Universidade de Aveiro Departamento de Biologia
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GENÉTICA POPULACIONAL DE *C. MAENAS*: OCEANOGRAFIA E DISPERSÃO LARVAR

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Professor Doutor Henrique José de Barros Brito Queiroga, Professor Auxiliar com Agregação do Departamento de Biologia da Universidade de Aveiro e co-orientação científica do Professor Doutor Gary Robert Carvalho, Professor de Ecologia Molecular do Departamento de Ciências Biológicas da Universidade de Bangor, Reino Unido.

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Doutor Henrique José de Barros Brito Queiroga
Professor Auxiliar com Agregação da Universidade de Aveiro

Doutor Jesus Manuel Pedreira Dubert
Professor Auxiliar da Universidade de Aveiro

Doutora Maria Marina Pais Ribeiro da Cunha
Professora Auxiliar da Universidade de Aveiro

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palavras-chave

análise de séries temporais, *Carcinus maenas*, conectividade de populações, dispersão larvar, DNA microssatélite, estrutura genética, fluxo genético, modelação numérica, parentesco, recrutamento, sucesso reprodutivo, variabilidade oceanográfica.

resumo

Decifrar a complexa interacção entre os ciclos de vida de espécies marinhas e a oceanografia revela-se fundamental para a compreensão do fluxo genético e da conectividade no meio marinho. Nas espécies marinhas com desenvolvimento indirecto o fluxo de genes entre populações depende da distância que separa as populações, bem como da interacção entre a duração do desenvolvimento larvar, do comportamento das larvas e dos padrões de circulação oceânica. A conectividade larvar influencia uma variedade de processos como a dinâmica de stocks e de populações, a distribuição e limites geográficos das espécies, a estrutura genética das populações e a dispersão de espécies invasivas e reveste-se consequentemente de uma importância fundamental na identificação das unidades populacionais evolucionariamente relevantes e para a gestão e conservação marinhas. Os marcadores genéticos e os Modelos Individuais Acoplados a Modelos Físico-Biológicos ("ICPBMs") são actualmente ferramentas fundamentais para o estudo dos padrões de dispersão larvar e para avaliar o nível de conectividade populacional. A presente tese respeita à avaliação das escalas espaciais de conectividade de populações de uma espécie costeira, o caranguejo *Carcinus maenas*, e utiliza conjuntamente informação de marcadores genéticos, análise de séries temporais de fornecimento de larvas e um modelo numérico de circulação oceânica.

O primeiro capítulo introduz a temática da conectividade em espécies marinhas e inclui algumas referências aos métodos moleculares, analíticos e de modelação seguidos ao longo da tese. Através da utilização de múltiplas ferramentas – avaliação da estrutura genética geográfica de *C. maenas* na sua distribuição nativa com recurso a marcadores de DNA (microssatélites) (Capítulo 2), avaliação da estrutura genética temporal das larvas que formam os eventos de fornecimento larvar à Ria de Aveiro, NW Portugal (Capítulo 3), descrição da variabilidade inter-anual do fornecimento larvar à Ria de Aveiro, NW Portugal (Capítulo 4) e validação de um modelo ICPBM que descreve os padrões observados de fornecimento (Capítulo 5) – esta tese espera poder contribuir para uma melhor compreensão dos mecanismos que regulam o fluxo de genes e a conectividade entre populações de organismos marinhos. No Capítulo 6 são apresentadas as principais conclusões da investigação.

A análise genética com recurso a microssatélites indicou que as populações de *C. maenas* são geneticamente homogéneas ao longo de várias centenas de km, dentro da distribuição nativa da espécie. Paralelamente, não foram encontrados indícios da existência de reprodução por "sweepstakes" em *C. maenas* de populações da costa oeste da Península Ibérica, visto que não se obtiveram diferenças genéticas significativas entre os eventos larvares. Também não se encontrou qualquer estrutura familiar entre as larvas que formam cada episódio de fornecimento, e não houve nenhuma redução significativa da variabilidade genética das larvas quando comparada com a de caranguejos adultos. A análise de séries temporais de suprimento de larvas na

Resumo (cont.)

Ria de Aveiro em cinco anos estudados indica que este é um fenómeno episódico e variável, sendo os maiores episódios de fornecimento coincidentes com as marés vivas e acentuados por fortes ventos de sul. O modelo ICPBM foi validado com sucesso e parece fornecer uma estimativa realística das escalas espaciais e temporais de dispersão larvar, de acordo com as observações da estrutura genética e da ausência de reprodução por "sweepstake" em *C. maenas* da costa oeste da Península Ibérica.

keywords

Carcinus maenas, gene flow, genetic structure, larval dispersal, microsatellite DNA, numerical modelling, oceanographic variability, population connectivity, recruitment, relatedness, reproductive success, time series analysis.

abstract

Unravelling the interactions between life-history strategies and oceanographic processes is central to the understanding of gene flow and connectivity in the marine environment. In particular, for marine species with indirect development gene flow between populations depends on the distance separating the populations and on the interaction between duration of the larval phase, larval behaviour and current patterns. Larval connectivity affects many processes, including stock and population dynamics, species ranges, population genetic structure, and the spread of invasive species and is therefore an important consideration to identify evolutionary relevant population unit and for marine management and conservation efforts. Genetic markers and Individual-based Coupled Physical-Biological Models (ICPBMs) are two of the tools currently available for tracking dispersal pathways of larvae and to assess the degree of population connectivity. The present thesis concerns the spatial and temporal scale assessment of population connectivity of a coastal marine species, the shore crab *Carcinus maenas*, making use of genetic markers, time series larval supply analysis and an oceanographic numerical model.

Chapter 1 introduces the thematic of marine species connectivity, including a brief reference to the molecular, analytical and modelling methods followed during the study. Making use of an interdisciplinary approach – assessment of genetic geographical structure with microsatellite markers within *C. maenas* native range (Chapter 2), assessment of temporal genetic structure of larvae forming each supply event to the Ria de Aveiro, NW Portugal (Chapter 3), description of interannual variability of larval supply to the Ria de Aveiro, NW Portugal (Chapter 4) and validation of an ICPBM to describe the observed time series of supply (Chapter 5) – the aims of this thesis is to contribute to our understanding of the mechanisms regulating gene flow and connectivity among marine populations. Finally, in Chapter 6 the main results and conclusions achieved are presented.

Microsatellites analysis indicated that *C. maenas* populations were genetically similar across hundreds of km, within the species native range. Additionally, there was no evidence of sweepstakes reproduction in *C. maenas* from western Iberian coast populations since there were no significant differences amongst larval events. Among larvae in each episode, no genetic relatedness was found, and larvae did not present reduced genetic variability when compared to adult crabs. On a long time scale, larval supply to the Ria de Aveiro was episodic and variable throughout five different studied years, with highest supply numbers generally occurring around spring tides and enhanced by strong southerly winds. The ICPBM was successfully validated and appears to provide a realistic estimate of the observed spatial and temporal scales of the larval dispersal, consistent with the observations on genetic structure and lack of sweepstake reproduction in *C. maenas* from western Iberian coast.

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Chapter 1

Introduction

1.1 General introduction

The early life history of most marine benthic invertebrate and fish species involves a planktonic larval stage of development. Successful dispersal of pelagic larvae determines population connectivity which influences significantly patterns of population structure and dynamics, and consequent management of marine ecosystems and biodiversity, and the monitoring and control of invasive species (Hastings & Harrison 1994, Botsford et al. 2001). Because of its importance for marine ecosystem functioning, larval dispersal and population connectivity have been the target of several literature reviews covering various aspects such as methods of study, larval transport, role of biological and physical processes, coupled biological and physical models and conservation of marine biodiversity (e.g. Bradbury & Snelgrove 2001, Levin 2006, Cowen et al. 2007, Jones et al. 2007, Werner et al. 2007, Selkoe et al. 2008, Cowen & Sponaugle 2009, Weersing & Toonen 2009).

During the planktonic period, larvae spend hours, days, weeks or months developing in oceanic currents and may be transported very distant from natal populations and suitable settlement sites (Thorson 1950), thus creating the need for a return migration. Successful cross-shelf transport of larvae is a critical component for both demographic and genetic connectivity between marine populations and it is influenced by several physical transport mechanisms (reviewed in Shanks 1995). The most relevant ones previously described are: wind driven surface currents (Willis & Oliver 1990), Ekman transport (Goodrich et al. 1989, Little & Epifanio 1991, McConnaughey et al. 1992), onshore convergence following relaxation of upwelling winds (Farrell et al. 1991) and internal waves (Shanks 1983, Pineda 1991). However, inherent spatial and temporal variability in larval transport and connectivity have been attributed to the chaotic nature of coastal circulations (Siegel et al. 2008).

Dispersal by pelagic larvae does not depend exclusively on physical transport mechanisms but relies on the complex interaction between the physics of the ocean and larval behaviour, and may also be influenced by the duration of the planktonic stages. Larval behaviour plays a crucial role, especially of their vertical position in the water column as a result of ontogenetic depth preference or selective tidal stream transport (reviewed in Queiroga & Blanton 2005), foraging behaviour (Woodson & McManus 2007) and orientation to environmental cues (Kingsford et al. 2002). The amount of time spent in

the plankton (pelagic larval duration – PLD) was usually accepted as proportional to the distance larvae may be transported (e.g. Shanks et al. 2003, Siegel et al. 2003), thereby influencing population genetic differentiation (Bohonak 1999). However, while in theory there should be a strong positive correlation between PLD and population connectivity, numerous studies (reviewed in Weersing & Toonen 2009) showed that the strength of the correlation is far lower, and its nature is more complex than reported previously. Moreover, Galarza et al. (2009) found no significant relation between PLD and gene flow patterns in seven littoral fish species with contrasting early-life-history traits. Such studies suggest that PLD and dispersal potential cannot predict connectivity patterns alone. PLD is influenced by several environmental conditions and additional mechanisms such as larval and adult behaviour, and reproductive and recruitment strategies (Cowen & Sponaugle 2009).

The high mobility of marine species and the absence of obvious barriers to dispersal in the ocean might be expected to limit the division of species' range. Under this scenario, larvae would be widely dispersed among a set of open, local populations, which interact through exchange of individuals (i.e. metapopulation), potentially over hundreds to thousands of kilometres (e.g. Roughgarden et al. 1985, Caley et al. 1996). This viewpoint was supported by studies that reported little genetic structure over large distances in species with relatively long PLDs. Recent research, however, showed evidence that this perspective is likely to be inaccurate for many species (Swearer et al. 2002, Jones et al. 2005), contributing a change in the paradigm of homogenous marine populations and large-scale connectivity (reviewed in Hauser & Carvalho 2008).

1.2 Aims and thesis rationale

The general aim of this PhD thesis was to employ genetic markers, namely microsatellite loci, to investigate the population genetic structure and the larval allelic composition of the shore crab *Carcinus maenas* in European waters, with particular focus in the west Iberian coast. Simultaneously, an oceanographic numerical model coupled with an individual based model was developed, which simulates larval behaviour, in order to describe the advection history of the larvae forming each supply event.

Making use of genetic information provided by a set of highly informative microsatellite DNA markers, the oceanographic model and detailed information concerning time series analysis of larval supply to the Ria de Aveiro, several objectives were addressed:

i) To characterize the genetic variability of populations of *C. maenas* within the species native range, with a special focus in the west Iberian coast, evaluate their degree of genetic differentiation and understand the forces shaping the patterns found;
ii) To determine the genetic structure, variability and relatedness of temporally separated samples of *C. maenas* larvae from different supply episodes to the Ria de Aveiro;
iii) To describe the interannual variability of larval supply of *C. maenas* to Ria de Aveiro along a time series of five different years and understand the interactions of bio-physical forcing that control the supply of larvae to coastal and estuarine systems;
iv) To validate an individual-based coupled physical biological model (ICPBM) to describe the observed time series of *C. maenas* larval supply to the west coast of the Iberian Peninsula.

The present thesis is linked to the project "Connect" - *Connectivity of marine populations assessed with genetic and numerical modelling tools* funded by Fundação para a Ciência e a Tecnologia (PTDC/BIA-BDE/65425/2006). Excepted when stated otherwise, I carried out the field sampling and all the associated laboratory work. Apart from supervisory input all analysis, interpretation and discussion are my own work.

The following provides the content of each chapter. Each chapter except the introduction and conclusion represents different units with specific and well defined objectives and individual Introduction, Methods, Results, Discussion and References section.

The purpose of Chapter 1, the introduction, is to give a brief state-of-the-art overview on the research topic of marine connectivity and to identify the main and specific objectives. The thesis includes another five chapters, the compositions of which are as follows:

Chapter 2 describes the genetic structure of *C. maenas* within the species native range using microsatellite markers, and corresponds to a manuscript published in *Marine Ecology Progress Series*. H. Queiroga and G. Carvalho supervised the study and S. Creer and M. Taylor helped in the molecular genetic studies, data analysis and revised the manuscript.

- ✓ Domingues CP, Creer S, Taylor MI, Queiroga H, Carvalho GR (2010). Genetic structure of *Carcinus maenas* within its native range: larval dispersal and oceanographic variability. Mar Ecol Prog Ser 410:111-123.

Chapter 3 describes the temporal genetic structure among *C. maenas* larval cohorts supplied to the Ria de Aveiro, and corresponds to a manuscript accepted for publication in *Heredity*. H. Queiroga and G. Carvalho supervised the study and S. Creer and M. Taylor helped in the molecular genetic studies, data analysis and revised the manuscript.

- ✓ Domingues CP, Creer S, Taylor MI, Queiroga H, Carvalho GR (accepted). Temporal genetic homogeneity among shore crab (*Carcinus maenas*) larval events supplied to an estuarine system on the Portuguese northwest coast. Heredity.

Chapter 4 describes the interannual variability patterns of *C. maenas* larval supply to the Ria de Aveiro, and attempts to identify the main bio-physical processes that control intra-year variability of larval supply. It corresponds to a manuscript submitted to *Marine Ecology Progress Series*. H. Queiroga supervised the study, J. Dubert and R. Nolasco supplied physical data and helped in the interpretation of results, M.J. Almeida, A. Sequeira and S. Tavares supervised larval sampling collections in 2002, 2008 and 2009, respectively.

- ✓ Domingues CP, Almeida MJ, Dubert J, Nolasco R, Sequeira A, Tavares S, Queiroga H (submitted). Crab larval supply to an estuary in the Eastern Atlantic upwelling system: predictability and idiosyncrasy at multiple temporal scales. Mar Ecol Prog Ser.

Chapter 5 describes the use and validation of an ICPBM in two subsequent years to describe the observed time series of *C. maenas* larval supply to the Ria de Aveiro. Additionally, the model will also be used to predict the origin of *C. maenas* larvae that are supplied to the Ria de Aveiro during the recruitment season corresponding to the observations, and to build a connectivity matrix between estuaries and Rias along the Iberian Peninsula. Such data will provide a comparative account of patterns of connectivity along the range of studied populations. This chapter corresponds to a manuscript in preparation to be submitted to an international journal. H. Queiroga

supervised the study and collaborated in the analysis, J. Dubert and R. Nolasco developed the ICPBM, ran the simulations and provided the model results based on biological data supplied by C. Domingues and H. Queiroga.

- ✓ Domingues CP, Nolasco R, Dubert J, Queiroga H (in preparation). Use and validation of an Individual Based Coupled Physical Biological Model to describe dispersal and supply of invertebrate larvae.

Chapter 6 summarises the main findings of the individual data chapters and points out future directions on the study of marine population connectivity.

1.3 Approach

In the vast marine environment it is difficult to determine the origin and fate of larvae in order to measure population connectivity. Conditions allowing for the direct observation of realized larval dispersal are rarely afforded (Gilg & Hilbish 2003, Kinlan & Gaines 2003) because the larval stages are often too small to follow individually or to track them with conventional tags (Levin 2006). Instead, dispersal patterns and marine connectivity has been analysed by indirect techniques. Of these, the use of molecular markers to track genetic differences between populations has allowed significant insights in understanding the dynamics of marine populations (Hellberg et al. 2002, Hellberg 2006). However, as stated in Selkoe et al. (2008), "molecular tools perform at their best when integrated with other data and approaches". Among others, the following approaches have been identified to help elucidating marine population genetics (reviewed in Selkoe et al. 2008): i) recruit and larval time series; ii) behavioural studies; (iii) natural and artificial tags, and iv) oceanographic simulation.

1.3.1 Molecular markers

The use of molecular markers has increased the ability to identify species, subspecies and populations (reviewed in Avise 2004). Until the 1980s, protein electrophoretic methods such as allozyme polymorphisms were the major approach to assess population diversity, but the main drawback of these markers was that they tended to underestimate levels of genetic variability, and imposed strict constraints on sample collection and storage. The fact that they showed relatively low variability, slow evolutionary rates and sometimes deviated from selective neutrality hindered their effective application to

discriminate among populations and to estimation of gene flow (Carvalho 1998). With advances in molecular biology, especially the visualisation and manipulation of nucleic acids (Carvalho 1998), many different biological domains became driven by the interest in the ability to study DNA variation directly. Technical advances on DNA sequencing and the development of the polymerase chain reaction (PCR) enabled genetic analysis of populations at the gene level with increasing resolution, allowing non-invasive sampling, as well as minute amounts and even degraded DNA to be analysed successfully. DNA sequencing is the optimal method for population comparison both in terms of high resolution and of interpretation of the results, but while the technique is not yet suitable for the analysis of large numbers of individuals in a short period of time, several classes of molecular markers such as mitochondrial DNA (mtDNA) and microsatellite polymorphisms, exhibiting high levels of polymorphism, have become extremely widespread in marine ecological studies in recent years (Cowen & Sponaugle 2009).

There are hundreds or thousands of mitochondria per cell containing mtDNA molecules in high copy numbers. Consequently, mtDNA is ideal for the analysis of highly degraded material which tends to be devoid of genomic DNA. In most animals, mtDNA is inherited through the maternal line, preventing recombination, and passing from generation to generation relatively unchanged. This condition confirms that non-recombining haplotypes of mtDNA are useful for the analysis of phylogenetic relationships and this is why it is commonly used in studies of phylogeography (Avise 2004). However, some regions of the mtDNA genome evolve rapidly at the sequence level making them of special utility in the analysis of recent population structure (Avise 2004). Because of the reduced effective population size in mtDNA relative to nuclear genes, and its uniparental inheritance, the main drawback of using mtDNA haplotypes in population genetics is that it may underestimate levels of genetic diversity and may not be representative of populations as a whole.

Microsatellite markers, also known as short tandem repeats (STRs), are polymorphic DNA loci consisting of a repeated nucleotide sequence (2-7 nucleotides) that varies in a population, thereby creating multiple alleles for a microsatellite locus. The importance of microsatellites as genetic markers lies in the properties they exhibit (Estoup & Angers 1998, Avise 2004). Microsatellites are very abundant, distributed randomly throughout eukaryotic genomes and typically exhibit high levels of variability in repeat number. Perhaps because the majority of microsatellite DNA is not subjected to selection

pressures, the mutation rate is very high, leading to extensive allelic variation and high levels of heterozygosity. Another advantage of microsatellites is that they follow a Mendelian inheritance pattern. Also the fact that they are co-dominant markers, allows the visualisation of all alleles present, and unambiguous assignment of genotypes. Microsatellite screening only requires minute amounts of tissue, even with highly degraded DNA, thus allowing for the screening of old samples and also live specimens. When compared to other DNA markers, routine assaying is technically straightforward and quick, although the initial work of detecting the repeats and isolation of primers can be laborious and time consuming (Estoup & Angers 1998). The characteristics of microsatellites as genetic markers to explore fine-scale ecological questions (Selkoe & Toonen 2006), and the ability to choose from a large selection of highly informative loci previously developed for *Carcinus maenas* (Tepolt et al. 2006, Pascoal et al. 2009) make microsatellites particularly appropriate to this thesis. They provide significant information about patterns of genetic variation among populations and individuals of a single species, facilitating the analysis of population genetic structure and gene flow among populations and genetic relatedness among individuals, even of small early life-history stages such as larvae (Estoup & Angers 1998). Other applications of microsatellite loci include forensic science, phylogeography, identification of individual organisms, and parentage assignment (e.g. Estoup & Angers 1998, Cornuet et al. 1999, Primmer et al. 2000, Jobling & Gill 2004).

Recently, the use of single nucleotide polymorphisms (SNPs) as genetic markers in population studies is emerging and seems to hold great potential especially because they are widespread throughout the genome (coding and non-coding regions), are less prone to technical error in scoring than microsatellites, and the methods for massive screening have been developed allowing their use for finding informative sequence data (Brumfield et al. 2003, Morin et al. 2004). However, the lower mutation rate of SNPs and consequently less information per unit locus, compared with microsatellites, prompted their application in molecular ecology to processes that occurred sometime in the past and to studies of comparative biogeography among different species (Brumfield et al. 2003). High throughout methods of analysis (Garvin et al. 2010) do however now allow analysis of hundreds of SNP loci simultaneously, thereby increasing overall statistical robustness.

In order to characterize genetic differentiation and to assess levels of connectivity among populations of *C. maenas*, traditional statistics such as F_{ST}, the fixation index

(Wright 1951), have been applied to microsatellite data. However, although yielding useful information for explore the magnitude of gene flow (Neigel 2002), a major shortcoming of this type of approach is that it is sometimes not possible to distinguish clearly between the influences of historical *versus* contemporary forces acting on population structure of a species and may be slow to detect recent or sudden shifts in genetic connectivity (Davies et al. 1999).

Advances in analytical tools such as new statistical approaches (e.g. Markov chain Monte Carlo algorithms) have led to the development of more rigorous assessments of population connectivity, yielding a more intuitive summary of the relationships between populations. Namely, Bayesian approaches, which provide multiple probabilities, allow more detailed inferences about both evolutionary parameters, such as mutation rate, and historical events, such as coalescence times (Luikart & England 1999). More recently developed methods known as assignment tests (reviewed in Manel et al. 2005) gained increasingly recognition for quantifying dispersal and to establish population membership of individuals or groups of individuals from genetic data. Presently, the use of assignment methods goes further than its initial application as a forensic tool, extending the field of application from conservation genetics to stock management (Hauser et al. 2006). Novel applications address identification of individual dispersers, estimation of interpopulation dispersal rate, parentage and genetic mixture analysis and identification of populations (Manel et al. 2005). Such application has been possible only with the discovery, in the last years, of more loci that are more polymorphic and abundant throughout genomes, together with the fact that the assignment method is based on the individual multilocus genotypes, rather than in population-wide descriptors (Davies et al. 1999).

Clustering Bayesian assignment methods are particularly useful when *a priori* data for potential source populations or species identity is not available whereby the method allows decomposing a mixture into its component parts, i.e. it searches for the most likely number of groups in the data (Manel et al. 2005). There are several examples in the literature describing the use of Bayesian clustering methods (e.g. Rannala & Mountain 1997, Pritchard et al. 2000, Eldridge et al. 2001, Falush et al. 2003). Their application have proven useful for the identification of populations, inference of population structure, assignment of individuals to populations, the study of hybrid zones and identification of migrants and admixed individuals. Nowadays, landscape genetics, defined as the combination of population genetics and landscape ecology (Manel et al. 2003,

Storfer et al. 2007) provide significant information on how landscape and environmental features influence gene flow, population structure, and local adaptation. In particular, landscape genetics may prove particular useful for determining the factors shaping patterns of genetic structuring among marine populations, traditionally assumed as genetically homogeneous across large distances (Hansen & Hemmer-Hansen 2007, Selkoe et al. 2008).

For the purpose of the present thesis, we applied a Bayesian clustering method that simultaneously integrates geographic and genetic information from high resolution genetic markers (Guillot et al. 2005). In this way it was possible to define clusters of individuals based on their multilocus genotypes, interpret the levels of genetic structure in the data set and suggest barriers to gene flow between clusters based on their geographical location (Chapter 2).

1.3.2 Larval time series analysis

Larval dispersal and supply determines the degree of connectivity between local populations and can be though affected by two sources of variation: variation in the spatial and temporal output of larvae produced by the local populations, which modifies the size and source of the larval pool, and subsequent spatial rearrangement of larvae in the pool and survival. Simultaneously, variation and unpredictability of physical processes during larval transport impose a high level of stochasticity on larval connectivity among marine nearshore populations (Siegel et al. 2008 and see also Chapter 4). Efforts to understand the mechanisms driving the patterns of spatial and temporal variability in larval supply, i.e. driving dispersal patterns, are essential to understanding and fully describing patterns of population connectivity. This is because the processes that control larval dispersal connect demographically benthic marine populations of species with a bi-phasic life cycle (Cowen & Sponaugle 2009).

Larval supply and larval settlement time series have been used to gain important insights into the nature of the oceanographic and biological processes that affect transport and dispersal mechanisms of coastal species (Hawkins & Hartnoll 1982, Pineda 1994, van Montfrans et al. 1995, Queiroga et al. 2006, Shanks & Roegner 2007). These studies usually implicitly assume an idealized pool of larvae that is available offshore and ready to be transported to their adult habitat. This larval pool assumption often is a misleading simplification, because it ignores the hierarchical nature of the processes that affect

dispersal and survival, where large-scale offshore processes have the potential to affect a very large number of larvae while small-scale nearshore processes then operate on fewer larvae (Pineda 2000).

The use of time series analyses in conjunction with genetic techniques may add significant valuable information for understanding the changes in connectivity and recruitment patterns that are crucial for the demography and population ecology (Burton 1996, Selkoe et al. 2008 and references therein). For example, genetic studies of larvae collected at different times often reveal genetic variation among larval cohorts that often exceeds that seen among geographic samples of adults and may elucidate the importance of seasonal shifts in current regimes that affect dispersal and connectivity patterns.

To enhance our understanding of marine population dynamics in *Carcinus maenas,* genetic patterns of variation among temporal samples of larvae were examined (Chapter 3). The relationship between patterns of larval supply abundance and shelf circulation and wind forcing were analyzed using a time series of five different years (Chapter 4).

1.3.3 Modelling tools

Thanks to advances in analytical and computational techniques as well improvements in the understanding of mesoscale ocean processes, over the last few years, is now possible to assess larval dispersal problems through physical modelling tools (Levin 2006). The use of models is especially useful given the impracticality of sampling over multiple temporal and spatial scales, and due to the difficulties and uncertainties associated with the use of natural tags. Physical modelling can actually predict the patterns of ocean circulation with high resolution and may be applicable to the study of ecosystem dynamics and particle transport, helping solving or giving directions for several questions related with population connectivity (Gallego et al. 2007, Werner et al. 2007). The use of physical modelling has, for example, contributed to a shift in the classic view of marine populations as open systems (Cowen et al. 2000). Because marine connectivity is intrinsically a coupled bio-physical problem, physical models are often combined with detailed biological variables (e.g. Siegel et al. 2003) to provide realistic estimates of mortality and larval behaviour. Namely, Individual-Based Coupled Physical–Biological Models (ICPBMs) are increasingly used for studying marine fish populations (reviewed in Miller 2007) to help explain patterns of distribution (Werner et al. 1993), make inferences about processes of

mortality (Brickman & Frank 2000) or feeding (Fiksen & MacKenzie 2002), identify potential spawning locations (Quinlan et al. 1999) and test complex hypothesis about evolution of life-cycle traits (Mullon et al. 2002). Similar approaches are also being used focusing in marine invertebrates (see Levin 2006 and references herein). Finally, the inclusion of population genetic information into models is presented in Galindo et al. (2006) that provided one of the first examples of integrated oceanographic and genetic models, to predict gene flow and population structure among locations of a reef-building coral across the Caribbean Sea.

Chapter 5 of this thesis presents the first results of the use and validation of an ICPBM to predict larval availability of *Carcinus maenas* in the Ria de Aveiro.

1.4 Study species: Carcinus maenas

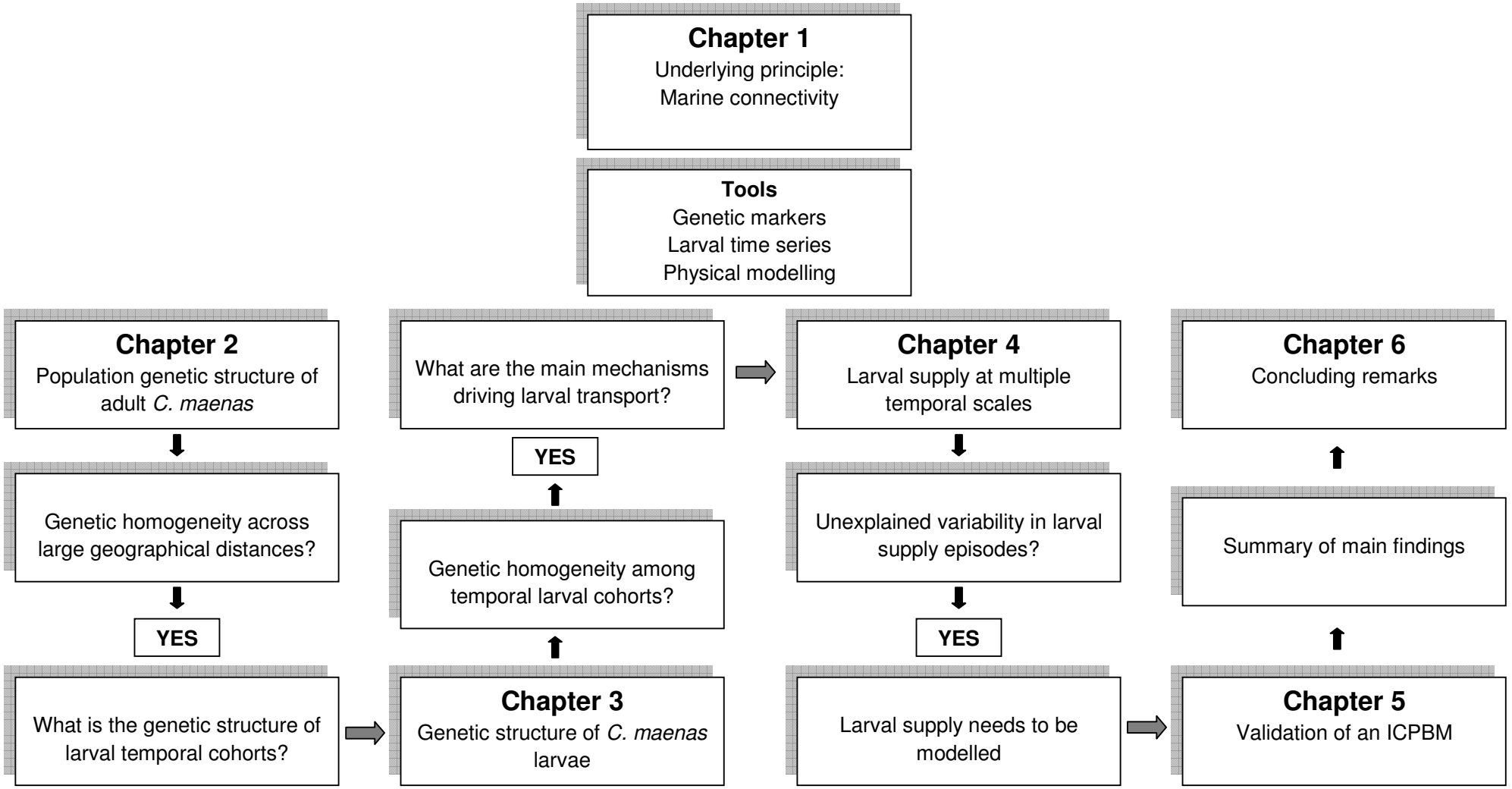
The common shore crab, *Carcinus maenas* (Decapoda, Portunidae), stands as one of the most characteristic and successful crustaceans that inhabit European estuarine systems and other coastal waters. There are several reasons for selecting *C. maenas* as a biological model to address a number of important issues in marine ecology related with gene flow, population connectivity and recruitment: i) wide geographical distribution, local high abundance and prime ecological importance; ii) ease of capture and suitability for laboratory studies; iii) significant information already available on life cycles, behaviour and larval ecology; and iv) passive plankton nets have been developed previously for the study of larval supply which allow the implementation of daily sampling programmes necessary to match the time scales of the forcing agents' variability. Nonetheless, several aspects of their life history still remain unclear, including information on genetic population structure.

The genus *Carcinus* has gained notoriety since it become a global invader during the last century, establishing populations along the east and west coasts of USA, South Africa, Japan, Australia and Argentina (Carlton & Cohen 2003, Darling et al. 2008). In some of these areas its range is still expanding, with predatory impacts on native communities (Yamada & Gillespie 2008). Its success as an invader species is due to its high adaptability, high fecundity and relatively long planktonic larval phase.

Along the native range, *Carcinus maenas* extends from 70° N in Norway to 22° N in Mauritania, including Iceland, the Faroe Islands, the British Islands and the western part of

the Baltic Sea (Almaça 1962, Crothers 1968). It is an iteroparous species with internal fecundation. It usually starts breeding at the age of one year and may spawn twice a year during its 3 to 4 years life span (Démeusy 1958). During each spawning event, a female may produce up to 200 000 eggs per brood (Broekhuysen 1936), which are carried attached to the pleopods during embryonic development. The reproductive season of *C. maenas* is affected by water temperature, and larval release and settlement therefore occur later at higher latitudes (Pihl & Rosenberg 1982) where the spawning season is shorter and occurs mainly in late spring and early summer. Close to the southern limit of the of the species distribution spawning occurs from mid winter to summer. In Portuguese estuaries, ovigerous females are most encountered from November to May (Queiroga 1995). During this period, larvae hatched inside estuaries during night ebbing tides are quickly flushed to the shelf, where most of the larval development takes place. The larval series includes four planktotrophic zoeae and one megalopa (Rice & Ingle 1975), and may extend four to six weeks, depending on temperature (Nagaraj 1993). Supply of megalopae to estuaries is an episodic phenomenon that depends on alongshore winds and tides (Almeida & Queiroga 2003, Queiroga et al. 2006). The current understanding of these processes, which are discussed in more detail in Chapter 4, is that diel vertical migration (dos Santos et al. 2008) helps in retention of the larvae in the inner shelf during spring and early summer, through interaction with the two-layered flow typical of upwelling circulation (Marta-Almeida et al. 2006). Relaxation of upwelling causes translocation of the larvae to the near-shore environment, and supply into estuaries then occurs by selective tidal stream transport (Queiroga et al. 2006). Settlement occurs from late winter to early summer with peaks found from mid March to July (Almeida & Queiroga 2003, Queiroga et al. 2006).

1.5 Schematic overview of thesis Chapters



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Chapter 2

Population genetic structure of Carcinus maenas

Domingues CP, Creer S, Taylor MI, Queiroga H, Carvalho GR (2010). Genetic structure of *Carcinus maenas* within its native range: larval dispersal and oceanographic variability. Mar Ecol Prog Ser 410:111-123

2.1 Abstract

Unravelling the interactions between life-history strategies and oceanography is central to our understanding of gene flow and connectivity in the marine environment. In the present study, we investigated the population genetic structure of the shore crab in its native range in relation to oceanographic characteristics and dispersal potential. Using ten microsatellite markers we surveyed, over two years, 18 locations distributed along ~ 4200 km within the species native range, from Sweden to Morocco, assessed the population structure by means of F_{ST} and Bayesian clustering analysis and tested the hypothesis of isolation-by-distance (IBD) with a Mantel test. We focused particular attention along a 1200 km stretch of the Iberian Peninsula. We found no evidence of genetic structure (F_{ST} = 0.0001, $P > 0.05$) along the Iberian coast, and patterns were temporally stable over two years. Across the more extensive geographic spatial scale, overall genetic differentiation was low (F_{ST} = 0.001) but statistically significant ($P < 0.001$). Furthermore, clustering analysis grouped the individuals into three genetic units, corresponding to samples from i) Sweden, ii) Wales and Iberian Peninsula and iii) Morocco. While the correlation between genetic and geographic distances was significant, the pattern was not consistent with an IBD pattern. Results suggests that, in the absence of barriers to gene flow, shore crab populations are genetically similar across thousands of kilometres, but isolated populations still may occur within the species native range. Local oceanography and larval behaviour may have a significant influence on the structuring of the populations under study.

2.2 Introduction

Most coastal marine invertebrates and fish develop from planktonic larvae that drift for days to months in the oceanic realm. Such development strategies therefore represent an important mechanism for the dispersal of marine species for adults that are either sedentary or exhibit limited mobility during the adult phases. Highly mobile larvae have the potential to be transported from several meters to hundreds of kilometres and in the apparent absence of barriers to gene flow, even distant regions might be connected genetically (Kinlan & Gaines 2003, Palumbi 2003, Thorrold 2006). Examples of ongoing long distance gene flow are widespread in marine systems (Bohonak 1999), though unrecognized barriers to dispersal often result in a disparity between the paradigm of large-scale connectivity and empirical observations (reviewed in Hauser & Carvalho 2008).

In fact, numerous studies report the presence of population subdivision in species with extensive potential for dispersal, and sometimes, across a surprisingly small scale (Shaw et al. 1999, Hutchinson et al. 2001, Taylor & Hellberg 2003, Bekkevold et al. 2005, Bilodeau et al. 2005, Weetman et al. 2007). Moreover, self-recruitment may be more common than previously recognized, as evidenced by genetic studies (e.g. Jones et al. 2005, Carreras-Carbonell et al. 2007).

Isolation-by-distance (IBD) theory states that the genetic distances between populations increases with greater geographic distance, and that genetic distance declines with increased dispersal radius, producing a clear geographic genetic structure (Hellberg et al. 2002, Palumbi 2003). Genetic IBD is evident in several species ranging from fish (Pogson et al. 2001, Purcell et al. 2006, Johansson et al. 2008), to marine invertebrates (Palumbi et al. 1997, Launey et al. 2002, Couceiro et al. 2007), and has proven to be a powerful approach to interpreting the dynamics of gene flow (Palumbi 2003). It is now recognized that the relationship between dispersal radius and population connectivity can be very complex and many factors such as the species' life history, behavioural adaptations, oceanographic circulation patterns or historical events (Pringle & Wares 2007) influence contemporary patterns of gene flow. The use of novel and more informative genetic markers and chemical tags, together with enhanced sampling design, data analysis and individual-based coupled physical–biological models incorporating oceanography and larval biology, provide a robust amalgam of tools to explore effectively larval dispersal and population dynamics (reviewed in Levin 2006, Cowen & Sponaugle 2009).

Here, we analyse microsatellite variation among populations of the decapod crustacean *Carcinus maenas* within the native species range. *C. maenas* is one of the most abundant and intensively studied invertebrates in the world and accordingly, is suited as a biological model to address a number of important issues in marine ecology related with gene flow and population connectivity. *C. maenas* has a native geographical distribution that extends from Norway to Mauritania, including Iceland, the Faroe Isles and the British Isles, where it inhabits estuaries and rocky shores during its juvenile and adult stages (Almaça 1962, Crothers 1968). Crabs from the genus *Carcinus* are very successful predators and tolerate a wide range of environmental conditions. *Carcinus* has become a global invader during the last century, establishing populations in the east and west coasts of North America, South Africa, Japan, Australia and Argentina (Carlton & Cohen 2003). In some areas its range is still expanding, with measurable impacts on native communities

(Yamada & Gillespie 2008). Dispersal is via a planktonic larval phase that consists of four zoeal stages and a megalopal stage that develops in the water column from late winter to early summer for four to six weeks depending on water temperature (Queiroga 1996). Larval *Carcinus* spend considerable time in the plankton, a life-history strategy that predicts long dispersal distances that will be strongly influenced by coastal and oceanic circulation regimes (Shanks et al. 2003, Peliz et al. 2007). In addition, several studies (Queiroga & Blanton 2005, Queiroga et al. 2007) show how shore crab larvae take advantage of the capacity to perform tidally-synchronized migrations to maximize their export out to the sea, followed by supply back to estuaries and rocky shores where adults live. Larvae are also known to perform extensive diel vertical migrations, exploiting currents at different depths that can be important for larval retention in shelf waters, especially in strongly vertically sheared flows (e. g. cross-shore upwelling circulation in stratified shelves), as described by Marta-Almeida et al. (2006).

Mitochondrial DNA variation across the European range of *Carcinus maenas* based on cytochrome c oxidase I (COI) gene was previously surveyed by Roman & Palumbi (2004) who found significant genetic differences between the off-shelf populations of Faeroe Islands and Iceland and the continental populations, as well as slight genetic structuring between the central North Sea and populations to the south along the Atlantic coast up to southern Spain. More recently, Darling et al. (2008) used both the COI gene and nine microsatellite loci to investigate the genetic patterns of *Carcinus* introductions around the globe. Some native *C. maenas* populations, from where just a few individuals were sampled, were analysed during the study but the data revealed little detectable genetic differentiation within the native species' range. The genetic structuring that was recovered was dominated by differences between the off-shelf population at Torshavn, Faeroe Islands, and the other native populations. Here, we analysed a broader sampling of populations, individuals and microsatellite loci covering the main distribution of *C. maenas* in its native European range, including one location in North Africa not studied previously.

The circulation of coastal waters of the western Iberian Peninsula is highly dynamic and characterised by seasonal and short term variations in current patterns. A predominantly equatorward flow occurs after the spring transition until the end of the summer, when northerly upwelling-favourable winds dominate the circulation (Fiúza et al. 1982), while in winter a predominantly poleward flow is observed (Iberian Poleward Current, IPC) (Peliz et al. 2005). During the non-upwelling season, the shelf is also under the influence of

another local feature resulting from river runoff of several rivers located on the northwest coast of Portugal and Spain that generates a low salinity surface layer, the western Iberia Buoyant Plume (WIBP) (Peliz et al. 2002). Previous studies in this region based on physical modelling indicate that larval transport processes can be strongly dependent on mesoscale features associated with both the WIBP and the IPC (Santos et al. 2004), as well as with different wind regimes, coastal orientation and river plumes (Peliz et al. 2007). Moreover, Peliz et al. (2007) hypothesized that dispersal conditions in northwest Iberia may be significantly different from the region to the south of the Estremadura promontory, and from north of Cape Finisterre due to changes in coastal topography and local oceanography. A previous survey carried out in the Portuguese coast found, indeed, weak but significant genetic structuring between *Carcinus maenas* populations from north and south of the Estremadura promontory (Pascoal et al. 2009). We then expect that variation in oceanographic features, which can vary seasonally or interannually, associated with different segments of the coast, should affect larval dispersal and population connectivity of the shore crab. To test this hypothesis, we surveyed the genetic connectivity in neighbouring populations sampled along 1200 km of the Iberian Peninsula coastline and examined the temporal stability of microsatellite allele frequencies from two consecutive years. In order to place regional differentiation into a broader geographic scale, we also surveyed the levels of population differentiation and tested the hypothesis of IBD in populations outside the Iberian Peninsula from samples collected in a third year. In total, our sampling comprised 18 locations distributed from Gullmarsfjord in Sweden, to Oued Tahadart in Morocco.

2.3 Materials and Methods

2.3.1 Sample collection

Carcinus maenas occurs in estuaries and rias along the Iberian Peninsula. We collected samples from 14 populations distributed along a 1200 km stretch of the southern, west and north coasts (Figure 1, Table 1) in 2006, and again from the same sites in 2007, allowing a test of temporal variation. To assess relationships across a broad geographic scale, four locations within the species' native distributional range, and one location in the Mediterranean were sampled in 2008: Gullmarsfjord (Sweden), Menai Strait (Wales, UK), Cadiz Bay (south west Spain), Oued Tahadart (Morocco) and Ebro Delta (Catalan coast). At each location, approximately 50 crabs were caught with baited hoop nets in the months of June to August, in order to minimize possible seasonal differences. From each

specimen, muscle tissue was removed from one periopod and preserved in 96% ethanol until DNA extraction.

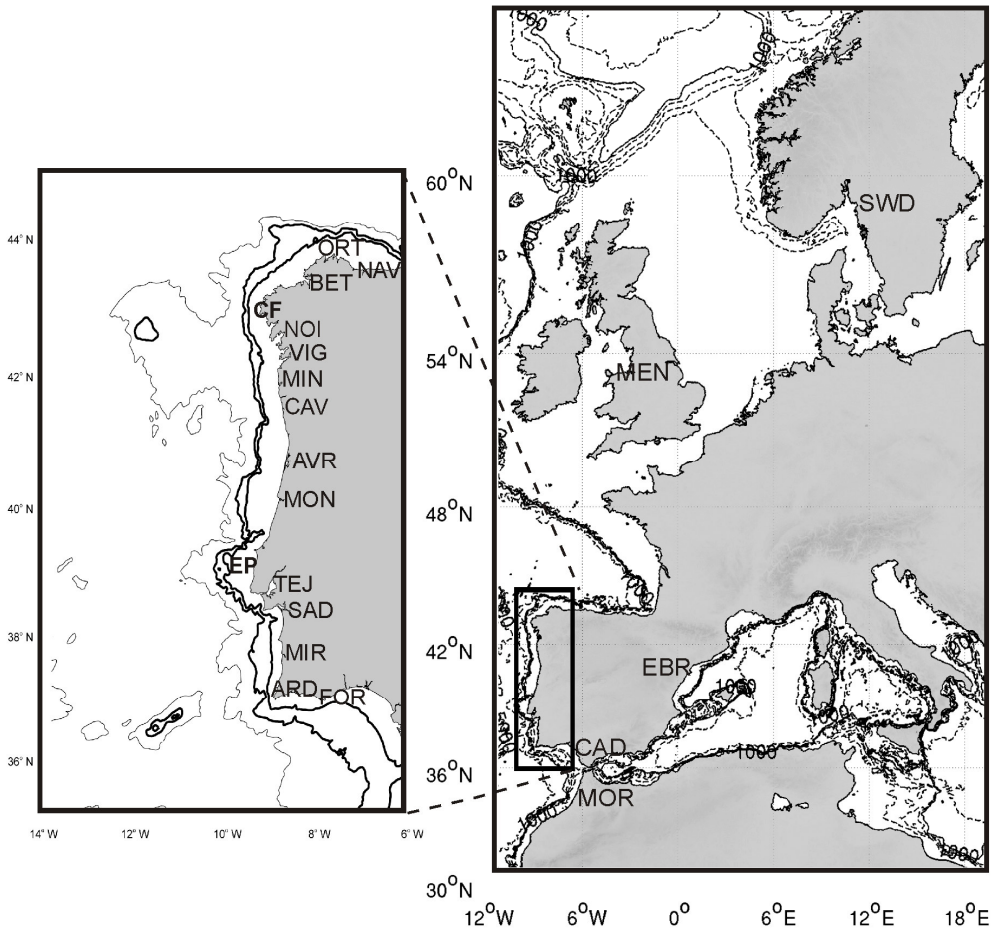

Figure 1 *Carcinus maenas*. Sampling sites. See Table 1 for location codes. CF: Cape Finisterre; EP: Estremadura Promontory.

2.3.2 DNA extraction and microsatellite genotyping

Total genomic DNA was extracted in 96-well format from muscle tissue using overnight digestion with Proteinase K following a modified salt extraction protocol (Aljanabi & Martinez 1997). DNA was resuspended in a volume of 100 µl of 1× TE buffer (10 nM Tris-Cl, 1 nM EDTA, pH 8.0) and stored at - 20 ºC. We selected 12 microsatellite loci developed for *Carcinus maenas*: ten loci from Tepolt et al. (2006) (Cma01EPA, Cma02EPA, Cma03EPA, Cma04EPA, Cma05EPA, Cma08EPA, Cma09EPA, Cma10EPA, Cma12EPA, Cma14EPA) and two loci from Pascoal et al. (2009) (SP107, SP495).

Table 1 *Carcinus maenas*. Sampling sites, position and number of individuals collected each year (2006 to 2008). Samples from the same location showing no genetic divergence were pooled, so that a total of 18 samples from *C. maenas* populations were included in the statistical processing. Code: sample location abbreviation

Location	Code	Geographic position	Sample size		
			2006	2007	2008
Gullmarsfjord	SWD	58º15'N 11º25'E	–	–	50
Menai Strait	MEN	53º14'N 4º10'W	–	–	50
Navia	NAV	43º32'N 6º43'W	39	20	–
Ortigueira	ORT	43º42'N 7º52'W	49	50	–
Betanzos	BET	43º21´N 8º12'W	48	50	–
Noia	NOI	42º48'N 8º54'W	50	50	–
Vigo	VIG	42º20'N 8º38'W	50	48	–
Minho	MIN	41º52'N 8º50'W	49	50	–
Cavado	CAV	41º31'N 8º46'W	50	50	–
Aveiro	AVR	40º37'N 8º44'W	50	50	–
Mondego	MON	40º08'N 8º50'W	50	50	–
Tejo	TEJ	38º44'N 8º55'W	49	50	–
Sado	SAD	38º24'N 8º45'W	27	50	–
Mira	MIR	37º39'N 8º43'W	–	49	–
Arade	ARD	37º09'N 8º29'W	34	50	–
Formosa	FOR	37º00'N 7º58'W	50	50	–
Cadiz	CAD	36º28'N 6º11'W	–	–	50
Oued Tahadart	MOR	35º34'N 6º00'W	–	–	30
Ebro Delta	EBR	40º38'N 0º43'W	–	–	40

Loci were amplified in two multiplex PCR reactions using forward 6-FAM, VIC, NED or PET fluorescently labelled primers. PCR amplifications contained approximately 20 to 100 ng of template DNA, 1× QIAGEN Multiplex PCR Master Mix (Qiagen) and 0.1-0.3 µM of each primer in a total reaction volume of 10 µL. Reactions were performed in Bio-Rad Tetrad2 Peltier Thermal Cyclers under the following conditions: 95 ºC for 15 min followed by 30 cycles of 94 ºC for 30 s, 55 ºC for 90 s and 72 ºC for 60 s followed by a final extension at 60 ºC for 30 min. Resulting products were then resolved on a ABI 3130xl Genetic Analyzer (Applied Biosystems) and sized using GeneScan LIZ-500 internal size standard and GENEMAPPER version 4.0 software (Applied Biosystems). During initial testing, 100 individuals were amplified independently two times across all loci to assess the reliability of PCR and genotyping error rate.

2.3.3 Statistical analysis

The potential presence of null alleles and scoring errors due to stuttering and large allele drop-out was tested using MICRO-CHECKER version 2.2.3 software (van Oosterhout et al. 2004). Allele frequencies and measures of genetic diversity such as expected heterozygosity (H_E), observed heterozygosity (H_o), number of alleles (N_A) and allelic richness (A) were calculated by FSTAT version 2.9.3.2 (Goudet 2001) and by GENETIX version 4.05 (Belkhir et al. 1996-2004). FSTAT was also used to assess deviations from Hardy-Weinberg equilibrium across all loci and populations using the inbreeding coefficient F_{IS} as estimated by f (Weir & Cockerham 1984) and to estimate overall levels of population differentiation using F_{ST} as estimated by θ (Weir & Cockerham 1984). The significance of F_{IS} and F_{ST} was tested based on a random permutation procedure, and confidence intervals (CI) calculated by bootstrapping over loci (Goudet 2001). Pairwise F_{ST} (θ) values between all population pairs were calculated with GENETIX, with their significances tested using 10 000 permutations. Annual, within-location samples that did not show significant genetic differentiation in these tests were pooled in subsequent analysis. Linkage disequilibrium between pairs of loci was tested using the exact test implemented in GENEPOP version 4.0 (Rousset 2008), with significance levels determined by the Markov chain method (dememorization = 5000, batches = 500, iterations = 10000). Where multiple comparisons were involved, we used the sequential Bonferroni procedure (Rice 1989) at the 5% level to adjust the statistical significance. IBD was assessed by plotting pairwise F_{ST} / (1 – F_{ST}) values (Rousset 1997) against the logarithm of the geographic distances (measured as the shortest distance by sea in km) between all sample sites. Mantel test (30 000 permutations) and reduced major axis (RMA) regression were conducted to assess the significance and strength of the relationship between genetic and geographic distances with the software IBDWS (Jensen et al. 2005). The same standard population genetic analysis described above was performed on the inferred populations identified by GENELAND (Guillot et al. 2005) (see below).

2.3.4 Power analysis

Statistical power for detecting genetic differentiation using the microsatellite markers characterised by given levels of allelic diversity and sample sizes was analysed with the program POWSIM (Ryman & Palm 2006). Computer simulations mimic sampling from populations at various levels of expected divergence under a classical Wright-Fisher model without migration or mutation. To test the power to detect an expected divergence

of F_{ST} = 0.001 among subpopulations, 1000 simulations, over 20 generations each, were run employing sample sizes corresponding to those from our sampling regions and the allele frequencies from the current data set as a starting point.

2.3.5 Bayesian clustering analysis

We used the Bayesian clustering methodology of GENELAND version 3.1.4 software (Guillot et al. 2005) in the R-PACKAGE (Ihaka & Gentleman 1996) to detect and determine the level of genetic structure in the data set. GENELAND integrates the spatial coordinates of individuals together with the genetic information and so provides an improved definition of the spatial genetic units when compared with non-spatial clustering methods. All the unknown parameters are processed simultaneously through Markov chain Monte Carlo (MCMC) computations. Due to substantial algorithm improvement implemented in the recent versions of GENELAND software (from version 3.0.0 onwards) we used the correlated frequency model that allowed us to detect subtle structures in the presence of low genetic differentiation that would probably remain undetected using an uncorrelated frequencies model (Guillot 2008). Additionally, improvements in the post-processing scheme allowed estimation of the number of populations (*K*), as well as the assignment of individuals to the inferred populations in a single step, treating the number of clusters as unknown. We placed an independent Gamma prior on the drift coefficients with parameters (2, 20). GENELAND was then run 50 times for each dataset with 500 000 MCMC iterations and a burn-in of 100 000 iterations in the post-processing. Next, we calculated the mean logarithm of posterior probability distribution of the data for each of the 50 runs and selected only the 10 with the highest posterior distribution to be considered in the analysis. We finally checked visually for the consistency of results across the 10 runs.

2.4 Results

2.4.1 Microsatellite amplification

The presence of null alleles, assessed with MICRO-CHECKER (van Oosterhout et al. 2004), was detected in two loci (Cma02EPA and Cma12EPA), and scoring errors due to stuttering was found at one locus (Cma12EPA). These two loci were therefore removed from subsequent analysis of population genetic structure. For the remaining ten loci, PCR products from repeated amplifications of the same individual consistently produced the same genotype and all reliably amplified in every sample except the locus SP495, which did not amplify in the samples collected in Ebro Delta. During our study, we identified that

individuals from this population are in fact *Carcinus aestuarii*, a sibling species of *Carcinus maenas* that occurs in the Mediterranean Sea. For this reason, the Ebro population was discarded from the analysis.

2.4.2 Genetic diversity and Hardy-Weinberg and linkage equilibrium

All microsatellite loci displayed moderate to high levels of polymorphism (Table 2): the number of alleles per locus ranged from 2 to 54 (mean = 19.8) and the expected heterozygosity from 0.377 to 0.959 (mean = 0.686). Global F_{IS} was 0.016 (95% CI = 0.004-0.029, $P < 0.001$) and there was a significant heterozygote deficiency over all loci, due to loci SP107 and SP495. The small but significant heterozygote deficiency suggests that inbreeding or a subtle spatial structure (i.e. Wahlund effect) exists within the data set (null alleles detected by MICRO-CHECKER were eliminated prior to analysis). Significant linkage disequilibrium was found in 42 out of 810 pairwise comparisons among ten loci for all populations however, none was significant after sequential Bonferroni correction (Rice 1989).

2.4.3 Power analysis

The simulations undertaken using POWSIM (Ryman & Palm 2006) indicate that the number of loci, the number of alleles per locus, their frequency distributions and the sample sizes used were sufficient to reveal population structure at a true F_{ST} as low as 0.001 with a statistical power of > 99%.

2.4.4 Population differentiation

Comparison of allele frequencies among samples collected along the Iberian Peninsula in 2006 and 2007, from the same locations, exhibited no significant genetic differences (F_{ST} ranged from –0.0030 to 0.0052). Since the signal of genetic differentiation detected appears to be stable, at least over two years, we pooled the samples from the same geographical location. Considering solely the Iberian Peninsula samples, no significant differentiation was detected using F_{ST} (F_{ST} = 0.000, 95% CI = –0.000 – 0.001, p > 0.05). Moreover, no structure was observed when these samples were pooled into north (NAV, ORT, BET, NOI, VIG, MIN, CAV, AVR, MON) and south (TEJ, SAD, MIR, ARD, FOR) of the Estremadura Promontory, allowing testing for regional differences of the coast (F_{ST} = – 0.000, 95% CI = –0.000 – 0.000, p > 0.05) (see Table 1 for sample location code).

Over a large geographical scale, an overall F_{ST} value of 0.001 (95% CI = 0.001-0.003, $P < 0.001$, Table 2), indicates low, but significant genetic differentiation among samples.

Table 2 *Carcinus maenas*. Summary statistics for 10 microsatellite loci pooled from 18 populations. Significant F_{IS} values of the tests for heterozygosity deficiency and significant F_{ST} values of the tests for genetic differentiation, following Bonferroni correction (Rice 1989), are denoted in **bold**. Asterisks refer to values that were significant before applying correction (*p < 0.05; **p < 0.01). N_A: number of alleles; *Ho*: observed heterozygosity; *He*: expected heterozygosity; F_{IS} (f) and F_{ST} (θ) values calculated after Weir & Cockerham (1984).

Locus	N_A	H_o	H_E	F_{IS}	F_{ST}
Cma01EPA	8	0.426	0.415	-0.010	0.001
Cma03EPA	16	0.798	0.806	0.007	0.002*
Cma04EPA	28	0.912	0.903	-0.012	0.000
Cma05EPA	2	0.414	0.425	0.034	0.003
Cma08EPA	33	0.934	0.952	0.009	0.000
Cma09EPA	22	0.778	0.792	0.021	0.001
Cma10EPA	54	0.953	0.959	0.008	0.000
Cma14EPA	11	0.365	0.377	0.022	0.004**
SP107	15	0.558	0.586	**0.049**	**0.006**
SP495	9	0.612	0.644	**0.051**	0.001
Overall	19.8	0.674	0.686	**0.016**	**0.001**

Pairwise comparisons were assessed among all samples collected from Sweden to Morocco (Table 3). Samples from the Iberian Peninsula together with the sample from Wales, UK, appeared homogeneous, with no significant differentiation observed from the Menai Straits to Cadiz following the use of sequential Bonferroni correction for multiple comparisons (pairwise F_{ST} ranged from -0.0025 to 0.0038). Comparisons between the previous samples (Menai Strait to Cadiz) with the Moroccan sample indicated significant genetic differentiation in 8 out of the 16 comparisons before sequential Bonferroni correction, though none were significant after correction (pairwise F_{ST} ranged from 0.0011 to 0.0110). Samples from Sweden, in the Skagerrak region, exhibited significant differences with all other locations, after correcting for multiple comparisons, except with the Menai Straits (Wales) sample: $F_{ST} = 0.0059$, $P < 0.05$ (SWD and MEN), $F_{ST} = 0.0289$, $P < 0.001$ (SWD and MOR) and against the Iberian Peninsula samples F_{ST} ranged from 0.0102 to 0.0193, $P < 0.001$. The Mantel test correlation between genetic and the logarithm of geographic distances was positive and significant ($Z = 1.20$, $r = 0.55$, $P < 0.01$), with geographic distance accounting for 30% of the variation in genetic

differentiation (Figure 2). When populations from the Iberian Peninsula were considered on their own, no significant IBD was observed (Z = 0.02, r = -0.03, P > 0.60) (Figure 2).

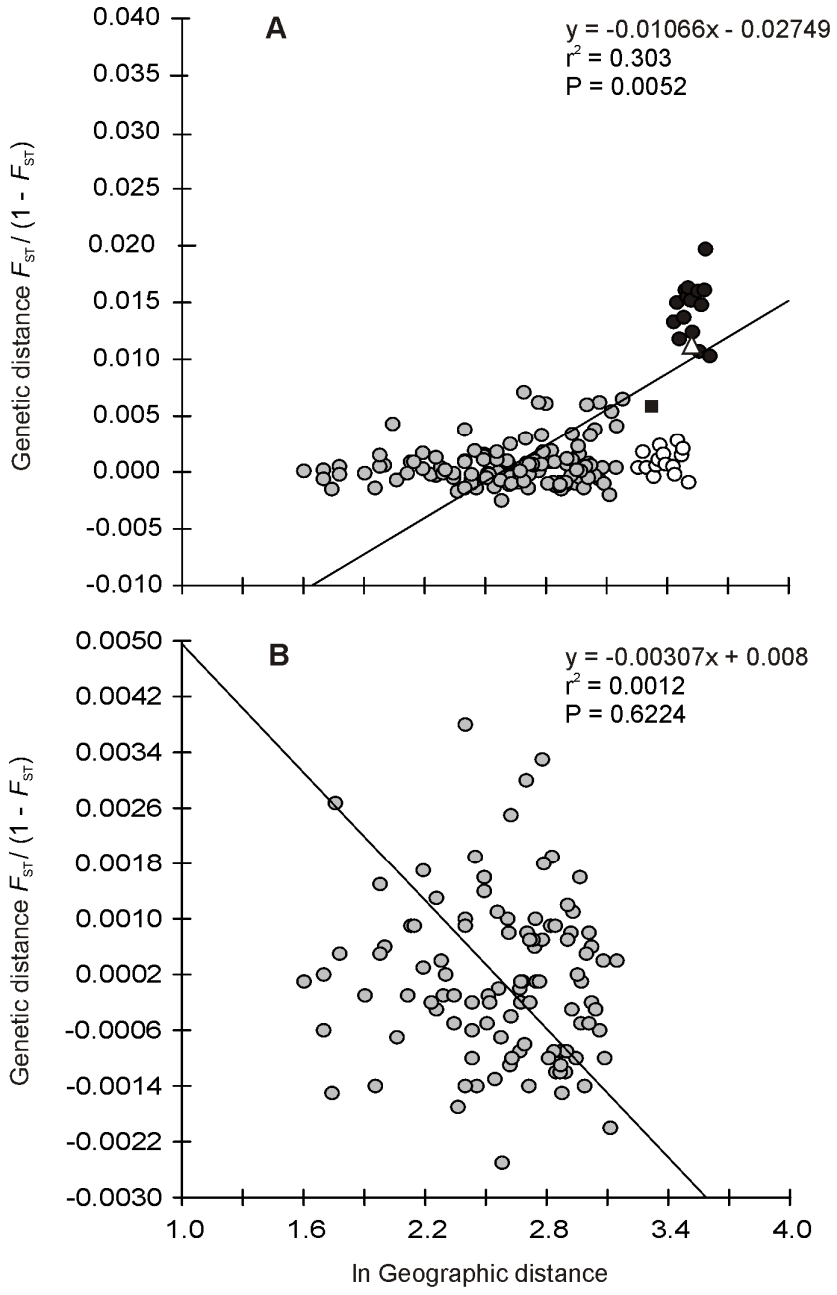

Figure 2 *Carcinus maenas*. Relationship between genetic differences (F_{ST} / [1 – F_{ST}]) and the logarithm (geographical distance) at (A) all sampling sites (SWD to MOR) and (B) at the Iberian Peninsula (NAV to CAD). Comparisons between (A) Sweden and Menai Strait (black square); Sweden and the remaining samples (black circles); Menai Strait and Morocco (white triangle); Menai Strait and the remaining samples (white circles); the remaining comparisons (grey circles), and (B) all comparisons between samples from Navia to Cadiz (grey circles). For abbreviations see Table 1.

Table 3 *Carcinus maenas*. Estimates of pairwise genetic differentiation (F_{ST} values estimated by θ) among 18 populations. Significant F_{ST} values following sequential Bonferroni correction (Rice 1989) for 153 multiple comparisons are in **bold**. Asterisks: values that were significant before applying correction (*p < 0.05; **p < 0.01). See Table 1 for sample location codes.

	SWD	MEN	NAV	ORT	BET	NOI	VIG	MIN	CAV	AVR	MON	TEJ	SAD	MIR	ARD	FOR	CAD
MEN	0.0059*																
NAV	**0.0132**	0.0004															
ORT	**0.0147**	0.0018	0.0006														
BET	**0.0117**	0.0004	-0.0003	-0.0001													
NOI	**0.0136**	-0.0004	-0.0013	0.0010	-0.0003												
VIG	**0.0159**	0.0006	0.0008	0.0016	-0.0017	0.0005											
MIN	**0.0153**	0.0011	0.0000	0.0000	-0.0014	-0.0007	-0.0015										
CAV	**0.0161**	0.0024	0.0008	0.0010	-0.0002	0.0017	0.0005	0.0001									
AVR	**0.0149**	0.0016	0.0032*	0.0030*	-0.0004	0.0038**	0.0004	0.0009	0.0015								
MON	**0.0122**	0.0007	0.0009	0.0001	0.0001	0.0014	0.0009	-0.0001	0.0003	-0.0002							
TEJ	**0.0158**	0.0005	-0.0010	-0.0012	-0.0012	0.0007	-0.0003	-0.0011	-0.0007	0.0019	-0.0005						
SAD	**0.0106**	-0.0003	-0.0005	0.0008	-0.0015	0.0001	-0.0002	-0.0009	-0.0010	-0.0002	-0.0006	0.0002					
MIR	**0.0146**	0.0028	0.0008	0.0016	-0.0003	0.0019	0.0018	0.0010	-0.0014	0.0025	0.0011	0.0009	-0.0014				
ARD	**0.0159**	0.0015	-0.0006	-0.0002	-0.0014	0.0007	-0.0009	-0.0009	-0.0010	0.0006	-0.0008	-0.0011	-0.0001	-0.0001			
FOR	**0.0193**	0.0021	0.0004	-0.0003	-0.0006	0.0011	-0.0009	-0.0012	0.0009	0.0007	0.0007	-0.0005	-0.0002	0.0013	-0.0006		
CAD	**0.0102**	-0.0010	0.0004	-0.0020	-0.0011	0.0006	0.0005	0.0001	0.0002	0.0012	-0.0012	0.0007	0.0001	-0.0025	-0.0014	0.0002	
MOR	**0.0289**	0.0110**	0.0064*	0.0041	0.0054*	0.0061*	0.0038	0.0032	0.0060*	0.0023	0.0034	0.0061*	0.0062*	0.0070*	0.0018	0.0011	0.0043

2.4.5 Bayesian clustering analysis

We investigated the number of clusters along the native range of *Carcinus maenas* based on 18 locations using GENELAND (Guillot et al. 2005), a Bayesian method that uses both genetic and spatial data. Posterior distributions of the estimated number of populations (*K*) displayed a clear mode at *K* = 3 across the 10 replicates (Figure 3).

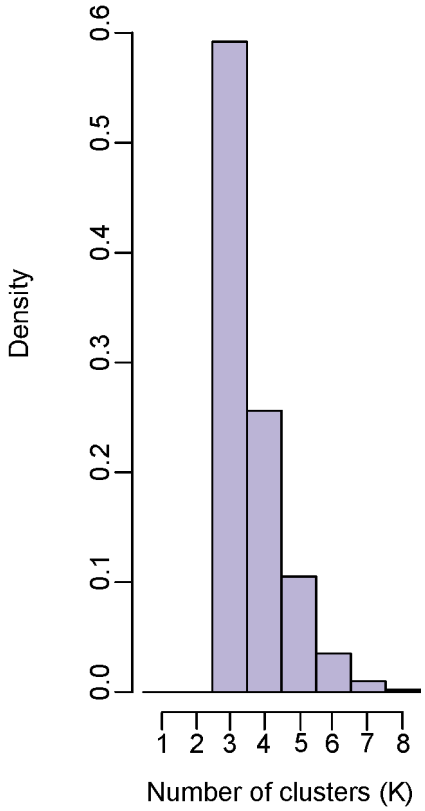

Figure 3 *Carcinus maenas*. Posterior density distribution of the number of clusters estimated from Geneland analysis in 10 replicates.

The Geneland model identified three spatially coherent clusters (Figure 4) in 8 out of 10 replicates: the first includes *C. maenas* from the Swedish population (SWD); the second, *C. maenas* from Menai Straits with the Iberian Peninsula (MEN-IP); and the third, *C. maenas* from the Morocco population (MOR). Each cluster had a probability of 0.8 of belonging to their regional group, thereby providing strong support to the respective cluster.

2.4.6 Population genetic parameters of the inferred populations

The three identified clusters displayed comparable levels of genetic diversity that were relatively high, they were assessed using expected heterozygosity and allelic richness, the

latter corrected for difference in sample size (Table 4). In the Swedish and Moroccan populations, F_{IS} was -0.003 and 0.053, respectively, and there was no evidence of departure from Hardy-Weinberg equilibrium. For the cluster comprising samples from Menai Straits and the Iberian Peninsula, F_{IS} was 0.016, with a significant deficiency in heterozygosity ($P < 0.001$). In the three inferred populations there was no significant linkage disequilibrium. Global F_{ST} was 0.011 (95% CI = 0.004-0.020, $P < 0.001$), and pairwise F_{ST} among clusters was 0.0141, $P < 0.001$ (SWD and MEN-IP), 0.0289, $P < 0.001$ (SWD and MOR) and 0.0045, $P < 0.05$ (MEN-IP and MOR).

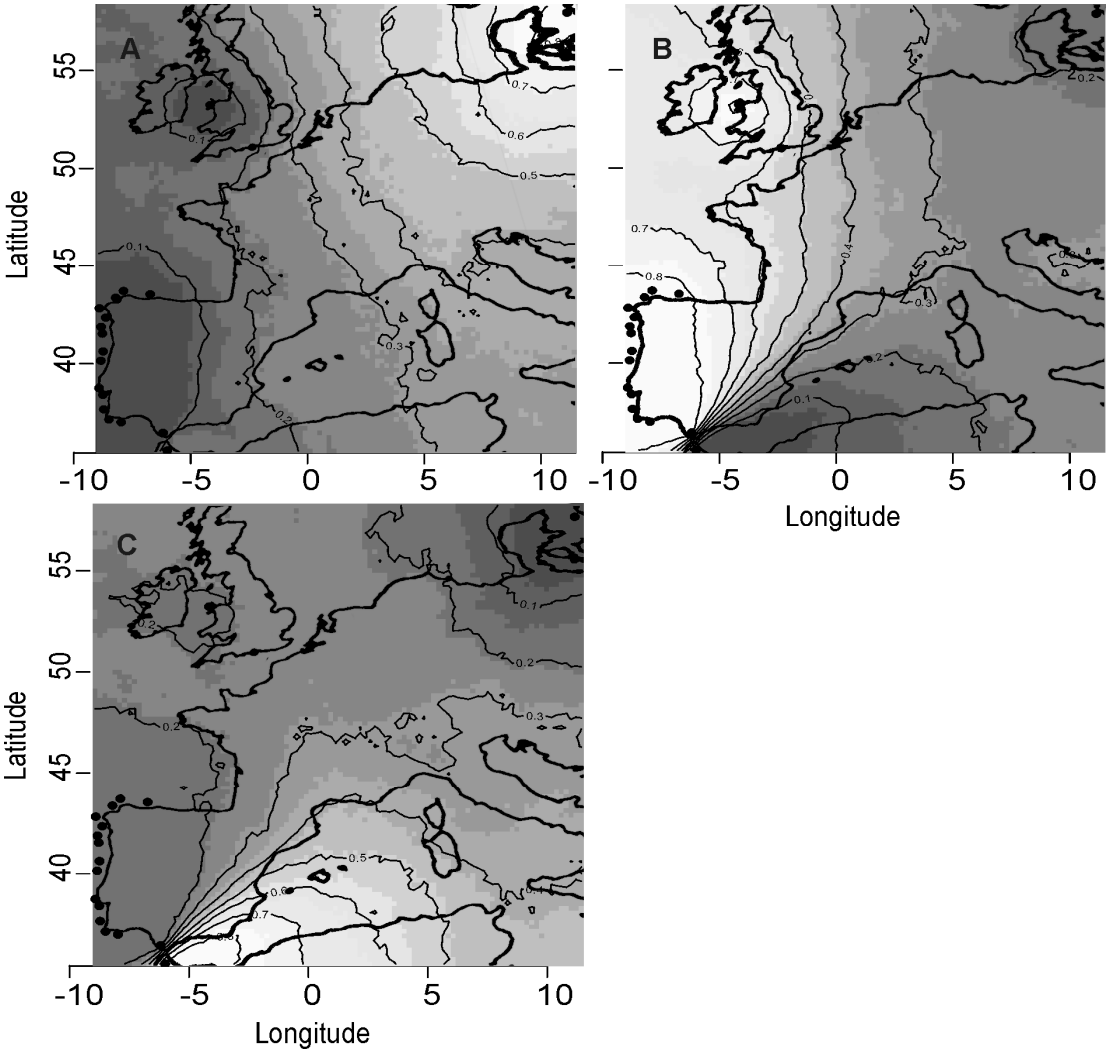

Figure 4 *Carcinus maenas.* Geneland assignment of individuals to clusters for K = 3 in the best run. The three plots represent the most likely cluster membership according to the Geneland algorithm. (A) Sweden cluster, (B) Menai Strait (UK) and Iberian Peninsula cluster, and (C) Morocco cluster. Highest population membership values are in white and level curves illustrate spatial changes in assignment values.

Table 4 *Carcinus maenas*. Summary statistics for 10 microsatellite loci for SWD, MEN-IP and MOR populations inferred from the cluster analyses. Significant F_{IS} values of the tests for heterozygosity deficiency, following Bonferroni correction (Rice 1989), are in **bold**. Asterisks: values that were significant before applying correction (*p < 0.05; **p < 0.01). n: sample size; A: allelic richness (estimated for a n = 25); *H*o: observed heterozygosity; *H*e: expected heterozygosity; F_{IS} (f) and F_{ST} (θ) values calculated after Weir & Cockerham (1984).

Locus	Sweden (n = 50)				Menai Strait & Iberian Peninsula (n = 1362)				Morocco (n = 30)			
	A	H_o	H_E	F_{IS}	A	H_o	H_E	F_{IS}	A	H_o	H_E	F_{IS}
Cma01EPA	2.8	0.520	0.466	-0.117	3.3	0.415	0.415	-0.001	2.8	0.433	0.352	-0.236
Cma03EPA	6.9	0.780	0.742	-0.052	9.1	0.802	0.806	0.005	10.6	0.733	0.852	0.141*
Cma04EPA	14.0	0.920	0.909	-0.012	14.0	0.914	0.903	-0.012	12.5	0.900	0.909	0.010
Cma05EPA	2.0	0.420	0.357	-0.176	2.0	0.411	0.429	0.040	2.0	0.467	0.499	0.067
Cma08EPA	19.4	0.880	0.948	0.072*	19.9	0.948	0.952	0.004	19.8	0.833	0.951	0.125*
Cma09EPA	8.9	0.900	0.826	-0.089	8.4	0.770	0.787	0.022	9.7	0.724	0.813	0.111
Cma10EPA	25.5	0.960	0.963	0.003	23.3	0.951	0.959	0.009	19.0	0.960	0.952	-0.009
Cma14EPA	5.7	0.440	0.488	0.099	5.7	0.366	0.371	0.020	6.4	0.266	0.250	0.060
SP107	5.8	0.620	0.645	0.039	6.8	0.560	0.590	**0.051**	5.6	0.448	0.433	-0.036
SP495	4.4	0.540	0.616	0.124	5.2	0.619	0.649	0.047*	3.9	0.536	0.603	0.114
Overall	9.5	0.698	0.696	-0.003	9.8	0.675	0.686	**0.016**	9.3	0.628	0.663	0.053

2.5. Discussion

2.5.1 Spatio-temporal structure across the Iberian Peninsula

In this study we used a combination of approaches (F_{ST}, IBD and Bayesian clustering analysis) to investigate genetic differentiation in *Carcinus maenas* within the Atlantic native species range and across the Iberian Peninsula in particular. Along the Iberian Peninsula, differentiation might be predicted across the Cape Finisterre and the Estremadura Promontory based on differences in oceanographic regime (Peliz et al. 2007). Diekmann et al. (2005) reported a split between northern and southern seagrass *Zostera noltii* populations along the west Iberian coast, coinciding with the Estremadura promontory, caused partly by geographical features that act as barriers to dispersal and by ocean surface currents. Pascoal et al. (2009) found weak genetic structure among *C. maenas* populations sampled during 2005 across a 450 km stretch of the Portuguese coast. Our data, based on samples collected with a spatial resolution of a few 10s to over 1000s of kilometres, indicates genetic homogeneity among sites separated by several hundreds of km along a 1200 km extent of the Iberian coast, suggesting that genetic exchange (effective migration) is occurring over this scale. Sampling in the same area over two consecutive years confirmed such a view, suggesting that apparent genetic similarity of populations is temporally stable and unlikely to be caused by sampling artifacts (Waples 1998). Although interaction of diel vertical migration behaviour with upwelling circulation may retain larvae inshore (Marta-Almeida et al. 2006), the prevailing current regimes and oceanographic features along the Iberian coast do not seem to act as barriers to larval dispersal. The close proximity of estuaries may also facilitate the exchange of migrants between rivers through larval drift, in accordance with dispersal distances in the order of 120 km during the larval life of the shore crab, on the western Iberian Shelf, as estimated by Peliz et al. (2007). *C. maenas* does not undergo philopatric migrations that could be a mechanism promoting population differentiation. Also, crabs collected by Pascoal et al. (2009) were sampled at the adult stage, precluding some "Allendorf-Phelps effect" (Waples 1998). We regard the results obtained by Pascoal et al. (2009) as reflecting the potentially transient nature of population structuring in local Portuguese shore crab populations caused by "sweepstakes" recruitment (Hedgecock 1994), that no longer persists in the absence of a strong physical barrier or diversifying selection (Pringle & Wares 2007). Additionally, in the Pascoal et al. (2009) study, the presence of null alleles was detected in 3 out of the 9 loci used to assess population

structure, which may have driven the differences between the present and the former study.

2.5.2 Population structure within the native range of *C. maenas*

Across larger geographical scales, F_{ST} estimates, IBD and Bayesian clustering analysis were all consistent in finding significant genetic heterogeneity among samples. Values of the pairwise tests of differentiation have shown significant differences between Sweden and the remaining Atlantic samples, with no significant differences for the group of samples extending from the Menai Straits in the UK, to Morocco. The IBD relationship found, although statistically significant is apparently non-linear, and essentially depends on the effect of the most divergent Swedish sample (Figure 2). Furthermore, results from GENELAND supported the existence of three genetic clusters in the data set: Sweden (SWD), Menai Strait together with Iberian Peninsula (MEN-IP) and Morocco (MOR) (Figure 4). These clusters were characterized by comparable levels of genetic diversity. Expected heterozygosity values were 0.696 (SWD), 0.686 (MEN-IP) and 0.663 (MOR), which are similar to values reported for microsatellites for other decapod crustaceans (e.g. *H*e = 0.5 to 0.6 for *Pachygrapsus marmoratus* (Silva et al. 2009); *H*e = 0.7 to 0.8 for *Maja brachydactyla* (Sotelo et al. 2008). The GENELAND model identified the Moroccan sample as an individual cluster, despite the failure of the F_{ST} approach to distinguish clearly Morocco from MEN-IP samples in individual comparisons. Some methods are expected to perform better under particular scenarios, such as high or low gene flow, and GENELAND spatial analysis may be a more sensitive approach especially when dealing with samples with very low levels of differentiation (Guillot 2008).

Our results suggest that shore crab larvae are not being exchanged continuously between the Sweden location and the remaining Atlantic samples. Such apparent isolation could arise from the geographical separation of populations following an IBD pattern, as stated above. However, the pattern of IBD between the Menai Straits sample with the other samples is not observed (except with Morocco), even for geographic distances ranging up to 2700-3200 km (Figure 2, white circles), the same distances that separates Sweden from some Atlantic locations. Such an observation indicates that, in the absence of barriers to gene flow, shore crab populations are genetically similar across thousands of kilometres. Therefore, in addition to geographical distance itself, the subdivision detected between Sweden and other sites is likely to be reinforced by regional current patterns and/or by the presence of an oceanographic barrier. The main inflow into the North Sea is

from North Atlantic water that spreads along the shelf break into the Norwegian Trench. Atlantic water also enters into the southern North Sea, through the English Channel (Danielssen et al. 1997). These water inflows may transport larvae that originate in Atlantic populations. However, these larvae may be retained by the counter-clockwise gyre formed in the northeastern end of the Skagerrak that can also block the inflow of waters from the southern part of the North Sea into the Skagerrak - Kattegat area (Danielssen et al. 1997). Such a scenario has been suggested previously to retain Norway lobster larvae (*Nephrops norvegicus*) along the Swedish west coast (Øresland 1998). Furthermore, the Norwegian Coastal Current that represents the main outflow from the Skagerrak, flows predominantly northward along the west coast of Norway (Danielssen et al. 1997), and may be flushing larvae to northern areas. Additional sampling between Sweden and the UK are needed to clarify whether the differentiation of the Swedish sample was actually due to limited larval exchange among populations in accordance with geographical distances or to a genetic break.

Tide-related vertical migratory behaviour is present in several brachyuran larvae and is known to influence the direction and extent of horizontal transport in estuarine species, mainly preventing larvae from being retained inside the estuary or the shore (Queiroga et al. 1997, DiBacco et al. 2001, Bilton et al. 2002, Queiroga & Blanton 2005). Nevertheless, *Carcinus maenas* larvae from Skagerrak lack a tidal rhythm because they inhabit areas where tides are of very small amplitude and have little effect on water circulation (Queiroga et al. 2002). Since tidal vertical migration was demonstrated to be inherited in crabs from meso-tidal areas as in the British Isles (Zeng & Naylor 1996), a possible effect of Skagerrak isolation may be reflected in the loss of tidal rhythm behaviour in *C. maenas* larvae from this region. The coincidence of a genetic break between Sweden and the rest of the Atlantic populations with the split in genetically determined behavioural patterns is interesting and deserves further investigation. Reproductive isolation can be promoted in populations that developed under different environmental conditions (Palumbi 1994). With our data we can only speculate if the populations from Sweden are, or may become, reproductively isolated from populations from meso-tidal systems. However, if a significant level of reproductive isolation was achieved in the latter scenario, there would clearly be enhanced opportunities for speciation. A parallel example, supported by genetic studies (Geller et al. 1997, Roman & Palumbi 2004, Darling et al. 2008), is seen in the sibling species relationship between *C. maenas* and *Carcinus aestuarii* from the Mediterranean Sea. The divergence between the two *Carcinus* forms was estimated to commenced

about 5 to 8 million years ago (Roman & Palumbi 2004) and is probably maintained by the Almeria-Oran front (Patarnello et al. 2007), which prevents frequent migration of larvae. The divergence between the Skagerrak and the Atlantic *C. maenas* is much shallower and may be the result of recently separated populations that have had insufficient time to diverge compared to the Atlantic and the Mediterranean forms. Indeed, the eastern North Sea coastlines where the Skagerrak is inserted had attained their present circulation about 8500 years ago following periods of change during the late Pleistocene and Holocene that included the deglaciation of the region (Gyllencreutz et al. 2006).

The Moroccan sample is also distinct from the other samples in the GENELAND analysis and is more closely related to the Arade ($F_{ST} = 0.0018$, $P > 0.05$) and Formosa ($F_{ST} = 0.0011$, $P > 0.05$) samples, in south Portugal, and more genetically distinct from the northernmost samples in Sweden ($F_{ST} = 0.0289$, $P < 0.001$) and the Menai Strait ($F_{ST} = 0.0110$, $P < 0.01$). Thus, IBD could be responsible for the genetic divergence of this sample from the European samples.

Although here we found evidence of restricted gene flow, it is worth highlighting the apparent genetic homogeneity observed among *Carcinus maenas* populations across oceanic distances as long as 3000 km, the approximate distance that separates Menai Straits from Cadiz Bay in SW Spain. These findings are consistent with weak genetic drift driven by presumed large effective population sizes, high fecundity and extended pelagic larval duration, which may translate into enhanced dispersal abilities. The presence of a larval export strategy from estuaries into shelf waters using selective tidal stream transport (Queiroga et al. 1997) enhances net transport of larvae between coastal populations that will drift with ocean currents. Also, *C. maenas* can spawn throughout winter and spring/summer seasons, depending on latitude, thereby ensuring that larvae are released under a wide variety of oceanographic conditions. Other marine crabs with high larval dispersal capacity show similar patterns of low genetic differentiation over extensive spatial scales (McMillen-Jackson & Bert 2004, Pfeiler et al. 2005, Cassone & Boulding 2006, Ungfors et al. 2009). Furthermore, weak or a complete lack of genetic differentiation along the European Atlantic coast has also been documented for other invertebrate species with high dispersal potential, such as the sea urchin *Paracentrotus lividus* (Duran et al. 2004), the European lobster *Homarus gammarus* (Triantafyllidis et al. 2005), the netted dog whelk *Nassarius reticulatus* (Couceiro et al. 2007), the spiny spider crab *Maja brachydactyla* (Sotelo et al. 2008), and the velvet swimming crab *Necora puber* (Sotelo et al. 2009). Although it is apparent therefore that taxa exhibiting similar life-history

parameters and high potential vagility may often show apparent panmixia along the Atlantic coast, exceptions continue to emerge (e.g. Shaw et al. 1999, Chiara et al. 2005, Diekmann et al. 2005). Such complexity is likely to arise from the interplay of local and regional oceanographic features with biological traits arising from variance in behaviour, developmental stage, predation and resource availability.

2.5.3 Comparison with previous studies

Previous studies of *Carcinus maenas* using mitochondrial DNA analysis indicated a significant genetic break between populations in western and northern Europe (Roman & Palumbi 2004, Darling et al. 2008), a pattern hereby confirmed with microsatellite markers. A recent study (Darling et al. 2008), examined genetic structuring of several native *C. maenas* samples when studying the invasion genetics of the genus *Carcinus* globally, using nine microsatellites, eight of which were also employed in the present study. Darling et al. (2008) reported the lack of regional geographic structure as shown by pairwise F_{ST} and R_{ST} analysis among samples from northern Europe (including one sample from Sweden) and western Europe (including samples from Betanzos, Aveiro and Cadiz), and also failed to identify any clustering in the data when using the Bayesian algorithm implemented in STRUCTURE (Pritchard et al. 2000). Such findings contrast with those reported here, where *C. maenas* from the Swedish region were clearly distinct from all other samples. The performance of the analytical tests is known to be sensitive to sample size and number and variability at loci screened, especially in samples with low differentiation (Ruzzante 1998, Cornuet et al. 1999), an observation typical of many marine taxa with high gene flow. In the study of Darling et al. (2008), as few as eight individuals were used in the analysis, potentially compromising their resulting observations. In our study, the employment of larger sample sizes are expected to enhance precision for differentiation estimators, as indicated by the power analysis, which yielded a power of > 99% of detecting even low levels of population structure.

2.6 Conclusions

In summary, estimates of F_{ST} were generally low among *Carcinus maenas* populations, in common with many marine species, and as expected for highly polymorphic microsatellite markers regardless of population structure (Slatkin 1995). Furthermore, traditional *F* statistics augmented by Bayesian methods can prove useful in assessing genetic differentiation in such circumstances. Microsatellite data from *C. maenas* across its native

range indicated the existence of genetically distinct populations across large geographic scales, providing evidence of a more complex population structure than suggested previously, and confirming the utility of microsatellite markers in detecting subtle genetic differentiation in a highly mobile species.

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Chapter 3

Genetic structure of Carcinus maenas larvae

Domingues CP, Creer S, Taylor MI, Queiroga H, Carvalho GR (accepted). Temporal genetic homogeneity among shore crab (*Carcinus maenas*) larval events supplied to an estuarine system on the Portuguese northwest coast. Heredity

3.1 Abstract

Despite the importance of larval biology in the life histories of many marine animals, relatively little information exists on the dynamics and genetic composition of larval cohorts. Supply of megalopae larvae of the shore crab, *Carcinus maenas*, was measured on a daily basis during eight months spread along two larval periods (2006 and 2007) at the Ria de Aveiro estuary, on the Portuguese northwest coast. Ten microsatellite DNA loci were employed to explore the genetic structure, variability and relatedness of temporally distinct megalopal events, selected from the major pulses of supply. Larval variation was also compared genetically to that of a previously studied adult crabs sample, at the same loci, collected in 2006 and 2007 along the Iberian Peninsula. Results revealed a lack of genetic differentiation and identical diversity levels among larval events over time. No evidence of reduced genetic diversity between megalopae relative to the diversity assessed from the pooled sample of adults was found. Moreover there was no evidence of any family relatedness among larvae from temporal events. The results obtained for *C. maenas* contradict predictions made by the sweepstakes reproduction hypothesis, where large variance in reproductive success is expected, which is presumably detectable as sharp genetic discontinuities among separate larval events. Data here indicate conversely a high degree of temporal genetic stability among larval supply to a given estuary under variable oceanographic conditions, consistent with the hypothesis that sampled larvae were drawn from a large number of adults that do not differ in reproductive success.

3.2 Introduction

The unpredictability of oceanographic conditions together with marine species' high fecundity and pronounced early life-stage mortality (type III survivorship curve) creates the potential for high variance in reproductive success among individuals, and is also referred to as sweepstakes reproduction (Hedgecock 1994). According to the sweepstakes hypothesis, relatively few adults in each generation succeed in reproducing, as a result of stochastic processes leading to their reproductive activity coinciding with the correct oceanographic conditions conducive to spawning, fertilization, larval survival, and recruitment (Hedgecock 1994). Sweepstakes reproduction has been described in both invertebrates and fishes (Li & Hedgecock 1998, Planes & Lenfant 2002, Pujolar et al. 2006, Hedgecock et al. 2007b, Liu & Ely 2009, Christie et al. 2010) . Large discrepancies between the effective (N_e) and the census (N) population sizes usually resulting in very

low N_e / N ratios, are often reported among marine species (reviewed in Hauser & Carvalho 2008) and are explained mainly by high variance in reproductive success. If this is the case, reduced genetic variability among cohorts of larvae or new recruits relative to the adult populations is expected (e.g. Hedgecock et al. 2007b) as well as genetic differentiation among cohorts arriving at a population over time (e.g. Li & Hedgecock 1998). Moreover, larvae travelling in batches may be related by kin (Planes et al. 2002, Veliz et al. 2006), increasing the likelihood of differences among cohorts, though such relatedness may not always be evident (Hernbinger et al. 1997).

Several cases of temporal genetic heterogeneity among larval or recruit cohorts have been reported previously (Kordos & Burton 1993, Ruzzante et al. 1996, Li & Hedgecock 1998, Moberg & Burton 2000, Planes & Lenfant 2002, Pujolar et al. 2006, Selkoe et al. 2006, Liu & Ely 2009), but such differences can be apportioned to variation in the adult reproductive contribution through random genetic drift within a single population only when immigration is ruled out (Li & Hedgecock 1998, Liu & Ely 2009). For instance, temporal variation of megalopal allelic frequencies of the blue crab *Callinectes sapidus* in the Gulf of Mexico suggested changes in larval source populations throughout the recruitment season that were attributed to seasonal changes in coastal current patterns or to timing differences in spawning season (Kordos & Burton 1993), rather than to transitory effects of variance in reproductive success. In other surveys (Ruzzante et al. 1996, Moberg & Burton 2000, Planes & Lenfant 2002, Pujolar et al. 2006, Selkoe et al. 2006), sweepstakes recruitment (Hedgecock 1994) best explains the differentiation among cohorts but additional factors (e.g. selection, spatial genetic variation, changes in larval delivery) could not be excluded. In contrast, some studies have found no reduced genetic diversity in recruits relative to estimates of diversity from adults (Flowers et al. 2002, Gilbert-Horvath et al. 2006, Rose et al. 2006, Calderón et al. 2009), no measurable genetic differentiation of recruits over time (Gilbert-Horvath et al. 2006, Calderón et al. 2009), no evidence of family structure (Hernbinger et al. 1997) and no reduction in effective population size (Poulsen et al. 2006) indicating little variance in reproductive success. It is evident therefore, that despite the prominence of stochastic larval supply events in the life histories of many marine animals no general trends can be derived from empirical observations. More studies analysing potential mechanisms driving differences among cohorts are needed and population structure analysis will benefit from the inclusion of a temporal genetic component and comparisons between the genetic composition of both adults and recruits.

In the present study, we investigated the temporal genetic structure and tested the sweepstakes hypothesis in samples of megalopae larvae of the shore crab *Carcinus maenas* collected in the Ria de Aveiro estuary, on the Portuguese northwest coast. *C. maenas* is widely distributed along the European coast and while in northern Europe it inhabits a wider ecological niche occurring both in estuaries and in rocky shores, in southern Europe it is mostly restricted to estuarine habitats, such as Ria de Aveiro, where it forms large populations. *C. maenas* is a portunid crab with a life cycle that alternates between benthic adults that inhabit estuaries and rocky shores and planktonic larval stages that develop in shelf waters. The species is characterised by high fecundity, with females producing up to 200 000 eggs per brood (Broekhuysen 1936), from late winter to early summer, in northwest Portugal (Queiroga 1995). Larvae are released as plankton to the open sea where they develop over four to six weeks, depending on temperature (Nagaraj 1993). The larval series comprises four zoeae and terminates with one megalopa (Rice & Ingle 1975), which is the stage that settles into benthic habitats before metamorphosis to the first crab stage. Supply of megalopae back to estuaries occurs mainly during highest amplitude tides around full and new moons, with peak abundance recorded from mid March to the end of July, and is enhanced by southerly winds or by northerly winds relaxation (Queiroga et al. 2006). Throughout larval development they are exposed to a combination of factors that affect their survival. Estimates of larval mortality in the field for decapod larvae are equivalent to an average instantaneous mortality rate of 7% day^{-1} (Rumrill 1990).

High dispersal potential would contribute to maintain genetically homogeneous populations connected through larval dispersal along the Iberian Peninsula (Domingues et al. 2010). Despite this fact, it is reasonable to expect high variance in reproductive success, given that only a few individuals may successfully reproduce each year, which genetic differences might be enough to cause genetic structure among larval events but not sufficient to impact adult population structure. In addition, the species' type III survivorship characteristics may result in the recruitment of genetically variable larval patches that can potentially contribute to transitory genetic differences among populations. These differences are illustrated by the divergence in the results observed by Pascoal et al. (2009) and Domingues et al. (2010), for the same geographical region. Finally, the oceanographic conditions along the western Iberian Peninsula ecosystem are variable throughout the year, and more unstable than previously thought (reviewed in Relvas et al. 2007). For example, during the winter months transient nearshore poleward

flows, eddy shedding from the poleward slope current and even intermittent upwelling events are observed. After the spring transition, which is marked by a change on the general direction of the wind from poleward to equatorward, upwelling events increase in frequency, intensity and length and continue throughout the summer. Spatial and temporal heterogeneity in oceanographic conditions affect larvae during development and promote episodic and variable supply back to adult populations (Almeida & Queiroga 2003, Queiroga et al. 2006), thereby influencing which larvae actually recruit to the next generation. The current understanding of the oceanographic mechanisms coupled with larval behaviour shows that diel vertical migration (dos Santos et al. 2008) promotes retention of crab larvae in the inner shelf during spring and early summer, through interaction with the two-layered flow typical of upwelling circulation (Marta-Almeida et al. 2006). Relaxation of upwelling causes translocation of the larvae to the near-shore environment; supply into estuaries then occurs by selective tidal stream transport (e.g. Queiroga et al. 2006). Available laboratory and field studies indicate that pulses of decapod larvae of the same age react coherently to environmental stimuli (Queiroga & Blanton 2005). This supports the view that larvae resulting from the same hatching event are subjected to essentially the same advection history. Indeed, individual patches of larvae may maintain their integrity throughout larval development (Natunewicz & Epifanio 2001), allowing aggregations of larvae from the same family group to settle in close proximity. This process is supported by oceanographic predictions along the California Upwelling System (Siegel et al. 2008) and might also occur in a similar oceanographic system, along the Portuguese west coast.

Here, we analyse the genetic structure, variability and genetic relatedness of temporally separated *Carcinus maenas* megalopal events from different supply episodes and examine if these larval events are genetically differentiated and have reduced genetic diversity when compared with the putative spawning populations. In this way, we hope to elucidate whether relatedness within larval events or large variance in reproductive success is a common feature in the shore crab. These hypotheses were investigated using ten microsatellite markers that were amplified in megalopae selected from eight major pulses of larval supply episodes that took place in the Ria de Aveiro estuary, Portugal, during two consecutive larval periods (2006 and 2007).

3.3 Materials and methods

3.3.1 Sample collection

Megalopae were collected from March to June from supply events that occurred in the 2006 and 2007 larval periods in Ria de Aveiro, a shallow coastal lagoon on northwestern Portugal (40º 37'17''N, 8º 44'56''W; Figure 1).

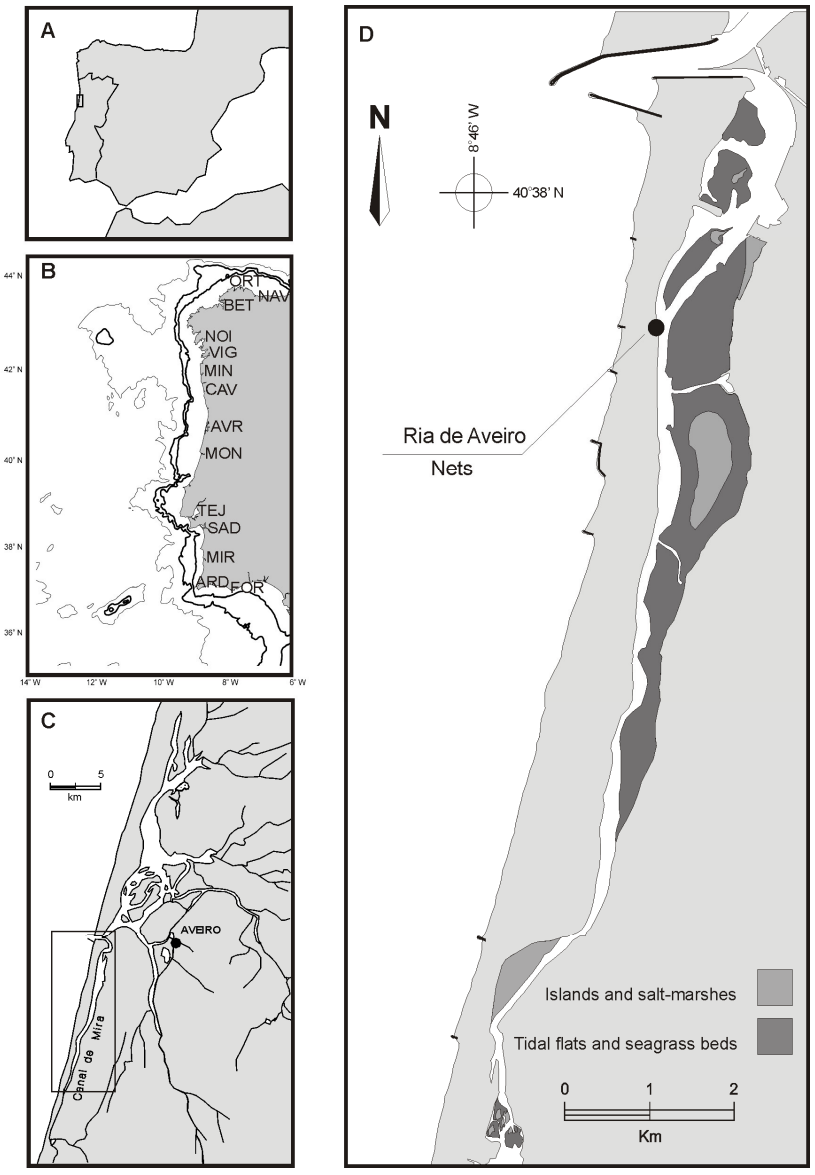

Figure 1 Sampling location for *Carcinus maenas* megalopae and adult crabs collection. (A) Iberian Peninsula, (B) Sampling location for adult crabs, AVR: Ria de Aveiro (for details see Domingues et al. 2010) (C) Ria de Aveiro, NW Portugal and (C) Sampling location for megalopae.

Megalopae were collected with the use of two passive plankton nets described by Queiroga et al. (2006). The nets were deployed at a station inside the Ria de Aveiro facing the inlet, one below the surface and one above the bottom, within one tidal excursion from the inlet. The nets measured the absolute supply of megalopae to the Ria de Aveiro during flood. Every day, live megalopae were removed from the plankton samples and preserved in 96 % ethanol for DNA extraction. A total of 2800 and 1106 megalopae were collected in 2006 and 2007, respectively, across eight main discrete megalopal supply events (Figure 2). Each event lasted three to five days and, whenever possible, 80-100 megalopae *per* event were randomly selected, proportionally to abundance in the surface and bottom nets, from the day of maximum supply, or from proximate days in events with a low number of larvae (Table 1). Genetic variation of megalopae was compared with 1262 previously analysed adult crabs collected from 14 estuaries along a 1200 km stretch of the Iberian Peninsula in the summer of 2006 and 2007 (Domingues et al. 2010), pooled in a single sample, referred to as "Iberian Peninsula" adult population (IP) (Table 1).

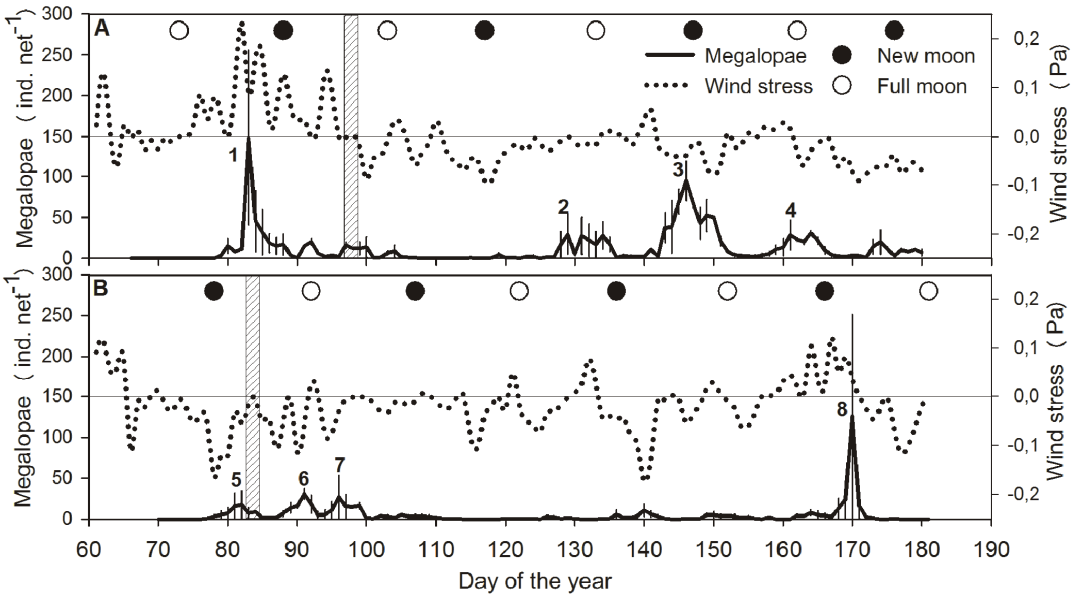

Figure 2 Average daily numbers of megalopal supply and along-shore wind stress values, in Ria de Aveiro. (A) 2006 and (B) 2007. Since the Portuguese west coast has an approximate north-south orientation and according to the upwelling theory we computed the along-shore component of wind stress (for details see Queiroga et al. 2006). Wind stress with a negative component indicates northerly winds, which favour upwelling; wind stress with a positive component indicates southerly winds, which favour downwelling. Numbers identify supply events from where megalopae were selected for genetic analysis. Shaded bars indicate the spring transition according to the intensity and magnitude of along-shore winds. Error bars: ±1SE.

Table 1 Identification of samples including larval supply events, adult population, collection periods and sample sizes. Each larval event is made up of a group of megalopae collected from each larval supply event according to Figure 2. IP: Iberian Peninsula.

Larval event	Supply	Collection period	Sample
E06.1	1	24 March 2006	98
E06.2	2	11-12 May 2006	94
E06.3	3	26 May 2006	100
E06.4	4	12-13 June 2006	91
E07.5	5	22-25 March 2007	75
E07.6	6	6-7 April 2007	75
E07.7	7	19-22 May 2007	37
E07.8	8	19 June 2007	82
Adult population IP		Summer 2006 & 2007	1262

3.3.2 DNA extraction and microsatellite genotyping

Prior to DNA extraction, megalopae were placed in distilled water to rehydrate larvae and wash possible contaminants attached to the animals. Total genomic DNA was extracted from whole megalopa using overnight digestion with Proteinase K following a modified salt extraction protocol (Aljanabi & Martinez 1997). DNA was resuspended in a volume of 100 µl of 1× TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) and stored at - 20 ºC. The samples were genotyped using the same ten species-specific microsatellite loci selected by Domingues et al (2010), to allow comparisons with results from adult population: Cma01EPA, Cma03EPA, Cma04EPA, Cma05EPA, Cma08EPA, Cma09EPA, Cma10EPA, Cma14EPA (Tepolt et al. 2006), SP107 and SP495 (Pascoal et al. 2009). Microsatellite loci were amplified in two multiplex PCR performed in 10 µL reactions containing 1 µL of the template DNA, 1x QIAGEN Multiplex PCR Master Mix (QIAGEN) and 0.1-0.3 µM of each primer (forward 6-FAM, VIC, NED or PET fluorescently labelled primers). Reactions were carried out in Bio-Rad Tetrad2 Peltier Thermal Cyclers under the following conditions: 95 ºC for 15 min followed by 30 cycles of 94 ºC for 30 s, 55 ºC for 90 s and 72 ºC for 60 s followed by a final extension at 60 ºC for 30 min. Fragments were separated on a ABI 3130xl Genetic Analyzer (Applied Biosystems) and alleles were scored relative to the internal size standard GeneScan LIZ-500 using GENEMAPPER version 4.0 (Applied Biosystems).

3.3.3 Genetic analysis

Scoring reliability was ensured by running MICRO-CHECKER version 2.2.3 (van Oosterhout et al. 2004) to test for null alleles, stuttering or large allele drop-out. FSTAT version 2.9.3.2 (Goudet 2001) was employed for calculating Wright's F_{IS} and F_{ST} (Weir & Cockerham 1984), number of alleles (N_A), allelic richness (A) and for testing Hardy–Weinberg proportions using F_{IS}, after 10 000 randomizations. Genetic differences among larval events were tested using global Fisher's exact tests based on allelic (genic) and genotype (genotypic) frequencies performed in GENEPOP version 4.0 (Rousset 2008), with significance levels estimated using the Markov chain method. GENETIX version 4.05 (Belkhir et al. 1996-2004) was used for calculating expected (H_E) and observed (H_o) heterozygosity and for comparing pairwise genetic differences (F_{ST}) among larval events and among larval events and the adult population, with significances tested using 10 000 permutations. Linkage equilibrium between pairs of loci was tested using the Markov chain exact probabilities obtained from GENEPOP version 4.0 (Rousset 2008). Sequential Bonferroni corrections were applied to account for multiple comparisons (Rice 1989). The simulation procedure of Ryman et al. (2006) implemented in the software POWSIM version 4.0 (Ryman & Palm 2006) was used to assess the alpha error and statistical power of our microsatellite loci to detect various true levels of divergence (F_{ST} = 0.0001, 0.0010, and 0.0100) among larval events, employing sample sizes corresponding to those from our events and the allele frequencies from the current data set. Each simulation was run 1000 times and power was determined as the proportion of simulations that Fisher's exact test detected significant at the 0.05 level. Differences in genetic diversity (in terms of expected heterozygosity and allelic richness) between larval samples and relative to the adult population were tested with Friedman ANOVA in STATISTICA version 8.0 StatSoft, Inc. A hierarchical analysis of molecular variance (AMOVA) (Excoffier et al. 1992) was performed in GenAlex version 6.2 (Peakall & Smouse 2006), computed on the proportion of variation among larval events, the proportion of variation among larvae within events and the proportion of variation within larvae, and tested for significance with 9999 permutations. We then performed a second AMOVA but grouped larval events according to the sampling year (2006 and 2007). Finally, in order to assess the potential seasonal effect of wind-driven circulation on larval genetic variation we performed two more AMOVAs, separately for each year, but pooling larvae collected before (less upwelling favourable) and after (upwelling favourable) the spring transition, which was measured by the intensity and frequency of along-shore winds (Figure 2). Estimates of average relatedness of all individuals to each other, across samples, were

assessed using Queller & Goodnight (1989) statistic r_{xy} obtained using the program IDENTIX version 1.1 (Belkhir et al. 2002). The mean and the variance of r_{xy} were estimated for each larval event, year and season and compared with their expected distribution, generated by 1000 permutation of alleles among individuals, under the null hypothesis of no relatedness (full-sibs, $r = 0.50$; half-sibs, $r = 0.25$; unrelated individuals, $r = 0.00$).

3.4 Results

3.4.1 Genetic diversity within and between larval events and adult sample

Standard genetic estimates for each larval event and adult population are summarised in Table 2. In total, 652 megalopae were analysed at ten microsatellite loci. Of the 197 alleles recovered in both adult and larval samples, 24 were exclusively from the adult population and 5 were only present in the larval sample. MICRO-CHECKER found no evidence of scoring errors or null alleles in any sample. No departure from Hardy-Weinberg expectation after correcting for multiple tests (Rice 1989) was detected in any of the eight larval events as well when we pooled samples from all events. In the adult population, a significant deficiency in heterozygosity was observed (Table 2). No evidence of linkage disequilibrium was found after sequential Bonferroni correction (Rice 1989) among any of the loci within samples.

All larval samples exhibited comparable genetic diversity (Table 2) as no significant differences were found between temporally distinct larval events in expected heterozygosity (Friedman ANOVA, $\chi^2 = 2.57$, *d.f. = 7,* $P = 0.922$) and allelic richness (Friedman ANOVA, $\chi^2 = 6.33$, *d.f = 7,* $P = 0.502$). Moreover, we found no evidence of reduced genetic diversity between larval events and the adult spawning population, assessed with expected heterozygosity (Friedman ANOVA, $\chi^2 = 3.01$, *d.f = 8,* $P = 0.934$) and allelic richness (Friedman ANOVA, $\chi^2 = 6.89$, *d.f. = 8,* $P = 0.548$) (Table 2).

Table 2 Summary statistics for each larval event and adult population of *Carcinus maenas*. Larval events were collected in Ria de Aveiro according to Figure 2 and adult population from pooled samples collected along the Iberian Peninsula. Asterisks indicate $P < 0.05$ before correction for multiple tests. **Bold** indicate values that remain significant following Bonferroni correction. *N:* sample size; N_A: average number of alleles; A: allelic richness; *H*o: observed heterozygosity; *H*e: expected heterozygosity; F_{IS}: inbreeding coefficients, calculated after Weir and Cockerham (1984).

Larval event	*N*	N_A	A	H_o	H_E	F_{IS}
E06.1	98	13.3	9.691	0.679	0.680	0.002
E06.2	94	12.2	9.280	0.681	0.689	0.012
E06.3	100	13.1	9.510	0.691	0.692	0.001
E06.4	91	13.5	9.707	0.689	0.684	-0.007
E07.5	75	12.6	9.561	0.673	0.676	0.004
E07.6	75	12.4	9.482	0.658	0.682	0.034*
E07.7	37	9.9	9.202	0.675	0.676	0.001
E07.8	82	12.6	9.583	0.688	0.685	-0.004
Overall	652	12.5	9.502	0.679	0.683	0.005
Adult population IP	1262	19.2	9.626	0.675	0.686	**0.016**

3.4.2 Genetic structure within and between larval events and adult sample

Overall genetic differentiation among larval events was very low (global $F_{ST} = 0.0003$) and not statistically significant according to Fisher's exact test on both allelic ($P = 0.164$) and genotypic ($P = 0.302$) frequencies. Similarly, none of the pairwise F_{ST} comparisons among larval events showed significant differences after sequential Bonferroni correction (Table 3). AMOVA, computed when considering all samples separately, revealed no significant variance among larval events (0.16%, $P = 0.268$). Similarly, only a small proportion of variation (0.22%) was associated with differentiation according to the sampling year (2006 and 2007), which was not significant ($P = 0.400$). No significant differentiation was attributable to differences among larvae within larval events (0.40%, $P = 0.260$) or within larvae (99.44%, $P = 0.246$). AMOVA of larval events pooled according to season revealed no significant differentiation between samples for both 2006 (0.00%, $P = 0.568$) and 2007 (0.14%, $P = 0.180$) years. Concerning differentiation between the adult population, Iberian Peninsula, and the larval events, pairwise F_{ST} values revealed no significant genetic differentiation (Table 3).

The results of power analysis in POWSIM were estimated as 0.100 ($F_{ST} = 0.0001$), 0.948 ($F_{ST} = 0.0010$) and 1.000 ($F_{ST} = 0.0100$). Therefore, the simulations indicate that the number of individuals and the numbers of loci used in the present study provide strong

support to identify genetic structure at very low levels of true divergence in the range of 0.0001-0.0100. The alpha error (the probability of obtaining significant genetic structure when the true $F_{ST} = 0$) were consistently about 5% in all simulations.

Table 3 Measures of genetic differentiation based on F_{ST} analysis. Below diagonal, pairwise F_{ST} (θ) estimates of genetic differentiation between all samples (larval events and adult population). Above diagonal, *P*-values of the tests of genetic differentiation. Asterisks indicate $P < 0.05$ before correction for multiple tests. None of the values remain significant following sequential Bonferroni correction.

	E06.1	E06.2	E06.3	E06.4	E07.5	E07.6	E07.7	E07.8	IP
E06.1		0.4120	0.7794	0.5714	0.6675	0.3140	0.3262	0.4445	0.7721
E06.2	0.0001		0.8533	0.1940	0.4610	0.0070*	0.7286	0.9326	0.4353
E06.3	-0.0009	-0.0012		0.6948	0.5149	0.1303	0.2259	0.8901	0.5630
E06.4	-0.0003	0.0010	-0.0007		0.7560	0.1181	0.0074*	0.4642	0.1281
E07.5	-0.0007	0.0000	-0.0002	-0.0010		0.1079	0.2208	0.5739	0.3168
E07.6	0.0005	0.0042*	0.0015	0.0016	0.0020		0.0936	0.0684	0.0584
E07.7	0.0007	-0.0014	0.0014	0.0063*	0.0017	0.0033		0.7930	0.3882
E07.8	0.0000	-0.0017	-0.0014	0.0000	-0.0004	0.0023	-0.0018		0.8482
IP	-0.0005	0.0000	-0.0002	0.0007	0.0003	0.0014	0.0003	-0.0007	

3.4.3 Genetic relatedness within and between larval events

Average relatedness was concordant and close to zero over all individuals within each larval event (ranged from -0.0246 to -0.0105). Within years and seasons the values of genetic relatedness were also low (Table 4). Analysis of significance based on a permutation resampling test did not allow rejection of the null hypothesis of no relatedness within samples.

3.5 Discussion

The processes that regulate larval dispersal, recruitment and genetic variation within marine populations through time are not completely understood. Elucidation of processes requires the analysis of both spatial and temporal genetic variation of larvae or recruits and adult populations (Selkoe et al. 2008). Microsatellite DNA is a powerful genetic tool to detect differentiation and provenance of individuals recruiting to a given population (Selkoe & Toonen 2006), even in species exhibiting high dispersal capacity, as in the shore crab *Carcinus maenas.*

Table 4 Estimates of average relatedness (r_{xy}) from IDENTIX based on 10 microsatellite loci. Standard deviations are in parenthesis. None of the values were significant after 1000 permutations. E06.1 and E07.5 correspond to the samples taken before the spring transition in 2006 and 2007, respectively. 2006 AST: samples pooled after the spring transition in 2006 (E06.2, E06.3 and E06.4); 2007 AST: samples pooled after the spring transition in 2007 (E07.6, E07.7 and E07.8).

Sample	r_{xy}
E06.1	-0.0107 (0.0011)
E06.2	-0.0105 (0.0011)
E06.3	-0.0105 (0.0009)
E06.4	-0.0110 (0.0010)
E07.5	-0.0145 (0.0015)
E07.6	-0.0141 (0.0016)
E07.7	-0.0246 (0.0022)
E07.8	-0.0116 (0.0014)
2006	-0.0027 (0.0005)
2007	-0.0030 (0.0008)
2006 AST	-0.0035 (0.0006)
2007 AST	-0.0041 (0.0009)

An intensive sampling, over two years, of 14 *Carcinus maenas* populations distributed along southern, western and northern Iberian Peninsula found genetically homogeneous populations (Domingues et al. 2010), and it is thus not possible to assign larvae recruiting to a particular estuary to putative source populations. We would expect a reduction of genetic diversity within larvae arriving at a given location relative to the adult source populations if only a small proportion of adult crabs contribute to reproduction at each spawning event. We would also expect genetic differences between temporally distinct larval supply episodes given that the mechanisms of larval dispersal and recruitment over time are strongly dependent on the interactions between larvae and variable aspects of wind-, tidal- and density-driven circulations (Queiroga & Blanton 2005). For example, Hedgecock et al. (2007b) reported genetic divergence at four microsatellite loci between recruited juveniles and adults of the European flat oyster *Ostrea edulis* from western Mediterranean despite the genetic homogeneity observed among the adult populations. Interestingly, Planes et al. (2002) found significant allozyme variation between the larvae of the reef fish *Naso unicornis* relative to the juveniles and the adults, while juveniles and adults were similar. More recently, an exhaustive study of Selkoe et al. (2006) reported significant genetic differentiation at seven microsatellite loci among cohorts of settlers of the kelp bass *Paralabrax clathratus*. This differentiation was explained by family structure

and by differences in larval distribution due to shifts in marine currents. In contrast, our results did not show reduced genetic variability among *C. maenas* megalopal events relative to the spawning population and also failed to show any significant temporal and seasonal (upwelling related) trend in genetic variability and in allele and genotype frequencies among distinct larval supply events, what is supported by the reasonable statistical power of our microsatellites to detect genetic structure. Average genetic relatedness analysis showed that each larval event is composed of a large group of unrelated megalopae and the same is true when we pooled larval events before and after the spring transition. Moreover, no departure from Hardy-Weinberg expectations was observed within or across all events. A slight deficit in heterozygotes was observed in the adult sample. This could be due to a Wahlund effect caused by small fluctuations in allele frequencies when pooling temporal samples, as well as heterogeneity resulting from temporary slight genetic differences among *C. maenas* populations along the Portuguese coast (Pascoal et al. 2009, Domingues et al. 2010).

The use of multiple approaches and the inclusion of oceanographic and behavioural information into genetic analysis will lead to a better understanding of the mechanisms regulating gene flow and connectivity among marine populations (reviewed in Selkoe et al. 2008). Data here were obtained under diverse oceanographic conditions such as: northerly winds favouring upwelling, as well as northerly winds relaxation and southerly winds favouring downwelling, which enhanced onshore supply of megalopae (Figure 2). The acquisition of daily data on larval supply during the entire larval period simultaneously with the acquisition of detailed information on physical variables allowed us to identify the discrete larval pulses and to select megalopae from the relevant episodes. At the same time, we conducted a detailed sampling of adult crab populations, comprising a distance of approximately 600 km to the south and to the north of Ria de Aveiro (Domingues et al. 2010). In this way, we maximised representative sampling of all putative spawning populations contributing to the Ria de Aveiro populations (Peliz et al. 2007). The results presented in this study indicate no evidence of marked variance in reproductive success in *Carcinus maenas*, at least using our markers and across the temporal and spatial scales examined. Nevertheless, we have noticed a recent decline in the supply of megalopae to Ria de Aveiro (for the same sampling period, with passive plankton nets: 2800 megalopae in 2006; 1106 megalopae in 2007; 1078 megalopae in 2008; 756 megalopae in 2009, unpublished data). The observed decline may result from variation in the output of larvae produced by the local populations, from fluctuations in phytoplankton

production or from variation in transport processes along the coast. Ultimately a reduced larval supply may contribute to the reduction of *C. maenas* abundance in Ria de Aveiro, where the species undertakes an important ecological and socio-economic role, despite being an ecologically problematic species in non-native locations (Carlton & Cohen 2003). High mortality is definitely occurring in early *C. maenas* life stages. Indirect estimates from data on abundance of zoea-1 and the megalopa larvae in the Ria de Aveiro indicates a total mortality rate exceeding 90% during the larval phase (Queiroga et al. 1994). A lack of temporal genetic differences among samples was reinforced by data indicating a lack of significant genetic relatedness within larval events, suggesting that larvae were indeed drawn from a large number of adults. As a consequence, local populations are not constrained by the genetic makeup of the larval pool that must subsequently recruit in order to maintain large populations. Evidence shows that larval aggregations or "parcels" (Siegel et al. 2008) may be retained in shelf waters after emission from estuaries (e.g. Natunewicz & Epifanio 2001, Marta-Almeida et al. 2006) and remain cohesive until settlement, making possible the detection of family structure within parcels if they were produced in the same spawning event. However, the longevity of such parcels may well depend locally on environmental heterogeneity, such as turbulence or small scale features of circulation that will disrupt the aggregations and mix larvae from different origins.

On the other hand, early post-settlement processes may have a significant role in the regulation of local populations (Hunt & Scheibling 1997). Young juveniles experience high mortalities, especially due to predation, and as such represent a critical phase in the life-history of the shore crab (Moksnes et al. 1998). Studies examining the genetic composition of young recruits are then necessary to provide information about the contribution of the various life stages on population structure of *Carcinus maenas*. Since juvenile crab fitness may be under genetic control caused by intense selection in the field, the use of genetic markers under selection could also be useful for determining whether pre or post-settlement selection is taking place among different spatial and temporal scales (Hauser & Carvalho 2008).

Contrary to what could be expected by the sweepstakes hypothesis we found no genetic differences and no relatedness among *Carcinus maenas* larvae as well as no reduced genetic diversity in larvae when compared to adults along the western Iberian coast. This suggests that the phenomenon might not be as widespread among marine species as previously acknowledged (Hedgecock et al. 2007a). High temporal genetic homogeneity

and stability of both larvae and adults suggest great connectivity among populations, which may explain the invasive success of the species around the globe. The lack of variance in reproductive success may indicate that the species has evolved to cope with a large variability of environmental conditions leading to high fitness across a large range of habitats. It is thus of relevant important to continue surveying shore crab population using multiple approaches and integrating demographic, genetic, behavioural and oceanographic data.

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Chapter 4

Crab larval supply at multiple temporal scales

Domingues CP, Almeida MJ, Dubert J, Nolasco R, Sequeira A, Tavares S, Queiroga H (submitted). Crab larval supply to an estuary in the Eastern Atlantic upwelling system: predictability and idiosyncrasy at multiple temporal scales. Mar Ecol Prog Ser

4.1 Abstract

We measured variability in daily supply levels of shore crab megalopae, at an estuary on the Portuguese northwest coast, Ria de Aveiro, located in the Eastern Atlantic upwelling system. The five-year study covered the shore crab larval season (generally February to July) in 2002 and from 2006 to 2009. We attempted to address the relationships between wind- and tide- driven circulation, number of flood hours during darkness and chlorophyll-a concentration in coastal waters on larval supply variation, which may be affected by a few or by a combination of these processes. Megalopae supply measured over the years was and episodic phenomenon and observations showed some predictable and idiosyncratic patterns. In some episodes, supply was highest around spring tides and was enhanced by southerly winds, as predicted, although unclear patterns were observed among years. Concerning the relationship of supply levels with number of flood hours during darkness and chlorophyll-a concentration, results indicated ambiguous patterns throughout time series although in some years increased levels of supply were positively correlated with number of flood hours during night. Analysis of multiple years conducted in this study showed that megalopae supply patterns of the shore crab to the Ria de Aveiro are more variable than previously supposed, suggesting the participation of several delivery mechanisms that vary within and among years. Still, there exists a proportion of supply variation that cannot be explained, at least with the mechanisms we proposed.

4.2 Introduction

Transport by coastal currents has been shown to affect larval supply, settlement, recruitment, population genetic structure, species ranges and the spread of invasive species of coastal marine invertebrate and fish with complex life cycles (Gaines & Roughgarden 1985, Farrell et al. 1991, Pineda 1991, Alexander & Roughgarden 1996, Byers & Pringle 2006, Pringle & Wares 2007, Cowen & Sponaugle 2009). Diverse physical forcing features occurring in nearshore and coastal environments cause variation in circulation patterns that may result in temporal variation of larval supply to coastal marine populations. The causes of larval supply variation are not definitely clarified and assessing the relative contribution of different mechanism is almost unattainable and contrasting results are reported among time, space and taxons (e.g. Roughgarden et al. 1988, Poulin et al. 2002, Miller & Shanks 2004, Shanks & Brink 2005, Mace & Morgan 2006).

Wind circulation patterns drives upwelling and downwelling events that have long been established to affect the timing and distribution of larval supply and settlement. For instance, populations from upwelling systems, along the western margin of continents, tend to be viewed as recruitment-limited (Connolly et al. 2001). Traditionally, it has been assumed that during upwelling-favourable wind periods planktonic larvae dwelling in surface waters are transported offshore and the opposite occurs during upwelling-favourable winds relaxation or downwelling-favourable winds (e.g. Roughgarden et al. 1988, Farrell et al. 1991, Wing et al. 1995, Connolly et al. 2001, Almeida & Queiroga 2003, Queiroga et al. 2006). However, while some larvae behave as passive particles that may follow ocean current shifts, others are capable swimmers that can actually control their vertical distribution in the water column and subsequently their cross-shelf transport (Queiroga & Blanton 2005, Queiroga et al. 2007) remaining close to the shore and able to be transported shoreward independently of upwelling or downwelling conditions (Poulin et al. 2002, Miller & Shanks 2004, Shanks & Brink 2005, Morgan & Fisher 2010). The complex interaction between environmental wind-forcing and species larval behaviour is then one of the reasons contributing to larval supply variation to coastal habitats, but other phenomena such as tide-driven circulation, number of flood hours during darkness and chlorophyll concentration may act together to produce high variability in larval supply levels.

Tidal and lunar cycle patterns of supply to estuarine and mangrove populations, occurring around spring tides, have been described for some brachyuran megalopae (van Montfrans et al. 1990, Moser & Macintosh 2001, Paula et al. 2001, Miller & Shanks 2004, Queiroga et al. 2006) using selective tidal stream transport (STST) to travel up estuaries. These patterns have been related to larval response to augmented hydrostatic pressure, salinity and turbulence during spring tides (e.g. Queiroga et al. 2006) or to a combination of onshore transport by internal waves and diel vertical migration (Miller & Shanks 2004). When returning to estuaries, decapod late stage larvae use STST to travel upstream, occupying a higher position in the water column during the night and a position close to the bottom during daytime, triggered by chemical cues present in waters and presumably to avoid visual predators (Queiroga 1998). Therefore, the number of flood hours during darkness can also influence supply intensity.

Phytoplankton is a direct or indirect source of food for most marine animals and may constitute an important component of the natural diet of decapod larvae (Factor & Dexter 1993,

Meyer-Harms & Harms 1993), especially when nutrition is limited in the field (Harms et al. 1994). Chlorophyll-a (Chl-a) concentrations are an indicator of phytoplankton abundance and biomass in estuarine, coastal and oceanic waters (Cullen 1982) and therefore may be correlated with the abundance of larvae. Larvae in good nutritional condition may be more capable of surviving and successfully recruit inside estuaries. Temporal and spatial variation in the concentrations of Chl-a in surface waters may be related to nutrient concentrations, wind-driven turbulence intensity, hydrodynamics and turbidity (Franks 1992).

Addressing the mechanism driving larval supply and settlement variation is challenging and requires data sampling over long time series, including interannual comparisons, in order to describe the relevant processes with sufficient detail, and only few studies (e.g. Goodrich et al. 1989, van Montfrans et al. 1990, Jones & Epifanio 1995, van Montfrans et al. 1995, Wing et al. 2003, Giménez & Dick 2007, Roegner et al. 2007) were able to perform such samplings.

In the present work, we examined the potential influence of some mechanisms and features on observed patterns of larval supply (late stage larvae – megalopae) to an estuary in the Eastern Atlantic upwelling system for a coastal marine invertebrate, *Carcinus maenas* (Decapoda, Portunidade). We combined detailed oceanographic data with daily larval supply information obtained during the reproductive season of five different years. *C. maenas* has a complex life cycle with four pelagic zoeae and a megalopa and is widely distributed on both hard and soft intertidal and shallow habitats of coasts and estuaries along its European native range.

We investigated the effect of wind- and tide-driven circulation, number of flood hours during darkness and Chl-a concentration on levels of larval supply. Specifically, we tested the hypothesis that: i) larval supply should increase after downwelling-favourable or relaxation of upwelling-favourable winds; ii) larval abundance should vary with the fortnightly tidal cycle; iii) larval supply should increase with increased number of flood tide hours occurring at night, and iv) larval abundance should increase after high levels of Chl a concentration in the coastal ocean.

4.3 Materials and methods

4.3.1 Field sampling and environmental data

Research was conducted at Canal de Mira located within Ria de Aveiro estuary, on northwestern Portugal (40º 37'17''N, 8º 44'56''W; Figure 1). Circulation in Ria de Aveiro is dominated by tides, which are semidiurnal with an average range of 2.1 m. Field sampling was conducted from March to June of 2006 and 2007 and from February to July of 2008 and 2009, covering the peak times of megalopae supply for *Carcinus maenas* at the northwest Portuguese coast. Additionally, in order to increase the number of data for interannual comparison, we used information from a previous study of Queiroga et al. (2006) in which field sampling took place from April to July of 2002.

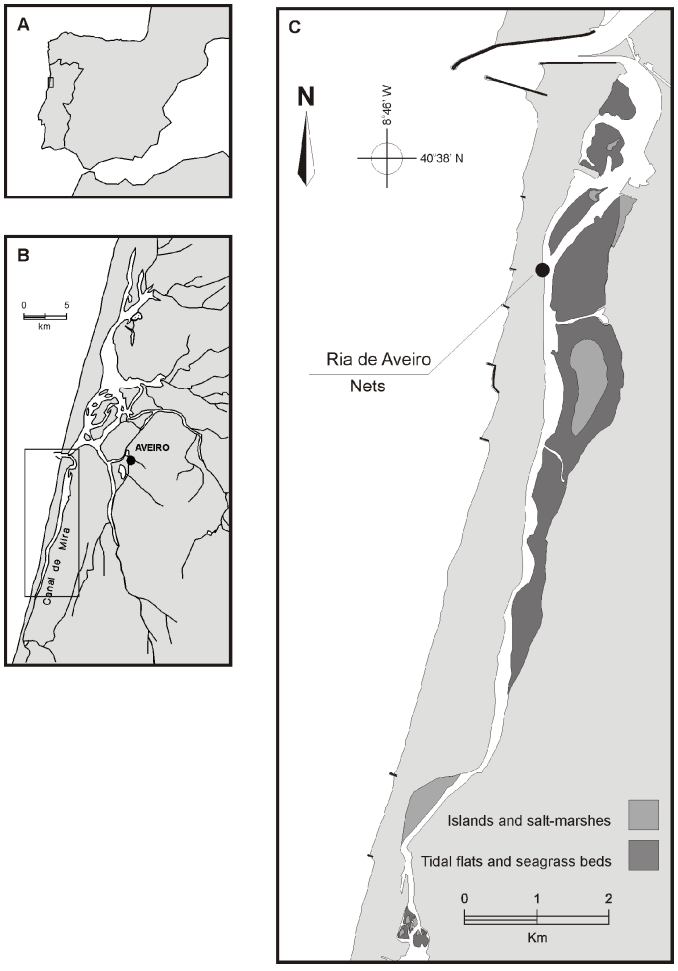

Figure 1 Sampling location for *Carcinus maenas* megalopal supply. (A) Iberian Peninsula, (B) Ria de Aveiro, NW Portugal and (C) Sampling location.

Larval supply was measured with the use of two passive plankton nets which provide a standardized form for sampling megalopae only during the flood tide (cf. Queiroga et al. 2006). One net was deployed at the water surface and the other was deployed above the bottom and both were censured on a daily basis, each morning, when the contents were recovered and the number of megalopae counted. Wind velocity for the region was obtained from the widely used National Centers for Environmental Prediction (NCEP) Reanalysis data project provided by the NOAA/OAR/ESRL PSD, Boulder, Colorado, USA, from their web site at http://www.esrl.noaa.gov/psd/ (Kalnay et al. 1996). Both components of wind, *u* (positive for westerly winds) and *v* (positive for southerly winds) were extracted for the point 40º 53'N; 9º 22'W, every six hours. From these data we calculated the wind stresses (c.f. Queiroga et al. 2006) for both north-south (along-shore) and east-west (cross-shore) components, which were then averaged over a 24 hour period. Sea level data were obtained from the Aveiro tide gauge, which is located at the inlet channel of the Ria de Aveiro. Daily tidal range was recorded as the change in tidal height between the average of the two daily maxima and the average of the two daily minima water levels. Daily subtidal sea level (SSL) was computed by running a 13 h moving average over the hourly sea level values in order to remove the tidal signal, followed by the calculation of the average of the filtered values for each day. Daily sea surface temperature (SST) data were obtained from a weather station of the Portuguese Instituto de Meteorologia located at Leixões, 60 km north of the Ria de Aveiro. Series of tidal elevation were extracted and analysed from tides prediction for the western Iberian region (Marta-Almeida & Dubert 2006). These data together with the sunrise and sunset times published by the Observatório Astronómico de Lisboa for the period in analysis, allowed to calculate the number of flood hours that take place every day and to obtain the number of flood hours during darkness. Series of Chl-a concentration were extracted from satellite observed Chl-a in the sea water (mg m^{-3}) obtained by optimized interpolation of the MODIS, SeaWiFS and MERIS OC5 Chl-a fields, and supplied by CERSAT/IFREMER, France, through their ftp server (ftp://ftp.ifremer.fr/ifremer/cersat/products/gridded/ocean-color/atlantic/EUR-L4-CHL-ATL-v01). Based on the daily mean distribution of the Chl-a within a pixel of 0.015º (nearly 1.65 km latitude x 1.28 km longitude), a time series of spatially averaged Chl-a for a rectangle extending to 100 km to the north and south of the Aveiro inlet and 40 km offshore (the shelf break, approximately) was calculated.

4.3.2 Statistical analysis

In order to describe the mechanisms that control larval supply to coastal habitats it is necessary to identify the time lag between environmental forcing and the subsequent

response of the coastal ocean and of larval supply. In the present study this was done using cross-correlation (Chatfield 1996) and least squares multiple regression (Sokal & Rohlf 1995), where the forcing variable preceded the response variable, indicated throughout this paper by negative numbers. Effects of wind on SSL, SST, Chl-a and supply were tested for lags ≥ - 5d, because it is difficult to meaningfully interpret situations where winds or tides precede hydrographical variables or supply by ≤ - 5d. The effect of tides on megalopal supply was investigated down to lags of - 15 d, in order to detect possible cases of fortnight periods only evident in shorter segments of the data series. Because the expected effect of Chl-a on supply is through changes of larval mortality associated with food conditions, which would take longer than the effects of other variables on larval advection, we tested this effect with lags down to -14 d. In the case of night flood hours the effect on supply was tested down to lags of -2 d. This option is justified on the base that maximum values of night flood hours in the Portuguese coast coincide either with the lowest (in autumn and winter) or highest (in spring and summer) amplitude tides along the semilunar cycle (Queiroga & Blanton 2005), and because megalopal supply was found to be dependent on tidal range at lags ≥ -2 d (see below). All cross-correlations were computed over the differenced series for each variable using a time lag of -1 d to remove the autocorrelation that was present in all data series (Chatfield 1996) and which could increase the probability of Type I errors. The auto-correlation present in the time series at the time lag of -1 d, reflecting the influence of megalopal supply of any one day on that of the next day, violates the assumption of independence required by least squares regression and would also result in increased probability of Type I errors. Because of that auto-correlation the residuals produced by the adjustment of the multiple regression models presented an autocorrelation structure that followed a first order autoregressive process. Therefore, in order to remove dependence of the data we applied the transformation $x_d - \Phi x_{d-1}$ to all the independent variates x where Φ represents the autoregressive coefficient at that time lag (Alpuim & El-Shaarawi 2008), similarly to the methodology applied by Queiroga et al. (2006).

According to upwelling theory, which predicts a drop of the SSL and a decrease in SST at the coast during an increase in upwelling favourable winds (equatorward wind stress), if the coastal ocean is responding to wind-driven circulation then SSL and SST should be correlated with the alongshore component of wind stress. We used cross-correlations to test this hypothesis and demonstrate that the upwelling-downwelling mechanism is operating during the sampling periods.

The relationship between environmental variables and daily supply of megalopae was analysed using cross-correlation and a least squares multiple regression model, applied separately to the total data series obtained in each year. In a exploratory approach to detect relationships between pairs of variables and time lags of effects we used cross-correlation analysis in which megalopal abundance was separately lagged against tidal range, number of flood hours during darkness and Chl-a concentration. We then used a least squares multiple regression model of daily supply against wind and tidal forcing, number of flood hours during darkness and Chl-a concentration at different time lags, as a predictive approach similar to that described in Queiroga et al. (2006). We applied multiple regression separately for each year, in a stepwise forward fashion starting with a set of sine and cosine functions that account for the semilunar cycle of tidal range and with along-shore wind stress, which are the main factors previously known to control supply of megalopae to estuaries on the Portuguese west coast (Queiroga et al. 2006). We decided to model tidal range with sine and cosine functions (with periods of 14 or 15 d, depending on which produced a better adjustment) using the property that any periodic function with a period equal to an entire number can be described by a linear combination of sine and cosine functions with periods equal to submultiples of the period of the main cycle (Wei 1990). This option was taken instead of using observed tidal range because i) cross-correlation indicated that delays between megalopal supply and tidal range were short (≥ -2 d), ii) the automatic phase adjustment of the sine and cosine functions dispenses from the need to test different lags and iii) the observed tidal range produced worse adjustments, probably due to the range inequality in consecutive fortnight cycles. Whenever there was a significant effect of wind or tidal range on megalopal supply we introduced the other variables: Chl-a concentration and number of flood hours during darkness. SSL and SST were not used in the regression model because they ultimately depend on the wind variable and co-vary linearly with it. When running the regression model variables that were not significant were discarded. Because of the existence of the autocorrelation structure in the residuals, the use of the determination coefficient r^2 to evaluate the overall fit of the regression model is not appropriate. Instead we used we used a modification of this statistic, r^{*2}, where the sum of squares of residuals was replaced by the sum of squares of the white noise sequence produced by the adjustment of an autoregressive process to the residuals (Queiroga et al. 2006). This sum of squares is a more realistic measure of the variability due to unexplained error because it accounts for the day to day dependence of the observations. From all the models that were fitted we retained the one with the highest fit, according to the respective r^{*2}.

Along the west Iberian Peninsula the number of hours of flood tide that take place during darkness is maximized at spring tides during spring and summer, whereas during autumn and winter it is maximised during neap tides (Queiroga & Blanton 2005). Available evidence shows that supply of crab megalopae to estuaries typically occurs during night flood tides (Little & Epifanio 1991, Olmi 1994, Queiroga 1998), but that tidal range also influences supply of crab and other invertebrate larvae to coastal habitats, either through internal wave transport (Shanks 1983, Shanks 1988, Pineda 1991) or through short-term behavioural responses to changes in hydrostatic pressure (Tankersley et al. 1995). In an attempt to separate the influence of night flood from tidal range we split the time series obtained in each year at Julian day 90, which marks the transition between the periods when maximization of nocturnal floods co-occurs with neap or spring tides, and applied cross-correlation and multiple regression also on the time series collected before and after day 90. Hereafter we refer to these periods as the complete, before day 90 and after day 90 data sets.

4.4 Results

Typical upwelling favourable winds (northwesterly) dominated during the sampling period of all years, particularly in 2002, followed by 2009, 2008, 2007 and 2006 as observed in Figures 2-6 B by wind stresses with a negative along-shore component and a positive cross-shore component. Downwelling favourable winds (southwesterly), evidenced by wind stresses with a positive along- and cross-shore component were less frequent especially in 2002 and 2009 although events of 6 to 7 days were observed during the studied period of these years (Figures 2 & 6 B). Southwesterly wind episodes were more frequent in the remaining three years when strong and extended events occurred in the beginning of spring in 2006, from days 77 to 91 (Figure 3 B), just prior to the beginning of summer in 2007, from days 158 to 170 (Figure 4 B), and during spring in 2008, from days 107 to 114 (Figure 5 B).

SST values fluctuated between 12 to 19-20 ºC (Figures 2-6 C) and, with the exception of 2006, SST was always associated with wind stress at lags of -1 to -4 d, as confirmed by maximum significant positive cross-correlations between alongshore wind stress and SST. Slightly stronger correlations were found for the split data series obtained both before and after day 90 (Table 1).The response of the ocean to wind stress in terms of SSL occurred for time lags of 0 and -1d as evidenced by maximum significant positive cross correlations between alongshore wind stress and SSL. For the complete data series and for the data

obtained before day 90 significant relationships were observed for all years, but not for the period after day 90 (Table 1).

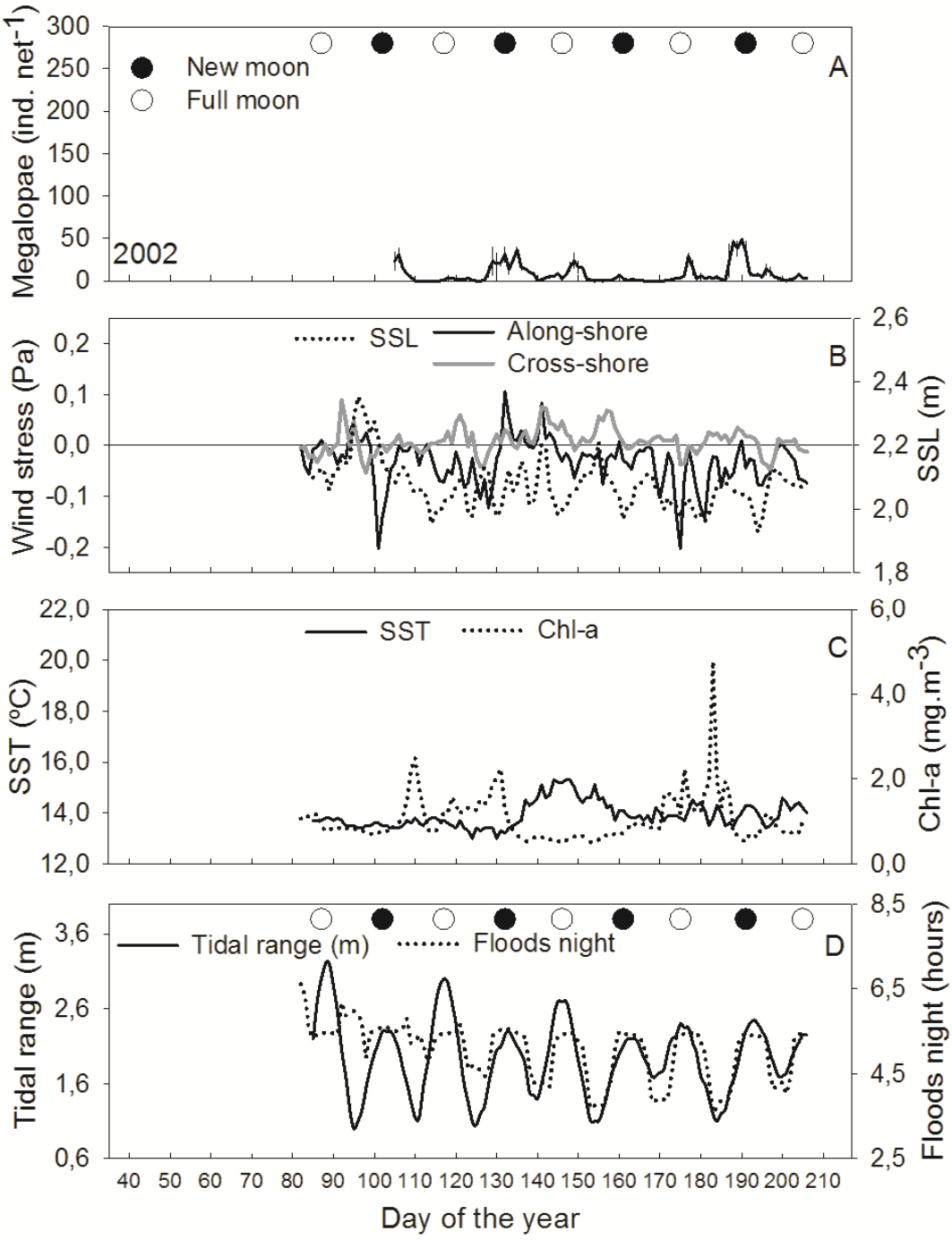

Figure 2 Time series at Ria de Aveiro for year 2002. Daily averages of (A) megalopal supply, (B) alongshore and cross-shore wind stress and subtidal sea level (SSL), (C) sea surface temperature (SST) and chlorophyll a (Chl-a) concentration, (D) tidal range and number of flood hours occurring at night.

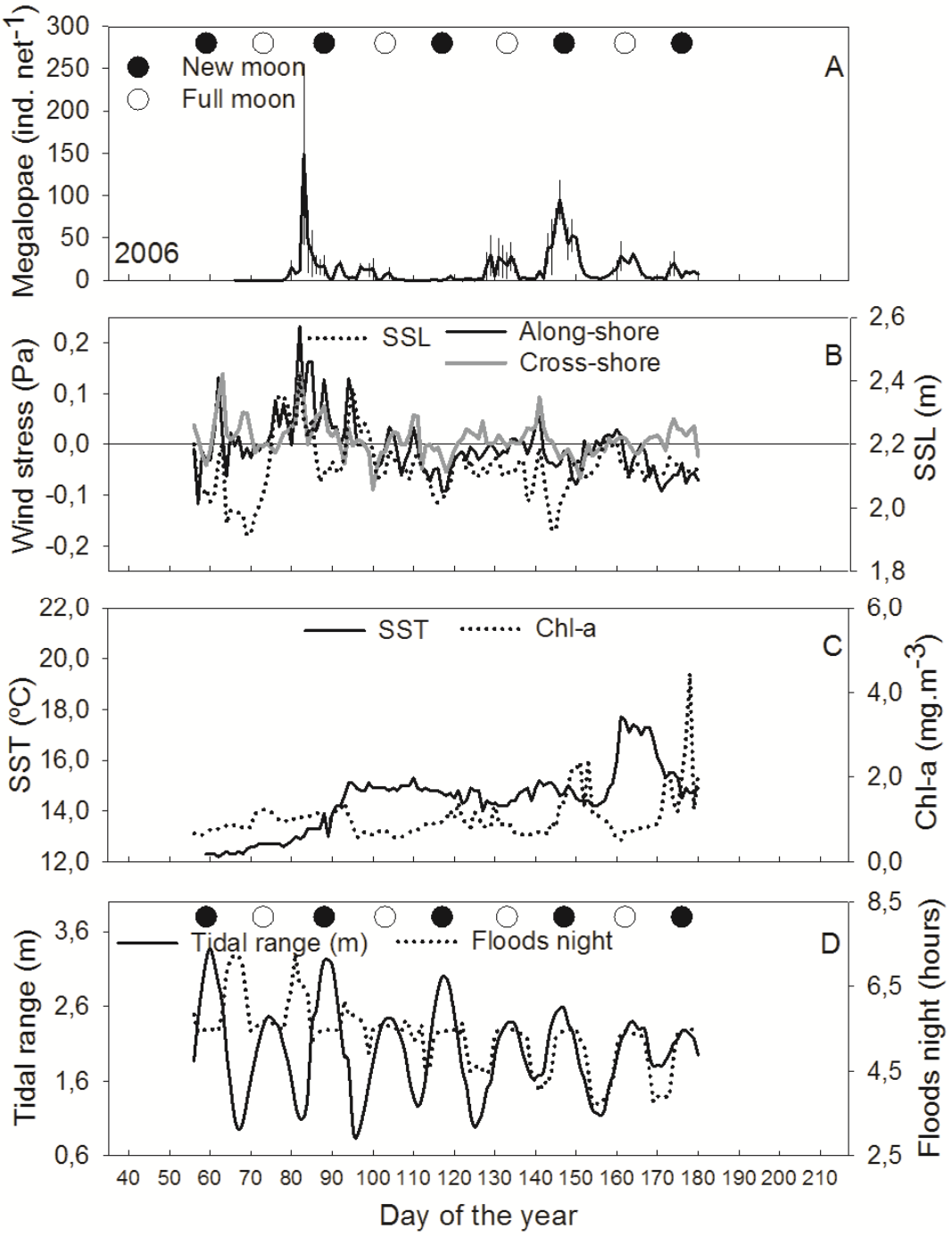

Figure 3 Time series at Ria de Aveiro for year 2006. Daily averages of (A) megalopal supply, (B) alongshore and cross-shore wind stress and subtidal sea level (SSL), (C) sea surface temperature (SST) and chlorophyll a (Chl-a) concentration, (D) tidal range and number of flood hours occurring at night.

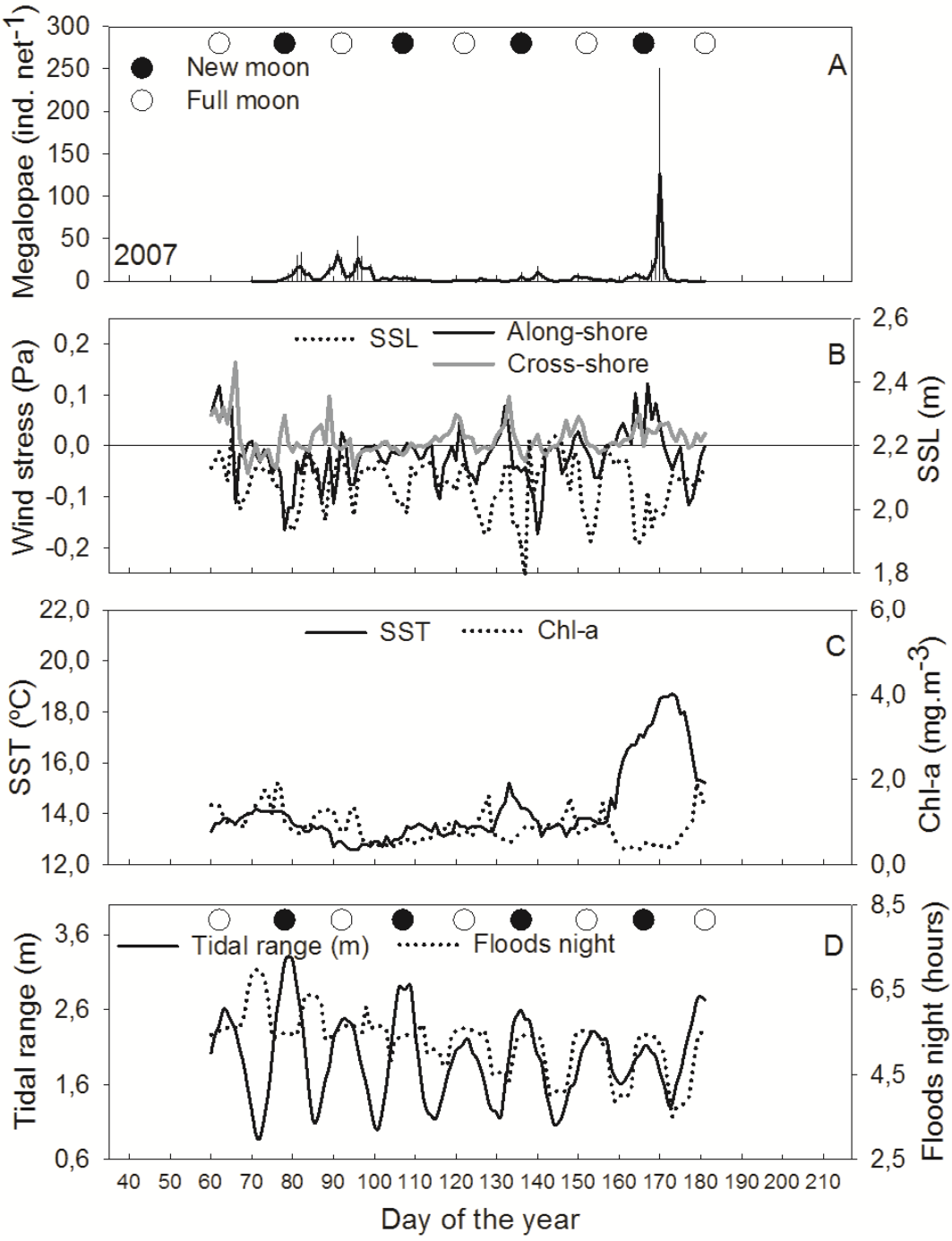

Figure 4 Time series at Ria de Aveiro for year 2007. Daily averages of (A) megalopal supply, (B) alongshore and cross-shore wind stress and subtidal sea level (SSL), (C) sea surface temperature (SST) and chlorophyll a (Chl-a) concentration, (D) tidal range and number of flood hours occurring at night.

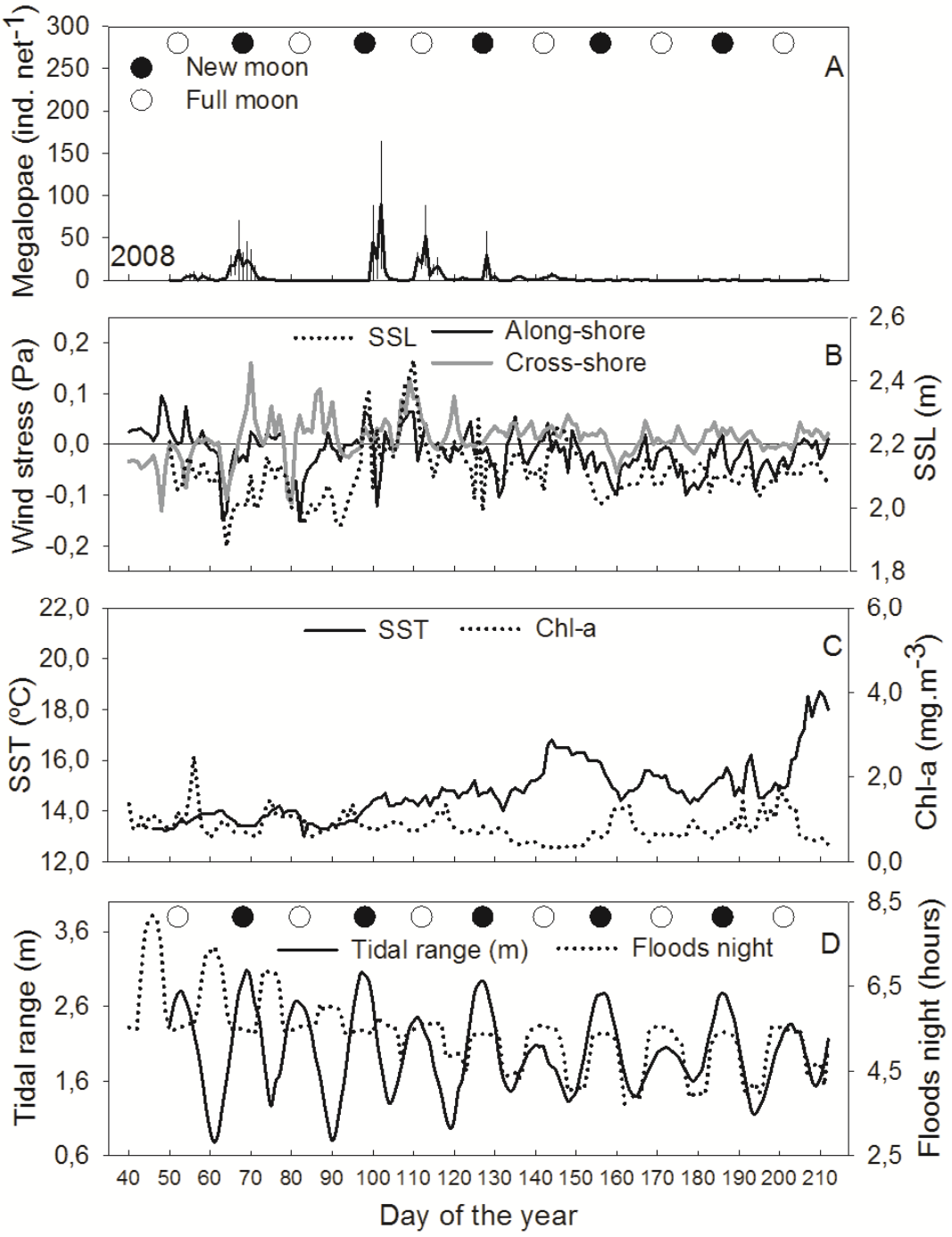

Figure 5 Time series at Ria de Aveiro for year 2008. Daily averages of (A) megalopal supply, (B) alongshore and cross-shore wind stress and subtidal sea level (SSL), (C) sea surface temperature (SST) and chlorophyll a (Chl-a) concentration, (D) tidal range and number of flood hours occurring at night.

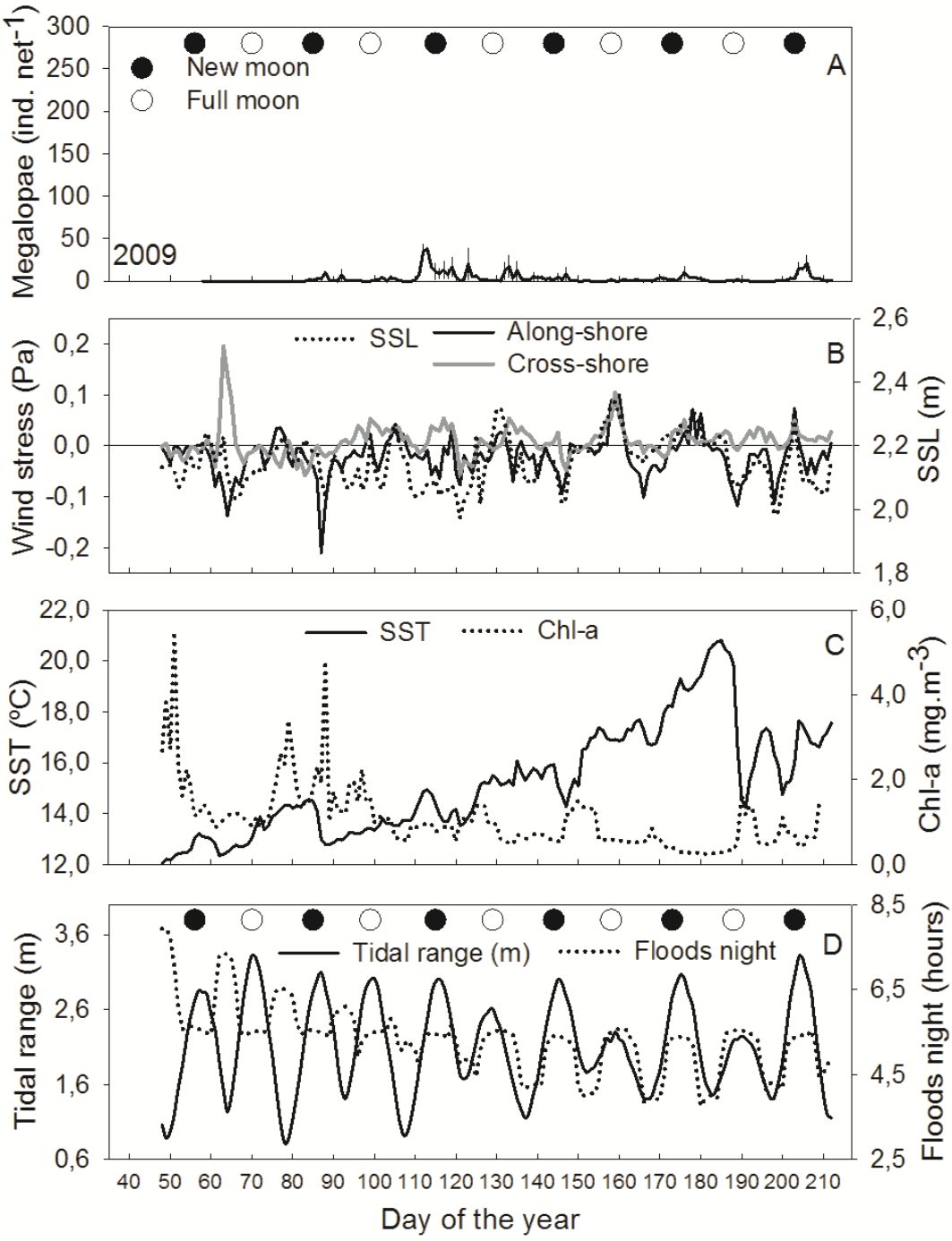

Figure 6 Time series at Ria de Aveiro for year 2009. Daily averages of (A) megalopal supply, (B) alongshore and cross-shore wind stress and subtidal sea level (SSL), (C) sea surface temperature (SST) and chlorophyll a (Chl-a) concentration, (D) tidal range and number of flood hours occurring at night.

Table 1 Time lags (d) and cross-correlations (*r* in parentheses) between alongshore component of wind stress (WS) and sea surface temperature (SST) and subtidal sea level (SSL) applied on the complete data series (All) and on the data obtained before (B90) or after (A90) day 90. Reported values refer to maximum significant ($P < 0.05$) cross-correlations in each case; nd: no data; ns: not significant.

Year		WS	
		SST	SSL
2002	All	nd	nd
	B90	nd	nd
	A90	-1 (0.368)	0 (0.257)
2006	All	ns	0 (0.516)
	B90	ns	0 (0.591)
	A90	ns	0 (0.403)
2007	All	-1 (0.209)	0 (0.232)
	B90	ns	-1 (0.442)
	A90	-1 (0.338)	ns
2008	All	-1 (0.247)	0 (0.245)
	B90	-4 (0.366)	0 (0.500)
	A90	-1 (0.254)	ns
2009	All	-1 (0.171)	0 (0.488)
	B90	0 (0.496)	-1 (0.465)
	A90	-3 (0.204)	0 (0.552)

Abundance of megalopae recorded in the nets varied by a factor of 4 among years, from a maximum average of 12 ind net^{-1} d^{-1} in 2006 and a minimum average of 3 ind net^{-1} d^{-1} in 2009 (Figures 2-6 A). If larval abundance should vary with the fortnight tidal cycle, with number of flood tide hours during darkness and with levels of Chl-a concentration we should find significant cross-correlations between megalopae catches and the cited variables. Significant correlations between abundance in the plankton nets and tidal range were never observed when the complete time series were analysed, but were present in the split data sets in 2002 and 2006, after day 90, and in 2007, before day 90, with maximum values at time lags of -2 or -15 d (Table 2). Megalopal abundance was positively correlated with flood hours during darkness in 2002 and 2006, at time lags ranging from -2 to +2 d. In 2002 significant associations were only found after day 90, while in 2006 all periods analysed showed significant values. In 2007, 2008 and 2009,

number of flood hours during darkness was not associated with larval supply at any lag (Table 2).

Table 2 Time lags (d) and cross-correlations (*r* in parentheses) between megalopal supply (Meg) and tidal range (TR), night flood hours (NF), and chlorophyll-a concentration (Chl-a), applied on the complete data series (All) and on the data obtained before (B90) or after (A90) day 90. Reported values refer to maximum significant ($P < 0.05$) cross-correlations in each case; nd: no data; ns: not significant.

Year		Meg		
		TR	NF	Chl-a
2002	All	nd	nd	nd
	B90	nd	nd	nd
	A90	-15 (0.261)	+2 (0.232)	-5 (0.227)
2006	All	ns	+1 (0.232)	ns
	B90	ns	-2 (0.532)	-12 (0.622)
	A90	-15 (0.272)	+1 (0.247)	ns
2007	All	ns	ns	-14 (0.308)
	B90	-2 (0.491)	ns	-5 (0.790)
	A90	ns	ns	-14 (0.350)
2008	All	ns	ns	ns
	B90	ns	ns	-11 (0.447)
	A90	ns	ns	-7 (0.240)
2009	All	ns	ns	ns
	B90	ns	ns	0 (0.761)
	A90	ns	ns	-14 (0.199)

Maximum values of Chl-a were obtained at the end of June in 2002 and 2006 and during February and March in 2009 (Figures 2-6 C). In 2007 and 2008 Chl-a levels were generally slightly lower compared with the other years (Figures 4 & 5 C). After analysis of the entire series, we found a significant positive correlation between larval abundance and Chl-a concentration at a lag of -14 d, only in 2007 (Table 2). Before day 90, significant correlations were found in all years at time lags ranging from 0 to -12 d and after day 90 cross-correlograms between megalopal supply and Chl-a concentration indicated delays ranging from -5 to -14 d, except in 2006 when the correlation was not significant (Table 2).

Results of the application of the regression model are reported in Table 3 that shows the best combination of variables to explain supply of *Carcinus maenas* megalopae in each year and period (complete, before day 90 and after day 90 data sets). Application of the multiple regression models in general improved the ability to detect significant associations of megalopal supply with forcing variables relative to the use of cross-correlations. This is apparent from the larger number of cases where a significant effect of individual variables was detected (compare Table 2 with Table 3) and results from simultaneously accounting for the effect on supply of the remaining variables in the models. Significant effects of tidal range were detected in all years but data sets when effects were detected were not consistent among years (Table 3). Because the use of a linear combination of sine and cosine functions automatically adjusts to the correct phase the results are reported in Table 3 without time lags. The results of the cross-correlations (Table 2) and visual inspection of the fit of model (not shown) to the observed data series indicated time lags between maximum supply and maximum tidal range of -2 to 2 d. Along-shore wind stress significantly affected megalopal supply in all years and data sets, at time lags between -4 and 0 d, except in 2006 and 2009 when significant results were obtained only in some of the data sets (Table 3). These time lags are within the expected response times of the coastal ocean to along-shore winds in terms of along- and across-shore circulation. However, the sign of the regression coefficient was not consistent among years and data sets. When a significant effect was found in the complete data sets (2006, 2007 and 2008) the sign was always positive, meaning that southwesterly, downwelling-favourable winds promoted an increase in megalopal supply. The coefficient was also positive in the three cases where a significant effect was detected in the data collected after day 90 (2002, 2007and 2008). In the data collected before day 90 the coefficient was positive in one (2006) but negative in the other cases (2007, 2008 and 2009), indicating augmented supply with northerly, upwelling-favourable winds. Chl-a was related with supply at time lags ranging from -7 to 0 d in several cases. As with wind stress, the sign of the effect was not consistent, indicating positive as well as negative effects of Chl-a on megalopal supply. Number of flood hours during darkness was never significantly related with megalopal supply. The modified coefficient of determination r^{*2} ranged from 0.198 to 0.937. Usually the split of the time series resulted in better fits of the models, indicated by consistent increases of the r^{*2} statistic relative to the complete data sets in all years.

Table 3 Result of periodic regression models of megalopae supply on sine and cosine functions describing the semilunar cycle of tidal range, alongshore wind stress (WS) and chlorophyll-a concentration (Chl-a), applied on the complete data series (All) and on the data obtained before (B90) or after (A90) day 90. Format of data is lag (regression coefficient, probability level). Lags for sine and cosine are not reported because they are always equal to zero. r^{*2} is the modified determination coefficient.

Year		Sine	Cosine	WS	Chl-a	r^{*2}
2002	All	nd	nd	nd	nd	nd
	B90	nd	nd	nd	nd	nd
	A90	(4.765, 0.0064)	(3.811, 0.0321)	0 (53.905, 0.0055)	-5 (7.721, 0.0001)	0.641
2006	All	ns	ns	-1 (99.671, 0.0140)	ns	0.412
	B90	(12.637, 0.0175)	ns	-1 (316.720, 0.0001)	ns	0.618
	A90	ns	(8.656, 0.0075)	ns	ns	0.704
2007	All	(-5.041, 0.0191)	ns	-3 (59.513, 0.0314)	ns	0.198
	B90	ns	ns	-2 (-90.870, 0.0006)	-3 (-10.627, 0.0032)	0.733
	A90	ns	ns	-3 (89.442, 0.0053)	ns	0.256
2008	All	ns	ns	-4 (45.007, 0.0328)	ns	0.228
	B90	ns	ns	-3 (-54.211, 0.0100)	-7 (-6.752, 0.0234)	0.640
	A90	(-3.302, 0.0286)	ns	-4 (108.467, 0.0000)	-7 (7.982, 0.0270)	0.288
2009	All	ns	ns	ns	ns	ns
	B90	(0.492, 0.0001)	(1.000, 0.0001)	-1 (-19,648, 0.0001)	0 (1.478, 0.0001)	0.937
	A90	ns	ns	ns	ns	ns

4.5 Discussion

Supply of *Carcinus maenas* megalopae to the Ria de Aveiro over five separate years during the recruitment season (February to July) was irregular, exhibiting periods of zero or low supply punctuated by episodic peaks. Cross-correlation and multiple regression analysis applied to series of daily observations indicated significant effects of tidal range, along-shore wind stress and Chl-a concentration on supply, at several time scales within each recruitment season. Peaks of larval supply were often associated with spring tides but effects of wind stress and Chl-a concentration were not consistent in sign. Moreover, large unexplained variation remained in most cases. Similar investigations have reported tide- and wind-driven transport as the main mechanisms responsible for supply and settlement of crab megalopae in other coastal systems of the world (van Montfrans et al. 1990, Little & Epifanio 1991, Moser & Macintosh 2001, Paula et al. 2001, Miller & Shanks 2004). However, the results of the present multi-year study show that the response of *C. maenas* megalopae supply to variation in physical variables is more complex and difficult to generalize than previously acknowledge by Queiroga et al. (2006), whose data from larval abundance collected by the passive nets at Ria de Aveiro in 2002 were included in the present study. Queiroga et al. (2006) sampled only after Julian day 90 and reported an increase in megalopae supply levels at the time of maximum tidal amplitude and after periods of southerly winds or equatorward wind relaxation. In the present study, results obtained with a similar multiple regression approach for the complete data set within each year (2002 series excluded) showed a significant association between larval supply, tidal range and wind stress only in one year (2007) and many instances of non-significant associations or of effects of opposite sign in the split data sets.

4.5.1 Tide-driven supply

Using cross-correlations we found a fortnightly period of oscillation of megalopae supply at time lags of -15 d (after day 90 in 2002 and 2006) and -2 d (before day 90 in 2007). These time lags indicate an immediate response of supply to maximum amplitude tides around new and full moons as previously identified (Queiroga et al. 2006). The use of sine and cosine functions in the multiple regression increased the detection of significant associations of supply to maximum amplitude tides (in total 8 data sets out of 13 possible). The sampling programme that we used does not allow us to discriminate between the two hypothesis usually considered to explain fortnight periods in larval abundance in coastal habitats, viz. the internal wave hypothesis, which is invoked to

explain shoreward transport of neustonic larvae over the shelf (Shanks & Wright 1987, Pineda 1999), or the flood tide hypothesis, which pertains to the immediate behavioural response of crab megalopae to changes in salinity or hydrostatic pressure during flood (DeVries et al. 1994, Tankersley et al. 1995, Welch & Forward 2001). However, because of the very short delays between maximum tidal range and maximum supply, and because *C. maenas* megalopae are not full neustonic organisms (dos Santos et al. 2008), we would favour the second hypothesis, which implies that the megalopae are already close to shore in order to suffer the influence of the tidal current.

4.5.2 Number of flood hours occurring at night

Most studies describing colonization of estuaries by crab megalopae refer to transport mainly during nocturnal flood tides (e.g. Christy & Morgan 1998, Queiroga 1998). The co-occurrence of darkness and flood in western Iberia is maximized during spring tides after day 90 (Queiroga & Blanton 2005) and indeed during the studied period the correlation between tidal range and the number of flood hours occurring at night was maximized during the spring and summer months (not shown). Positive cross-correlations between night floods hours and larval supply were significant only in two years (2002 and 2006) for the period after day 90, at time lags of +1 and +2 days. However, if megalopae are responding also to the length of flood tides under the cover of darkness during supply to, and migration up, estuaries (Christy & Morgan 1998, Queiroga 1998), then we would expect a reinforcement of a semilunar oscillation in supply during spring and summer. A simple visual inspection of the supply data series (Figures 2 to 6, panel A) indicates that repeated peaks at periods of 14-15 days, which were also identified by the cross-correlation and multiple regression analyses, did only occur during spring and summer (of 2002, 2006 and 2008).

4.5.3 Wind-driven supply

Overall, the coastal ocean responded quickly to wind-driven circulation as evidenced by the significant cross-correlations between along-shore wind stress with SST and SSL, which dropped or rose following periods of northwesterly or southwesterly winds, respectively. The sign of the effects of wind stress on SST and SSL and the response time detected are within the predictions of upwelling theory and of previous observations (Wooster et al. 1976, Jorge da Silva 1992).

We found some larval events clearly predicted by strong southwesterly winds, which caused an immediate increase in SSL produced by coastal convergent currents. The clearest examples were the supply peaks that took place from days 82 to 88 in 2006 and from days 167 to 171 in 2007, which yielded in a single day ca. 150 and 125 megalopae per net, respectively. Significant effects of wind stress on supply were always positive for the complete data set and for observations made after day 90 in accordance with previous observations (Queiroga et al. 2006), but negative effects were usually detected before day 90. Interpretation of these results requires some speculation in the light of the current knowledge about larval behaviour and direction of transport over the shelf. *Carcinus maenas* larvae are known to perform extensive diel vertical migrations in shelf waters, being more abundant close to the surface during the night (dos Santos et al. 2008). Results of a modelling study (Marta-Almeida et al. 2006) showed that this behaviour should promote retention of larvae in the inner shelf during upwelling, caused by the extended period of time spent by the larvae in the shoreward undercurrent. Retention in the inner shelf should favour increased recruitment, but the present data do not support this simple interpretation. If diel vertical migration would directly affect supply to estuaries, then the negative effect of wind stress on supply detected in winter would be explained, but a change in migration behaviour from nocturnal migration in winter to reverse migration during spring and summer would have to be assumed. An alternative explanation is that the along-shore component of ocean circulation is driving most of the variability in supply. Results from a different experiment using the same numerical model (Peliz et al. 2007) predicted a dramatic northward transport driven by downwelling favourable winds when compared with the net southward transport during upwelling favourable winds of similar magnitude. Unpublished observations (J. Dubert, personal communication) indicate that the wind events of the end of March 2006 and end of June 2007 can cause a northward flow over the inner shelf of up to 35 km d^{-1}. Given the time lags identified (-1 to -4 d) this interpretation would account for the strong supply peaks, provided that pools of competent megalopae are available in the coastal ocean a few 10s to 100 km to the south of the Ria de Aveiro. Since estuaries and rias are distributed along the western Iberia at 20 to 60 km intervals and that the planktonic larval duration of *C. maenas* is around four to six weeks this interpretation seems more plausible than invoking a change in larval behaviour with season, which has never been documented (Queiroga & Blanton 2005). Interpretation of variation of supply strength with along-shore currents at intra-year time scales would also be compatible with the negative effects of along-shore wind stress detected in winter. The predominance of southerly winds during winter but

alternating with northerly winds at periods < 9 d in the western Iberian coast (Fiúza et al. 1982) could result in transient pools of megalopae to the north of the Ria, which could be transported southward and recruit to the Ria with northerly winds events.

4.5.4 Chlorophyll-a concentration

Upwelling conditions bring up nutrient-rich waters promoting high levels of phytoplankton production, influencing the variability of Chl-a concentration along the Portuguese west coast (Oliveira et al. 2009). The multiple regression analysis showed variable relationships between Chl-a concentration and larval supply levels, with lags of 0 to -7 d and negative as well as positive effects. In general we would expect positive effects of Chl-a concentration on supply, as an indirect result from greater primary and secondary production that would enhance survival, but negative effects were also observed. Oliveira et al. (2009) provide evidence that shows patchy distribution of Chl-a and an asymmetry with surface temperature caused by northerly winds forcing. This complex response of the surface ocean causes temporal and spatial variability that contributes to increase the complexity in the interpretation of larval supply levels.

4.5.5 Other possible causes of variability

As with eastern boundary currents in general, the two-mode circulation model over the Iberian shelf (reviewed in Relvas et al. 2007), with a poleward current during winter and an equatorward current during summer, is a simplification. During winter the Iberian Poleward Current (IPC, Peliz et al. 2005) interacts with the Western Iberia Buoyant Plume (WIBP, Peliz et al. 2002) and cause eddy shedding and the occurrence of transient low salinity lenses, fronts and retention zones that may accumulate and transport larvae (Santos et al. 2004). Moreover, intermittent upwelling events do occur that increase the complexity of the circulation. During summer the influence of the WIBP is reduced but the response of the coastal ocean to upwelling winds is asymmetrical in terms of along-shore and vertical components of circulation, and eddy formation still occurs as a results of the cross-shelf displacement of the IPC with wind events of variable intensity and duration. These mechanisms may be at the base of the elusive relationships between ocean circulation and supply events that we detected in the present study, and that are a characteristic feature of recruitment patterns in many marine populations (Jones & Epifanio 1995, van Montfrans et al. 1995, Roughgarden & Smith 1996, Wing et al. 2003, Shanks 2006, Giménez & Dick 2007, Siegel et al. 2008). The idiosyncratic nature of many

of the supply events detected may also be connected with spatial and temporal differences in larval production, as well as with the length of our observational program. Highest densities of ovigerous females of *Carcinus maenas* are found from January to June in the Portuguese coast, with smaller densities in July and August (Queiroga 1995), and maximum densities of early juveniles have been observed from May to July in the Ria de Aveiro (Queiroga 1993). The time series we collected cover most of the species' larval season in all years except 2002. However, seasonality of reproduction implies that the period before day 90 corresponds to a shorter series than the subsequent period, with possible consequences on our detection of the significance and magnitude of the effects between seasons. Additionally, the long periods with very low or zero supply detected in some of the years suggests that availability of competent megalopae off the Ria de Aveiro may be very small during extended periods, possibly connected with temporal and spatial variations of larval hatching interacting with the subsequent advection history.

Finally, we would like to note what appears to be a regime shift in *Carcinus maenas* megalopal supply to the Ria de Aveiro, indicated by higher fits of the regression models to the split data series relative to the complete data series. This regime shift seems to be connected with the co-occurrence of darkness and flood with spring tides between the spring and autumn equinoxes, but could also be associated with the spring transition from a predominantly downwelling to a predominantly upwelling regime that occurs in March-April in the west Iberian margin (Huthnance et al. 2002).

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Chapter 5

Use and validation of an ICPBM to describe dispersal and supply of invertebrate larvae

Domingues CP, Nolasco R, Dubert J, Queiroga H (in preparation). Use and validation of an Individual Based Coupled Physical Biological Model to describe dispersal and supply of invertebrate larvae

5.1 Abstract

Description of the spatial and temporal patterns of marine larval dispersal and supply requires the development of novel tools capable of encompassing the inherent variability of the mechanisms involved. In this study we use and validate an individual-based coupled physical biological model to describe observed time series of the crab *Carcinus maenas* larval supply to the west coast of the Iberian Peninsula, collected with daily frequency during the reproductive season of two years. The time series of observed supply show the influence of tide- and wind-forced circulations despite of large unexplained variability and the occurrence of idiosyncratic supply events. Our model is a nested offline version of ROMS forced by observed winds and river input and by a general circulation model that provides heat exchanges with the atmosphere. To this oceanographic model an individual-based model simulating periodic hatching, environmentally-controlled growth and mortality, as well as vertical migration behaviour, is coupled. Our model is able to describe large supply events associated with strong SW winds, with time lags of -5 to +6 d (best fit: lag = -2 d, r = 0.79). Interpretation of the time course of the events shows that during these periods there is a general convergence to the coast and a strong northward flow. The model also predicts that recruiting larvae are retained in the inner shelf, independently of supply event, and that larvae transported to the outer shelf and beyond do not recruit. The model appears to provide a realistic estimate of the observed spatial and temporal scales of the dispersal, and may be used to understand population connectivity in coastal invertebrate species with a bi-phasic life cycle.

5.2 Introduction

According to highly cited estimates (Thorson 1950) most extant marine animals posses a planktotrophic larva in their life cycles. Because larvae are usually small and have poor swimming capacities, they are subjected to high mortality rates (Morgan 1995) and are potentially transported by marine currents over large distances (Scheltema 1971). These characteristics of marine larvae have major consequences for the dynamics of marine populations since i) variation in mortality rates affects the number of larvae supplied to local populations and subsequent recruitment, and ii) many marine populations are made of spatially separated local populations connected by larval dispersal. These problems have been identified since the beginning of the last century (Hjort 1914) but have been

difficult to address due to the practical problems posed by the small size of the larvae, which makes them difficult to tag or otherwise follow individually (Thorrold et al. 2002). Therefore, information on dispersal ranges and pathways (Grantham et al. 2003) is lacking for virtually every species or, at best, is based on circumstantial evidence.

Genetic (Palumbi 2003, Hedgecock et al. 2007) and chemical fingerprinting (Becker et al. 2007, Carson et al. 2008) methods provide direct or indirect measures of larval dispersal distance and population connectivity. However, both methods are rarely unequivocal giving the impracticality of sampling over multiple temporal and spatial scales and to the uncertainties associated with assignment methods. One alternative is to study the ecology of early life history stages using numerical modelling methods (Gallego et al. 2007, Miller 2007). Over the last years, the use of oceanographic numerical models coupled with individual based models (individual-based coupled physical biological model - ICPBMs) have been the focus of several researches to study the dispersal of invertebrate and fish larvae or eggs in the ocean (see reviews of Levin 2006 and Miller 2007 and references herein). ICPBMs provide a simulation environment that integrates abiotic variability of the marine environment with biological processes at multiple temporal and spatial scales, and are therefore a powerful tool to understand dispersal of larvae and connectivity of populations, especially if used in conjunction with other tools able to provide independent estimates of these processes (Levin 2006, Selkoe et al. 2008).

Besides good descriptions of advective and turbulent processes pertaining to spatially-explicit circulation models, knowledge of a hierarchy of biological factors is necessary in order to parameterize unresolved biological processes (Dippner 2006) such as behaviour, growth, and mortality, all of which influence dispersal of larvae. Many of these biological factors are precisely those for which observations in the marine environment are difficult to make, and therefore ICPBMs need careful calibration and validation, including comparison of model predictions with measurements made with independent methods (Hannah 2007). Therefore, a precise assessment of whether model predictions approximate well enough the real data is essential before their use for practical applications, as identified in a recent assessment of future directions in modelling physical–biological interactions (Hannah & Peters 2006, Hannah 2007). Previous exercises to validate larval dispersal models were based on qualitative comparisons of predicted larval spatial distributions against *in situ* observations (e.g. Ellien et al. 2004, Paris & Cowen 2004, Peliz et al. 2007b, Erftemeijer et al. 2009). However, if a dispersal

model to assess connectivity is correct, then it should be able to predict also temporal changes of supply of competent larvae at specific locations. This would be a much more strict validation of the model because instantaneous predictions of larval concentration average out high frequency variation during the advection history, whereas accumulation of small advection and diffusion errors at each time step along the time series accumulates at the end of the larval life. Quantitative validation of biological models is generally relatively undeveloped (Arhonditsis & Brett 2004) and as far as we know, validation of ICPBMs of larval dispersal against time series of observed supply has never been attempted. This study aims to fill this gap by quantitatively validating an ICPBM of larval dispersal of the shore crab *Carcinus maenas* in western Iberia against observed time series of supply at the Ria de Aveiro estuary.

Results obtained from a study that investigated megalopae supply levels of the shore crab to the Ria de Aveiro, northwest Portugal, in five different years during the species larval season (Domingues et al. submitted) revealed that supply is an episodic and variable phenomenon. Larval supply levels depend on the past advective history based on larval source, hatching periodicity, forcing history and behaviour. The advective component is essentially determined by the ambient currents and diel vertical migration, which can be very important in strongly vertically sheared flows, as in cross-shore upwelling/downwelling circulation in stratified shelves (Marta-Almeida et al. 2006). Domingues et al. (submitted) showed that while some megalopal supply events could be predicted by tide- and wind- forcing, unclear and unexplained patterns were also observed within and among years. Concerning population genetic structure, Domingues et al. (2010) showed that *Carcinus maenas* populations along the western Iberian coast are genetically homogeneous, over two years, possibly as result of high dispersal potential, although slight genetic differences between northern and southern Portuguese locations were reported before (Pascoal et al. 2009). Moreover, shore crab populations appear to be a large “panmictic” unit, from Cadiz Bay, South Spain to Wales, UK. This could imply that populations along a 3000 km range freely exchange individuals mediated by larval transport. However, given the species larval development time and the alternation of wind-driven coastal currents, that seems unlikely to occur, at least frequently. For these reasons, we need to model *C. maenas* larval dispersal and supply in an attempt to find explanations or directions to better understand the patterns previously reported and, ultimately, marine population connectivity. ICPBMs can also be very important for the monitoring of *Carcinus* spread in recently invaded locations. This is exemplified in the

work of Banas et al. (2009) that studied shore crab larval retention in Willapa Bay, Washington, USA, and in the work of See & Feist (2010) which modelled larval development and transport of *C. maenas* along the USA west coast.

The aim of this study was to use and validate an ICPBM for the western Iberia to predict *Carcinus maenas* larval dispersal and supply to the Ria de Aveiro. *C. maenas* has a keystone role and forms large populations in coastal ecosystems worldwide, and significant information on life cycle, behaviour, ecology and genetics has been accumulating (e.g. Queiroga et al. 1994, Queiroga et al. 1997, Yamada 2001, Queiroga et al. 2006, Darling et al. 2008, Domingues et al. 2010). *C. maenas* in southern Europe lives mostly in estuarine systems. The larval phase comprises four planktotrophic zoeae and one megalopa (Rice & Ingle 1975) that develop in the water column from four to six weeks, depending on water temperature (Dawirs 1985, Mohamedeen & Hartnoll 1989, Nagaraj 1993). First zoeae hatch inside estuaries and are quickly exported to the ocean, and the megalopa is the stage that reinvades estuaries. The ocean simulations were performed using the Regional Ocean Modelling System (ROMS) and a Lagrangian particle tracking submodel that includes advection and diffusion, as well as diel vertical migrations and temperature-dependent growth to simulate larval emission and dispersal. The approach used in this study was to compare predictions of the model with empirical quantitative measurements of megalopal supply obtained during the larval season of two years, at the Ria de Aveiro. A successful validation of the ICPBM for the Iberian Peninsula allow the test of complex hypotheses about dispersal ranges and demographic, as well as genetic connectivity for the selected biological models, and would also demonstrate the ability of ICPBMs in general to analyse these processes.

5.3 Methods

5.3.1 The oceanographic model

The simulations were conducted using a 3-D (60 vertical layers) free-surface terrain-following primitive equation hydrostatic model configurable for fully realistic regional applications. The code is based on the Regional Ocean Modelling System (ROMS, Shchepetkin & McWilliams 2003, 2005) with embedded nesting capabilities through Adaptive Grid Refinement In Fortran (AGRIF, Penven et al. 2006). ROMS-AGRIF enables the use of online and offline nesting thus permitting the regional applications to be build based on basin and local-scale configurations, and to cover a wide range of time and

spatial scales. Examples of ROMS-AGRIF application to the western Iberia are reported in Peliz et al. (2007a) and in Oliveira et al. (2009). The present configuration represents an improvement and extension of the configuration used by Peliz et al. (2007a) and Oliveira et al. (2009) to the Atlantic margin of the Iberian Peninsula. A summarized description is given here and for full details about the model configuration the reader is referred to those papers.

For a realistic simulation of the western Iberian margin it is necessary to include local aspects like the Gibraltar Strait exchange (Mediterranean inflow/outflow) and the wind-driven dynamics. The remote circulation influencing the western limit of the study region associated with the Azores Current (AC) should also be accounted for. To resolve the large and small scales, two grids were used (Figure 1): a first domain grid (FD), with a resolution of 1/10 º (*ca.* 10 Km), from 32 ºW to 0.5 ºW and 30 ºN to 48 ºN. This FD was used to provide initial and boundary conditions, through offline nesting, to the large domain (LD), which has a resolution of 1/27 º (i.e. a mean resolution of 3.5 km) and includes the western Iberian margin, from the Gulf of Cadiz (34.5 ºN) to the Bay of Biscay 45.5 ºN, and from 12.5 ºW to the Strait of Gibraltar at 5.5 ºW. The LD covers an area of 1200 x 600 km and constitutes the target domain used for the dispersal simulations.

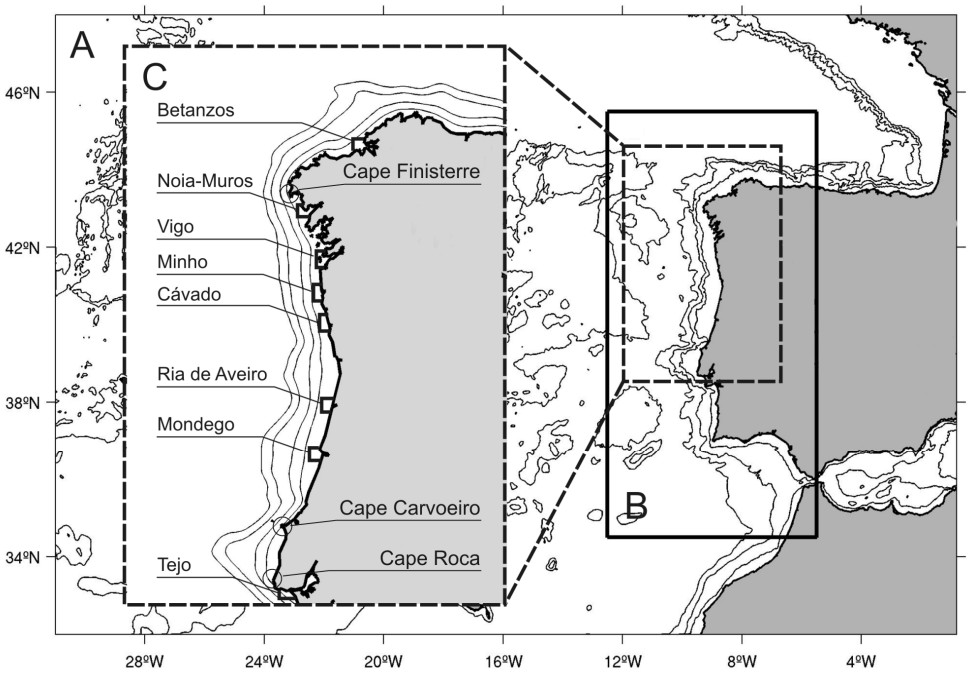

Figure 1 Map of the study zone and model domains. (A) first domain (FD), (B) large domain (LD), and (C) location of estuaries used in the model showing the shelf area adjacent to each estuary where larvae were emitted and recruited. Inset (C) also shows details of the 50, 100, 250 and 500 m isobaths of the smoothed bathymetry used in the model.

The FD was first initialized from rest using monthly temperature and salinity climatologies from Levitus & Boyer (1994) and Levitus et al. (1994) at the boundaries, and was forced using monthly surface fluxes from Comprehensive Ocean-Atmosphere Data Set (COADS; da Silva et al. 1994). Monthly geostrophic and Ekman velocities were applied along the open lateral boundaries. The methodology used is similar to that used in climatological studies of the coastal transition zone of the California current system (Marchesiello et al. 2001). This FD configuration reached equilibrium solutions after four years. At this stage, the Mediterranean Water (MW) was represented using a nudging term. After that period, and once the ocean reaches equilibrium, realistic forcing at the surface (instead of a climatological one) was used. The forcing consisted in the NCEP2 air-sea fluxes (www.ncep.noaa.gov) and QuikScat reanalyzed satellite winds from CERSAT (cersat.ifremer.fr) for the period 2001 to 2009, with a spatial resolution of 0.25 º. The outputs of the FD were used to initialize and provide boundary conditions to the target domain, LD, through offline nesting. Forcing of the LD was the same as of the FD, ensuring consistency of forcing for both domains and avoiding problems at the boundaries. For the target domain, LD, the Mediterranean Undercurrent (MU) was imposed by a boundary inflow/outflow condition at the Strait of Gibraltar (see Peliz et al. 2007a for details), and hence the Mediterranean outflow along the western margin of Iberian Peninsula is introduced. The inflow of freshwater to the ocean originated from the main rivers of the region was included in the form of real river outflow (provided by INAG, Water Institute of Portugal), when available. When there were no registers of river outflow during a period of time a climatological value for seasonal river outflow was imposed. The outputs of the model, consisting in temperature, salinity, and three-dimensional velocity field, were stored every four hours in order to be used for the Lagrangian module described below.

5.3.2 The Lagrangian offline Individual Based Model

An Individual Based Model (IBM) was coupled to the ROMS-AGRIF to simulate larval hatching, growth, mortality and behavior of *Carcinus maenas*. Coupling of the IBM and ROMS-AGRIF was made using a code developed by Xavier Capet (http://www.atmos.ucla.edu/~capet/Myresearch/my_research_floats.html), and successfully tested in Carr et al. (2008). This drifter-tracking code simulate larvae trajectories from stored ROMS-AGRIF velocity and hydrological fields, using a high order predictor corrector scheme to integrate the motion equation $dX/dt = U_{roms}(X,t)$, with X being the position vector (x,y,z), and U_{roms} being the modeled 3D velocity vector over time, given an

initial condition $X(t_0) = X_0$. We first tested, on a subset of the simulations, that this offline procedure yields qualitative and quantitative similar results to the online procedure, in which case the larvae were advected with the time step of the model (300s). This is so because no high frequency variability (like tides) is present in the modeled velocity fields. Additionally to the advection generated by the model velocities, and similarly to Peliz et al. (2007b), the particle movements included random velocities in the vertical direction, which were used to parameterize unresolved turbulent processes. A diel vertical migration (DVM) scheme inferred from Queiroga (1996) and dos Santos et al. (2008) was also explicitly introduced, in order to simulate the vertical movements of larvae within the water column depending of the hour of the day. The DVM scheme consisted of forcing the larvae to drift at a deep level between 06h and 20h, and at the surface between 22h and 04h, every day, while during the remaining periods (04h to 06h and 22h to 24h) the larvae migrated vertically from surface to the deep level and from this deep level to the surface. The deep level is the bottom layer of the model, if the local depth is shallower than 60 m, or 60 m if the local depth is deeper than this.

Larval development time (LDT), as well as mortality rate caused by non-optimum conditions of temperature and salinity, were modeled pooling information from laboratory experiments made by Nagaraj (1993), Dawirs (1985) and Mohamedeen & Hartnoll (1989) who reared larvae without simulated substrate. The proportional effects of temperature on LDT, and of temperature and salinity on mortality, based on the time a larva was exposed to a specific value of temperature in the case of LDT, or to a specific combination of temperature and salinity in the case of mortality, were estimated by linearly interpolating between the laboratory data for each larval stage. Age and the probability of death were assessed at each time step of the model (300 s). Larvae were killed randomly based on the proportional death rate during the previous time interval. If a larva survived non-optimal combinations of temperature and salinity they would grow from age 0 at hatching to 4 at the molt to megalopa; megalopae lived and remained competent until age 5 and then died. No other temporally or spatially distributed source of mortality (e.g. predation) was used because of lack of information. Results of the different model experiments indicated that mortality did reduce the number of larvae supplied to the Ria de Aveiro, but that i) temporal patterns of supply and ii) the estuaries that supplied larvae to the Ria did not change relative to the experiments without mortality. Therefore, we are only reporting the outputs without mortality.

A set of 900 virtual larvae, distributed over four days (225 larvae d^{-1}), was introduced in the surface layer in the neighborhood of each of eight estuarine systems (Betanzos, Muros-Noia, Vigo, Minho, Cávado, Aveiro, Mondego and Tejo, Figure 1), every fortnight during nocturnal ebb tides, from February to July of each year. This time schedule and vertical position were used in order to simulate periodic hatching and export of first stage larvae from estuaries during the larval season (Queiroga et al. 1994, 1997). The position, (latitude, longitude, depth), temperature, salinity, age and death probability of a total number of 86400 larvae per year (900 larvae $estuary^{-1}$ x 12 release periods x 8 estuaries) were stored in a single file with a time step of two hours. For simplicity we only used estuaries separated by at least 40 km between Betanzos and Tejo because, according to initial trial simulations larvae hatched from estuaries beyond those two would not recruit to the Ria de Aveiro. Tides were not used in the model. This option was taken for two reasons. The first is that behavior of crab larvae relative to tidal variables has only been detected very close or within estuaries and bays (Queiroga & Blanton 2005), and the resolution of the LD is not enough to model estuarine inlets and currents. The second is that previous studies have shown that the DVM detected in *Carcinus maenas* larvae (dos Santos et al. 2008) on a background of tidal currents does not affect the net horizontal advection of larvae over the west Iberian shelf (Marta-Almeida & Dubert 2006). Because estuarine inlets were not conveniently represented by the model we released and recruited larvae in an area of the shelf adjacent to each estuary (approximate 12 x 12 km), which roughly corresponds to the area of the influence of the estuarine plume, and defined supply to the Ria the Aveiro as the number of particles that crossed that respective area (Figure 1C).

5.3.3 Time series of supply

Time series of supply of *Carcinus maenas* megalopae were obtained in the Ria de Aveiro, a bar-built estuary located on the northwest coast of Portugal (40º 37'17''N, 8º 44'56''W; Figure 2). Circulation in the Ria de Aveiro is dominated by tides, which are semidiurnal with an average range of 2.1 m. Field sampling methods and original data are described in Queiroga et al. (2006) and Domingues et al. (submitted). Briefly, collections of larvae were made with two passive plankton nets (surface and bottom) that were deployed daily in the Canal de Mira facing the inlet and left fishing for 24 h. A trapping device inside each net prevented the loss of material during ebb tide. The sampling periods covered much of the species' larval season of five different years: April-July 2002, March-June 2006 and 2007 and February-July of 2008 and 2009. The data reported in the present paper are

daily averages of the number of megalopae collected by the two nets. The supply time series (Figure 2) showed periods of several weeks with zero or very low supply punctuated with supply events driven by wind events, as well as several instances when supply events occurred with semilunar periodicity and were associated with spring tides (Domingues et al., submitted). Because our numerical model does not simulate tides, it cannot describe the semilunar components of supply. For this reason we chose as the validation series those obtained in 2006 and 2007, which displayed very large supply events at the end of March and end of June of 2006 and 2007, respectively, coincident with extended periods (10 to 15 d) of predominantly southerly winds.

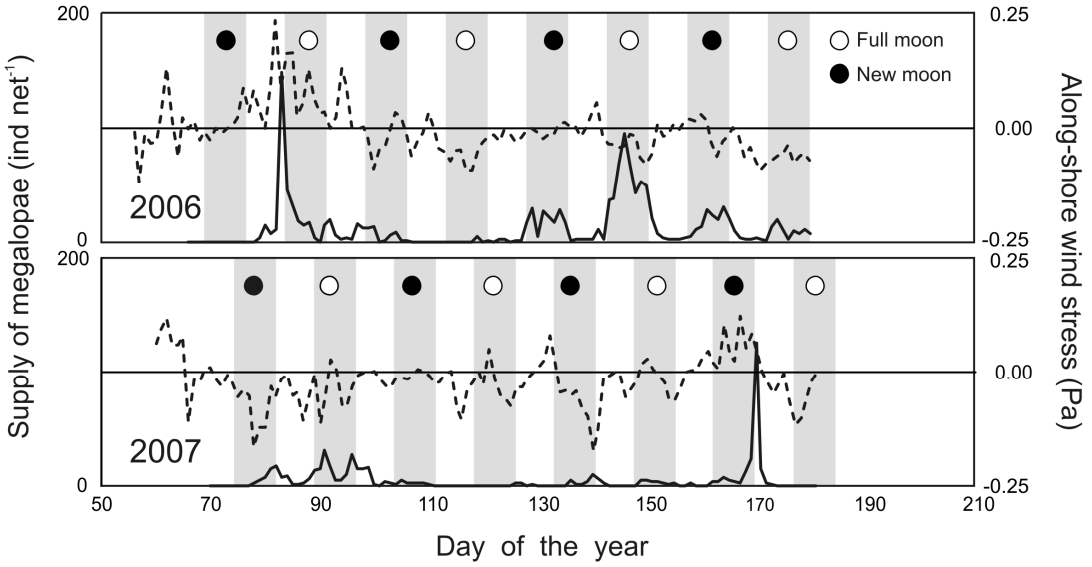

Figure 2 Daily numbers of observed megalopal supply (continuous line), along-shore wind stress (broken line) and spring tides (grey bars) in the Ria de Aveiro for 2006 and 2007.

3.4 Model experiments

In order to validate the model and to test its response to different growth rate and behavioural scenarios we performed several model experiments using the IBM model described above as our Base model. In the Base experiment we did not recruit the megalopae to the estuaries, but kept them in the model and recorded them every time they crossed the area adjacent to the Ria de Aveiro (Figure 1). Under this option we were interested in the potential trajectories of the larvae in the shelf, without imposing any behavioural constraint regarding proximity of the estuaries. Inspection of animations produced from the model (see below) indicated that the larvae that recruited to the Ria de Aveiro and other estuaries never left the inner shelf and that their trajectories were mostly parallel to the shore, increasing the probability of recruitment of competent megalopae to

estauries. This led us to the Invasion experiment, where the megalopae were considered to recruit to each estuary once they crossed the respective adjacent shelf area (i. e. the megalopae entered the estuaries and were not counted afterwards). Because the two large supply events recorded in 2006 and 2007 clearly stood out from the remaining and did not recur periodically, they also allowed us to inspect the sensitivity of the model to differences in growth rate. To this end we slowed the growth rate by 1 d per larval stage and repeated the Base and Invasion experiments with this new growth rate. In order to inspect the influence of DVM on dispersal trajectories and supply to the Ria de Aveiro, we ran the Base model with the slow growth rate without DVM. In this configuration the larvae were released as before but were treated as completely passive particles subjected to the 3-D velocities of the model. We henceforth refer to these experiments as the Base/Fast growth, Base/Slow growth, Invasion/Fast growth and Invasion/Slow growth experiments when we used larvae with DVM, and Passive/Base/Slow growth experiments when larvae did not have DVM. These experiments were repeated separately for 2006 and 2007.

5.3.5 Statistical analysis

The adjustment of the predicted time series relative to the observations was assessed using cross-correlation analysis (Chatfield 1996), separately for each year, by lagging the observed relative to the predicted series. This procedure produces negative lags when predicted maxima precede observations, and positive lags when observed maxima precede predictions. In order to reduce noise and remove strong autocorrelation at 1 d lag in both predictions and observations the series were smoothed, with a moving average of period 5 d, and differenced at a lag of -1 d.

5.4 Results

Figure 2 shows observed supply of *Carcinus maenas* megalopae to the Ria de Aveiro in 2006 and 2007. Inspection of the figure indicates that supply was affected by tidal range, especially in 2006 when 4 supply events recurred with fortnight periodicity, during spring tides in spring and summer, and by along-shore winds, with the highest supply events of both years following several days of strong southerly winds (see Domingues et al. submitted).

As expected from the fact that the model does not simulate tides or tidal behaviour, the time series of predicted supply do not reproduce the semilunar component present in the

observations (Figures 3 and 4). However, the model does predict increased supply within ± 10 d of the high supply peaks associated with southerly winds, in March 2006 and June 2007. The Base experiments show wide supply peaks (Figures 3 and 4, A and B), caused by successive passages made by individual larvae in the shelf area adjacent to the Ria, which contrast both with the Invasion experiments (Figures 3 and 4, C and D) and with the narrow wind-driven peaks in the observations. The Slow growth experiments resulted in delayed predicted supply peaks.

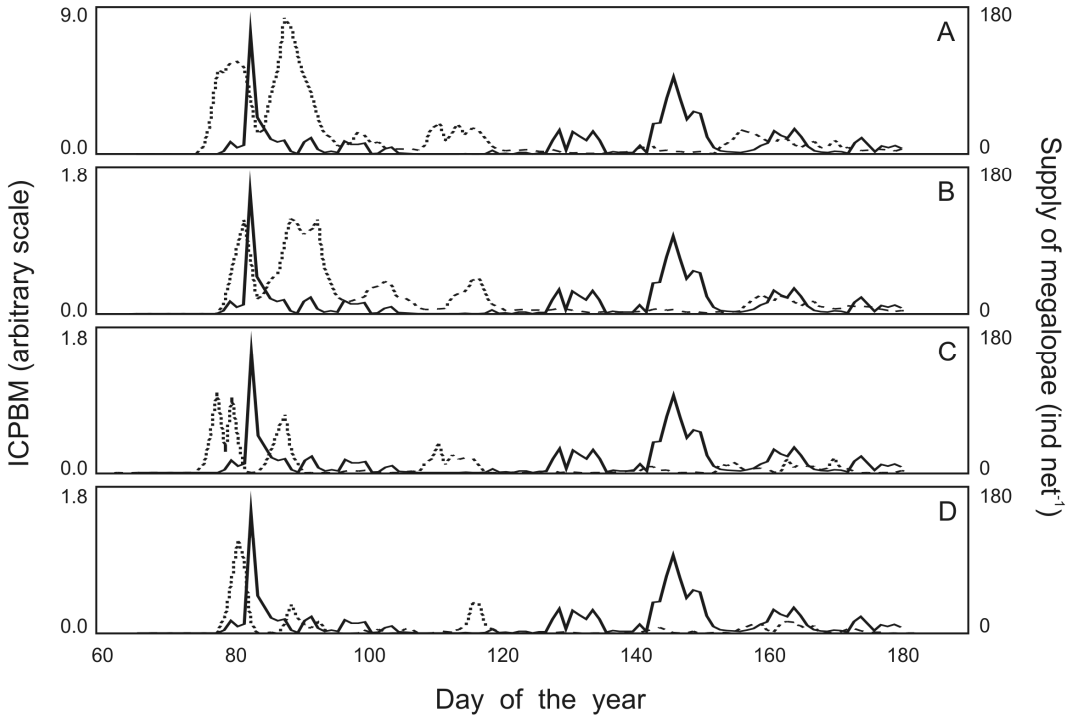

Figure 3 Observed megalopal supply (continuous line) and ICPBM predictions (broken line) for the different model experiments in 2006. (A) Base/Fast growth, (B) Base/Slow growth, (C) Invasion/Fast growth, and (D) Invasion/Slow growth.

Cross-correlation analyses (Table 1, Figures 5 and 6) indicate maximum significant correlations at time lags from -5 to +6 d. In 2006, a slower growth rate resulted in a smaller time lag between observations and predictions while in 2007 the opposite ocurred.

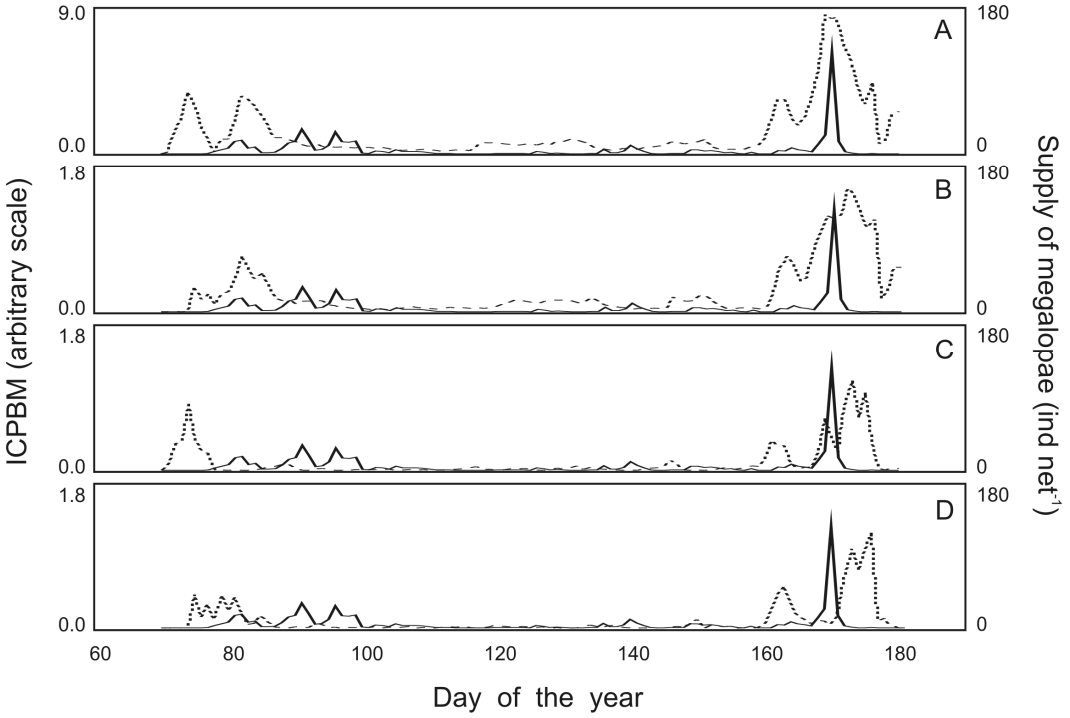

Figure 4 Observed megalopal supply (continuous line) and ICPBM predictions (broken line) for the different model experiments in 2007. (A) Base/Fast growth, (B) Base/Slow growth, (C) Invasion/Fast growth, and (D) Invasion/Slow growth.

Differences in growth rate increased (and decreased) the fit in 2 out of 4 cases, and differences in recruitment behaviour had a similar effect. The best fit, both in terms of time lag and correlation, was obtained in the Invasion/Slow growth experiment of 2006. In most experiments the cross-correlograms indicate significant correlations at positive and negative lags. This was caused by pairs of peaks in the predicted series surrounding the observed peak (e.g. compare Figures 3A and 4D with Figures 5A and 6D). In all experiments, most of the correlation was driven by the days of maximum supply.

Table 1 Time lags (d) and cross-correlations (*r* in parentheses) between observations and predicted time series, for each model experiment and year. Reported values refer to maximum significant ($P < 0.05$) cross-correlations in each case.

Growth rate	Base		Invasion	
	2006	2007	2006	2007
Fast growth	+6 (0.36)	+2 (0.57)	-5 (0.47)	+3 (0.55)
Slow growth	-1 (0.29)	+3 (0.62)	-2 (0.79)	+5 (0.51)

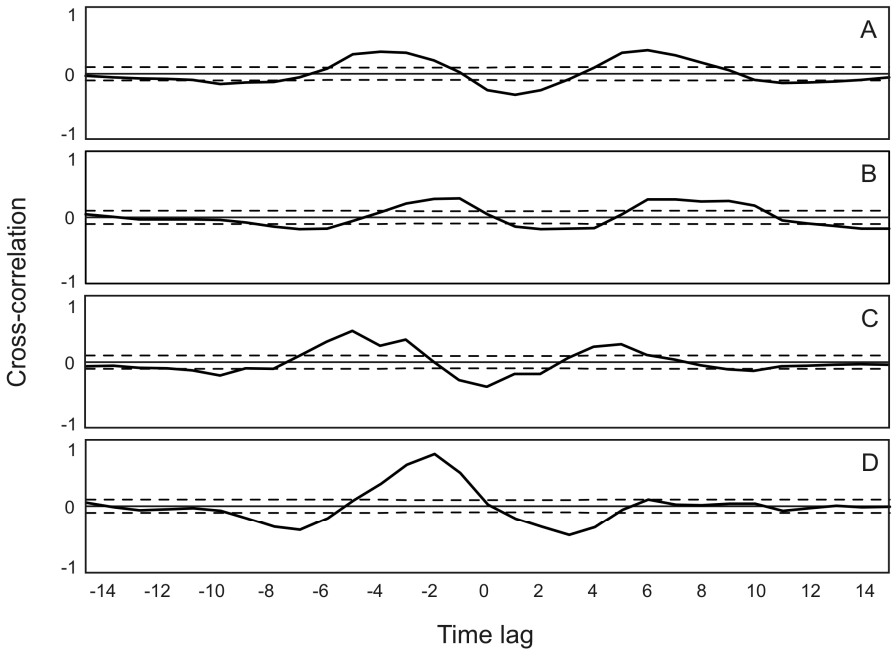

Figure 5 Cross-correlations between observed megalopal supply and ICPBM predictions for the different model experiments in 2006. (A) Base/Fast growth, (B) Base/Slow growth, (C) Invasion/Fast growth, and (D) Invasion/Slow growth. Continuous line: cross-correlograms, broken line: approximate 95% confidence intervals.

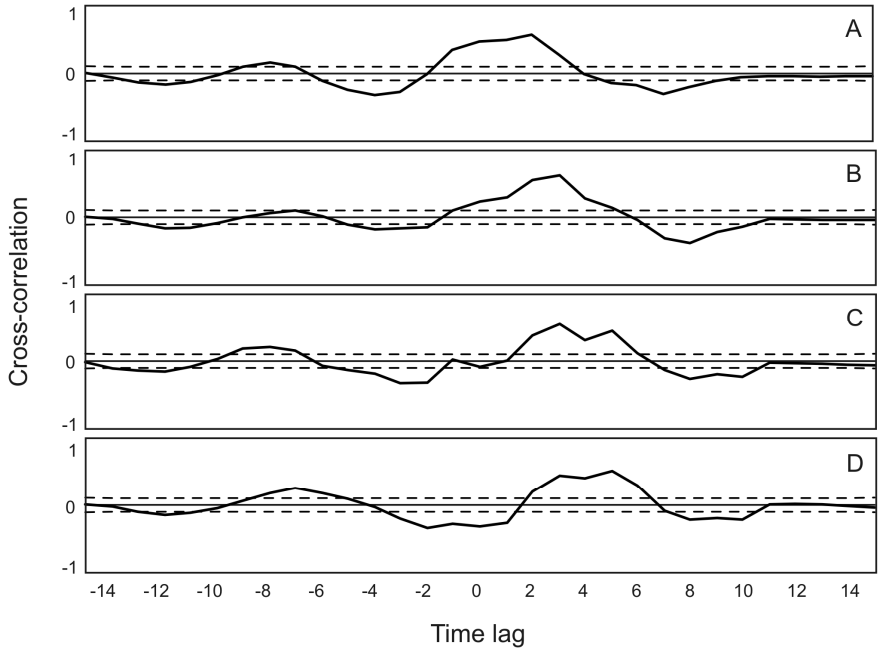

Figure 6 Cross-correlations between observed megalopal supply and ICPBM predictions for the different model experiments in 2007. (A) Base/Fast growth, (B) Base/Slow growth, (C) Invasion/Fast growth, and (D) Invasion/Slow growth. Continuous line: cross-correlograms, broken line: approximate 95% confidence intervals.

In all experiments there was a clear difference in the pathways of the larvae that recruited to the Ria de Aveiro (or to the other estuaries; not shown) relative to the larvae that never recruited. While the larvae that recruited were very seldom advected beyond middle shelf (100 m isobath) and had trajectories clearly dominated by an along-shore component, larvae that never recruited to estuarine habitats were consistently advected beyond middle shelf. Examples of trajectory densities (vertically integrated number of times a larva entered each cell of the model) can be found in Figures 7 and 8, for the Base/Slow growth model experiments of both years.

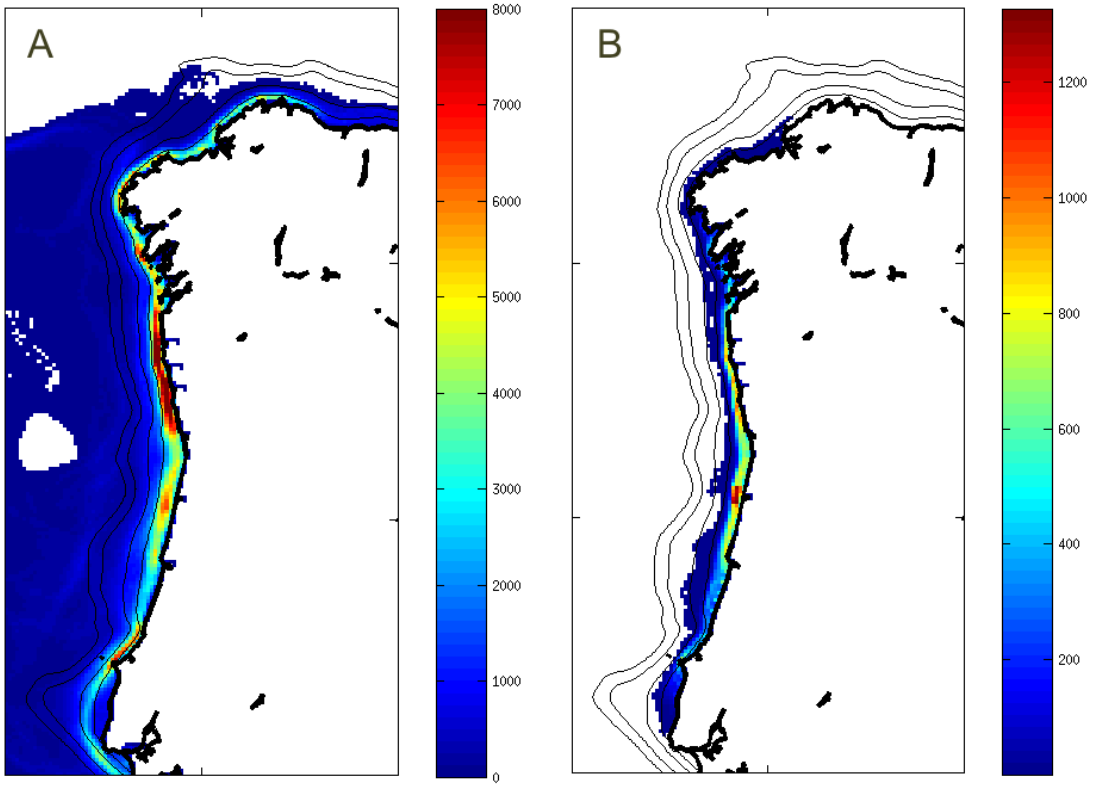

Figure 7 Maps of larval trajectory densities for the Base/Slow growth model experiment in 2006. (A) trajectories of the larvae that never recruited to the Ria de Aveiro, and (B) trajectories of the larvae that recruited to the Ria de Aveiro. Colour scale represents the vertically integrated number of times a larva entered each cell of the model.

Removal of DVM behaviour in the Passive/Base/Slow growth experiments produced a temporal pattern of supply that also reproduced the wind-driven maximum supply events at time lags identical to the remaining experiments. However, the level of larval wastage from the shelf was much larger than with DVM, with a decrease of one order of magnitude in supply (not shown) and only a few tens of particles successfully recruiting to the Ria. Moreover, the average daily offshore distances of the larvae within a section delimited by

the 39.5 and 41.0° N was much greater in simulations without DVM (Figure 9). The average distance ranged from 100 to 200 km without DVM, but from 10 to 80 km with vertically migrating larvae.

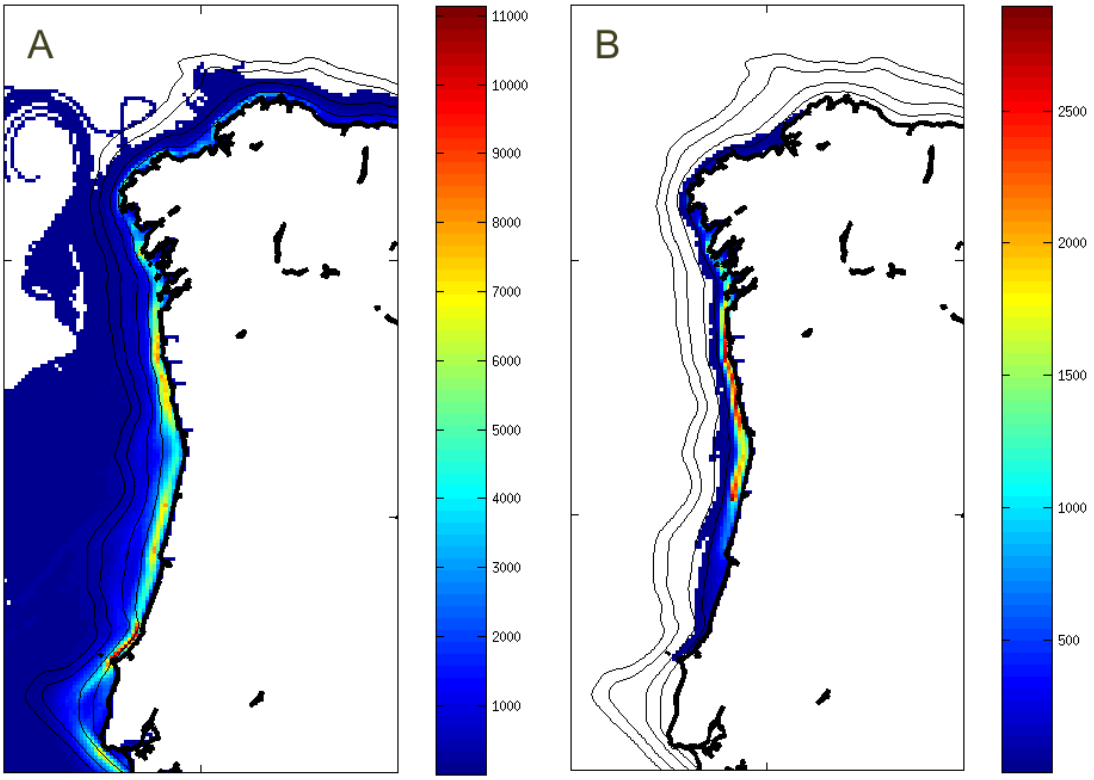

Figure 8 Maps of larval trajectory densities for the Base/Slow growth model experiment in 2007. (A) trajectories of the larvae that never recruited to the Ria de Aveiro, and (B) trajectories of the larvae that recruited to the Ria de Aveiro. Colour scale represents the vertically integrated number of times a larva entered each cell of the model.

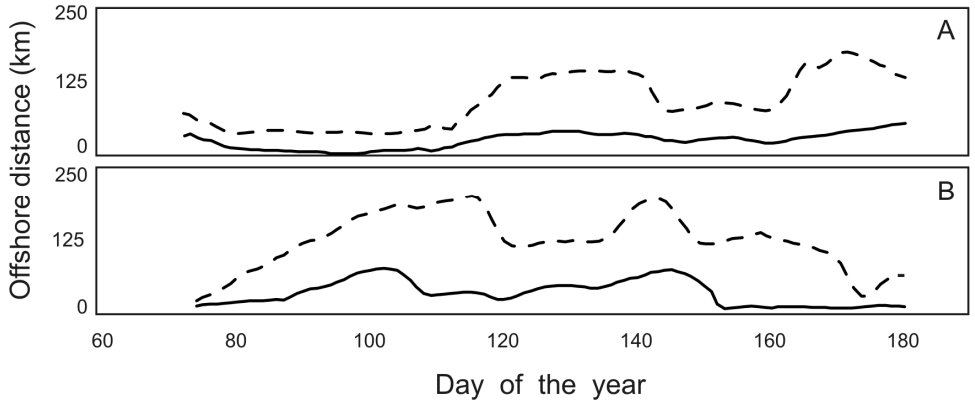

Figure 9 Daily larval offshore distances for the Base/Slow growth (continuous line) and Passive/Base/Slow growth (broken line) experiments of both years (A) 2006 and (B) 2007.

Examples of the time course of the advection processes and supply can be seen in the animations produced with daily distributions of larvae predict by the model in the Base/Slow growth and Passive/Base/Slow growth experiments of both years (Additional files 1-4).

Table 2 shows the average daily contribution of each estuary to the Ria de Aveiro (arbitrary scale), as well as the average distance travelled by the recruiting larvae predicted by the Base model with fast and slow growth rates, during the period of the observations. We used the Base model because, since the larvae were not constrained to enter the estuaries, this experiment would produce the largest estimates of larval dispersal. Distance was calculated as the sum of the products of the contributions by each estuary by distance to the Ria de Aveiro, divided by the sum of the contributions. The data indicate that larvae supplied to the Ria de Aveiro come from a mixture of estuaries and that the largest contributors are located to the north of the Ria: Vigo and Noia in 2006 and Minho in 2007 were larger contributors to the Ria than the Ria itself. The average estimated distances that larvae coming from north and south estuaries were about 70 and 160 km, respectively.

Table 2 Average daily contribution of each estuary to the Ria de Aveiro (arbitrary scale) and average distance (km) travelled by the recruiting larvae as predicted by the Base model with fast and slow growth rates. All represents distances including all estuaries and South and North represent distances including only south or north estuaries, respectively. In parenthesis the estuary distances to the Ria de Aveiro is given, with negative values for the estuaries to the south and positive values for the estuaries to the north.

Growth rate	Source Estuary								Distance		
	TEJ (-245)	MON (-57)	AVR (0)	CAV (96)	MIN (137)	VIG (174)	NOI (232)	BET (377)	All	South	North
2006 Base/Fast	0.0007	0.0444	0.2273	0.1447	0.1702	0.2457	0.2284	0.0000	123	-60	169
2006 Base/Slow	0.0073	0.0388	0.1227	0.1012	0.1652	0.2554	0.2104	0.0000	135	-87	172
Average	0.0040	0.0416	0.1750	0.1230	0.1677	0.2505	0.2194	0.0000	129	-73	170
2007 Base/Fast	0.0133	0.1856	0.2119	0.2076	0.2629	0.2448	0.2450	0.0000	103	-70	162
2007 Base/Slow	0.0138	0.1942	0.2550	0.2308	0.2971	0.1545	0.0800	0.0000	77	-70	142
Average	0.1350	0.1899	0.2335	0.2192	0.2800	0.1996	0.1625	0.0000	90	-70	152

5.5 Discussion

For each of the two years analysed in the present study our results show a broad agreement between observations of daily supply of *Carcinus maenas* larvae to the Ria de Aveiro and simulations produced by a numerical model coupling oceanographic circulation with information on larval biology. In particular, the model predicted larval events driven by strong southwesterly winds with an error of ± 6 days, independently of growth rate and recruitment behaviour. During the recruitment events the model indicated a strong northward flow and convergence to the coast consistent with downwelling theory and observations made on the Portuguese coast (Wooster et al. 1976, Jorge da Silva 1992)

The role of DVM behaviour in larval dispersal patterns was investigated by contrasting larval dispersal pathways from simulations with and without DVM. The results showed that most of the passive larvae were advected offshore and those that recruited were sometimes advected to the shelf break and returned during downwelling periods. The proportion of recruits also decreased about 10 times when compared with larvae migrating vertically. Successfully recruiting vertically migrating larvae, on the other hand, never left the inner shelf. Field observations indicate that *Carcinus maenas* larvae display strong vertical migration behaviour, being closer to the surface during the night (dos Santos et al. 2008) and a previous modelling attempt indicated that, for a vertically migrating larvae, upwelling could be beneficial for retention given the time that larvae would spend in the compensating bottom undercurrent (Marta-Almeida et al. 2006). The coastal distribution and abundance of *C. maenas* larval stages were studied along the northwest Portuguese coast by Queiroga (1996) and dos Santos et al. (2008), who sampled along the water column from the coast to offshore distances of approximately 170 and and 70 km, respectively. Both studies showed that *C. maenas* larval stages were confined to the inner and middle shelf stations, with higher concentrations found at less than 20 km offshore. Larval dispersal patterns in simulations ran without DVM strongly depart from the observed field larval distributions described above since larvae in the model were advected to average distances as far as 200 km off the coast. Dispersal patterns obtained with DVM highlight the importance of the inner-shelf environment for larval development, and are consistent with recent findings from other upwelling systems indicating that larval development of many coastal invertebrates takes place in the nearshore strip (Morgan et al. 2009).

The prediction that most of the non-periodic changes in larval supply results from along-shore advection is a major suggestion of the model. Along-shore circulation has been associated with changes in supply and recruitment of coastal invertebrates by interacting with coastal topography and redistributing the larvae regionally. This was demonstrated with crab and sea urchin larvae in the upwelling system of northern California, where release of larvae trapped in the lee of Point Reyes and in the Gulf of the Farallones following relaxation of upwelling winds resulted in transport of larvae and increased settlement to northward locations (Wing et al. 1995). The mechanism operating on the northwest coast of Iberia seems to be different. In the stretch of coast where larvae recruiting to the Ria de Aveiro develop, which extends from the Estremadura promontory to Cape Finisterre, there are no major capes nor gulfs that could act as a larval trap. Instead, it is the alternating along-shore circulation that advects the larvae up and down a mostly rectilinear coast and delivers competent larvae to places depending on advection history during the whole development period. This pattern of advection and the inner-shelf retention of a significant faction of the larval pool also suggests a conceptually new mechanism for estuarine reinvasion of crab megalopae. Instead of being dependent on mechanisms for cross-shore advection, competent megalopae have an increased probability of being transported across the estuarine plumes during along-shelf dispersal. Crab megalopae have been shown to react to estuarine water by arresting swimming and dropping to the bottom with high light levels (Forward & Rittschof 1994). This reaction results from the inhibitory effect of chemicals present in estuarine water resulting from the decomposition of organic matter. The successive passage of megalopae close to estuarine inlets and the effect of chemical cues could result in the accumulation of competent larvae in the bottom close to estuaries and provide a trapping mechanism that would enhance supply into estuaries during night flood tides (De Vries et al. 1994, Tankersley et al. 1995, Welch & Forward 2001). This type of mechanism could also operate for coastal species from other habitats because of the generality of chemically-induced behaviour during settlement of invertebrate and fish larvae (e.g. Pawlik 1992, Davis & Moreno 1995, Kopin et al. 2001).

The model was sensitive to changes in growth rate equivalent to an increase of 5 d in larval development time, which took four to six weeks at the temperatures predicted by the model. This slow growth rate usually resulted in a decrease in the time leg between observations and predictions, suggesting that the laboratory data (Dawirs 1985, Mohamedeen & Hartnoll 1989, Nagaraj 1993) underestimate growth rate. A better

understanding of the influence of growth rate on the fit of the model requires however an exploration of the predicted intra- and inter-annual changes in temperature and its effect on growth, which is not possible in the present study.

The initial experiments made with mortality rates caused by exposure to non-optimum conditions of temperature and salinity (Nagaraj 1993) decreased the overall levels of predicted supply to the Ria de Aveiro by about 30%. Any spatially variable factor of mortality has the potential to affect dispersal and connectivity (Cowen et al. 2000, Watson et al. 2010), especially in the presence of strong environmental gradients. However, mortality from abiotic conditions did not affect predictions of realized larval dispersal because there are no strong environmental gradients in the area to which dispersal is constrained. Predation, non-optimum abiotic conditions, starvation, disease and wastage form areas appropriate for settlement and metamorphosis are usually considered the major mortality factors affecting larvae of marine fish and invertebrates, with values that may surpass 90% (Morgan 1995). Intensive sampling of *Carcinus maenas* larvae in the Ria de Aveiro in one single year indicated that megalopae were 100 times less abundant than newly hatched first zoeae (Queiroga et al. 1994). This would imply a total mortality rate of 99%. If mortality rates derived from laboratory rearings of larvae fed *ad libitum* give realistic approximations of true mortality due to exposure to temperature and salinity conditions, then the results of the model experiments suggest that a significant part of larval mortality is due to exposure to non-abiotic conditions.

Results of the model are also consistent with previous observations on genetic structure and on the lack of sweepstake reproduction in *Carcinus maenas* from this region. Domingues et al. (2010) found that *C. maenas* forms a genetically homogeneous unit from Gulf of Cadiz, South Spain to Wales, UK over a gradient of approximately 3000 km. Since the model predicts maximum dispersal distances of 250 km, connectivity among these populations in order to homogenise allele frequencies must take several generations. The model also predicts that each larval event is a mixture of larvae from different estuaries and therefore it lends support to the observations of no genetic differences among supply events and between larvae and the adult population found in Domingues et al. (accepted).

The individual based model used in the present study does not include tidal behaviour, nor seasonal or spatial variation in larval hatching. Therefore, the model cannot reproduce semi-lunar or seasonal patterns of larval supply. This limitation of the model is likely to be

the cause of significant cross-correlations with the observations driven by only the few points when strong changes in supply levels were observed, following SW winds. Nevertheless, the model appears to provide realistic estimates of the spatial and temporal scales of the dispersal driven by along-shore winds, which are the main factor regulating variability of oceanic currents in upwelling continental shelves. In our opinion the results of the present study lend strong support to the applicability of ICPBMs to understand and describe population connectivity in coastal invertebrate species with a bi-phasic life cycle, and point possible ways to improve these models to obtain still better estimates in the future. We wish also to note that this study indicates that, provided the relevant information is available, spatial and temporal patterns of larval dispersal and supply can be predicted by mechanistic bio-physical models, challenging the paradigm that dispersal and recruitment in marine populations is essentially a stochastic phenomenon (Siegel et al. 2008).

Acknowledgements

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Legend to additional files

Additional file 1 Time evolution of larval trajectories for the complete larval series (first zoea to megalopa) for the Base/Slow growth model experiment in 2006. Left panel: trajectories of the larvae that never recruited to the Ria de Aveiro, right panel: trajectories of the larvae that recruited to the Ria de Aveiro. Arrows close to estuaries indicate periodic hatching of larvae and moving arrows represent direction and intensity of the wind. Larvae are colour-coded according to natal estuary and become grey at moult to megalopae. Red, blue and green flashing circles indicate recruitment events in the observations and the model, in the observations only, and in the model only, respectively. Recruitment

events are defined as when daily observed and predicted supply of megalopae exceeded average levels for at least 3 consecutive days.

Additional file 2 Time evolution of larval trajectories for the complete larval series (first zoea to megalopa) for the Base/Slow growth model experiment in 2007. Left panel: trajectories of the larvae that never recruited to the Ria de Aveiro, right panel: trajectories of the larvae that recruited to the Ria de Aveiro. Arrows close to estuaries indicate periodic hatching of larvae and moving arrows represent direction and intensity of the wind. Larvae are colour-coded according to natal estuary and become grey at moult to megalopae. Red, blue and green flashing circles indicate recruitment events in the observations and the model, in the observations only, and in the model only, respectively. Recruitment events are defined as when daily observed and predicted supply of megalopae exceeded average levels for at least 3 consecutive days.

Additional file 3 Time evolution of larval trajectories for the complete larval series (first zoea to megalopa) for the Passive/Base/Slow growth model experiment in 2006. Left panel: trajectories of the larvae that never recruited to the Ria de Aveiro, right panel: trajectories of the larvae that recruited to the Ria de Aveiro. Arrows close to estuaries indicate periodic hatching of larvae and moving arrows represent direction and intensity of the wind. Larvae are colour-coded according to natal estuary and become grey at moult to megalopae. Red, blue and green flashing circles indicate recruitment events in the observations and the model, in the observations only, and in the model only, respectively. Recruitment events are defined as when daily observed and predicted supply of megalopae exceeded average levels for at least 3 consecutive days.

Additional file 4 Time evolution of larval trajectories for the complete larval series (first zoea to megalopa) for the Passive/Base/Slow growth model experiment in 2007. Left panel: trajectories of the larvae that never recruited to the Ria de Aveiro, right panel: trajectories of the larvae that recruited to the Ria de Aveiro. Arrows close to estuaries indicate periodic hatching of larvae and moving arrows represent direction and intensity of the wind. Larvae are colour-coded according to natal estuary and become grey at moult to megalopae. Red, blue and green flashing circles indicate recruitment events in the observations and the model, in the observations only, and in the model only, respectively. Recruitment events are defined as when daily observed and predicted supply of megalopae exceeded average levels for at least 3 consecutive days.

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Chapter 6

Concluding remarks

6.1 Concluding remarks

The purpose of this chapter is to summarize the main findings of individual data chapters. Resolving the scope and processes involved in larval dispersal and population connectivity require combined application of biological and physical oceanographic approaches, with the inclusion of a variety of techniques.

This thesis integrated genetic tools and oceanographic data to the study of marine connectivity along the west Iberian coast, using as a biological model the well studied common shore crab *Carcinus maenas*. The following summarizes the main findings:

Chapter 2 – "**Population genetic structure of *Carcinus maenas***"

This chapter examined the genetic structure of *Carcinus maenas* in its native range from Sweden to Morocco with a special focus in populations along the Iberian coast. Population structure was assessed using F_{st} and Bayesian clustering analysis, and the hypothesis of isolation-by-distance (IBD) was tested. The amount of data presented is substantial and the analysis using multiple statistical methodologies was sufficiently robust with respect to population structuring analysis. The results revealed low levels of genetic differentiation indicating that in the absence of barriers to gene flow, shore crab populations can be genetically similar across thousands of kilometres. Nonetheless, isolated populations do occur in the limits of the species range, such as those sampled in Sweden and Morocco, probably due to an oceanographic barrier in the case of Sweden and to IBD in the case of Morocco, mainly driven by differences between the latter population and Wales.

Chapter 3 – **"Genetic structure of *Carcinus maenas* larvae"**

This chapter tested the sweepstakes recruitment hypothesis by examining whether there is temporal genetic variability in *Carcinus maenas* larvae supplied to an estuary in northwest Portugal. For this purpose the population genetic structure between two larval recruitment years, among larval events within a year, and before/after a shift in the dominant oceanographic conditions in the region that occurs in late spring/early summer, as well as the genetic relatedness within and between larval events, were analysed. Each larval event was genetically compared with to 1262 genotyped adults across a 1200 km stretch of the Iberian Peninsula coast. This study represents the first direct test of the sweepstakes hypothesis in marine animals during their dispersing phase, as opposed to after settlement. The results showed no support for sweepstakes reproduction in *C.*

maenas as there were no significant differences amongst larval events, no evidence for genetic relatedness among larvae in each episode, and the larvae from each event were representative of the population at large.

Chapter 4 – "Crab larval supply at multiple temporal scales"

This chapter investigated the relative importance of various mechanisms of shoreward and estuarine transport of *Carcinus maenas* larvae to Ria de Aveiro, northwest Portugal. The daily abundance of *C. maenas* megalopae was measured in five different years allowing interannual comparisons. Megalopae supply was an episodic phenomenon and time series analysis techniques revealed that supply was highest around spring tides and was enhanced by southerly winds, as predicted, although unclear patterns were observed among years. The results confirm previous observations that transport of the shore crab to the nearshore northwest Portugal may be due to physical phenomena associated with wind forcing and tides. However, extended larval time series among various years showed that a proportion of larval supply could not be explained, probably due to the inherently stochasticity of larval dispersal.

Chapter 5 – "Use and validation of an individual-based coupled physical biological model to describe dispersal and supply of invertebrate larvae"

This chapter illustrated the use and validation of an Individual-Based Coupled Physical Biological Model (ICPBM) to describe the observed time series of *Carcinus maenas* megalopae supplied to the Ria de Aveiro in two of the years studied in Chapter 4. The model was able to describe large supply events associated with strong SW winds, with time lags of -5 to +6 d. The model also predicted that recruiting larvae were retained in the inner shelf, independently of supply event, and that larvae transported to the outer shelf and beyond never recruited. Results from the model appeared to give a realistic estimate of the time course of the processes that resulted in the observed supply events. This emphasizes the hypothesis that larval supply can indeed be predicted and may not be intrinsically stochastic as stated in Chapter 4. Comparison of modelling predictions with empiric observational studies helped in validating the model and open good prospects for the use of IBPBMs to study scenarios of larval dispersal and population connectivity.

In summary, the information generated by this study indicated: i) high genetic homogeneity among *Carcinus maenas* populations across oceanic distances as long as 3000 km, ii) the presence of some significant barriers to gene exchange in this species

within its native range, in the present day environment; iii) larval genetic homogeneity among larvae supplied to a particular population and no family structure among larvae from each supply episode, although the supply was temporally variable and mainly dependent of tide- and wind-forcing; iv) realistic description of megalopal supply to the Ria de Aveiro driven by winds, which are one of the major factors controlling oceanic circulation in upwelling systems; and v) genetic and modelling data appear to provide a coherent picture of observed connectivity patterns.

6.2 Future Perspectives

The future of larval dispersal and marine population connectivity studies is to continue using and improving currently available tools as well as expanding the coupling between multidisciplinary areas such as physics, modelling, statistics, larval ecology and genetics (Levin 2006, Selkoe et al. 2008, Cowen & Sponaugle 2009). Along with these approaches there is a strong necessity to clearly define sampling strategies and to carry out intensive temporal sampling using adequate sample sizes, in order to effectively describe marine population connectivity.

Further development should include improving conceptual and predictive models for the ecology of *Carcinus maenas* and for the functioning of oceanic eastern boundary systems. The tests of relatedness within larval cohorts seem to hold much promise to understand spatial and mechanistic patterns of larval dispersal and would now be interesting to screen the level of relatedness among recently settled juveniles. As illustrated by Nielsen et al. (2009) with Atlantic cod, adaptive population divergence may be prevalent despite apparently high levels of gene flow in most marine species. The use of genetic markers under selection, for example gene associated single nucleotide polymorphisms (SNPs), could be useful to look at genetic variation. Loci under selection can indeed display high levels of genetic differentiation among populations that would not be uncovered by neutral microsatellites markers.

In the future, the use of high-throughput genotyping and sequencing technologies (Coombs 2008) will make available information of whole genome what is likely to dramatically change the accumulated knowledge and will certainly change the scope of future research on population dynamics of marine species.

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