricultural and food chemistry*, **57**, 3397–403.

Savina, M. & Ménesguen, A. (2008) A deterministic population dynamics model to study the distribution of a benthic bivalve with planktonic larvae (*Paphia rhomboides*) in the English Channel (NW Europe). *Journal of Marine Systems*, **70**, 63–76.

Shakir, F.K., Audilet, D., Drake, A.J. & Shakir, K.M.M. (1994) A Rapid Protein Determination by Modification of the Lowry Procedure. *Analytical Biochemistry*, **216**, 232–233.

Shumway, S.E., Sherman, S.A., Cembella, A.D. & Selvin, R. (1994) Accumulation of paralytic shellfish toxins by surfclams, *Spisula solidissima* (Dillwyn, 1897) in the gulf of maine: Seasonal changes, distribution between tissues, and notes on feeding habits. *Natural Toxins*, **2**, 236–251.

Sloan, N. & Breen, P. (1988) Northern abalone, *Haliotis kamtschatkana*, in British Columbia: fisheries and synopsis of life history information. *Canadian Special Publication of Fisheries and Aquatic Sciences*, **103**, 1–46.

Soares, A.M.M. (2004) *Variabilidade Do "Upwelling" Costeiro Durante o Holocénico Nas Margens Atlânticas Ocidental e Meridional Da Península Ibérica*. Universidade do Algarve.

Sousa, M. & Oliveira, E. (1994) Ultrastructural and cytochemical study of spermatogenesis in *Donax trunculus* (Mollusca, Bivalvia). *Journal of Submicroscopic Cytology and Pathology*, **26**, 305–311.

Steiner, G. & Muller, M. (1996) What can 18S rDNA do for bivalve phylogeny? *Journal of Molecular Evolution*, **43**, 58–70.

Stenton-Dozey, J.M.E. & Brown, A.C. (1994) Exposure of the sandy beach bivalve *Donax serra* Roding to a heated and chlorinated effluent. 1. Effects of temperature on burrowing and survival. *Journal of Shellfish Research*, **13**, 443–449.

Talmage, S.C. & Gobler, C.J. (2011) Effects of elevated temperature and carbon dioxide on the growth and survival of larvae and juveniles of three species of northwest atlantic bivalves. *PLoS ONE*, **6**, e26941.

Taris, N., Baron, S., Sharbel, T.F., Sauvage, C. & Boudry, P. (2005) A combined microsatellite multiplexing and boiling DNA extraction method for high-throughput parentage analyses in the Pacific oyster (Crassostrea gigas). *Aquaculture Research*, **36**, 516–518.

Taylor, M., Fox, C., Rico, I. & Rico, C. (2002) Species-specific TaqMan probes for simultaneous identification of (*Gadus morhua* L.), haddock (*Melanogrammus aeglefinus* L.) and whiting (*Merlangius merlangus* L. *Molecular Ecology Notes*, **2**, 599–601.

Tebble, N. (1976) *British Bivalve Seashells. A Handbook for Identification*. British Museum, Edinburgh.

Theologidis, I., Fodelianakis, S., Gaspar, M.B. & Zouros, E. (2008) Doubly uniparental inheritance (DUI) of mitochondrial DNA in *Donax trunculus* (Bivalvia: Donacidae) and the problem of its sporadic detection in Bivalvia. *Evolution*, **62**, 959–70.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. & Higgins, D.G. (1997) The CLUSTAL_X Windows Interface: Flexible Strategies for Multiple Sequence Alignment Aided by Quality Analysis Tools. *Nucleic Acids Research* , **25** , 4876–4882.

Thompson, C.M., Hare, M.P. & Gallager, S.M. (2012) Semi-automated image analysis for the identification of bivalve larvae from a Cape Cod estuary. *Limnology and Oceanography-Methods*, **10**, 538–554.

Thomsen, J., Casties, I., Pansch, C., Körtzinger, A. & Melzner, F. (2013) Food availability outweighs ocean acidification effects in juvenile *Mytilus edulis*: laboratory and field experiments. *Global change biology*, **19**, 1017–27.

Tirado, C. & Salas, C. (1998) Reproduction and fecundity of *Donax trunculus* L., 1758 (Bivalvia : Donacidae) in the littoral of Malaga (Southern Spain). *Journal of Shellfish Research*, **17**, 169–176.

Tiwari, S. & Gallager, S. (2003) Semi-automated image analysis for the identification of bivalve larvae from a Cape Cod estuary. *Ninth IEEE International Conference on Computer Vision, Vols I and II, Proceedings* pp. 494–501. IEEE COMPUTER SOC, 10662 LOS VAQUEROS CIRCLE, PO BOX 3014, LOS ALAMITOS, CA 90720-1264 USA.

Tlili, S., Métais, I., Ayache, N., Boussetta, H. & Mouneyrac, C. (2011) Is the reproduction of *Donax trunculus* affected by their sites of origin contrasted by their level of contamination? *Chemosphere,* **84**, 1362–70.

Tlili, S., Métais, I., Boussetta, H. & Mouneyrac, C. (2010) Linking changes at sub-individual and population levels in *Donax trunculus*: assessment of marine stress. *Chemosphere,* **81**, 692–700.

Troedsson, C., Simonelli, P., Nägele, V., Nejstgaard, J.C. & Frischer, M.E. (2008) Quantification of copepod gut content by differential length amplification quantitative PCR (dla-qPCR). *Marine Biology,* **156**, 253–259.

Uriarte, I., Farías, A., Hernandez, J., Schäfer, C. & Sorgeloos, P. (2004) Reproductive conditioning of Chilean scallop (*Argopecten purpuratus*) and the Pacific oyster (*Crassostrea gigas*): effects of enriched diets. *Aquaculture,* **230**, 349–357.

Utting, S.D. & Millican, P.F. (1997) Techniques for the hatchery conditioning of bivalve broodstocks and the subsequent effect on egg quality and larval viability. *Aquaculture,* **155**, 45–54.

Vadopalas, B., Bouma, J. V., Jackels, C.R. & Friedman, C.S. (2006) Application of real-time PCR for simultaneous identification and quantification of larval abalone. *Journal of Experimental Marine Biology and Ecology,* **334**, 219–228.

La Valle, P., Nicoletti, L., Finoia, M.G. & Ardizzone, G.D. (2011) *Donax trunculus* (Bivalvia: Donacidae) as a potential biological indicator of grain-size variations in beach sediment. *Ecological Indicators,* **11**, 1426–1436.

Videla, J.A., Chaparro, O.R., Thompson, R.J. & Concha, I.I. (1998) Role of biochemical energy reserves in the metamorphosis and early juvenile development of the oyster *Ostrea chilensis*. *Marine Biology,* **132**, 635–640.

Viles, F.J. & Silverman, L. (1949) Determination of Starch and Cellulose with Anthrone. *Analytical Chemistry,* **21**, 950–953.

Vite-García, N. & Saucedo, P. (2008) Energy Storage and allocation during reproduction of Pacific winged pearl oyster *Pteria sterna* at Bahía de la Paz, Baja California Sur, Mexico. *Journal of Shellfish Research,* **27**, 375–383.

Voliani, A., Auteri, R., Baino, R. & Silvestri, R. (1997) Settlement and growth of Donax trunculus L. along Tuscanian coast. *Biologia Marina Mediterranea,* **4**, 458–460.

Waldock, M. & Nascimento, I. (1979) The triacylglycerol composition of *Crassostrea gigas* larvae fed on different algal diets. *Mar. Biol. Lett,* **1**, 77–86.

Walne, P. & Mann, R. (1975) Growth and biochemical composition in Ostrea edulis and *Crassostrea gigas*. *Proc. 9th European Marine Biological Symposium* pp. 587–607.

Weinberg, J. (1999) Age-structure, recruitment, and adult mortality in populations of the Atlantic surfclam, *Spisula solissima*, from 1978 to 1997. *Marine Biology,* **134**, 113–125.

Weiss, I.M., Tuross, N., Addadi, L. & Weiner, S. (2002) Mollusc larval shell formation: amorphous calcium carbonate is a precursor phase for aragonite. *The Journal of experimental zoology,* **293**, 478–91.

White, M.M., McCorkle, D.C., Mullineaux, L.S. & Cohen, A.L. (2013) Early exposure of bay scallops (Argopecten irradians) to high CO2 causes a decrease in larval shell growth. *PloS one,* **8**, e61065.

Whyte, J., Bourne, N. & Ginther, N. (1990) Biochemical and energy changes during embryogenesis in the rock scallop *Crassadoma gigantea*. *Marine Biology,* **106**, 239–244.

Wu, X., Cheng, Y., Zeng, C., Wang, C. & Cui, Z. (2010) Reproductive performance and offspring quality of the first and the second brood of female swimming crab, *Portunus trituberculatus*. *Aquaculture*, **303**, 94–100.

Yan, T., Zhou, M., Fu, M., Wang, Y., Yu, R. & Li, J. (2001) Inhibition of egg hatching success and larvae survival of the scallop, *Chlamys farreri*, associated with exposure to cells and cell fragments of the dinoflagellate *Alexandrium tamarense*. *Toxicon*, **39**, 1239–1244.

Yawetz, A., Fishelson, L., Bresler, V. & Manelis, R. (2010) Comparison of the effects of pollution on the marine bivalve *Donax trunculus* in the vicinity of polluted sites with specimens from a clean reference site (Mediterranean Sea). *Marine pollution bulletin*, **60**, 225–9.

Zar, J. (1984) *Biostatistical Analysis*, 2nd Ed. New-Jersey.

Zeichen, M.M., Agnesi, S., Mariani, A., Maccaroni, A. & Ardizzone, G.D. (2002) Biology and Population Dynamics of *Donax trunculus* L. (Bivalvia: Donacidae) in the South Adriatic Coast (Italy). *Estuarine, Coastal and Shelf Science*, **54**, 971–982.