



Assessment of the aquacultural potential of the Portuguese oyster *Crassostrea angulata*

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Dissertação de doutoramento em Ciências do Meio Aquático

2007

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oyster *Crassostrea angulata***

Dissertação de Candidatura ao grau de Doutor
em Ciências do Meio Aquático submetida ao
Instituto de Ciências Biomédicas de Abel
Salazar da Universidade do Porto.

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Abel Salazar da Universidade do Porto.

ACKNOWLEDGEMENTS

First of all, I would like to thank Dr. Pierre Boudry for accepting to supervise this work, for your commitment and for providing me all conditions that made possible this work. Thank you also for your guidance and at the same time for giving me the freedom to make my own decisions which helped me to become a more independent researcher.

Gostaria de agradecer à minha co-orientadora, a Professora Doutora Maria Armanda Henriques por me ter aceite como seu aluno de doutoramento e pela disponibilidade.

Gostaria de agradecer ao Dr. Carlos Costa Monteiro por ter permitido a realização de parte deste trabalho na estação experimental de moluscicultura de Tavira do Instituto Nacional de Investigação Agrária e das Pescas (INIAP/IPIMAR). Gostaria também de agradecer-lhe pela disponibilidade e incentivo durante o desenrolar do doutoramento.

Je remercie Dr. Philippe Goulletquer et Dr. Tristan Renault pour m'avoir accueillie au sein du Laboratoire de Génétique et Pathologie de la Station de La Tremblade de l'Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER).

I would like to acknowledge the Portuguese Foundation for Science and Technology for a PhD grant that made this work possible. This work was also made possible by a Marie Curie Training fellowship.

Gostaria de agradecer ao Dr. Francisco Ruano pelo seus preciosos ensinamentos de patologia, pelas várias conversas sobre a ostra Portuguesa, disponibilidade, interesse, camaradagem e incentivo durante todo o desenrolar do doutoramento.

Je tiens à exprimer toute ma gratitude à Dr. Tristan Renault pour m'avoir donné goût à la Pathologie et pour son implication au cours de ce travail. Il a toujours été disponible pour passer du temps avec moi malgré ses nombreuses activités.

Merci également à Sylvie Lapègue et à Serge Heurtebise pour leurs nombreux conseils de génétique et son aide quotidienne.

Merci à l'équipe de la station de La Tremblade pour votre aide quotidienne: Delphine L ., Christopher, Véronique, Isabelle, Maeva, Céline, Florence R. (merci pour toute l' biblio), Pascal (le vrai Dr. des larve), Bruno, Raphaël, Christophe, Patrick, Jean-François, Helen, Jean-Cristophe, Nicole, Stéphanie, Florence C., Stéphanie, Jean-Pierre, Laurence et Martine.

Gostaria de agradecer às várias pessoas que tive o prazer de conhecer no IPIMAR e na Universidade do Algarve, nomeadamente: ao Maurício Teixeira por tudo o que me ensinou, pela sua camaradagem e ajuda constante; ao Dr. Luís Cancela da Fonseca pelo interesse e incentivo; ao Radhouan pelo interesse e ajuda no tratamento estatístico; à Sandrinha pela simpatia e apoio; à Senhora Augusta e à Senhora Graça pela simpatia e ajuda no laboratório; ao João, Ana, Tânia, Paula, Victor e Zé pelo apoio e bons momentos que passamos juntos.

Je voudrais adresser mes sincères remerciements à Sylviane et Albert Gass pour son accueil, amitié et extrême gentillesse.

Thank you Nicolas for your continuous encouragements, suggestions, and discussions during our coffee breaks. Thank you also and your family for helping me to settle and discover your marvellous country. But I would like to thank you most of all for your friendship.

Thank you Tim for your patience to answer my never ending questions, inspiration and friendship. Thank you also for sharing with me your passionate vision of science.

Un grand merci à toi Stéphane pour ton soutien, bonne humeur et extrême gentillesse.

Muito obrigado Alexandra e Teresa pela vossa amizade, camaradagem, generosidade e apoio constante.

Merci aux personnes rencontrées à La Tremblade, avec lesquels j'ai partagé de vrais instants d'amitié: Maeva, Delphine L ., Christopher, Véronique, Isabelle, Jean-Luc, Aude, Noélia, Adeline, Jean-Côme, Doris, Lionel, Céline, Béatrice, Christophe, Niklas, Gaele, Delphine R., Karine, Yohann et Pascal.

Um agradecimento especial aos meus amigos de sempre Costa, Garcia, Mário, Paulo, Pedro e Tiago por me terem sempre apoiado.

Gostaria de agradecer aos meus avós e sogros pelo apoio e incentivo.

E como não podia deixar de ser, gostaria de agradecer ao meu pai e irmão pelo apoio, paciência e encorajamento permanente.

E por fim gostaria de agradecer à pessoa que mais me ajudou, à Vera, a minha companheira, por tudo, e ao fruto do nosso amor, o pequenino Daniel, por me alegrar os dias.

RESUMO

A ostra Portuguesa *Crassostrea angulata* teve uma elevada importância comercial na Europa até finais de 1970, altura em que praticamente desapareceu devido a elevadas mortalidades. Actualmente a produção de ostras na Europa baseia-se sobretudo numa única espécie, a ostra Japonesa *C. gigas*, que foi introduzida para substituir a ostra Portuguesa. No entanto, algumas populações de *C. angulata*, localizadas sobretudo no Sul de Europa, resistiram à mortalidade massiva dos anos 70 bem como à introdução de *C. gigas*. O objectivo principal desta tese foi avaliar o potencial aquícola destas populações de *C. angulata* numa perspectiva comparativa com *C. gigas* e com os híbridos dos dois taxa.

A composição genética de duas populações de *C. angulata* (situadas no Rios Mira e Sado, Portugal) foi analisada através da utilização de um marcador de ADN mitocondrial. Os resultados obtidos confirmaram que *C. gigas* não foi introduzida nas duas populações estudadas, podendo estas ser consideradas como populações “puras” de *C. angulata*.

Efectuaram-se cruzamentos factoriais entre *C. angulata* (Rio Sado) e *C. gigas* (Rio Seudre, França) de modo a obter descendências puras e híbridos, tendo-se procedido ao seu cultivo na Ria Formosa (Portugal). Observou-se uma sobrevivência e taxa de crescimento superiores na descendência de *C. gigas* comparativamente com a descendência de *C. angulata*. Os híbridos tiveram uma performance intermédia, logo não se observou vigor dos híbridos para este dois parâmetros. A análise da proporção de cromossomas em falta e células aneuploides revelou níveis mais elevados em *C. angulata* do que em *C. gigas*, tendo-se observado valores intermédios nos híbridos. Verificaram-se igualmente diferenças na morfologia e pigmentação da concha entre *C. angulata* e *C. gigas*.

A análise do recrutamento de indivíduos do género *Crassostrea* na Ria Formosa através de marcadores de ADN mitocondriais e nucleares sugere que existe uma sobreposição no padrão temporal de recrutamento de *C. angulata* e *C. gigas*. Os resultados obtidos sugerem igualmente que houve recrutamento de híbridos durante o mesmo período. No entanto, observaram-se diferenças nas frequências de haplotipos e alelos em diferentes períodos da época de recrutamento o que sugere que existe um isolamento reprodutivo parcial entre *C. angulata* e *C. gigas*.

Verificou-se um surto de herpesvirus 1 de ostra associado a elevadas taxas de mortalidade durante o cultivo larvar de *C. angulata*, *C. gigas* e híbridos. Por seu turno, análises zoosanitárias em adultos demonstraram uma maior infestação parasitária (protozoários e metazoários) na descendência de *C. angulata* quando comparada com a de *C. gigas*. Observou-se nos híbridos infecções bacterianas e alterações citológicas associadas a infecções virais.

Os resultados obtidos são discutidos numa perspectiva de desenvolvimento sustentável do cultivo de ostras em Portugal e preservação de populações de *C. angulata*.

ABSTRACT

The Portuguese oyster *Crassostrea angulata* was a species of major economic importance in Europe up to the early 1970's, when mass mortalities almost led to its disappearance. Nowadays, the European oysters farming industry is mainly based on the production of *C. gigas* that was introduced to replace *C. angulata*. However, there are still a few populations of *C. angulata* in southern Europe that manage to survive and recuperate from the mass mortalities observed in the 1970's. Moreover, it is thought that *C. gigas* was not introduced in these *C. angulata* populations and hence they may constitute important genetic resources. The main objective of this work was to assess the aquacultural potential of *C. angulata* in comparison with *C. gigas* and their hybrids.

The genetic composition of two *C. angulata* populations (from Mira and Sado Rivers, Portugal) was analyzed using a mitochondrial marker to confirm that *C. gigas* had not been introduced in these populations. The analyses showed that these two populations can be regarded as being "pure" *C. angulata* populations.

Experimental crosses were carried out between *C. angulata* and *C. gigas* broodstocks to produce pure and hybrid progenies. Oysters from the different groups were grown in Ria Formosa (Portugal). A higher growth rate and survival was observed in the *C. gigas* progeny in comparison with the *C. angulata* progeny. Their reciprocal hybrids had an intermediate performance and hence no heterosis was observed for these traits. A higher proportion of aneuploid cells and missing chromosomes were recorded in the *C. angulata* progeny than in the *C. gigas* progeny, with hybrids showing intermediate values. Differences were also observed between *C. angulata* and *C. gigas* progenies in terms of shell morphology and pigmentation.

The analyses of the recruitment pattern of *Crassostrea* sp. in Ria Formosa using mitochondrial and nuclear DNA markers revealed an overlap in the temporal setting pattern of *C. angulata* and *C. gigas*. Moreover, the results obtained suggest that hybrids settled during all setting season. However, differences in haplotypic and allelic frequencies observed at different settling periods, which suggest that partial reproductive isolation may exist between the two taxa.

Ostreid herpesvirus 1 was detected during the larval rearing of *C. angulata*, *C. gigas* and their hybrids and associated with high mortality rates. Moreover, zoosanitary analyses on adults revealed a higher parasitic load (protozoans and metazoans) in *C. angulata* progeny in comparison with *C. gigas* progeny. Bacterial and virus-like infections were observed in the hybrids.

These results are discussed in terms of the sustainable development of oyster aquaculture in Portugal and of the preservation of *C. angulata* populations.

RESUME

L'huître portugaise *Crassostrea angulata* était une espèce majeure d'intérêt économique jusque dans les années 1970 où des mortalités en masse ont presque conduit à son extinction. Depuis, la production d'huîtres en Europe est principalement basée sur l'huître du Pacifique *C. gigas* qui a été introduite pour remplacer *C. angulata*. Cependant, il existe encore quelques populations de *C. angulata* dans le sud de l'Europe qui ont réussi à survivre et récupérer des mortalités en masse des années 70. De plus, il était supposé que l'huître *C. gigas* n'avait pas été introduite dans ces populations et donc celles-ci constituent d'importantes ressources génétiques. Le principal objectif de ce travail était d'évaluer le potentiel aquacole de ces populations de *C. angulata* par rapport à *C. gigas* et de leurs hybrides.

La composition génétique de deux populations de *C. angulata* (rivières Mira et Sado, Portugal) a été analysée à l'aide d'un marqueur du génome mitochondrial pour confirmer que *C. gigas* n'a pas été introduite dans ces populations. Les analyses ont montré que ces deux populations peuvent être considérées comme de « pures ».

Des croisements expérimentaux ont été réalisés à l'écloserie expérimentale de La Tremblade (France) entre des géniteurs *C. gigas* et *C. angulata* pour produire des descendants purs et hybrides. Les huîtres des différents groupes ont été mis en élevage dans la Ria Formosa (Portugal). Un plus fort taux de croissance et une meilleure survie ont été observés chez les descendants purs *C. gigas* par rapport aux descendants purs *C. angulata*. Leurs hybrides réciproques présentaient une performance intermédiaire et donc aucun effet hétérosis n'a été observé pour ces traits. Une plus forte proportion de cellules aneuploïdes et de chromosomes manquants a été observée chez les descendants purs *C. angulata* par rapport aux descendants purs *C. gigas*, les hybrides présentant des valeurs intermédiaires. Des différences ont été observées entre les descendants purs *C. angulata* et *C. gigas* en termes de morphologie de la coquille et de pigmentation.

Les analyses du patron de recrutement des espèces *Crassostrea* sp. dans la Ria Formosa à l'aide de marqueurs ADN mitochondrial et nucléaire ont révélé un chevauchement dans le temps de la sédentarisation de *C. angulata* et *C. gigas*. De plus, les résultats obtenus suggèrent que des hybrides se sont fixés durant toute la saison de recrutement. Cependant des différences dans les fréquences haplotypiques et alléliques ont été observées à différentes périodes de recrutement, ce qui suggère un possible isolement reproducteur partiel entre les deux taxa.

L'herpèsvirus OsHV-1 (Ostreid Herpesvirus type 1) a été détecté pendant l'élevage larvaire de *C. angulata*, *C. gigas* et de leurs hybrides et associé à de forts taux de mortalité. De plus, des analyses zoosanitaires au stade adulte ont révélé une charge parasitaire (protozoaires et metazoaires) plus élevée chez les descendants purs *C. angulata* par rapport aux descendants purs *C. gigas*. Des infections bactériennes et de type virus ont été observées chez les hybrides.

Ces résultats sont discutés en termes de développement durable de l'aquaculture de l'huître au Portugal et de conservation des populations de *C. angulata*.

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GENERAL OBJECTIVES AND THESIS STRUCTURE

The Portuguese oyster *Crassostrea angulata* sustained the European oyster production for almost one century until the 1970's when mass mortalities almost wiped-out this species from Europe. Some natural populations of *C. angulata* located in southern Europe managed to survive and recovered from these mortality episodes. Nowadays, the European oyster production is mainly based on a single species, the Pacific oyster *C. gigas*, that was introduced to replace *C. angulata*, although there is a small production of the European oyster *Ostrea edulis*. In this context, the use of existing biodiversity to diversify oyster farming can be important for the sustainability of the European oyster production. A better knowledge about the aquacultural potential of remaining populations of *C. angulata* in a comparative perspective with *C. gigas* can bring new insights. Given the history of *C. angulata*, it is also of great importance to define its current zoosanitary status, to develop tools for the detection of pathogens and to compare the susceptibility of *C. angulata* and *C. gigas* to various pathogens. In addition, since *C. angulata* and *C. gigas* hybridise under laboratory conditions the assessment of heterosis (or, alternatively, outbreeding depression) for traits of commercial importance or combined beneficial characteristics in their hybrids should be performed. Moreover, the commercial exploitation and valorisation of *C. angulata* can contribute for the preservation of remaining pure populations of this species that are endangered by the expansion of *C. gigas* farming. The main objective of this work was to contribute to a better knowledge of the aquacultural potential of *C. angulata*, *C. gigas* and their hybrids throughout a multidisciplinary approach.

In the first chapter the culture, genetics and pathology of mollusc bivalves are reviewed as well as more specific aspects concerning the comparative biology of *C. angulata* and *C. gigas*. The experimental procedure and the main results obtained are presented in the subsequent six chapters. For the majority of these chapters, part of the work conducted is presented in articles that have been published or submitted to peer-reviewed journals. The work and main results obtained in this thesis are organized in the following way:

- A mitochondrial marker was used to determine if previously identified wild populations of *C. angulata* remain "pure" (Article 1). In addition, mitochondrial DNA sequence variability was compared between a *C. angulata* population (Sado River, Portugal) and a *C. gigas* population (Seudre River, France). These studies ensure that these populations constitute a reliable source of *C. angulata* and *C. gigas* progenitors for the production of pure and hybrid progenies under laboratory conditions (Chapter 2);

- Progenies of *C. angulata* and *C. gigas*, and their hybrids were produced under laboratory conditions in order to identify genetically-based differences and heterosis for traits, such as growth, survival, shell morphology and pigmentation. Karyological disorders (aneuploidy) that are generally associated with a decrease in growth rate were also evaluated (Articles 2 and 3) (Chapters 3 and 4);
- Recruitment of cupped and flat oysters in Ria Formosa was evaluated in order to characterize their recruitment pattern and to know when and if they can be selectively collected. In addition, natural hybridization between *C. angulata* and *C. gigas* was evaluated in temporal and spatial scales throughout the use of a mitochondrial and a nuclear marker. This was done to determine to what extent there are reproductive barriers between the two taxa (Chapter 5);
- A DNA extraction method for the detection of ostreid herpesvirus 1 (OsHV-1) by PCR from small larval samples was developed (Article 4). Moreover, trials were conducted to detect irido-like virus DNA from known and presumed infected oyster specimens using molecular tools (Chapter 6);
- An outbreak of OsHV-1 during larval rearing of *C. angulata*, *C. gigas* and their reciprocal hybrids was described using different molecular tools (Article 5). In addition, the zoosanitary status of adults from the previous groups that were grown in Ria Formosa was analysed by macroscopic and microscopic examinations (Chapter 7).

CHAPTER 1. GENERAL INTRODUCTION

1.1. BIVALVE MOLLUSCS CULTURE

The culture of bivalve molluscs is one of the oldest forms of aquaculture and it is known since Roman times (Héral and Deslous-Paoli, 1991). However, only by the end of the 19th century reliable techniques for culturing bivalves were developed, which allowed the rapid growth of aquacultural production (Lubet, 1994). Approximately one century later the development of hatchery techniques (Loosanoff and Davis, 1963; Walne, 1974) greatly augmented the culture of bivalves and open new avenues for the expansion and sustainability of the bivalve culture industry.

1.1.1. Production statistics

According to FAO (2007) statistics, the total world fishery production (excluding algae and plants) from capture fisheries and aquaculture was approximately 132 million tonnes in quantity and 45.9 billion euros in value in 2003. The contribution of aquaculture to global supplies of aquatic animal organisms continues to grow (Figure 1), increasing from 4 % of total production by weight in 1970 to approximately 32 % in 2003 (FAO, 2007).

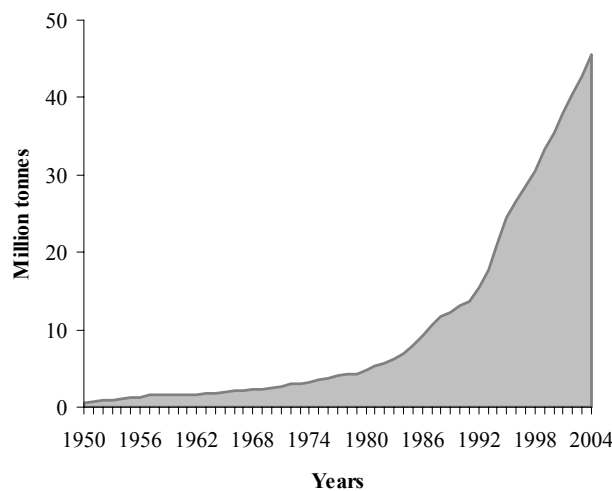


Figure 1. Global production of aquaculture (excluding algae and plants) from 1950 to 2004 (FAO, 2007).

In the last years, China was responsible for more than 2/3 in weight of the world aquaculture production being by far the largest producer. In 2004, molluscs represented approximately 22.3 and 14.0% in quantity and value, respectively, of the world aquaculture animal production (Figure 2).

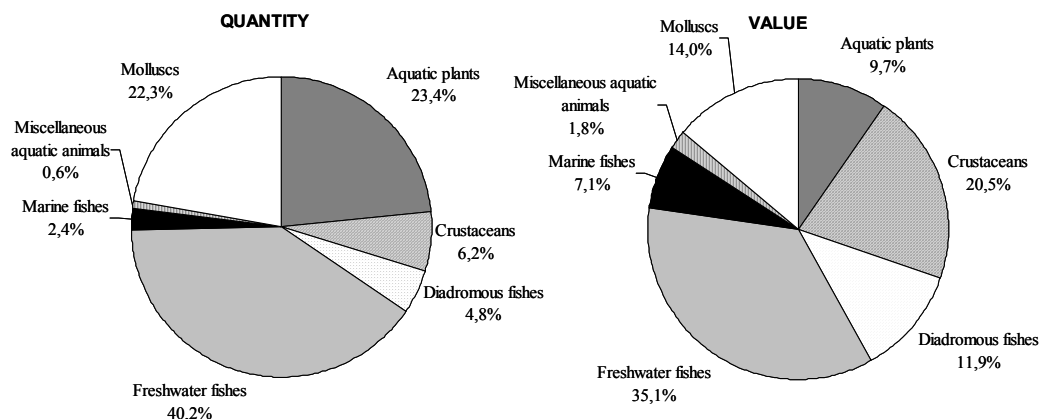


Figure 2. World aquaculture production of major species groups in quantity and value in 2004 (FAO, 2007).

The Pacific oyster, *Crassostrea gigas*, is produced in 27 countries and is the mollusc species most produced in the world. In 2004, the production of this species attained approximately 4.4 million tonnes (Figure 3).

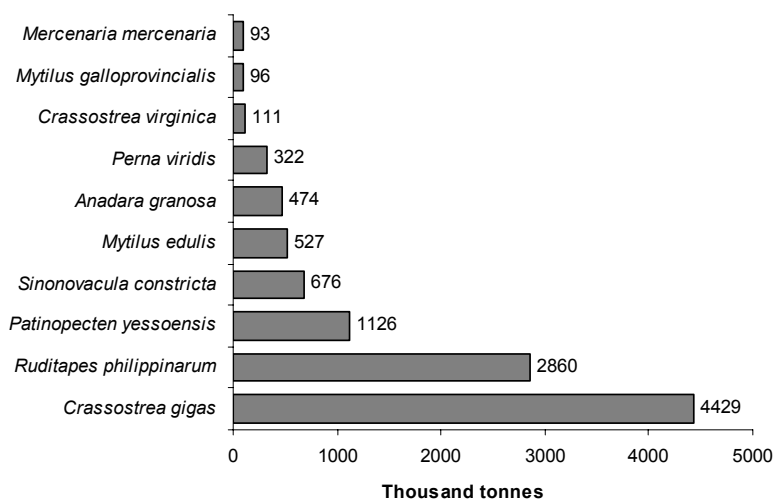


Figure 3. World aquaculture production of major mollusc species in 2004 (FAO, 2007).

China is the largest producer of *C. gigas*, with more than half of the world production (FAO, 2007). However, Guo *et al.* (1999) reported that others species of the genus *Crassostrea* are probably included in the *C. gigas* production statistics. According to the same authors, the zhe oyster *C. plicatula* and the suminoe oyster *C. ariakensis* together

account for 70 to 90 % of China; *Crassostrea* spp. and *C. gigas* only accounting for the remaining part.

The Portuguese oyster *C. angulata* sustained the European oyster production for almost one century until between 1967 and 1972 (Figure 4). During this period, high mortalities almost wiped out *C. angulata* from Europe (Comps, 1988). In order to overcome the crises, *C. gigas* was introduced in Europe to replace *C. angulata* and sustained the oyster industry (Grizel and Héral, 1991). Although the production of *C. angulata* in Europe reached 100 000 tons in 1953 (FAO, 2007), nowadays the production of *C. angulata* is less than 10 tons (F. Ruano, personal communication). According to Boudry *et al.* (1998) and Huvet *et al.* (2000), *C. angulata* is presumed to be the main oyster species produced in Taiwan, with a production of 20 750 tonnes in 2004 (FAO, 2007). It was also recently reported by Guo *et al.* (2006) that *C. angulata* is farmed in middle China, namely in Fujian and Zhejiang regions.

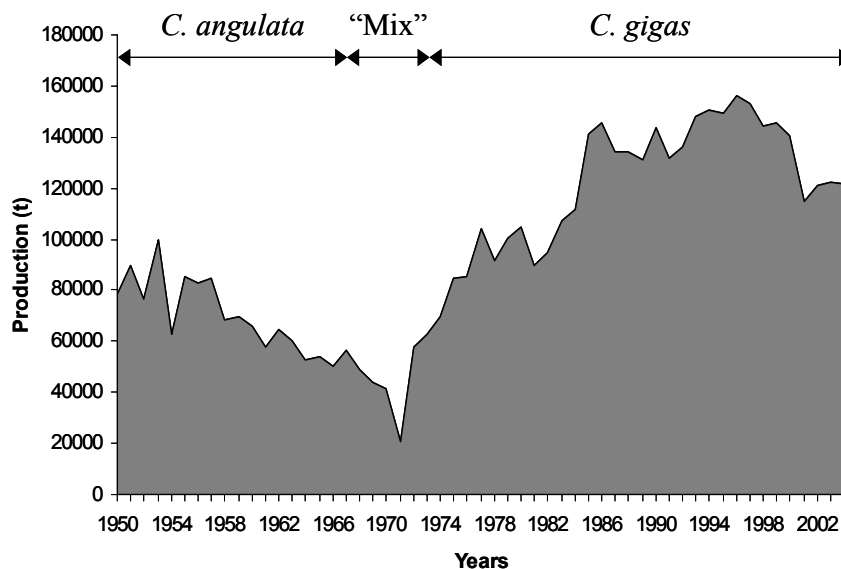


Figure 4. Aquaculture production of *C. angulata* and *C. gigas* in Europe between 1950 and 2004 (FAO, 2007).

1.1.2. Methods of bivalve culture

Seed supply

In bivalve culture a steady supply of seed is of paramount importance. Seed can be collected from the wild or produced in the hatchery. The majority of the seed used worldwide for bivalve culture is from natural collection.

Seed collected from the wild

Collection of seed from the natural environment can vary considerable from year to year because of fluctuations in environmental conditions (e.g., temperature, salinity, food). Furthermore, pollution of the natural environment (e.g., anti-fouling paints containing TBT) can drastically reduce natural spatfall (Quayle, 1988). These factors will affect the time and abundance of natural spatfall and hence collection of seed from the wild can be unreliable. The successful collection of seed from the wild greatly depends on the knowledge about the life cycle, spawning period, larval development and settlement process of the target species. This information is essential in order to forecast when and where to collect juveniles. Seed can be collected from natural beds or using different material (collectors). If collectors are put too early, they are often covered with fouling organism, which will reduce spat settlement. If the collectors are put too late, or in a less suitable place, it can result in low setting success.

Hatchery

In regions where natural spatfall is too variable, low or even absent, the use of hatchery produced seed is crucial for the bivalve aquaculture industry. In addition, it is possible to produce seed in hatcheries throughout most of the year, so during periods where there is no natural recruitment. On the other hand, breeding techniques and chromosomal manipulation can be used to produce seed genetically improved for desirable traits.. The operation of a bivalve hatchery can be divided in four distinct phases (Figure 5):

- 1- Microalgae production;
- 2- Broodstock conditioning and spawning;
- 3- Larval rearing;
- 4- Larval setting.

For all these phases, a *sine qua non* condition is a good biological quality of the water, especially for larval rearing (Robert and Gérard, 1999). The most common manifestation of poor water quality in a bivalve hatchery is the lack of development of fertilised ova to D-shaped veliger larva and reduced larval growth (Utting and Helm, 1985).

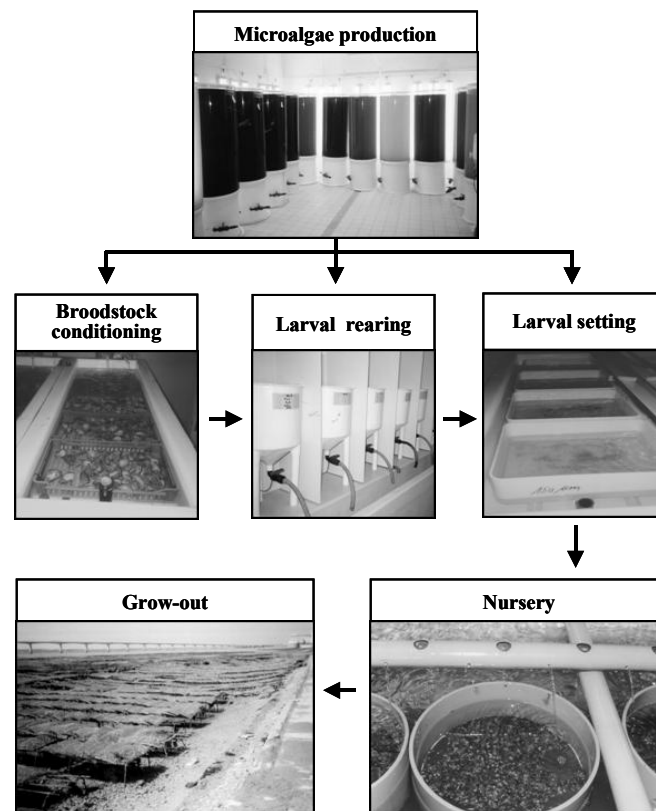


Figure 5. General flow chart of bivalve culture from hatchery seed.

1- Microalgae production

The development of culture techniques for several microalgae species was a crucial factor for the production of spat under controlled conditions. The microalgae species most commonly used for conditioning broodstock and larval and spat rearing are: *Chaetoceros calcitrans*, *C. gracilis*, *Isochrysis galbana*, *Pavlova lutheri*, *Phaeodactylum tricornutum*, *Rhinomonas reticulata*, *Skeletonema costatum*, *Tetraselmis suecica* and *Thalassiosira pseudonana* (Coutteau and Sorgeloos, 1992). These species have been widely used due to their ease of culture, cell size, cell wall structure and biochemical composition. Microalgae can be cultured in continues, semi-continues or batch systems (Breese and Malouf, 1975). Continuous culture (or chemostat culture) is characterized by continuous withdraw of cells and supply of medium to the culture. In semi-continues culture, a part of the culture is harvest periodically and replaced with fresh medium to retain the original volume. In batch cultures, the medium is inoculated with microalgae using successively larger containers, and the culture is harvested all at once when the culture reached the optimal phase and density. The continuous culture systems are more difficult to maintain

than batch culture systems, but allow the production of microalgae of uniform quality in large quantities (Breese and Malouf, 1975).

The biochemical composition of microalgae can be substantially altered by the culture conditions, namely the growth medium, temperature, light intensity and wavelength, photoperiod and growth stage at time of harvest (Utting, 1985; Brown *et al.*, 1989). Hence, microalgae culture conditions can be important since it is known that their biochemical composition has a strong influence on growth and survival of bivalve larvae and spat (Utting, 1986; Helm and Laing, 1987; Thompson *et al.*, 1993; Soudant *et al.*, 1998; Jonsson *et al.*, 1999). Moreover, many studies have shown that the broodstock diet composition can affect egg composition, hatching rate and larvae quality (Soudant *et al.*, 1996; Martinez *et al.*, 2000; Caers *et al.*, 2002). In order to overcome fluctuation in biochemical composition of microalgae due to variation in the culture conditions and to provide more balanced diets mixed algal diets are used (Helm, 1977). In the past decade, there has been an increasing interest for alternatives to on-site algal production. Nonetheless, alternative diets are still rarely used in hatcheries (Knauer and Southgate, 1999). For the larval phase only small quantities of microalgae are need but with a high quality in terms biochemical composition and microbiological properties. Broodstock requires large quantities of microalgae even when a small number of animals are being conditioned. Indeed, the total cost of algal production in hatcheries is about 30 % of the total seed production (Coutteau and Sorgeloos, 1992) and it is considered one of the main problems for the profitability of hatcheries (Myers and Boisvert, 1990).

2- Broodstock conditioning and spawning

Maximum egg quality and larval viability is realized when parent animals are in a optimum stage of gonadal development (Lannan, 1980; Lannan *et al.*, 1980; Gallagher and Mann, 1986; Utting, 1993). It was found that in many bivalve species normal development of gonads can be stimulated and spawning induced out-of their natural reproduction season under laboratory conditions (Loosanoff and Davis, 1963). Several factors influence gonadal development such as temperature, salinity, food ration and biochemical composition of the diet (Loosanoff and Davis, 1963; Mann, 1979; Devauchelle, 1989; Utting, 1993; Muranaka and Lannan, 1984; Chávez-Villalba *et al.*, 2003; Fabioux *et al.*, 2005), and photoperiod in some species (Devauchelle and Minguant, 1991). Water quality is also an important factor for the successful conditioning of the broodstock and open or semi-closed systems are generally used (Utting, 1993; Robert and Gérard, 1999). Recirculation systems can also be used (Robinson, 1992a) but they are considered less suitable (Utting and Millician, 1997). The gametogenic cycle in marine bivalves is linked to cycles of glycogen storage since *de novo* synthesis of lipids during vitellogenesis is made

at the expense of stored glycogen (Gabbott, 1975; Robinson, 1992b). The onset of broodstock conditioning at elevated temperatures may force the development of eggs before sufficient glycogen has accumulated for the synthesis of lipid (Gallager and Mann, 1986). This can result in eggs with lower quality and/or reduce fecundity. Hence, pre-conditioning of broodstock in high food regimes before the conditioning at elevated temperatures is used to increase glycogen reserves that will be required during the stage of vitellogenesis (Utting and Millician, 1997).

Spawning of sexually ripe bivalves under laboratory conditions may be induced by thermal (e.g., heat shock, cold shock) or chemical (e.g., ammonium hydroxide, potassium chloride) stimuli (Loosanoff and Davis, 1963; Galtsoff, 1964). Other stimuli that are used to induce spawning include addition of stripped gametes to the water, aerial exposure for a short period or injection of hormones into the adductor muscle. Another method for obtaining ripe eggs and sperm in some species of bivalves is by strip spawning (*i.e.*, mechanical removal from the gonad). The oocyte obtained by this method in some bivalve species (e.g., *C. gigas*) undergo spontaneous germinal vesicle breakdown and arrest at meiotic metaphase I when put in seawater (Stephano and Gould, 1988). Strip spawning is especially useful in controlled genetic crosses, where induced spawning can be problematic (Debrosse and Allen, 1991). However, the use of this method is not possible in several bivalve species (e.g., scallops, mussels, clams) since under normal conditions the passage of the oocytes through the oviduct is necessary to reactivate meiosis so the eggs can be fertilized.

3- Larval rearing

After fertilization, the embryos of bivalves first develop into trochophore larvae followed by the shelled larval stage. The duration of the larval stage greatly depends on the rearing conditions (*i.e.*, temperature, salinity, amount of food), but generally has a duration between 10 and 26 days. Some bivalve species (e.g., oysters of the genus *Ostrea*) incubate their larvae during the first larval stages, which considerably reduce the free swimming period. Various larval rearing techniques are used in commercial hatcheries such as (Robert, 1994; Robert and Gérard, 1999):

- Larvae are reared in close systems in cylindrical conical-based tanks with large rearing volumes (4 - 45 m³). The initial larval density is low (1 - 2 larvae per ml) and water is renewed every 2 - 5 days with partial renewal on some days;
- Larvae are reared as previously described, but in smaller rearing volumes (2 - 5 m³). Initial larvae density is higher ranging from 5 to 10 larvae per ml and is decreased over time down to 1-2 larvae per ml by culling;

- Larvae are reared in flow-through systems in tanks with small rearing volumes (0.2 m³) equipped with filters to prevent loss of larvae. Initial larval densities are very high at about 800 larvae per ml.

The two major parameters that are controlled during larval rearing are temperature and the amount of food (Davis and Calabrese, 1964; Breese and Malouf, 1977; Sprung, 1984; Beiras *et al.*, 1994). The optimum rearing temperature varies according to the species cultured and the strategy adopted. Higher temperatures will generally lead to faster growth, but the risk of bacterial proliferation and/or viral replication is higher. Nutrition is considered the factor that most explain variance in larval growth (His *et al.*, 1989). The microalgae species used and the amount of food given are generally adapted to the size of the larvae and should be rigorously controlled during larval rearing. Microalgae densities above the optimum can have a negative effect on growth and survival of bivalve larvae (Sprung, 1984). This is generally associated with: (1) dense microalgal densities that hamper the feeding apparatus despite the fact that larvae can adapt to different densities (Strathmann *et al.*, 1993) and/or; (2) negative effect of microalgae metabolites. Another parameter that also has some influence on larval performance in some bivalve species is salinity (Breese and Malouf, 1977; Helm and Millican, 1977; Nell and Holliday, 1988; His *et al.*, 1989). Moreover, the conditions at which the parent adults are maintained during maturation can influence the temperature and salinity tolerance of larvae (Helm and Millican, 1977; His *et al.*, 1989). The high densities and sometimes stressful conditions under which larvae are reared can lead to the appearance of bacterial diseases. Antibiotics are used in hatcheries to control bacterial proliferation and reduce mortality (Lemos *et al.*, 1994; Robert *et al.*, 1998). However, they can decrease larval growth (Helm and Millican, 1977), lead to the appearance of resistant bacterial strains (Robert *et al.*, 1998) and some are dangerous for humans and the environment. Antibiotics are especially used in the rearing of bivalve species that regularly show high mortalities associated with bacterial contamination (e.g., scallops). In order to avoid the proliferation of harmful bacteria and the subsequent use of antibiotics, prophylactic methods are used such as good hatchery hygiene practices, reduction of stressful conditions (e.g., overcrowding, high temperatures), maintenance of good water quality and the use of probiotics (Gibson *et al.*, 1998; Robert *et al.*, 1998; Riquelme *et al.*, 2000).

4- Larval setting

The appearance of the foot indicates that the larvae (pediveligers) will soon settle and metamorphose. During approximately the same period an eyespot becomes visible, which is more or less easy to observe depending on the species. For some bivalves (e.g.,

oysters), it is necessary to provide a substrate (cultch or collectors) on which the larva will settle. Some hatcheries use techniques in order to obtain single oyster spat that are required by some farmers (e.g., French oyster farmers), namely: (1) finely crushed shell chips small enough ($< 400\ \mu\text{m}$) to allow the settlement of only one larva per fragment and; (2) fibre glass panels or sieves from which the spat can be easily removed soon after setting. Cultchless oyster spat can also be obtained using the chemicals epinephrine and nor-epinephrine (Coon *et al.*, 1986). Using these chemicals, competent oyster larvae will metamorphose without settlement behavior or attachment, thus becoming cultchless spat. Larvae of some bivalve species (e.g., clams, mussels) attach by a byssal thread generally on the bottom or to the side of the rearing tanks and hence do not need cultch. For these species down-welling systems are generally used during the late phase of larval rearing and larvae will settle on the mesh screen fitted to the base of the rearing vessel. For efficient hatchery operation it is important that larvae settle in a short period. Chemicals such as certain catecholamines, potassium chloride and ammonium chloride can be used to induce and synchronizing metamorphosis in bivalve larvae (Nell and Holiday, 1986; Coon *et al.*, 1986; 1990b). Another strategy used in some hatcheries, is to not allow the larvae to settle, since at least some bivalve species can delay metamorphose during several days (Coon *et al.*, 1990a). These ready to settle larvae are then transported in containers at low temperatures (Holiday *et al.*, 1991). This technique allows farmers working in locations remote from the hatchery to receive and settle the larvae (remote setting). This process is widely used on the West coast of the United State of America and Canada. Some hatcheries initially maintain the newly settled spat in their larval rearing containers or in the setting tanks for some days (micro-nursery). Spat is then transferred from the hatchery to the nursery.

Nursery

Newly settled spat is generally not placed directly in the grow-out site to avoid high mortality due to adverse environmental conditions and predation. Spat are kept in some sort of protective culture systems (nursery) until they reach a large enough size to withstand these factors. Nursery systems are land-based or field-based and spat are fed partially, or exclusively, with natural phytoplankton. These greatly reduce the high cost of microalgae production (Coutteau and Sorgeloos, 1992). Land-based nursery systems can be either indoor or outdoor on which spat are usually reared in downwelling or upwelling systems (Utting and Spencer, 1991). Spat can also be reared in field-base nursery systems in protected areas in subtidal, low intertidal or in ponds. Once the seed attains planting size it is then transferred to the grow-out site.

Grow-out phase

During the grow-out phase seed is cultured up to market-size and is generally the longest phase of the culture process. Several techniques have been developed for growing bivalves, which greatly depends on the species cultured and the local environment (Landau, 1992). Nevertheless, a common feature of the different culture techniques is that the majority of the bivalves feed on microorganisms (mainly phytoplankton) as well as other sources of organic matter present in the natural environment.

The culture is carried out in intertidal or subtidal zones using on-bottom or off-bottom techniques. For some clams species cultured in the intertidal zone, the seed is directly planted in the ground and the young clams bury themselves. In order to reduce mortality caused by predators the grow-out area is protected with fences and/or covered by mesh. Another strategy to reduce predation is to eliminate or reduce the predators before planting or during the grow-out phase. Some farmers also prepare the culture beds before planting the seed (e.g., adding sand and gravel into the sediment) to facilitate the transfer of food and oxygen between the water-sediment interface and the clams (Ruano, 1997). During the grow-out phase, farmers keep the beds clean by removing undesirable organisms and debris. Some bivalves such as oysters are cultured on-bottom after the ground is hardened (e.g., using oyster shells) and the young oysters are sown on the ground attached to collectors or individually. In the intertidal zones bivalves are also culture off-bottom in trays or iron tables (rack culture) on which the collectors or oyster bags are fixed. Bouchot culture is another type of off-bottom culture in the intertidal zone on which ropes, branches or roots with the bivalve seed attached are put on poles that are vertically fixed in the ground (Landau, 1992).

Subtidal zones are also used for bottom culture of bivalves. The ground is usually initially clean (e.g., from rocks, detritus and predators) and the bivalves are put directly on the bottom or in racks and mesh bags. Bivalves are also grown in suspended culture in bags, nets, trays or ropes that are attached to floating systems (e.g., longline systems, raft culture). When the bivalves reach the market-size animals are dredged or collected. Boats can be specially equipped with mechanical devices for harvesting.

1.2. GENETICS IN BIVALVE MOLLUSCS CULTURE

Genetic improvement of plants and animals has been undertaken in the last thousand years without any real scientific knowledge of genetic principles. Only in the 20th century, based on the pioneering work of Gregor Mendel in the 19th century, the principles and concepts of genetics were intentionally used to improve the production of plants and animals. Bivalve culture has benefited relatively little from genetics comparatively with other aquaculture species (Hulata, 2001). Nevertheless, significant progress have been made in the last years, which is of paramount importance to keep the bivalve culture industry economy viable since prices of many products decrease as production increases (Newkirk, 1980; Beaumont, 1994). In addition, the use of genetic can be very important to counteract crises caused by diseases (see section 1.3.). Genetic can also be used in bivalve culture for the conservation of natural resources. This can be especially important for species in which there culture mainly depends on collection of seed from the natural environment (e.g., some mussel and clam species).

1.2.1. Molecular markers and their applications

Electrophoretically distinguishable protein variants (allozymes) were the first molecular genetic markers used in bivalve culture management. The main disadvantages of allozymes are that: (1) only a small fraction of enzyme loci appear to be allozymically polymorphic (limiting the potential number of markers); (2) untranslated genetic variation cannot be detected; and (3) high amount of tissue samples with a good quality (frozen at -20°C) are required. These markers have been used for decades, and addressed many questions of interest to bivalve culture. More recently, mitochondrial DNA (mtDNA) markers have been also used in bivalve studies. Three types of mtDNA markers have been used namely: (1) restriction fragment length polymorphism (RFLP) analysis of the entire molecule; (2) RFLP of mitochondrial genes or fragments amplified using polymerase chain reaction (PCR); and (3) mtDNA sequence polymorphism. The use of Nuclear DNA (nDNA) markers is most recent in bivalve studies, such as: (1) PCR-RFLP; (2) random amplified polymorphic DNA (RAPD); (3) amplified fragment length polymorphism (AFLP); (4) variable number tandem repeats (VNTR); and (5) single nucleotide polymorphism (SNP) markers. The main advantages of nuclear markers are their potential to detect high levels of polymorphism and their Mendelian inheritance. Molecular markers can be classified in type I (associated with genes of known function) and type II (associated with anonymous genomic fragments) markers (Liu and Cordes, 2004). Allozymes and mtDNA markers are generally type I markers and nDNA markers are mostly type II markers although there are some exceptions. The type of molecular markers to be used greatly depends on the specific objectives of the study. Molecular

genetic markers have been used to address a number of questions of importance in bivalve culture, such as:

- Genetic identification of taxa that cannot be easily discriminated based only on morphological traits [e.g., discrimination between *C. angulata* and *C. gigas* (Boudry *et al.*, 1998)];
- Genetic variation within and between populations (of both native and introduced taxa) to assess their potential as a source of progenitors or seed [e.g., assessment of the genetic variability in introduced populations of *C. gigas* in different regions (Smith *et al.*, 1986; Moraga *et al.*, 1989; Boom *et al.*, 1994)];
- Effect of hatchery and field culture practices on the genetic composition of the cultured species [e.g., to estimate effective population size and loss of genetic variation of hatchery-propagated stocks of *C. gigas* (Hedgecock and Sly, 1990) and *C. angulata* (Rebordinos *et al.*, 1999)];
- Relationship between fitness-related traits and genotype [e.g., correlation between heterozygosity and mean weight observed in the American oyster *C. virginica* (Singh and Zouros, 1981)];
- Genome mapping and identification of markers associated with quantitative trait [e.g., identification and mapping using AFLP markers of disease-resistance QTLs in *C. virginica* (Yu and Guo, 2006), and linkage maps using microsatellites in *C. gigas* (Hubert and Hedgecock, 2004)];
- Impact of introduced species [e.g., introduction of *C. gigas* in southern Portugal (Huvet *et al.*, 2004)];
- Assessment of the successful implementation of genetic improvement programs [e.g., assessment of contamination of *C. gigas* families with individuals from other families (Hedgecock *et al.*, 1995)].

1.2.2. Genetic improvement

The main objective of genetic improvement of cultured bivalves is to increase sustainability, productivity and profits, which may be achieved in a number of ways, such as enhanced survival, increased growth, or improved product quality (Wilkins, 1981). The development of artificial spawning techniques and procedures for larval rearing by Loosanoff and Davis (1963) and Walne (1974) allowed the control of the whole life cycle of several bivalve species. These techniques made feasible the study and application of different genetic improvement strategies. Traits of interest in bivalve culture can be divided in two groups: (1) qualitative traits, defined as characteristics that fall into one of two discrete categories and are usually controlled by one or few genes; and (2) quantitative traits, defined as characters that are measured and are influenced or

controlled by a larger number of genes. These latter genes are very rarely identified and it is thought that their number is high, each having a small effect of the trait. Only very few qualitative traits of commercial interest have been reported in bivalves, such as the golden coloured *C. gigas* from Tasmania or the notata shell marking of the northern hard shell clam *Mercenaria mercenaria* in USA (Newkirk, 1980; Beaumont and Hoare, 2003). The first step in setting up a breeding program that aims to select for a qualitative trait is to determine if it is a single-gene characteristic inherited in a simple Mendelian way. However, complications arise if dominance is incomplete or the trait is sex-linked. A further level of complexity occurs when the character is controlled by more than one locus. However, most of the traits of importance for bivalve culture are quantitative traits such as growth rate, survival, disease resistance, maturity and time of spawning, meat yield and shell shape (Newkirk, 1980). Different genetic improvement strategies have been used to improve these traits, namely:

- Selective breeding;
- Inbreeding and hybridization (crossbreeding);
- Chromosome manipulation.

Selective breeding

Selective breeding generally provides only long-term gains, since it produce small gains that accumulate each generation. Theoretical predictions of the success of selective breeding can be done throughout the quantification of the extent of the phenotypic and genotypic variation of the traits to be improved (Falconer and Mackay, 1996). This is generally done by determining the range, mean, variance and heritability of each trait. Heritability in the broad sense can be defined as the proportion of phenotypic variance (composed by both genetic and environment components) that can be attributed to genetic variation. The genetic variation can be further divided into: (1) additive genetic variation that refers to the presence or absence of particular alleles affecting the phenotype; (2) dominance genetic variation is due to the presence or absence of particular genotypes (*i.e.*, combinations of alleles) at the loci that affect the phenotype; and (3) interaction genetic variance is produced by the interaction between loci affecting the phenotype. Additive genetic variation is the part of the genetic variation that can be exploited by selective breeding. Non-additive genetic variation is not so easy to exploit by selective breeding since it results from genotypic combinations both within and between loci and cannot be transmitted from a parent to its offspring. The proportion of the phenotypic variance that can be attributed to additive genetic variation is called narrow-sense heritability. Broad-sense heritability can be estimated by analysis of variance of full-sib families whereas narrow-sense heritability can be estimated by parent-offspring

regression or by analysis of variance of half-sib families. Both type of heritabilities have been estimated for several traits (e.g., growth rate, survival, shell shape) in many commercially important bivalve species such as *C. gigas* (Ernande *et al.*, 2003), *C. virginica* (Haley *et al.*, 1975), the European oyster *Ostrea edulis* (Toro and Newkirk, 1990), *M. mercenaria* (Hilbish *et al.*, 1993) and the blue mussel *Mytilus edulis* (Newkirk, 1980). A high variation have been observed in both broad-sense and narrow-sense heritabilities estimates for different traits within and between bivalve species. It is noteworthy that an heritability estimate is valid only for the specific environmental conditions in which the trait has been recorded and the genotypes studied. Nevertheless, additive genetic variation indicated by significant narrow-sense heritability estimates indicate that selective breeding should be successful in improving those traits in a range of environmental conditions, unless strong genotype by environment interaction exists. Indeed, selection studies were in most cases successful in modifying the selected traits in the desired direction. These studies included selection for different traits such as resistance to MSX disease in *C. virginica* (Haskin and Ford, 1979), shell shape in the Pacific pearl oyster *Pinctada fucata* (Wada, 1986), tolerance to elevated temperatures in *C. gigas* (Hershberger *et al.*, 1984) and growth rate in different species such as *O. edulis* (Newkirk and Haley, 1982), *C. virginica* (Paynter and Dimichele, 1990), the Chilean oyster *O. chilensis* (Toro and Newkirk, 1991) and the Sydney rock oyster *Saccostrea commercialis* (Nell *et al.*, 1999). Different types of selective breeding programs (e.g., mass selection, family selection, within-family selection) can be used and there choice greatly depends on the value of narrow-sense heritability and information available on the trait for various families (Beaumont, 1994). Genetic correlation between traits has also to be considered in selective breeding to avoid unintentional selection against an important trait while selecting for another one. As mentioned above, molecular markers can also be used to select breeders in a process called marker assisted selection (MAS) which generally involves genetic linkage map and quantitative trait loci (QTL) analyses.

Inbreeding and hybridization

Inbreeding is the consequence of mating between related individuals (or self-fertilisation as an extreme form), which increases homozygosity by pairing alleles that are alike by descent. Inbreeding usually reduces the performance and viability of offspring, a phenomenon known as inbreeding depression. Inbreeding can lead to a greater likelihood of deleterious recessive alleles being paired and expressed leading to inbreed depression (Lynch and Walsh 1998). Hybridization can be defined as the mating between genetically distinct groups (e.g., families, strains, populations, sub-species, species). The term hybridization, strictly speaking, is generally used for interspecies crosses and the term

crossbreeding for within species crosses (Beaumont and Hoare, 2003). Due to the blurred species boundaries in close related taxa, the term hybridization will be used for both type of crosses in this document (Beaumont and Hoare, 2003).

Inbreeding can be beneficial when used as a mean of purifying lines (e.g., to produce animals for use in bioassay) or integrated in genetic improvement programs (e.g., linebreeding, crossbreeding). Hybridization can be undertaken to combine beneficial traits from two genetically distinct groups or/and to exploit heterosis (or hybrid vigor). Another potential application of hybridization is the production of sterile animals or unisex cohorts of animals, which generally increase productivity and/or avoid reproduction in the natural environment (e.g., to prevent uncontrolled reproduction of an introduced species). Heterosis (or hybrid vigor) can be defined as the phenomenon in which the performance (i.e., the mean score for a particular quantifiable character) of the hybrids is superior to the performance of best parent line (Griffing, 1990). The combination of both inbreeding and hybridization to exploit heterosis had a major impact in the production of some agricultural crops (Crow, 1998). For these species, the exploitation of heterosis generally involves three stages: (1) selection of the individuals to start the process; (2) development of inbred lines to increase homozygosity; and (3) crossing inbred lines. To a lesser extent, inbreeding and hybridization had also an important impact in the production of farm animals (Hulata, 2001; McAllister, 2002).

Inbreeding depression has been reported in several bivalve species, including *C. virginica* (Longwell and Stiles, 1973), the catarina scallop *Argopecten circularis* (Ibarra *et al.*, 1995), and *C. gigas* (Beattie *et al.*, 1987; Hedgecock *et al.*, 2001; Evans *et al.* 2004). In studies in which inbred lines of *C. gigas* were crossed, F1 hybrids showed hybrid vigour for growth and survival during larval and juvenile stages (Hedgecock *et al.* 1995, 1996; Bayne *et al.*, 1999; Pace *et al.*, 2006). These studies supported previous results that indicated substantial non-additive genetic variation for some *C. gigas* traits (Lannan, 1980; Hedgecock *et al.*, 1991). However, it should be pointed out that the main objective of a breeding program is to produce hybrids with a better performance than the outbred population and not a mere recover from the accumulated effects of inbreeding depression (Falconer and Mackay, 1996). Hence, further studies are needed to evaluate the putative use of heterosis in bivalves, as have been done in some plants and vertebrate animals (Crow, 1998). A high load of deleterious recessive mutations was reported in oysters, which supported the dominance theory of inbreeding depression and the complementary phenomenon of heterosis (Bierne *et al.*, 1998; Launey and Hedgecock, 2001).

Hybridization between individuals from closely related species, sub-species or genetically different populations of bivalves has also been conducted. Hybridization between genetically different groups (that have not been selected or inbred) can result in

hybrid vigour or in a combination of favorable characteristics. Several studies reporting hybridization between different bivalve taxa have been conducted, namely: *M. edulis* X *M. galloprovincialis* (Beaumont *et al.*, 2004); *M. mercenaria* X *M. campechiensis* (Menzel, 1986); *C. gigas* X *C. rivularis* (Allen and Gaffney, 1993); and *C. gigas* X *C. angulata* (Bougrier *et al.*, 1986, Soletchnik *et al.* 2002). Generally the hybrids produced did not grow faster or survive better than the best parental species at the different development stages analyzed. Hybridization between different populations were also conducted in several bivalve species, including *C. gigas* (Imai and Sakai, 1961), *C. virginica* (Mallet and Haley, 1983; Mallet and Haley, 1984), *M. mercenaria* (Camara *et al.*, 2006) and *A. circularis* (Cruz and Ibarra, 1997; Cruz *et al.*, 1998). No evidences of useful heterosis or combination of favourable qualities were observed in these studies, although some suggest the presence of non-additive genetic variation.

Chromosome manipulation

Like the majority of higher animals and plants, commercially important bivalve species are diploid. However, it is viable for these species to possess three (triploids) and four (tetraploids) chromosomes for each pair (Beaumont and Fairbrother, 1991). Most bivalve species are amenable to chromosomal manipulation since: (1) fertilization occurs externally, (2) eggs are spawned before meiosis I is completed, and (2) activation by spermatozoa is required before meiosis will proceed (Beaumont and Hoare, 2003). Chromosomal manipulation can be achieved by subjecting eggs to physical (e.g., temperature, pressure) or chemical (e.g., cytochasin B, 6-dimethylaminopurine) shocks. These techniques allow the production of triploids and tetraploids throughout the retention of the chromosomes destined to enter the polar bodies. Either meiosis I or meiosis II divisions can be targeted (for triploid production) as well as first cleavage (for tetraploid production). However, tetraploids produced by these techniques are usually non-viable. Another technique which can be used to produce tetraploids is by suppressing the meiosis II division in eggs of triploids and fertilizing them with haploid spermatozoa (Guo and Allen, 1994). This will produce different ploidies including tetraploids. Adult tetraploids will produce diploid gametes that combined with haploid gametes produce triploids which are called interploid triploids. This method is preferentially used since it produces 100 % triploid offspring whereas chemical shocks are seldom 100 % efficient and imply the use of toxic substances (Guo *et al.*, 1996). Triploids are created for three main reasons: (1) higher growth rate comparatively with diploids; (2) constant taste and texture; and (3) sterility. However, some studies have showed that some triploids can produce viable gametes and triploids can revert to diploidy (Beaumont and Hoare, 2003). Triploids have been produced in many bivalve species, including *C. gigas*, *C. virginica*, *P. fucata*, *A.*

irradians, *Pecten maximus*, *M. edulis*, *M. mercenaria*, *R. philippinarum* (Beaumont and Fairbrother, 1991).

1.3. PATHOLOGY OF BIVALVE MOLLUSCS

In this document disease is defined as any state in which the host is impaired in one way or another (e.g., growth rate decrease, immune system suppression, death) (Mortensen, 2000). Pathogenic agent (or pathogen) is defined as any organism which causes considerable damage to the host. The term virulence will be used to indicate the degree of pathogenicity of an organism and the expression symbiotic fauna to describe organisms (mostly commensals) that are thought to cause no damage or only minor lesions to the host. Disease results of the interaction between three main factors: host, pathogenic agent and environment (Snieszko, 1973). Certain organisms may be present in the host for a long time without causing considerable damage, but if the balance between the factors mentioned above is disturbed they can become pathogenic agents (e.g., some viruses and bacteria). Other organisms may cause negligible damage when present at low number, but can be highly virulent at high number (e.g., ciliates). Hence, in certain cases, the distinction between pathogenic and symbiotic agent can be unclear.

Bivalve culture has been adversely affected by mass mortality caused by diseases (Renault, 1996). Moreover, the growing rate of molluscan aquaculture production increases the risk of zoosanitary problems. Some diseases have become a primary constrain to the development and sustainability of the bivalve mollusc aquaculture industry, causing a severe socio-economic impact in many countries. It is thought that contaminants can influence immune functions in marine bivalve molluscs leading to enhanced susceptibility to disease agents (Pipe and Coles, 1995). In other cases of mass mortality where the cause was not clearly identified, pathogens have been indicated as the most probable cause (Renault, 1996). Others causes responsible for mass mortality of cultured bivalves have also been pointed out such as environmental contaminants and bad husbandry practices. A better knowledge on the defense mechanism of bivalves, pathogenic agents and host-pathogen relationship is of paramount importance to define strategies to mitigate or eliminate problems cause by disease agents.

In this section an overview of the defence mechanisms of bivalves, major diseases of known aetiology and methods used to detect pathogens will be described. A more detailed description of *C. angulata* and *C. gigas* pathogens and abnormal mortality events are left to section 1.4.

1.3.1. Defence mechanisms

Despite considerable progresses that have been done since 1940s, little is still known about the defence mechanisms of bivalves comparatively to other farmed species such crustaceans and fishes (Sparks, 2005). There is a tendency to fill this gap by using what is known in other invertebrate species. It is thought that bivalves, like other invertebrates

animals, have only an innate immune system (*i.e.*, defence mechanisms against invading organisms and foreign substances that an individual is born with) and lack an adaptive immune system (*i.e.*, characterized by immunological memory and high specificity) such as is found in mammals (Iwanaga and Lee, 2005). Nonetheless, evidence for specific memory in a copepod was recently reported (Kurtz and Franz, 2003), which suggests that in some invertebrates adaptive immune response may be present. Although the immune systems of invertebrate animals do have similarities (Iwanaga and Lee, 2005), generalisations about their immunity may lead to erroneous conclusions due to the heterogeneous composition of this group of animals (that include all non-chordate animals). Even within bivalves differences may exist in the immune system of the different taxa that constitute this group.

The first line of defence of bivalves is the shell that constitutes a physical barrier that protects the animals from threat that can cause lesions (*i.e.*, septic injury) or even death. Under adverse environmental conditions that can lead to a higher susceptibility to pathogens, bivalves can close their valves and avoid water to enter with the help of the mantle. Epithelial surfaces of the mantle and other tissues also constitute a physical barrier that tends to prevent the entry of pathogens into the organism. It is also thought that certain tissues can segregate substances (*e.g.*, mucus in the gills) that can entrap and eliminate undesirable microorganisms. Another defence mechanism in bivalves is nacreization where the parasite or foreign material that invades the space between mantle and shell is covered with nacre (Cheng, 1981).

Once pathogens or foreign material have entered the organism the internal defence system of bivalves, like in all metazoan animals, must be capable of: (1) recognise invading organism and foreign substances (effector mechanisms); and (2) eliminate those threats without injure the host (effector mechanisms). The factors involved in recognition of pathogens and foreign material are thought to include both cellular (whole cell) and humoral (cell product) components, and two main types of response are thought to be involved: (1) a cellular response involving phagocytosis and encapsulation; and (2) a humoral response throughout the secretion of antimicrobial substances and other molecules in the hemolymph.

Immune cells and cellular response

Hemocytes have an important role in the internal defence system of bivalves, but they also have others functions including wound repair, shell repair, excretion, and nutrient digestion and transport (Cheng, 1981). A uniform classification of hemocytes has been attempted based on morphological and functional criteria (Cheng, 1981; Hine, 1999). Hemocytes can be roughly divided in: (1) granular hemocytes (containing many granules);

(2) agranular hemocytes (with few or no granules); and (3) brown cells (also called serous cells or rhogocytes). Granular and agranular hemocytes circulate in hemolymph but can also migrate to other tissues since they are capable of amoeboid locomotion. Granular hemocytes usually comprise the majority of hemocytes in bivalves and they have generally a high phagocytic activity. Agranular hemocytes may be further divided in three types of cells: blast-like cells; basophilic macrophage-like cells; and hyalinocytes (Hine, 1999). Brown cells are essentially non-mobile and have large pigment globules (light to dark brown) in the cytoplasm (Cheng, 1981). Brown cells aggregate in lesions and around parasites and are also capable of phagocytosing particles. However, the high heterogeneity in morphology and function observed in different bivalve species makes the classification of immune cells very difficult (Hine, 1999). For example some bivalve species do not have granulocytes at all (e.g., in some scallop species), so generalizations on the types of hemocytes present in bivalves species can be misleading. In *C. gigas*, the following types of immune cells have been described (Grizel *et al.*, 2003): two types of granulocytes (basophilic and acidophilic granulocytes, having both types eccentric nucleus); agranular hemocytes (with an eccentric nucleus); and hyalinocytes (characterized by a high nucleus/cytoplasm ratio and a central nucleus).

Inflammation is the immediate response of the organism to a wound and foreign invasions, which involves the constriction of hemolymph vessels and sinuses as well as infiltration of hemocytes (hemocytosis) to the infected or injured tissues (Fisher, 1986). This is generally the first step prior to the two main cellular responses: (1) phagocytosis and (2) encapsulation.

Phagocytosis

Phagocytosis involves the uptake of foreign biotic or abiotic materials (e.g., debris, protozoa, fungi, bacteria) by immune cells. Although it is thought that all immune cells are phagocytic, granulocytes are considered the most active ones. Phagocytosis can be divided into a number of stages:

- Attraction. The encounter between hemocytes and foreign particles may occur randomly or hemocytes can be attracted towards the target. It is thought that chemoattractant substances secreted or present in target (chemotaxis) are involved in the attraction process (Cheng, 1981);
- Recognition and adherence. It is thought that non-self recognition and adherence between hemocytes and foreign particles occurs throughout cell-surface receptor;
- Internalisation. Different uptake mechanisms have been described such as uptake by gliding along filopodia, gliding in a funnel-like pseudopod and invagination of cell surface.

Throughout one of these mechanisms the particles are taken into the cell and is enclosed in a vesicle called a primary phagosome;

- Elimination of foreign particulate matter. The primary phagosome may then fuse with lysosomes, also called secondary phagosomes, to form phagolysosomes. Cytoplasmic granules of hemocytes have been interpreted as being lysosomes. Digestible material is then degraded by the enzymes and antimicrobial peptides present in the lysosomes. Foreign particles entrapped in the primary phagosome can also be destroyed throughout the generation of reactive oxygen intermediates. Intact particles may also be physically removed through migration of laden hemocytes, which mainly occurs in the digestive organs and labial palps.

During phagocytosis, hydrolytic enzymes and antimicrobial peptides present in lysosomes are used to eliminate foreign particles as mentioned above. Several enzymes have been identified in bivalve hemocytes that are thought to be involved in intracellular digestion of foreign particles. Another mechanism used by immune cells to eliminate phagocytose particles is the production of reactive oxygen intermediates (ROIs) namely, O_2^- , H_2O_2 , OH^- and OCl^- . In order to protect tissues from oxidative damage caused by ROIs specific antioxidant enzymes are produced (Bachère *et al.*, 2004).

Encapsulation

Encapsulation is the mechanism by which hemocytes eliminate a pathogen or particle that is too large to be phagocytosed. It is thought that hemocytes differentiate into fibro-like cells that surround the pathogen or particle forming a thin capsule of cells which is then ringed by a very thick capsule composed of fibrous (Fisher, 1986). It is thought that hemocytes are not preconditioned to either encapsulate or phagocytose, since the first hemocytes that reach the target try to phagocytosis it and once when they fail the process of encapsulation is initiated.

Humoral response

Hemocytes and other cells secrete a variety of substances into the hemolymph that are used to recognize and eliminate foreign particles (Cheng, 1983). It is thought that during phagocytosis lysosomal enzymes are secreted by degranulation, but it is also possible that other cells besides hemocytes can also be involved in the humoral response. Several substances are thought to be released into the hemolymph. Hemolymph lectins are thought to be synthesized by immune cells binding to carbohydrate determinants of membrane glycoproteins or glycolipids resulting in agglutination of foreign particles (Pipe and Coles, 1995). Recently, it was reported an antiviral effect in the hemolymph of *C. gigas*, but the putative substances involved remain unknown (Olicard *et al.*, 2005). The

existence of tissue inhibitors of metalloproteinases (TIMPs) in *C. gigas* was suggested by Montagnani *et al.* (2001). These proteinases have been reported to play an important role in pathogen invasion (e.g., viruses, bacteria). The expression of a TIMP-like in *C. gigas* (called Cg-TIMP) was observed only in hemocytes of animals exposed to bacterial challenge (*Vibrio* sp.) and shell damage (Montagnani *et al.*, 2001).

Apoptosis

Apoptosis is the process of programmed cell death that is used in several metazoan to eliminate unneeded, infected or deleterious cells. It was observed that *C. gigas* hemocytes are able to induce apoptosis, which may have an important role not only in preventing pathogen dissemination but also participate in the maintenance of the immune system homeostasis (Lacoste *et al.*, 2002).

Phenoloxidase activity

The prophenoloxidase (ProPO) system has been suggested has one of the important defence mechanisms of invertebrates. The phenoloxidase (PO) is an enzyme responsible for production of melanin that exists in the hemolymph under the inactive form of ProPO. Melanin is involved, among other physiological processes, in encapsulation of foreign particles, wound healing and generation of toxic intermediates such as reactive oxygen species (ROS) that may combat infections (Kim and Kim, 2005). The activation of the ProPo system occurs in response to microbial substances (e.g., exogenous proteases, microbial cell). Evidence of the presence of phenoloxidase-like activity has been reported in several bivalve such as *M. edulis*, the carpet shell clam *Ruditapes decussatus* and the Manila clam *R. philippinarum* (Coles and Pipe, 1994; Muñoz *et al.*, 2006). Recently, a true phenoloxidase activity was demonstrated in *C. gigas* by Hellio *et al.* (2007).

Signaling pathway

Recent studies in *C. gigas* have identified genes related to the Rel/NF- κ B signal pathway, which is a key component of pathogen recognition and activation of the response that is conserved throughout much of the animal kingdom (Montagnani *et al.*, 2004). These findings support the concept of a Rel/NF- κ B signal pathway in bivalves.

1.3.2. Major diseases

Pathogens of bivalves are known to include viruses, bacteria, fungi and protozoa. Some metazoan such as helminths, annelids and crustacean are considered to be only slightly pathogenic to bivalves or even merely symbiotic fauna. Several viruses belonging to different families have been observed in bivalves since the 1970s. The pathogenicity of

these viruses in most cases was not demonstrated due to difficulties in their isolation and culture mainly due to the lack of bivalve cell lines. Nevertheless, mass mortality of bivalves associated with these viruses has been reported. In Europe, mass mortalities of *C. angulata* were associated with two irido-like viruses between late 1960s and 1970s (Comps, 1988). More recently high mortalities caused by Ostreid herpesvirus 1 (OsHV-1) were also observed. This virus affect larvae and juveniles of different species namely, *C. gigas*, *C. angulata*, *C. rivularis*, *O. edulis*, *R. decussatus*, *R. phillipinarum* and the King scallop *Pecten maximus* (Renault and Novoa, 2004). Experimental transmission of OsHV-1 was demonstrated under experimental conditions (Arzul *et al.*, 2001). Disseminated neoplasia, consisting of proliferation of abnormal circulating cells, although of uncertain aetiology, is thought to be caused by viral infections (Elston *et al.*, 1992). Other viruses have also been observed in bivalves, interpreted as members of the *Papovaviridae*, *Togaviridae*, *Reoviridae*, *Birnaviridae* and *Picornaviridae* (Renault and Novoa, 2004). Several diseases caused by bacteria have been described, mainly belonging to the genus *Vibrio*, *Pseudomonas*, *Aeromonas*, *Nocardia* and *Rickettsia* (Paillard *et al.*, 2004). Bacteria that provoke high mortalities belong generally to the genus *Vibrio* and mostly affect larval cultures (e.g., *V. alginolyticus*, *V. anguillarum*, *V. tubiashi*, *V. splendidus*). One of the few well-documented bacterial disease that affects adults and juveniles of bivalves is the Brown ring disease. This disease is caused by *V. tapetis* and is responsible by mass mortality of adult and juvenile *R. phillipinarum* (Paillard *et al.*, 1990). Few pathogenic fungi have been described in bivalves (e.g., *Ostracoblabe implexa*, *Sirolpidium zoophthorum*,) and the ones observed were not generally associated with high mortalities (Bower, 1994). Among the different pathogens, protozoan are the most common cause of the diseases in bivalves that result in high mortalities of adult animals. The production of the oyster *C. virginica* in USA has been seriously affected by the protozoan pathogens *Perkinsus marinus* (Dermo disease), *Haplosporidium nelsoni* (MSX disease) and *H. costale* (Seaside disease) (Renault, 1996). In Europe, the protozoan *Bonamia ostreae* (Bonamiasis) and *Marteilia refringens* (Aber disease) were responsible for mass mortalities of *Ostrea edulis* (Renault, 1996). Another protozoan responsible for high mortalities is *M. sydneyi* that can cause high mortality in the mangrove oyster *S. glomerata* in Australia (Perkins and Wolf, 1976).

1.3.3 Control measures and detection tools

The transfer and introduction of live aquatic organisms has been recognized as the major cause of disease outbreaks and epizootics. One of the few ways to mitigate the impact of diseases is throughout the implementation of programs to avoid the introduction of pathogens from a region or country where they are present into geographic areas free of

these pathogens (Berthe *et al.*, 1999). In the case of hatchery produced seed it is possible to implement control measures to prevent vertical and horizontal transmission of infectious disease agents, including: (1) selection of non-infected progenitors; (2) disinfection of eggs (in cases where the pathogen is only attached to the surface of the eggs); (3) control of the environmental conditions that can lead to disease outbreaks; and (4) implementation of cleaning and disinfection practices. During the nursery and grow-out phase it is very difficult or even impossible to apply traditional measures used in other culture aquatics species. Nevertheless, some measures can be implemented such as the use of good husbandry practices that reduce the impact of diseases.

The use of adequate diagnostic tools is a key point to determine the cause of diseases as well as the mode of action of pathogenic agents. Most of the investigation and routine detection of pathogens has been done by histological examination using light microscopy. This technique provides a large amount of information since several tissues are examined. Histology allows not only the detection of pathogenic agents but also the observation of histopathological alterations and assessment of the physiological condition of the animal (*e.g.*, maturation stage). Other complementary techniques such as tissue imprints, squashes and smears have also been used. Although these techniques are not as informative as histology, they can provide quick answer and in some cases more detailed information (*e.g.*, tissue imprints for the detection of bonamiosis). Transmission electron microscopy (TEM) allows the ultrastructural examination of pathogenic agents and hence its used in confirmatory identification in most cases. The use of TEM in the last decades led to an explosion of knowledge of diseases of bivalves (Sparks, 2005). Some techniques are also used to culture putative pathogens and facilitate their detection such as the fluid thioglycollate medium assay for perkinsosis and specific media to grow and isolate bacteria. However, histology and TEM are time consuming and some pathogens can be difficult to detect and recognise, particularly at low number. More recently, the use of molecular tools such as immunoassays and nucleic acid-based techniques allowed the development of quick, sensitive and specific diagnostic tests. Some DNA-based techniques (*e.g.*, *in situ* hybridization, PCR) have been developed to detect some of the major pathogens of bivalves such viruses, bacteria and protozoan. The sequencing of DNA from these pathogens can be very informative in order to help clarify their taxonomy and biology. However, the use of these techniques can result in false negative and positive results. Hence, it is of central importance to develop, validate and standardise molecular tools for detection of bivalve pathogens.

1.4. THE STUDIED SPECIES: *CRASSOSTREA ANGULATA* AND *C. GIGAS*

In this section a bibliographic review about different aspects of the biology of *C. angulata* and *C. gigas* is presented with special emphasis on the former taxon. Most of the studies done on *C. angulata* have been performed with animals introduced in France from Portugal, but some studies have also been conducted in animals from populations in Portugal and Spain.

1.4.1. Systematic description

The high intraspecific variation of oyster shells, which is highly influenced by the environment, greatly complicate classification of oysters based on morphological characters, especially in some genera such as *Crassostrea* and *Saccostrea*. Stenzel (1971), in the “Treatise on Invertebrate Paleontology”, provided a review of the most important works in ostreid taxonomy. He pointed out the difficulties to identify and classify oysters correctly, especially on the various hierarchical levels above species and superspecies. The classification of Stenzel (1971) was reviewed and somewhat modified by Harry (1985) and is shown in Figure 6.

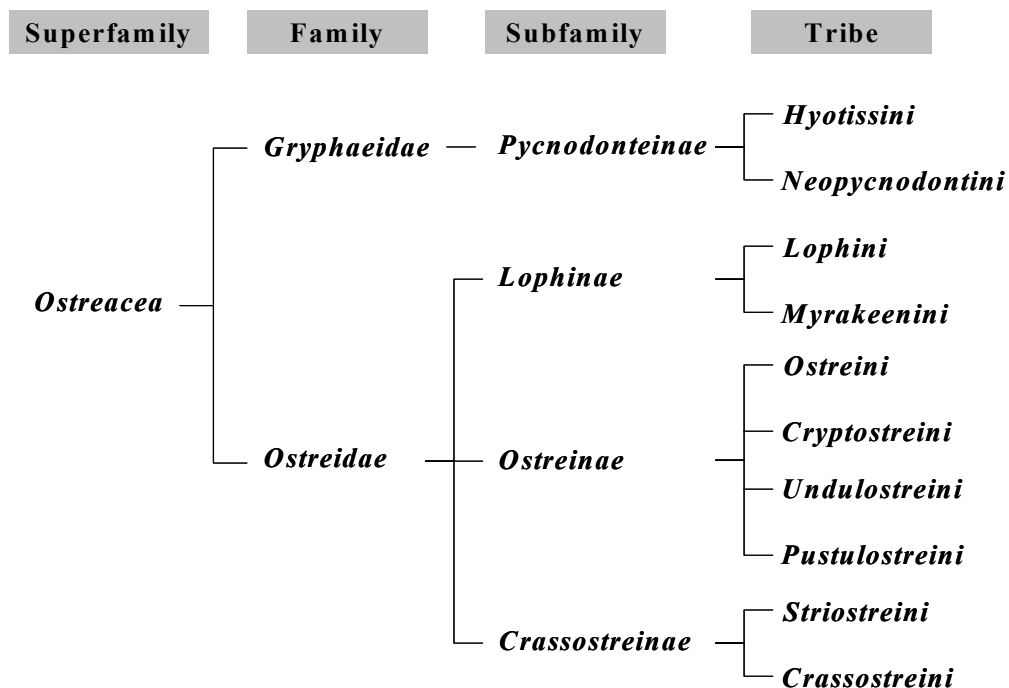


Figure 6. Classification of living oysters according to Harry (1985).

In species of the superfamily *Ostreacea* the left valve is usually attached to a firm substrate, during at least early post-larval life. Shells are inequivalve and somewhat inequilateral. There are no hinge teeth in adults comparable to those of most other

bivalves, but in *Ostreacea* some form of ridges or pustules called chomata (Stenzel, 1971) are present in some species. The ligamental area is divided into three parts with resilifer in middle. The functional ligament is elongate and thin extending before and behind the umbos along the hinge. After the larval phase, only the posterior shell adductor muscle is retained and the foot is lost. Mantle opens all along margins, except at the point of palliobranchial fusion. In the family *Ostreidae* the shape of the scar of the adductor muscle is frequently reniform and the commissural shelf is usually absent. The left promyal passage is always closed and the accessory heart is present. The rectum passes completely behind the pericardium and does not pierce the heart. In the subfamily *Crassostreinae* shells have a medium to large size, usually elongate dorsoventrally. The left valve is usually deeply cupped and the right one is nearly flat. The mantle lobes are thickened and the right promyal passage is open, but narrow. In the tribe *Crassostreini* the chomata is absent throughout life. The adductor muscle scars of both valves tend to have a dark pigmentation and the interior of the valves is white. In the genus *Crassostrea*, valves of the prodissoconch are unequal, hinge has two teeth at each end and the internal anterior ligament extends beyond the hinge (Ranson, 1960). Adult shell is very variable, usually elongated and the left valve is cuplike. The adductor muscle scar is displaced in dorsolateral direction and promyal chamber on the right side is large. The rectum ends at posteroventral curve of the adductor muscle and the anus is usually without any appendage. Species of this genus do not brood their larvae and eggs are relatively small.

The Portuguese oyster was first called *Gryphaea angulata* by Lamarck (1819) due to similarity of the shell of this species with fossil *Gryphaea* species, and latter designated *Ostrea angulata* and by other authors *O. (Gryphaea) angulata*. The Pacific oyster was initially described as *O. gigas* by Thunberg (1793). Based on conchological features, anatomical and spawning habits these two taxa are now included in the Genus *Crassostrea* (Sacco, 1897) and known as *C. angulata* and *C. gigas*. The systematic position of the Portuguese and Pacific oysters according to Harry (1985) is shown in Table 1.

Table 1. Systematic position of *C. angulata* and *C. gigas*.

Phyla	Mollusca
Classe	Bivalvia
Order	Pterioida
Superfamily	Ostreacea
Family	Ostreidae
Subfamily	Crassostreinae
Tribe	Crassostreini
Genus	Crassostrea
Species	<i>Crassostrea gigas</i> (Thunberg, 1793) <i>Crassostrea angulata</i> (Lamarck, 1819)

1.4.2. Geographic distribution and origin

The Portuguese oyster *C. angulata* was initially described by Lamarck to be native of southwestern Europe. In 1867, a small number of *C. angulata* from Portugal was introduced in the Arcachon region (France) (Edwards, 1976). One year after, it is thought that the whole shipment of boat called “Le Morlaisien”, transporting *C. angulata* oysters from the mouth of the River Tagus (Portugal), was thrown overboard in the Gironde estuary (France) (Edwards, 1976). Soon after, *C. angulata* colonized several regions of the Atlantic French coast and it was subsequently introduced in other European countries (Zibrowius, 1991). Nowadays, populations of this species can be found along the south and east coasts of Portugal and Spain, and in the Atlantic coast of Morocco (Fabioux *et al.*, 2002). Surprisingly, populations of *C. angulata* were also reported in Taiwan (Boudry *et al.*, 1998).

The Pacific oyster or Japanese oyster *C. gigas* is presumed to be native to the North West Pacific region. Due to its commercial value, fast growth and resistance to diseases, *C. gigas* was successfully introduced in several regions and has now a worldwide distribution. The first unofficial introductions of *C. gigas* in Europe probably occurred in the late 1960's in response to the declining production of *C. angulata* due to high mortalities as well as the fast growth of *C. gigas* (Grizel and Héral, 1991). At that time *C. gigas* was introduced in France and Britain (Grizel and Héral, 1991; Steele and Mulcahy, 1999). The official introduction of *C. gigas* in France occurred between 1971 and 1975, where large quantities of oysters were imported from British Columbia (Canada) and Japan (Grizel and Héral, 1991). To date, naturalized populations of *C. gigas* can be found in Europe, west coast of North America (including Canada and USA; Quayle, 1988), South African coast (Robinson *et al.*, 2005), New Zealand (Smith *et al.*, 1986) and

Australia (English *et al.*, 2000). The present known distribution of naturalised populations of *C. angulata* and *C. gigas* is shown in Figure 7.

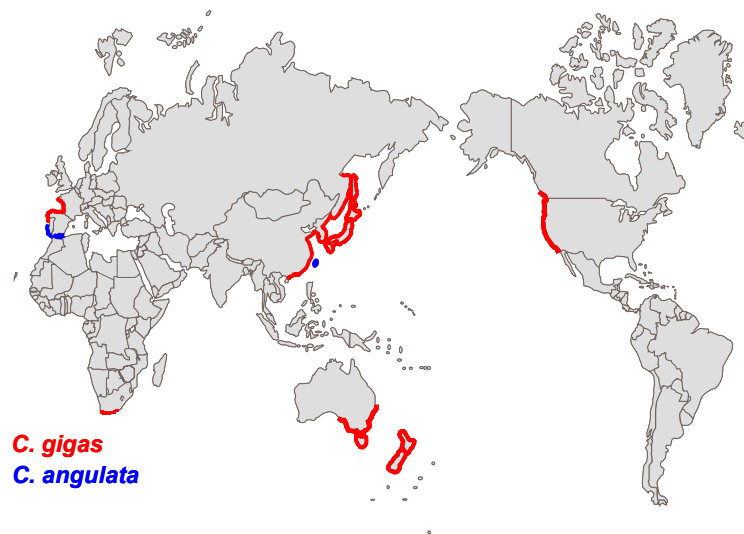


Figure 7. Geographic distribution of native and introduced populations of *C. angulata* and *C. gigas*.

The disjunctive geographical distribution of the genetic close related oysters *C. angulata* and *C. gigas* lead to the question of their origin. Three hypothesis have been proposed to explain their geographic distributions: (i) *C. angulata*, *C. gigas* and *C. cattuckensis* (India) derived from a fossil ancestor *C. gryphoides* and toward the end of the Miocene and later, tectonic events produced land barriers, isolating three populations, resulting in the distribution of the three species (Stenzel, 1971); (ii) *C. angulata* was transported from Europe to Asia some centuries ago probably by European merchants ships (Menzel, 1974); (iii) *C. angulata* was introduced in Europe by undocumented anthropogenic transfer during the earliest days of circumglobal navigation from Asia (Ranson, 1960). Based on mitochondrial DNA sequence data, O'Foighil *et al.* (1998) estimated a divergence time of 1 to 2 million years for the Portuguese and Pacific oysters, long after closure of the Tethyan Seaway estimated in approximately 7 million years ago. Although this study may overestimate oysters divergence times, it clearly undermine Stenzel's hypothesis, suggesting that the closure of the Tethyan Seaway occurred before the appearance of the last common ancestor of the Portuguese and Pacific oysters. Indeed, Hedgecock *et al.* (2004) suggested that the divergence between *C. angulata* and *C. gigas* is less then one million year, which indicate that their divergence is even more recent. According to Ranson (1948, in Edwards, 1976) there are no evidences of the presence of *C. angulata* on the Miocene, Pliocene and Quaternary beds of Portugal,

which supports the hypothesis of a recent introduction of *C. angulata* in Europe from another region. On the other hand, Lawrence (1995) argued that the fossil record does not promote the notion that the Portuguese and Pacific oysters may have been imported into the eastern Atlantic by humans. In fact, *C. angulata* have been reported to exist for at least 2200 years BP in Spain (Ruiz *et al.*, 2004). It is also possible that other *Crassostrea* species, native of the eastern Atlantic, have derived from *C. gryphoides*, but are no longer present in Europe, could have been incorrectly identified in the late Holocene as *C. angulata*. Another possibility is that in some palaeontological studies other species of oysters were mistakenly classified as *C. angulata* because of the high morphologic plasticity of oysters which is highly influenced by the environment. The close genetic relationship between *C. angulata*, *C. gigas*, and other *Crassostrea* species from Asia such as the Kumamoto oyster *C. sikamea* and *C. ariakensis* suggests that *C. angulata* has an Asian origin (Buroker *et al.*, 1979; O'Foighil *et al.*, 1998; Boudry *et al.*, 2003). Karyotype and chromosomal location of the major ribosomal genes determined by fluorescence *in situ* hybridization also support an Asian origin of *C. gigas* and *C. angulata* (Wang *et al.*, 2004). Moreover, studies using mitochondrial markers reported the presence of pure populations of *C. angulata* in Taiwan (Boudry *et al.*, 1998). Consequently, Taiwan has been proposed as a possible origin of European *C. angulata* populations (Boudry *et al.*, 1998; Huvet *et al.*, 2000). All these findings suggest that *C. angulata* has an Asian origin and is probably a case of recent undocumented anthropogenic introduction (see Article 6 in Appendices).

1.4.3. Habitat

Crassostrea angulata and *C. gigas* are found in the low intertidal to subtidal (low depths) levels in estuaries, lagoons and rias. Populations of *C. angulata* in Portugal are also observed several kilometres upstream (Ruano, 1997). These two taxa are commonly found on hard and soft bottoms of mud, hard silt, muddy sand, rocks, muddy gravel with shells, or on different man-made materials. Both *C. angulata* and *C. gigas* adults are euryhaline, being able to survive in low salinities for short periods (2-6 ‰) to fully marine conditions (Héral and Deslous-Paoli, 1991; Ruano, 1997), but are more commonly found in brackish waters.

1.4.4. Comparative anatomy

The anatomy of oysters after metamorphosis is simpler than most bivalves due to the absence of various organs and structures (Galtsoff, 1964). Moreover, several organs have different functions which greatly reduce the number of specialised organs. The anatomy and function of the organs of *C. angulata*, *C. gigas* and other *Crassostrea* species are

quite similar and have been described by several authors namely, Leenhardt (1926, in Stenzel, 1971), Nelson (1938, 1960 in Evseev *et al.*, 1996), Galtsoff (1964), Stenzel (1971), Vilela (1975), Quayle (1988), and Evseev *et al.* (1996). Hence, the anatomy and function of the main organs of *C. angulata* and *C. gigas* will be jointly described and whenever the case differences between the two will be highlighted.

Morphology and structure of the shell

Crassostrea angulata and *C. gigas* possess a shell that covers the soft body, protecting the animal during the different stages of their life cycle.

Larval shell

The shape of larval shell is less irregular and presents a more complex hinge apparatus than adults. Although the morphology of *C. angulata* and *C. gigas* larval shell varies during its development it can be characterized by the following features according to Ranson (1948, 1960), Pascual (1971a) and Hu *et al.* (1993): (1) the shell is inequivalve; (2) at an early phase, the length (L) of the left valve is superior to the height (H); (3) after approximately 110 μm in left valve length, $H > L$; (4) internal ligament anterior to the hinge; (5) two rectangular teeth on each side of the provinculum on both left and right valves; (6) relative short provinculum with a well-defined central apparatus; and (7) a knobby umbo directed posteriorly (Figure 8).

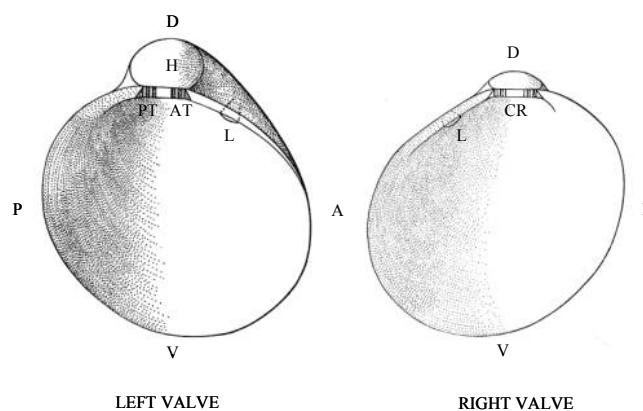


Figure 8. Left and right valves of a late *C. angulata* larva. A, anterior margin; AT, anterior provincular teeth; CR, central apparatus ridge; D, dorsal margin; H, hinge; L, ligament; P, posterior margin; PT, posterior provincular teeth; V, ventral margin. X 165 (adapted from Ranson, 1960).

Ranson (1960) reported the absence of morphological differences between late larval shells of *C. angulata* and *C. gigas*, whereas he could differentiate other species of the genus *Crassostrea* using the same approach. However, Galtsoff (1964) stated that differences in shell morphology allowed the identification of oysters only at the genus level (*i.e.*, *Ostrea*, *Crassostrea*, *Pycnodonta*), since he could find no differences among species within the genus *Crassostrea* (*e.g.*, *C. virginica* in comparison with *C. gigas*). More recently, Hu *et al.* (1993) reported the existence of morphological differences between *C. virginica* and *C. gigas*. The previous authors pointed out that quantitative measurements are needed to differentiate species of the same genus whereas qualitative characters (*e.g.*, hinge dentition, shell shape) are only useful to separate species of different genera.

Adult shell

In *C. angulata* and *C. gigas* adults the two valves that constitute the shell have different size and shape and hence the shell is said to be inequivalve. The left valve is the bigger one and is frequently deeply cupped (Figure 9).

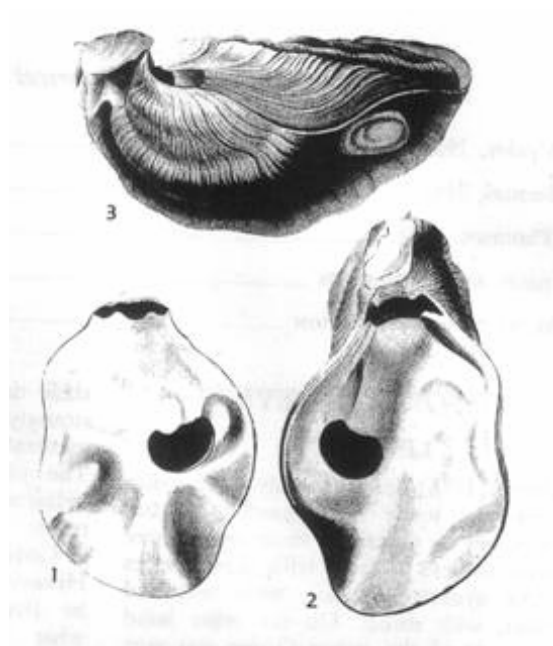


Figure 9. Adult *C. angulata* (Lamarck, 1819) shell (after Delessert, 1841 in Stenzel, 1971).
(1) Right valve; (2) left valve; (3) both valve together viewed from the posterior side.

The right valve is usually flat or slightly convex. The distance between the dorsal and ventral margins is generally longer than the distance between the anterior and posterior

margins. The shape of oysters shell is highly variable and is generally a result of the type of substrate on which the individuals grow as well as other factors such as the degree of crowding (Quayle, 1988). In some cases, the outside surface configuration of one valve closely resembles the substratum on which the other valve (generally the left valve) is attached which is called xenomorphism (Stenzel, 1971). It is important to point out that shell shape is influenced by the substratum in two separate ways: (1) in a non heritable way where shell shape variation is merely due to the subtract and environmental conditions under which the oysters grow; and (2) in a heritable way where the adaptation to a specific substratum and/or environment may result in variation in shell shape within or between species (Stenzel, 1971).

According to Galtsoff (1964), the shell of *Crassostrea* species consists of four layers: (1) the periostracum, an outer layer extremely thin; (2) the median prismatic layer, which is especially well developed on the right valve; (3) the ostracum layer, an inner layer; and (4) the hypostracum, a very thin layer that is present only under the place of attachment of the adductor muscle. The different layers are composed mainly by calcium carbonate (CaCO_3) crystals and proteins. The type of CaCO_3 depends on the protein framework of the different layers, for example calcite is present on the prismatic layer and aragonite in the ostracum layer (Sudo *et al.*, 1997). The hinge ligament is located on the umbral region and has a different structure and function from the shell. It is formed of conchyolin similar to that of the main shell, but the amount of calcification is much reduced resulting in a elastic material (Quayle, 1988). The function of hinge ligament is to spring the valves apart and, jointly with the action of the adductor muscle that olds the valves together, control the opening and closing of the shell.

Menzel (1974) and Biocca and Matta (1982) mentioned that the adult shells of *C. angulata* and *C. gigas* are indistinguishable, albeit no morphometric analysis were performed and the criteria's used to differentiate them were not specified. On the other hand, Galtsoff (1964) reported that the adductor muscle scar of *C. angulata* was highly pigmented whereas in *C. gigas* pigmentation was either absent or very light.

Body and function of the main organs

Larval stage

The anatomical structure of an oyster larva suffers considerable changes during its development and is very different from an adult oyster. Some organs and structures are only present during the larval phase such as the velum, foot, byssus gland, statocysts and the anterior adductor muscle. The main organs of a fully developed larva of a species of the genus *Crassostrea* is shown in Figure 10. The velum is a circular platform covered with cilia, which can be protruded between the valves. By means of the velum, the larva is

able to swim and to collect food. The larval sense organs comprise a pair of statocysts and a pair of dark pigmented eyes. A foot, which is covered with strong cilia, appears in ready to settle larvae and allow them to crawl in order to find a suitable surface for settlement. At the base of the foot is the byssus gland whose function is to produce a cement that will attach the larva to the substratum. All these structures as well as the anterior adductor muscle disappear or become dysfunctional after metamorphosis. The other structures have a similar function as during the adult phase and will be described below.

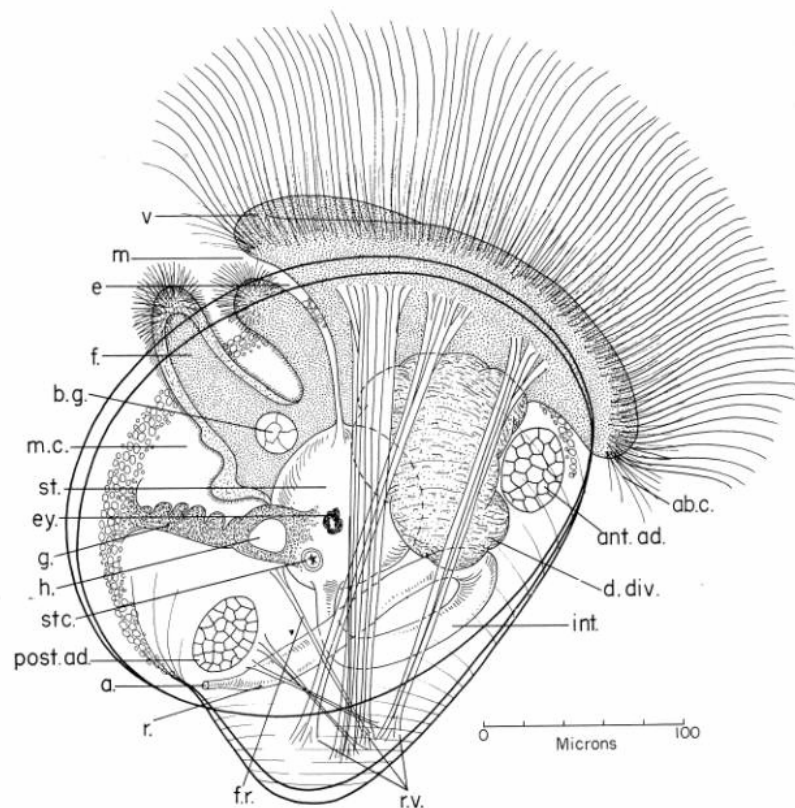


Figure 10. Fully developed larva of *C. virginica* (after Galtsoff, 1964). a., anus; ab.c., aboral circle of cilia; ant.ad., anterior adductor muscle; b.g., byssus gland; d.div., digestive gland; e., esophagus; ey., eye; f., foot; f.r. foot retractor muscles; g., gill rudiment; h., heart; int., intestine; m., mouth; m.c., mantle cavity; post.ad., posterior adductor muscle; r., rectum; r.v., velar retractor muscles; st., stomach; stc., statocysts; v., velum.

Adult stage

In an adult *Crassostrea* sp. the fusion of the body with the shell occurs mainly at the adductor muscle, but also at the Quenstedt's muscles (a pair of tiny muscles) located just

back to the hinge. The body is covered by the right and left folds of the mantle, which are joined together at the dorsal edge and at a point on the ventral margin. This point serves to separate the area of the exhalant water current from the inhalant. The exhalant area is divided by the adductor muscle, with water being discharged through two passages: (1) the cloacal passage, ventral to the adductor muscle; and (2) the promyal passage, dorsal to the adductor muscle. Under the free edges of the mantle lie several organs and structures that are shown in Figure 11.

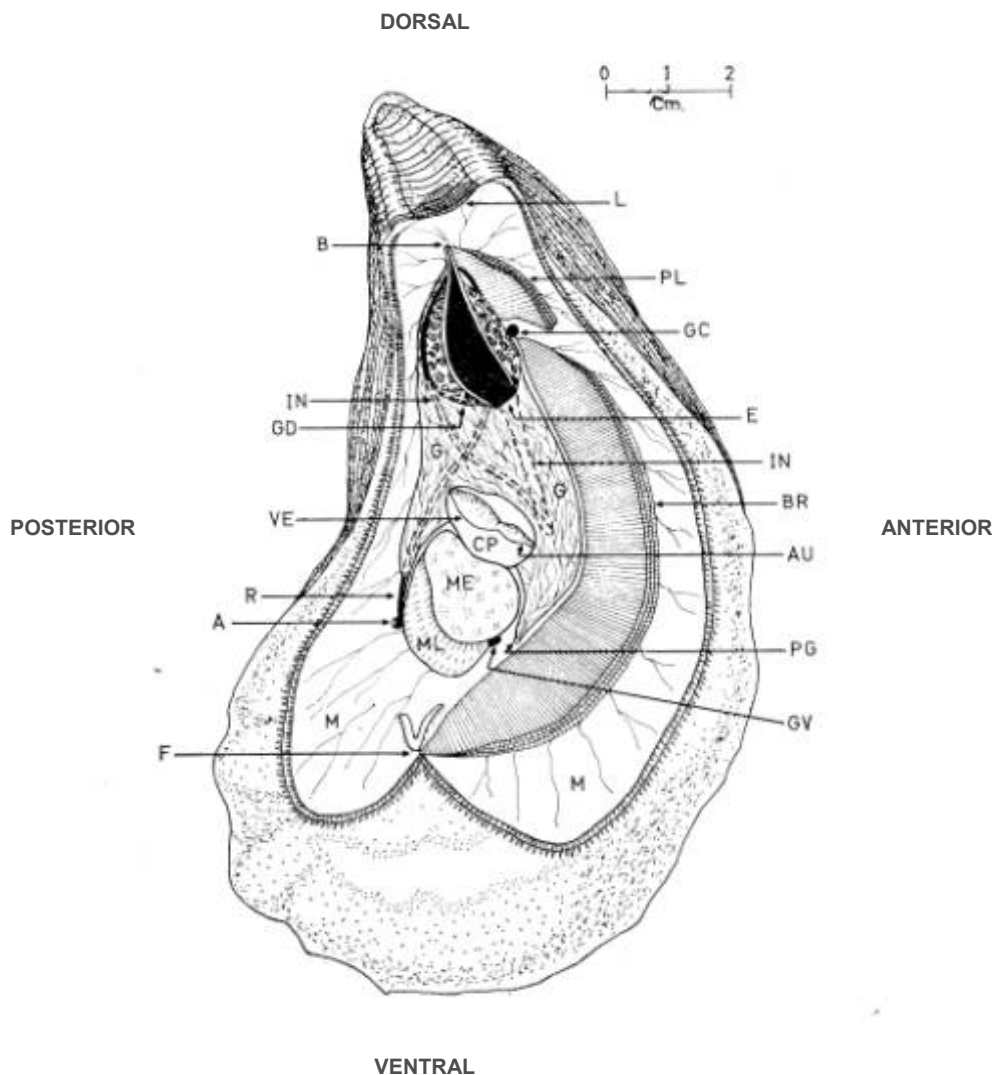


Figure 11. Adult *Crassostrea* sp. with right valve and part of mantle removed (after Vilela, 1975). A, anus; Au, auricle; B, mouth; Br, gills; CP, pericardial cavity; E, stomach; F, fusion of two mantle lobes and gills; G, gonad; GC, cerebral ganglia; GD, digestive gland; GV, visceral ganglia; IN, intestine; L, hinge ligament; M, mantle; ME, striated part of the adductor muscle; ML, smooth part

of the adductor muscle; PG, opening of the urinogenital vestibule; PL, labial palps; R, rectum; V, ventricle.

1- Mantle

As indicated previously, the mantle consists of two lobes joined at a dorsal and ventral position. At the dorsal region the fusion of the two lobes form the cephalic hood over the mouth and labial palps. The mantle consists of a sheet of connective tissue containing muscles, nerves and blood vessels. The mantle edge is formed by three folds: (1) the inner fold; (2) the middle fold; and (3) outer fold. The inner fold is highly mobile, well supplied with muscles and blood sinuses, with equally tentacles. The middle fold is heavily tentacled with tentacles with two sizes. The outer fold is separated from the middle one by the periostracal groove. The main functions of the mantle are: (1) formation of the shell and ligament; (2) storage of reserve materials (glycogen and lipids); (3) reception of sensory stimuli (physical and chemical); (4) respiration (particularly in young oysters); (5) excretion of hemocytes and waste products; (6) participation in the dispersal of eggs during spawning; and (7) to avoid the passage of water over the whole length of the mantle edge or in certain areas.

2- Gills and labial palps

The oyster gills (or ctenidia) consist of two pairs of demibranchs, one pair at each side of the animal. Each pair is composed of four lamellae arranged to form a "W". Inter-lamellar junctions joined the lamellae of each demibranch, forming the water tubes. Lamellae are formed of filaments that are joined together by interfilamentary tissue, leaving spaces (ostia) which allow water to pass between filaments. Filaments are the structural unit of the gill and are folded in groups called plicae. To support the filaments and the various junctions, small rods of chitinous material are embedded in the tissue. The filaments are mainly composed of connective tissue, blood vessels and muscles. The whole gill system is provided with cilia which create a flow of water from inhalant to the exhalant chamber. Gills fulfill several functions such as: (1) respiration; (2) capture of food particles; (3) uptake of nutrients and dissolved organic matter; and (4) dispersal of gametes during spawning. Most of these functions depend on the coordinate performance of gills apparatus and adductor muscle.

Closely associated with the gill system are the labial palps located in the antero-dorsal part under the cephalic hood of the mantle and close to the mouth. The palps consist of four triangular-shaped bodies, with the bases attached dorsally to the body mass. They are mainly composed of connective tissue, muscle fibers and a ciliated epithelium. Labial

palps are implicated in reducing mucus volume, sorting and guiding food particles into the mouth (Bernard, 1974).

Evseev *et al.* (1996) described the anatomy of *C. gigas* and found anatomical differences in the arrangements of the interlamellar septa when compared with the observations for the same features of *C. angulata* by Nelson (1960, in Evseev *et al.*, 1996). According to these authors the interlamellar septa are at 5 and 6 plica intervals in *C. gigas* and *C. angulata*, respectively. A direct comparison of the anatomy of the two taxa using animals from different populations is still lacking in order to clarify these observations.

3- Digestive system

The digestive system includes the mouth, oesophagus, stomach, crystalline style sac, digestive gland, intestine (midgut and rectum) and anus. It begins with the mouth that opens into a short oesophagus that leads to an elaborate stomach. In the stomach there are a number of ducts leading to the digestive gland. Opposite to the oesophagus is an entrance to the intestine and close to this entrance there is a narrow tube that leads to the crystalline style sac. The intestine loops around the stomach to end in the anus, where it opens into the cloacal passage of the exhalant chamber. The food is moved from the mouth toward the anus by a ciliated epithelium. Food particles that arrive in the stomach are mixed and grinded by the crystalline style (a stiff gelatinous rod) that rotates and rubs against a hard chitinous structure called the gastric shield. The crystalline style is produced in the style sac and it is a temporary structure. When the crystalline style dissolves it release digestive enzymes, which jointly with other enzymes present in the stomach digest food particles. The particles are also conveyed to the opening of the digestive ducts and propelled into the digestive gland where both extracellular and intracellular digestion occurs. During digestion, hemocytes migrate in and out of the stomach and are able to ingest and digest individual particles of food. Subsequently, digestion and absorption continues in the intestine. Another function of the intestine is to compact waste material into solid strings to facilitate its elimination via the anus.

4- Excretory system

The excretory system consist of two parts: one located between the pericardium and the adductor muscle; and the other underneath the mantle forming the kidney that consists of two nephridial lobes. The two lobes open to the outside via the urinogenital vestibule. The main activity of the excretory system is to excrete the end products of the catabolism (e.g., ammonia, urea).

5- Cardio-vascular system

A heart, arteries, open sinuses, veins and an accessory heart form the circulatory system of oysters. The heart consists of one ventricle and two lateral auricles and is enclosed in a chamber called pericardial cavity. Dark pigmented cells give the auricles a darker coloration in relation to the ventricle. Oxygenated hemolymph flows from the gills to the auricles and passes through auriculo-ventricular valves into the ventricle. The hemolymph is then pumped through the anterior and posterior aortas, arteries and finally to arterioles. The hemolymph flows to sinuses of variable size with no walls and is then collected in veins and carried to the gills for oxygenation or to the kidney for depuration. Hemocytes are not only confined to the vessels and they flow throughout tissues, accumulating in the sinuses. Hence, the cardio-vascular system is described as being open or semi-closed. An accessory heart located on the wall of the cloacal chamber, which is slowly pulsating, helps the circulation of the hemolymph. The function of the cardio-circulatory system is to carry nutrients, oxygen and waste products to the different parts of the body.

6- Nervous system

In bivalves, there is no major nervous control center and neurosecretory cells are located in cerebral, pedal and visceral ganglia. The nervous system of oysters is simpler than most bivalves, mainly due to their sedentary mode of life and regression of the foot after metamorphosis. The pedal ganglion is absent and oysters only have the cerebral ganglion near the mouth and the visceral ganglia that occur under the adductor muscle. The two ganglia are joined by the cerebro-visceral connectives. After metamorphosis, the only known organs of sense in oysters of the genus *Crassostrea* are the tentacles of the mantle and the pallial organ inside the cloaca. The specific function of the latter organ remains unknown.

7- Reproductive system

The reproductive anatomy of oyster is relatively simple. The paired gonads are located underneath the mantle epithelium embedded in a layer of connective tissue and cover the outer surface of the digestive gland. In a sexually mature animal the gonad may account for more than 50 % of the body volume, becoming by far the largest organ. The gonad is composed of follicles where the germ cells develop during gametogenesis. The only sign of the paired origin of the gonad is revealed by the two separate systems of gonadal tubes, one at each side of the body. The follicles merge along the dorsal side of the body to form gonadal tubes that became larger and finally uniting in a wider gonoduct. The gonoduct opens into the urinogenital vestibule, which also receive the urinal duct.

1.4.5. Reproduction and early development

Sexuality

The sexes in *C. angulata* and *C. gigas* are normally separate, although a small frequency (between 0 and 3 %) of individuals exhibiting simultaneous hermaphroditism have been observed (Amemiya, 1925; 1929; Pascual, 1971b; Vilela, 1975; Steele and Mulcahy, 1999). In simultaneous hermaphrodites of *C. angulata* ripe eggs and sperm were found in the same tubule of the gonad (Amemiya, 1925). However, these two taxa can be mainly described as being alternative hermaphrodites since they can change sex one or more times throughout life (Amemiya, 1929; Le Dantec, 1968). Nevertheless, it was suggested that in *C. gigas* there are individuals that do not change sex and stay males during their entire life (Guo *et al.*, 1998). The juveniles of both taxa usually mature as males and after change to females (Vilela, 1975; Guo *et al.*, 1998), but sex-changes have been observed in both directions (Amemiya, 1929; Le Dantec, 1968). In a group of 3 to 4-old *C. angulata*, Le Dantec (1968) observed that changes of sex only affected a small fraction of the population and the proportion of females that changed to males (18 %) was only slightly smaller than the proportion of males that changed to females (24 %). It is though that the sex ratio of *C. angulata* and *C. gigas* populations depends on individual age and in older groups females are generally dominant (Guo *et al.*, 1998). However, various sex ratios have been reported by several authors. Le Dantec (1968) observed that in *C. angulata* populations males were dominant on Western Atlantic coast of France. However, Vilela (1975) observed that the sex ratio in *C. angulata* populations on western Atlantic coast of Portugal was around 1:1. For *C. gigas* different sex ratios have also been observed which varied not only among populations but also temporally within populations (Steele and Mulcahy, 1999). Genetic control of sex in *C. gigas* was proposed by Guo *et al.* (1998), but environmental and demographic factors have also been reported to be involved in sex determination (Le Dantec, 1968; Buroker, 1983).

Gametogenesis

Like most oyster species, *C. angulata* and *C. gigas* undergo reproductive cycles characterized by a period of gametogenesis, spawning and gonad redevelopment. Oogenesis begins with the appearance in the germinal epithelium of oogonia which are recognized by their relatively large nuclei with conspicuous nucleoli. Oogonia divide repeatedly by mitosis and then enter meiosis that are arrest at the prophase stage of meiosis I. These produce oocytes that are recognized by the presence of fibrillar mitochondrial bodies and conspicuous nucleoli. Oocytes undergo a period of vitellogenesis that is characterized by an increase in cell volume and accumulation of lipids globules as well as other reserves. In spermatogenesis, the spermatogonia (which

are smaller than oögonia) undergo repeated mitotic divisions. Subsequently, spermatocytes are formed followed by spermatids and finally develop to flagellated spermatozoa. Contrary to oögenesis, where meiosis is only completed at fertilization, spermatogenesis meiotic divisions are completed in the follicles.

Gametogenesis in bivalves is usually controlled by both exogenous (e.g., temperature, salinity, food) and endogenous factors (e.g., neuro-endocrine cycles, genotype). Among the different exogenous factors that influence gametogenesis in bivalves, temperature is considered one of the most important. There is generally an optimal temperature range for gametogenesis which is species-specific and outside this range the rate of gametogenesis is strongly reduced or even arrested. Mann (1979) estimated under experimental conditions that below 10 °C there is no gamete development in *C. gigas* and below 15 °C male development is slower in comparison with females. However, Fabioux *et al.* (2005) observed maturation of germ cells at 8 °C in both female and male *C. gigas*. Chávez-Villalba *et al.* (2003) suggested that the beginning of gametogenesis is not only dependent on thermal conditions but also on reserve stock. On the other hand, according to Lubet (1994) temperatures above 25 °C can have a negative effect on gonad development of *C. gigas*. Nevertheless, the relationship between temperature and gametogenesis is complex. Muranaka and Lannan (1984) observed that the rate of gametogenesis in *C. gigas* is a function of temperature intensity and time rather than accumulated thermal exposure. Gametogenesis in *C. gigas* is also influenced by salinity and a salinity below 30 ‰ exerted a negative effect on the rate of gonadal development (Muranaka and Lannan, 1984). The amount of food can also influence the onset of gametogenesis as well as fecundity (Muranaka and Lannan, 1984; Chávez-Villalba *et al.*, 2003). To my knowledge, there is no experimental data on the effect of temperature or other factor on gametogenesis in *C. angulata*.

Fecundity and spawning

The number of gametes produced by an oyster greatly depends, among other factors (e.g., physiological condition), on the size of the specimens. The number of eggs released in one spawning in *C. gigas* may reach more than 100 millions (Galtsoff, 1964) whereas in *C. angulata* lower values have been reported (Vilela, 1975). Spawning in oysters of the genus *Crassostrea* is generally induced by thermal stimulation and/or by sexual products emitted by conspecifics or close-related species (Galtsoff, 1964; Gaffney and Allen, 1992). The type and level of tide as well as changes in salinity are all thought to induce spawning behavior (His *et al.*, 1999). Generally more than one stimuli is involved the induction of spawning in the natural environment. Male oysters are generally more sensitive to stimulation and usually spawn first then females.

Fertilization and embryogenesis

The oocytes of both taxa when just spawn have an irregular shape (often with a pear-like outline) and have a very thin membrane surrounding them. After a little while in seawater oocytes become spherical with a diameter around 50 μm . Oocytes undergo spontaneous germinal vesicle breakdown when they are incubated in seawater even without sperm. Oocytes can be fertilized regardless of whether or not germinal vesicle breakdown has occurred and in the latter case sperm induce the vesicle breakdown (Stephano and Gould, 1988). After fertilization the first and second polar bodies are expelled and cleavage begins. The embryonic and larval phases in *C. angulata* were described by Amemiya (1926) and Vilela (1975) and in *C. gigas* by Fujita (1929 in Galtsoff, 1964). According to the previous authors, both phases are identical in the two taxa. The first division of the egg is unequal and perpendicular to point of polar body extrusion. The second division, leads to the formation of three blastomeres at the animal pole and a larger one at the vegetal pole. At the third division, cleavage becomes spiral. Subsequent divisions occur, leading to the formation of the morula. As divisions proceed the blastula stage is reached. Subsequently, gastrulation begins and an invagination gives rise to the archenteron and to a small blastopore. The embryo gradually develops into a ciliated larva called trochophore. During this first larval stage the shell is secreted. The second larval stage is the veliger, which is formed around 24 hours after fertilization. At the beginning the larva has a straight dorsal hinge, giving it the characteristic shape of a capital D letter. Hence, during this phase the larva is often called straight-hinge larva or D-larva. The duration of the embryonic phase until the appearance of the D-larva depends mainly on temperature, but also on other factors such as salinity and food availability. Embryos are generally more sensitive than larvae to environmental factors. Amemiya (1926) observed that optimum salinity for the development of *C. angulata* from fertilization until the D-larval stage ranged between 28 and 35 ‰. The same author observed that below 21 ‰ numerous embryos are abnormal and almost no shell-larvae are produced. For *C. gigas*, Robert and His (1985) observed that the majority of embryos developed normally and the D-larval stage was reached between a salinity of 20 and 30 ‰. At a salinity of 35 ‰ the previous authors observed a high proportion of abnormal D-larvae. It is worth noting that the salinity at which the progenitors were kept prior to spawning can influence the optimum salinity for the development of the eggs and larvae (Helm and Millican, 1977).

Early D-larvae of *C. angulata* and *C. gigas* have a shell height (the maximum distance between dorsal and ventral margins) of approximately 60 μm and begins to feed. As previously mentioned, the larval shell consists of two valves and the first larval shell is called prodissoconch I. As larvae grows beyond 100 μm the prodissoconch II is formed

and the umbo begins to form (the umbo stage). When larvae reach a shell height of approximately 300 μm the foot appears and they enter the pediveliger stage, which indicate the end of the larval phase and the start of benthic life after metamorphosis (Pascual, 1971a; Hu *et al.*, 1993). The duration of the larval phase and survival at this phase depends on several environmental factors such as temperature, salinity, pH, oxygen, turbidity and food availability (His *et al.*, 1999). Another determinant of larval growth and survival is pollutants (e.g., heavy metals, tributyltin), which can have a drastic effect. The quantity of quality of food is of paramount importance for larval survival and growth, but salinity and temperature are also crucial factors. *Crassostrea angulata* and *C. gigas* larvae can tolerate a large range of environmental conditions and are less sensitive than embryos. *Crassostrea gigas* larvae can tolerate temperatures between 15 and 34 °C and salinities between 15 and 39 ‰ (His *et al.*, 1999). Nevertheless, *C. gigas* larvae appear to grow most rapidly under experimental conditions at a temperature from 28 to 30 °C and a salinity from 19 to 30 ‰ (Helm and Millican, 1977; Nell and Holliday, 1988; His *et al.*, 1989). For *C. angulata*, Le Dantec (1968) observed that in the natural environment larval development takes place with a water temperatures between 16 and 23 °C and a salinity between 18 and 35 ‰. Moreover, the previous author reported higher survival at temperatures superior to 22 °C and a salinity between 28 and 32 ‰. Marteil (1976 in Héral *et al.*, 1986) suggested the optimum temperature for larval growth was 22 °C for *C. angulata* and 23 °C for *C. gigas*.

Larval development and settlement

At the pediveliger stage *C. angulata* and *C. gigas* larvae develop two lateral eyespots, a foot and positive geotaxis. In *C. gigas*, the eyespot appears first on the right side and only after on the left side (Coon *et al.*, 1990a). At the pediveliger stage competent larvae respond to appropriate cues from the environment exhibiting a settlement behavior, which includes swimming with the foot protruded and crawling maneuvers. Once a suitable habitat is found the larvae settle by gluing their shell to the substratum with a proteinaceous cement produced by the pallial gland in the foot (Quayle, 1989). However, the induction of the settlement behavior does not obligatorily lead to settlement and metamorphosis. *Crassostrea gigas* larvae can delay metamorphosis, but after a certain period of time they eventually die or metamorphose spontaneously under unfavorable conditions, which usually lead to their death (Coon *et al.*, 1990a). Settlement behavior and metamorphosis is induced by environmental cues which are associated with a suitable habitat. In *C. gigas*, it is though that two distinct control pathways exist, one for settlement behavior and one for metamorphosis (Coon *et al.*, 1990a). Some substance have been reported to induce settlement behavior in *C. gigas* larvae (e.g., ammonia and L-3,4-

dihydroxyphenylalanine) whereas others induce metamorphosis (e.g., epinephrine) (Coon *et al.*, 1990b). Usually *C. angulata* and *C. gigas* larvae settle in oyster shell, which may be due to the release of pheromones by juvenile and adult oysters. Bacterial supernatants are also thought to play a role in attracting oyster larvae (Fitt *et al.*, 1990). For *C. gigas*, maximal larval settlement occurs at seawater temperatures between 25 and 30 °C and salinity of 30 ‰ (Jones and Jones, 1982). Metamorphosis is characterized by several anatomical modifications which include among others the loss of the velum, foot and anterior adductor muscle, and the secretion of the adult dissoconch. According to Quayle (1989), *C. gigas* prodissoconch remains attached to the dissoconch for some time after metamorphosis in a position near the umbo. This feature is used to differentiate young *C. gigas* shells from shells of other species of the genus *Crassostrea* (Quayle, 1988). Immediately after metamorphosis *C. angulata* spat can tolerate a salinity between 10 and 45 ‰ (Le Dantec, 1968). Nell and Holliday (1988) reported that the optimum salinity range for the growth of young *C. gigas* spat was 15-30 ‰, although they could survive even in a wider range of salinity.

1.4.6. Growth during juvenile and adult phase

Like in other bivalves, growth in oysters leads to: (1) increase in shell weight; and (2) increase in body weight. Shell and body size are associated, but their growth can be partly uncoupled. Increase in shell size is highly dependent on water temperature whereas food is the driving force behind body growth. Very often, growth is expressed in terms of increase in shell size, since it is more easily measured and it also contains shell growth history. Increase in body size, although considered a more appropriate indicator of growth, is more difficult to measure and is subject to seasonal fluctuation linked to the reproductive cycle. Shell growth may be studied in terms of linear measurements (height, length and depth), volume or weight. Differential growth among shell variables may result in changes in shell shape with increasing body size or in response to environmental constraints (Quayle, 1988).

Temperature also influences growth and it is thought that below 10 °C there is no shell growth in *C. gigas* (Quayle, 1988). Under optimal environmental conditions *C. gigas* can reach a total weight between 60 and 92 g in 12 months (Héral and Deslous-Paoli, 1991). Salinity has apparently a low effect on growth of juveniles and adults of *C. gigas* within the range tolerated (Héral and Deslous-Paoli, 1991). Other factors such as water flow rate, time of immersion and stocking density have mainly influence the amount of food ingested by oysters and hence affect growth (Quayle, 1988). Due to differences in these factors considerable differences in *C. gigas* growth rate have been observed among grow-out sites (Héral and Deslous-Paoli, 1991). Significant differences in growth rate have been

reported between *C. angulata* and *C. gigas*. Production yield is an important economic trait in oyster culture that takes into account both survival and growth (His, 1972; Bougrier *et al.*, 1986; Soletchnik *et al.*, 2002). These studies showed that when oyster reach market sizes (with an individual total weight around 65 g) the individual weight of *C. gigas* was around two times higher than *C. angulata*.

1.4.7. Feeding behaviour and ecophysiological characteristics

Crassostrea angulata and *C. gigas* like the vast majority of bivalves are filter-feeders using their gills to remove suspended particles from the water. *Crassostrea gigas* can capture a high variety of particles from the water such as bacteria, protozoa, microalgae, invertebrate larvae and detritus (Quayle, 1988). Only some of these particles are digested (e.g., some microalgae species and bacteria strains) and others are eliminated in faeces and pseudofaeces. *Crassostrea gigas* can also acquire nutrients by direct absorption of dissolved organic material from seawater (Manahan, 1990). Selection of the particles captures allows the enrichment of the organic content of ingested matter by preferentially rejecting inorganic matter prior to ingestion as pseudofaeces (Hawkins *et al.*, 1998). Larvae and adults of *C. gigas* regulate the amount of food ingested when present at different concentrations by adapting filtration rate to food concentrations (Gerdes, 1983a). In order to limit the quantity of food ingested *C. gigas* can also produce pseudofaeces, where particles combined with mucus are rejected by the gills and labial palps (Hawkins *et al.*, 1998).

Temperature and body size have an important effect on *C. gigas* oxygen consumption (Bougrier *et al.*, 1995) and a seasonal effect on oxygen uptake was also reported in *C. gigas* (Gerdes, 1983b). It was also observed that *C. gigas* can spent a considerable part of the time without consume oxygen during immersion time (Bougrier *et al.*, 1998). During that period it is though that *C. gigas* initiate anaerobic pathways to provide energy to maintain basal metabolism (Bougrier *et al.*, 1998).

In order to understand the physiological basis for the growth rate differences observed between *C. angulata* and *C. gigas*, some ecophysiological parameters have been compared. His (1972) observed that *C. gigas* had a higher clearance rate than *C. angulata* as well as higher valve activity. This author also observed that below around 10 °C the difference in valve activity between the two taxa increased. However, it is noteworthy that His (1972) analysed a very small number of animals. It was also observed that *C. gigas* juveniles had higher oxygen consumption rates than *C. angulata* juveniles (Goulletquer *et al.*, 1999). Haure *et al.* (2003) did not observe the same differences in the clearance and oxygen consumption rates between the two taxa. However, Haure *et al.* (2003) observed that *C. gigas* had a higher feeding time activity than *C. angulata* (89 vs.

73 %) as well as a higher scope for growth (167.8 vs. 123.1 J.h⁻¹.g⁻¹). This study suggested that *C. angulata* and *C. gigas* have different strategies for the allocation of available energy, which could explain partially their dissimilar growth.

1.4.8. Genetics

Cytogenetics

Karyotype and chromosomes characterization

Oysters of the genus *Crassostrea* have a diploid chromosome number of $2n = 20$ which is a common feature in the *Ostreidae* family (Thiriou-Quévèreux, 2002). According to Leitão *et al.* (1999a), the karyotype of *C. angulata* (animals collected in Sado estuary, Portugal) consist of 9 metacentric and 1 submetacentric (pair number 8) chromosome pairs. However, Cross *et al.* (2003) observed only 10 metacentric chromosome pairs in *C. angulata* individuals collected in Guadalquivir estuary, Spain. The karyotype of *C. gigas* (animals collected in Seudre estuary, France) consists of 10 metacentric chromosome pairs (Thiriou-Quévèreux and Insua, 1992).

The nucleolar organizer regions (NORs), which correspond to a chromosome segment containing major (18S-5.8S-28S) ribosomal RNA genes (rDNA), were observed terminally on the metacentric pair 10 in *C. angulata* and *C. gigas* using silver nitrate staining (Leitão *et al.*, 1999a). The location of rDNA in pair 10 was later confirmed by fluorescence *in situ* hybridization (FISH) in both *C. angulata* (Cross *et al.*, 2003) and *C. gigas* (Guo *et al.*, 2001). Recently, it was observed that the minor genes encoding the 5S rRNA in *C. angulata* are located in two metacentric chromosome pairs of large size (pair 4 and 5) and hence not in pair 10 (the smallest one) where the major rDNA genes were observed (Cross *et al.*, 2005). G-banding (Leitão *et al.*, 1999b) and restriction enzyme digestion banding (Leitão *et al.*, 2004) have been successfully used in *C. angulata* and *C. gigas*, which contributed to a better characterization of their chromosomes. The G-banding study highlighted the high similarity between *C. angulata* and *C. gigas*, but differences were observed on the two arms of chromosome pair 7 which agrees with their taxonomic separation (Leitão *et al.*, 1999b). Different banding patterns using restriction enzyme revealed were observed between *C. angulata* and *C. gigas* for all chromosomes pairs with the exception of chromosome pair 10 (Leitão *et al.*, 2004). These cytogenetics studies suggest that *C. angulata* and *C. gigas* are indeed very similar, but can be distinguished.

Somatic aneuploidy

Several studies have revealed the occurrence in *C. gigas* of somatic aneuploid cells, which are cells with an abnormal number of chromosome of $2n = 19$, 18 and 17. Somatic

aneuploid cells have been observed not only in hatchery-produced individuals but also on natural populations (Thiriot-Quiévreux *et al.*, 1992; Zouros *et al.*, 1996; Leitão *et al.*, 2001c). Chromosome loss has been observed also in somatic cells of triploids (Wang *et al.*, 1999) and tetraploids of *C. gigas* (McCombie *et al.*, 2005). Experimental evidence has suggested that there is a genetic basis for the aneuploidy level (Leitão *et al.*, 2001c) and certain environmental contaminants can increase it (Bouilly *et al.*, 2003, 2004). Furthermore, there is evidence of differential chromosome loss in somatic cells, since only in 4 of the 10 chromosome pairs of *C. gigas* are affected by the loss of one homologous (Leitão *et al.*, 2001b). A negative correlation between somatic aneuploidy level and growth rate has been observed in both juvenile and adults of *C. gigas* (Thiriot-Quiévreux *et al.*, 1988, 1992; Zouros *et al.*, 1996; Leitão *et al.*, 2001a).

Marker-based differences

Allozymes markers were used to evaluate the genetic similarity between *C. angulata* and *C. gigas* populations by several authors (Mathers *et al.*, 1974; Buroker *et al.*, 1979; Mattiucci and Villani, 1983). Mathers *et al.* (1974) using 2 allozyme loci found no significant differences between *C. angulata* and *C. gigas* samples. Further allozyme studies supported previous findings, but using a higher number of allozyme polymorphic loci, namely 18 (Buroker *et al.*, 1979) and 13 loci (Mattiuci and Villani, 1983). These three studies concluded that the genetic distance observed between populations of the two taxa was within the levels usually reported between populations of the same species. Huvet *et al.* (2000) revealed low but significant genetic differences between *C. angulata* and *C. gigas* populations using microsatellite markers (mean Wright's fixation index; $F_{st} = 0.022$). These authors reported that the genetic differentiation observed in pairs of populations of the two different taxa were twice as large as in pairs of populations of the same taxon. PCR-RFLP analysis of a ITS-1 fragment using 4 restriction enzymes as well as sequencing analysis of the same fragment revealed no differences between *C. angulata* and *C. gigas* (Huvet, 2000). More recent studies based on satellite DNA (López-Flores *et al.*, 2004) and 5S rRNA gene (Cross *et al.*, 2006) highlighted the genetic similarity between *C. angulata* and *C. gigas*. All these genetic studies clearly indicate close phylogenetic ties between the two taxa. However, two studies on the COI gene revealed clear genetic differences between the two taxa (Boudry *et al.*, 1998; O'Foighil *et al.*, 1998). An average of 2.3 % differences in mitochondrial COI nucleotide sequence was observed between *C. angulata* and *C. gigas* (O'Foighil *et al.*, 1998). Recently, Kim *et al.* (2006) identified single nucleotide polymorphisms (SNPs) that allow the differentiation between *C. angulata* and *C. gigas* populations.

Genetic variability

The existence of genetic variability is of paramount importance for the successful implementation of genetic improvement programs. In the last decades some allozyme studies were done in which the genetic variability was estimated in native and introduced populations of *C. angulata* and *C. gigas*. When a species is introduced into a new area, a reduction of genetic variability and/or a change in allelic frequency may occur (Smith *et al.*, 1986). This is generally due to different selective pressures of the new environment and/or a small group (founder effect) or non representative individuals of the original population. Several allozymes studies revealed high genetic variability for *C. angulata* and *C. gigas* populations in areas where these taxa were introduced (Table 2).

Table 2. Summary of the levels of genetic variability estimated using allozyme markers in wild populations of *C. angulata* and *C. gigas*. NL, number of loci studied; MNA, mean number of alleles detected per locus; P, proportion of polymorphic loci (at 95 %* and 99 % ** levels) and mean proportion of heterozygotes observed.

Taxon/population	NL	MNA	P	H _o	Reference
<i>C. angulata</i>					
Portugal (Sado)	25	2.52	0.600 *	0.234	Buroker <i>et al.</i> (1979)
Spain (Cadiz)	8	2.50	0.625 **	0.297	Michinina and Rebordinos (1997)
Taiwan (Yunan) ^M	7	2.14	0.429 *	0.057	Liu <i>et al.</i> (1995)
<i>C. gigas</i>					
Japan (Miyagi)	27	2.59	0.630 **	0.222	Buroker <i>et al.</i> (1979)
France (Atlantic coast)	13	3.09	0.597 **	0.154	Moraga <i>et al.</i> (1989)
New Zealand (East coast)	9	2.82	0.471 *	0.195	Smith <i>et al.</i> (1986)
USA (West coast)	15	2.70	0.533 *	0.210	Buroker <i>et al.</i> (1975)
Australia (Tasmania)	17	3.30	0.735	0.267	English <i>et al.</i> (2000)

Huvet *et al.* (2000) using microsatellites markers also did not observed reduction in the genetic variability of European populations of *C. angulata* and *C. gigas* comparatively with populations in Taiwan and Japan, respectively.

Boom *et al.* (1994) observed substantial mitochondrial DNA variability in four populations of *C. gigas* in British Columbia (Canada) transplanted from the Miyagi

Prefecture (Japan). The previous authors used a RFLP approach to study the entire mitochondrial genome, including areas that may show higher mutation rates. In contrast, O'Foighil *et al.* (1998) observed that all the individuals from two wild populations in Japan exhibit the same nucleotide sequence of a fragment of the mitochondrial cytochrome oxidase subunit I (COI) gene. In the same study, a high genetic variability was observed in a *C. angulata* population from Sado estuary (Portugal) even though only a small number of individuals were analyzed.

1.4.9. Pathogens susceptibility and symbiotic fauna

Part of the success of *C. gigas* as an aquaculture species is due to its high resistance to diseases, unlike other commercially important oysters species like *O. edulis* and *C. virginica* that suffered mass mortalities due to different pathogenic agents. Nevertheless, high mortalities of known and unknown etiology have been reported in larvae, juveniles and adults of *C. gigas*. In addition several microorganisms and metazoan of doubtful pathogenicity have been reported in this species (Table 3).

Several mortality episodes during larval rearing of *C. gigas* associated with bacterial and viral infections have been described. In some case mortalities have been associated with bad husbandry practices and/or to low egg quality which eventually led to disease outbreaks caused by opportunistic pathogens. Nevertheless, experimental transmission assays have showed that several pathogens can cause mass mortality of healthy *C. gigas* larvae, which is the case of OshV-1 (Renault and Arzul, 2001) and some strains of *V. splendidus* biovar II (Sugumar *et al.*, 1998). The phenomenon known as summer mortality, characterized by high mortalities of both juveniles and adults of *C. gigas* when water temperature increases, has been reported in different regions of the world (e.g., Japan, USA, France) in the last decades (Imai *et al.*, 1965; Perdue *et al.*, 1981; Dégremont *et al.*, 2005). This phenomenon is one of the major constraints to *C. gigas* production. There are several hypothesis to explain summer mortality such as adverse environmental conditions (*i.e.*, high temperature and salinity), physiological stress induced by reproduction and pathogens. Several pathogens have been associated with summer mortality namely, OshV-1 (Friedman *et al.*, 2005), *Nocardia* sp. (Friedman *et al.*, 1991) and *V. splendidus* (Lacoste *et al.*, 2001). Nevertheless, the etiology of summer mortality remains complex but it is presumed to be a combination of several factors.

Table 3. Pathogens and symbiotic fauna of *C. gigas* and their significance.

Organism	Common name	Life stage	Significance	Reference
Viruses				
Irido-like virus	Oyster velar virus disease (OVVD)	Larval	OVVD was only reported in rare occasions in hatcheries, but was associated with high mortalities	Elston and Wilkinson (1985)
Ostreid herpesvirus 1 (OsHV-1)		Larval and juvenile	High mortalities observed during larval rearing that can reach 100 %. Mortality also observed in juveniles	Renault and Arzul (2001)
Irido-like virus	Gill necrosis virus disease	Adult	Low mortality associated. Putative cause of gill disease	Comps (1988)
Irido-like virus		Adult	Up to 15 % mortality	Comps and Bonami (1977)
Papova-like virus	Viral gametocytic hypertrophy or Ovacystis	Adult	No mortality associated	Choi <i>et al.</i> (2004)
Bacteria				
<i>Alteromonas</i> spp.		Larval		Garland <i>et al.</i> (1983)
<i>V. tubiashi</i>		Larval		Hada <i>et al.</i> (1984)
<i>Vibrio splendidus</i> II	Bacillary necrosis	Larval	High degree of virulence that can cause mortalities that can reach 100 % in a short period of time	Sugumar <i>et al.</i> (1998)
<i>Cytophaga</i> -like	Hinge ligament disease	Juvenile	Heavy mortality associated	Dungan and Elston (1988)
Extracellular giant rickettsia		Adult		Azevedo and Villalba (1991)
<i>Rickettsia</i> -like		Adult	Occasional mortalities were reported	Renault and Cochenne (1994)
<i>Chlamydia</i> -like		Adult	No mortality associated	Renault and Cochenne (1995)
<i>Nocardia crassostreae</i>	Pacific oyster nocardiosis	Adult	High mortality associated around that can reach 35 %	Friedman <i>et al.</i> (1998)
<i>V. aestuarianus</i> 01/32		Adult	High mortality associated	Garnier <i>et al.</i> (in press)

Table 3. (continued) Pathogens and symbiotic fauna of *C. gigas* and their significance.

Organism	Common name	Life stage	Significance	Reference
Fungi				
<i>Ostracoblabe implexa</i>	Dutch shell disease	Adult	Low mortality due to infection of the adductor muscle attachment area	Bower <i>et al.</i> (1994)
Protozoan				
<i>Mikrocytos mackini</i>	Denman Island disease	Adult	Responsible for low to medium mortalities in older individuals	Farley <i>et al.</i> (1988)
<i>Marteilioides chugmuensis</i>	Marteilioides of oocytes	Adult	It affects reproduction of infected individuals. No mortality associated	Comps <i>et al.</i> (1987)
<i>Haplosporidium nelsoni</i>	Haplosporidiosis	Adult	No mortality associated	Renault <i>et al.</i> (2000)
<i>Ancistrocoma</i> -like		Adult	No mortality associated	Bower <i>et al.</i> (1994)
<i>Nematopsis</i> sp.		Adult	No mortality associated	Bower <i>et al.</i> (1994)
<i>Sphenophrya</i> -like		Adult	No mortality associated	Bower <i>et al.</i> (1994)
<i>Trichodina</i> sp.	Gill trichodina	Adult	Associated with high mortalities when present at high number	Boussaïd <i>et al.</i> (1999)
Metazoan				
<i>Mytilicola orientalis</i>	Mytilicoliasis	Adult	Rarely associated with mortalities	Sparks (1962)
<i>Mytilicola intestinalis</i>	Mytilicoliasis	Adult	Rarely associated with mortalities	Dare (1982)
<i>Polydora</i> sp.	Shell-boring polychaetes	Adult	Rarely associated with mortalities	Quayle (1988)
<i>Mycicola ostreae</i>		Adult	No mortality associated	Hoshina and Sugiura (1953)
<i>Gymnophalloides tokiensis</i>	Oyster trematode disease	Adult	No mortality associated	Bower <i>et al.</i> (1994)
<i>Urastoma</i> sp.	Oyster gill turbellarian	Adult	No mortality associated	Bower <i>et al.</i> (1994)

Major mortalities between 1966 and 1973 almost led to the disappearance of *C. angulata* from Europe (Renault, 1996). During this period two distinct epizootic mortalities were described. The first one called “gill disease” characterized by the appearance of gill lesions was described for the first time in France in 1966 (Arvy and Franc, 1968; Alderman and Gras, 1969; Marteil, 1969; Comps, 1970). This was followed by a second period of mass mortalities that started in 1970, but this time without distinctive clinical signs (Comps, 1988). Different agents were associated with these mortalities such as fungi, protozoans and viruses (Arvy and Franc, 1968; Besse, 1968; Gras, 1969; Comps and Duthoit, 1976). Irido-like viruses was regarded as one of the most probable causes of the mortalities of *C. angulata* and these viruses were designated as: (1) gill necrosis virus (GNV) for the first mortality episode (Comps and Duthoit, 1976); and (2) haemocyte infection virus (HIV) for the second one (Comps, 1988). Nonetheless, these viruses were not isolated or biochemically characterized neither experimental transmission studies were performed to demonstrate their pathogenicity (Comps, 1988). It was also reported that the oyster stocks were very high in some regions prior to the beginning of the mortalities, therefore, increasing the probability and impact of presumed epizooties (Héral *et al.*, 1986). Official large-scale introduction of *C. gigas* in France was decided in 1970 in order to overcome the crisis caused by *C. angulata* mortality (Grizel and Héral, 1991). It was pointed out that smaller scale unofficial introductions of *C. gigas* that started in the 1960's may have led to the introduction of the putative pathogenic agent that caused the mortalities (Renault, 1996). The irido-like viruses associated with the mortalities were also observed in *C. gigas* (Comps and Bonami, 1977; Comps and Duthoit, 1976), but no major losses were detected in this taxon during a period when *C. angulata* suffered mass mortalities (Comps, 1988). Hence, these results suggest that *C. angulata* and *C. gigas* have different susceptibility to the putative agents responsible for the mortalities that almost wiped out *C. angulata* from Europe. Several mortality episodes during larval rearing of *C. angulata* of unknown etiology have been reported. Indeed, a higher mortality of *C. angulata* larvae is generally observed in comparison with *C. gigas* during hatchery rearing (unpublished data). Experimental transmission assays suggest that OsHV-1 may be implicated in these mortality episodes (Arzul *et al.*, 2001), but no disease outbreaks associated with this virus were reported. Besides irido-like viruses and OsHV-1, other organisms have also been observed in *C. angulata* (Table 4).

Table 4. Pathogens and symbiotic fauna of *C. angulata* and their significance.

Organism	Common name	Life stage	Significance	Reference
Viruses				
Ostreid herpesvirus 1 (OsHV-1)		Larval	High mortalities in larvae infected with OsHV-1	Arzul <i>et al.</i> (2001)
Irido-like virus	Gill necrosis virus	Adult	Associated with gill disease and mortalities up to 70 % .	Comps (1988)
Irido-like virus	Hemocytic infection virus	Adult	Associated with high mortalities	Comps (1988)
Bacteria				
<i>Chlamydia</i> -like		Adult		Comps <i>et al.</i> (1979)
Fungi				
<i>Ostracoblabe implexa</i>	Dutch shell disease	Adult	Low mortality due to shell malformation on the adductor muscle attachment area	Bower <i>et al.</i> (1994)
Protozoan				
<i>Trichodina</i> sp.		Adult	High number of <i>Trichodina</i> sp. associated with mortality. Also associated with gill disease	Comps (1988)
<i>Ancistrum</i> -like		Adult		Ruano and Dias (1994)
Metazoan				
<i>Ostrincola similis</i>		Adult		Lin and Ho (1999)
<i>Myicola ostreae</i>		Adult	No mortality associated	Bower <i>et al.</i> (1994)
<i>Polydora</i> sp.	Shell-boring polychaetes	Adult	Rarely associated with mortalities	Ruano (1996)

1.4.10. Reproductive isolation

Despite significant genetic and phenotypic differences observed between *C. angulata* and *C. gigas* the two taxa are unquestionably very close related. This raises the questions if there are reproductive barriers between the two taxa. In order to address this question, several studies were performed to determine if there are pre- and/or post-zygotic mechanisms that prevent their genomes from merging.

Pre-zygotic mechanisms

The analysis of sexual maturation in *C. angulata* and *C. gigas* individuals of the same age and grown under similar conditions suggest that the two taxa have slightly different reproductive strategies, which can result in asynchronism spawning (Huvet, 2000; Soletchnik *et al.*, 2002). In addition, differences between these two taxa in the minimum temperature at which gametes are released were suggested by Lubet (1994) based on the work of Le Dantec (1968). The hypothesis of different reproductive strategies is supported by the putative temporal discrepancy on the occurrence of larvae of the two taxa based on the analysis of chronological series (P. Soletchnik, personal communication). The failure of sperm to fertilize eggs of a different taxa and preferential fertilization are two other pre-zygotic mechanisms. Interspecific fertilization often occurs in the genus *Crassostrea* with at least moderate success (Gaffney and Allen, 1993), although asymmetric fertilization success was observed between two sympatric species, namely *C. gigas* and *C. sikamea* (Banks *et al.*, 1994). High fertilization rates in crosses of *C. angulata* with *C. gigas* indicate that there is no sperm/egg incompatibility between the two taxa (Imai and Sakai, 1961; Menzel, 1974; Huvet *et al.*, 2001). In addition, molecular analysis of six-hour old embryos revealed no evidence of preferential fertilization between gametes from the same taxon when spermatic competition was allowed between taxa (Huvet *et al.*, 2001).

Post-zygotic mechanisms

Interspecific crosses in the genus *Crassostrea* generally yield non-viable larvae (Gaffney and Allen, 1993). However, normal viability of F1 hybrids of *C. angulata* and *C. gigas* was observed by several authors (Imai and Sakai, 1961; Menzel, 1974; Bougrier *et al.*, 1986; Huvet *et al.*, 2002). According to Menzel (1974) meiosis in the F1 hybrids and mitosis in the F2 hybrid embryos appeared normal. However, Numachi (1966, in Gaffney and Allen, 1993) have previously reported that F1 hybrids of both taxa display normal viability and fertility, but the F2 progeny did not survive to settlement. These results were not corroborated by Huvet *et al.* (2002) that managed to produce F2 hybrids that showed normal fertilization rates, developmental yields, and settlement rates. Recently hybridization between the two taxa was cytogenetically confirmed using restriction enzyme digestion banding (Leitão *et al.*, 2007). A study by Huvet *et al.* (2004) provided evidence for the existence of hybridization between *C. angulata* and *C. gigas* in the natural environment, which suggest that the reproductive barriers are weak or even absent.

CHAPTER 2. MITOCHONDRIAL DNA VARIATION IN WILD POPULATIONS OF *CRASSOSTREA ANGULATA* AND *C. GIGAS*

2.1. GENETIC CONFIRMATION OF TWO PRESUMED WILD POPULATIONS OF *CRASSOSTREA ANGULATA*

2.1.1. Background and objectives

The use of a polymerase chain reactions (PCR) with restriction fragment length polymorphism (RFLP) analysis of the mitochondrial cytochrome oxidase subunit I (COI) gene revealed differences between populations of *C. angulata* and *C. gigas* (Boudry *et al.*, 1998). Using this technique, presumed “pure” populations of *C. angulata* and *C. gigas* were identified in Europe (Boudry *et al.*, 1998; Huvet *et al.*, 2000). However, these populations of *C. angulata* (see Table 5) are endangered by the expansion of *C. gigas* aquaculture (Ruano, personal communication). Indeed, *C. gigas* has been observed in populations that until recently were presumed to be constitute only by *C. angulata* individuals (e.g., some populations in Cadiz, Spain; see Table 5). In addition, evidence for natural hybridization in southern Portugal (Ria Formosa) between the two taxa was reported by Huvet *et al.* (2004), which endangered the genetic integrity of the few remaining populations of *C. angulata* in Europe.

The objective of this study was to determine if the populations of *C. angulata* in Sado and Mira Rivers remain “pure” using the COI PCR-RFLP technique developed by Boudry *et al.* (1998). The second objective of this study was to further document the geographic range of *C. angulata* not only in Europe but also in Asia throughout bibliographic research.

2.1.2. Experimental procedure

In order to determine if *C. gigas* haplotypes were still absent in Mira and Sado populations, a large sample (109 individuals per population) was analysed using the COI PCR-RFLP technique described by Boudry *et al.* (1998). The sample size used, at a 95 % confidence level, allows the detection of *C. gigas* haplotypes if present at a frequency above 0.03. The experimental procedure is detailed in Article 1.

2.1.3. Results and Discussion

Restriction fragment polymorphism of the COI fragment was observed for 3 (*TaqI*, *Sau3A*, and *MseI*) of 4 endonucleases used. Hence, no variability was detected on restriction

profiles using *Hha*I. In addition, new restriction profiles were observed for *Sau*3A and *Mse*I that were called b and c, respectively. A schematic representation of restriction fragment patterns obtained from each digestion as well as the other patterns previously described by Boudry *et al.* (1998) are shown in Figure 12.

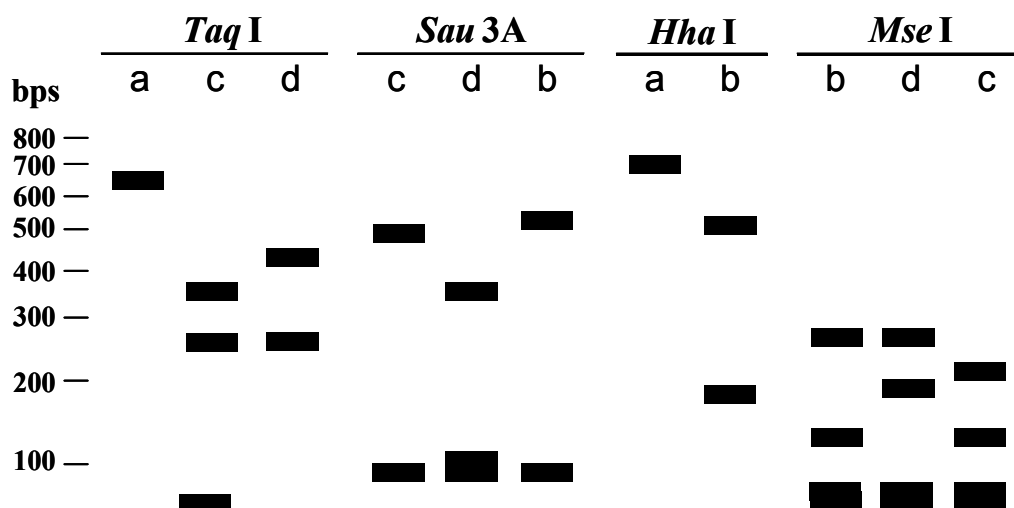


Figure 12. Representation of restriction fragment patterns of a COI gene fragment digested with *Taq*I, *Sau*3A, *Hha*I and *Mse*I based on Boudry *et al.* (1998). The two new restriction profiles observed in the present study for *Sau*3A (restriction profile b) and *Mse*I (restriction profile c) are also showed. Restriction fragments shorter than approximately 70 bp are not shown.

The restriction profiles observed allowed 5 haplotypes to be distinguished among the 218 oysters studied. Three of the haplotypes were the same as previously described by Boudry *et al.* (1998), namely haplotypes A (ccab), B (cdab) and J (acab). Two new haplotypes called F (ccac) and G (cbab) were observed. The haplotype frequencies in each population is shown in Table 5 as well as results from previous studies using the same PCR-RFLP approach.

Table 5. COI PCR-RFLP haplotype frequencies of *C. angulata* and *C. gigas* European populations.

Country	Population	Status*	Reference	Haplotypes (based on 4 endonucleases)								
				N	A	B	J	F	G	D	C	E
Portugal	Sado	A	Boudry <i>et al.</i> (1998)	13	0.85	0.15						
	Ria Formosa	A/G	"	11	0.82	0.09					0.09	
	Ria Formosa	A/G	"	11	0.82						0.18	
	Mira	A	Huvet <i>et al.</i> (2000)	30	1							
	Sado	A	Fabioux <i>et al.</i> (2002)	50	0.94		0.06					
	Faro	A/G	"	42	0.83	0.10	0.02				0.05	
	Tavira	A/G	"	40	0.72		0.05				0.23	
	Mira	A	Present study	109	0.91	0.04		0.05				
	Sado	A	Present study	109	0.87	0.07	0.04		0.02			
Spain	Cadiz	A	Boudry <i>et al.</i> (1998)	26	0.92	0.08						
	Cadiz	A/G	Huvet <i>et al.</i> (2000)	50	0.96	0.02				0.02		
	Orio	G	Fabioux <i>et al.</i> (2002)	50							0.98	0.02
	Islares	G	"	48							1	
	Ribadesella	A/G	"	50	0.04						0.93	0.03
	Castropol	G	"	45							1	
France	Seudre	G	Boudry <i>et al.</i> (1998)	20							0.95	0.05
	Bonne Anse	A/G	"	9			0.11				0.88	
	Charente	A/G	"	15	0.07						0.93	
	Arcachon	G	"	15							1	
	Rade Brest	G	"	24							0.92	0.08
	Gravelines	G	"	23						0.04	0.91	0.04
	Vieux-Boucau	A/G	Huvet <i>et al.</i> (2000)	49	0.08						0.90	0.02
	Arcachon	G	"	50							0.98	0.02
	Seudre	G	"	49							0.98	0.02
	Roz estuary	G	Fabioux <i>et al.</i> (2002)	50							0.96	0.04
UK	Guernsey	G	Boudry <i>et al.</i> (1998)	16							0.87	0.12
	Bangor	G	"	10							0.60	0.40

* presumed status: A, "pure" *C. angulata* population; G, "pure" *C. gigas* population; A/G "mix" population

The Mira and Sado populations showed a high frequency of haplotype A (0.91 and 0.87, respectively). Haplotype B was observed in both populations at low frequencies (< 0.07) whereas haplotypes J and G were only observed in Sado population (0.04 and 0.07,

respectively). On the other hand, haplotype F was observed only in Mira population (0.04). Haplotype B was observed for the first time in Mira River, which was previously thought to be monomorphic for the mitochondrial marker used (Huvet *et al.*, 2000). Boudry *et al.* (2003) sequenced COI fragments from individuals showing 5 of the previous described PCR-RFLP haplotypes, allowing a clear separation of haplotypes C, D and E (associated with *C. gigas*) from haplotypes A and B (associated with *C. angulata*). The nucleotide sequence of haplotypes F, G and J as well as the two other *C. angulata* haplotypes (A and B) previously described (Boudry *et al.*, 2003) showed a higher similarity between them when compared with *C. gigas* haplotypes (E, C and D). The different haplotypes observed for *C. gigas* and *C. angulata* populations are summarized in Figure 13.

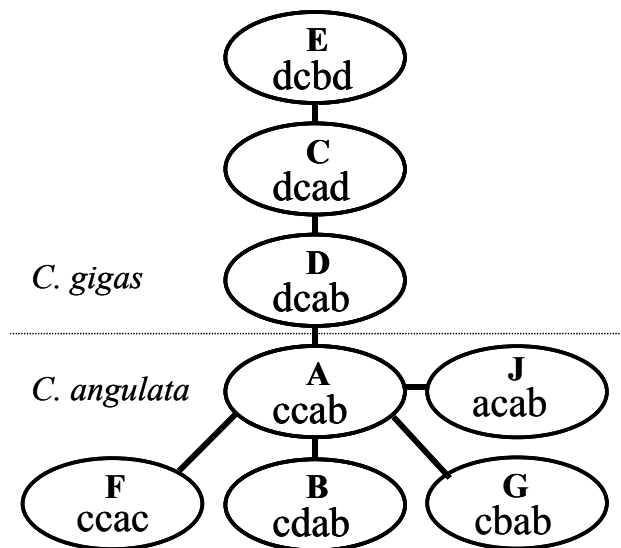


Figure 13. Minimum spanning tree of COI PCR-RFLP haplotypes from *C. angulata* (A, J, F, G and B) and *C. gigas* (E, C and D) populations. The haplotypes E, C, D, A, J and B were described by Boudry *et al.* (1998) and haplotypes F and G were observed in the present study. Each haplotype is separated from adjacent one by a single restriction site.

Article 1 (published in Journal of Shellfish Research)

Journal of Shellfish Research, Vol. 23, No. 3, 759–763, 2004.

EVIDENCE FOR THE PRESENCE OF THE PORTUGUESE OYSTER, *CRASSOSTREA ANGULATA*, IN NORTHERN CHINA

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ABSTRACT The Pacific oyster, *Crassostrea gigas* (Thunberg), and the Portuguese oyster, *C. angulata* (Lamarck), are two closely related taxa. Although these two taxa were both introduced from Asia into Europe, one (*C. gigas*) was voluntarily introduced in the early 1970s, whereas the other (*C. angulata*) was presumed to be present in Europe for at least four centuries, but nearly disappeared because of disease. Few *C. angulata* populations remained in southern Portugal, Spain and Morocco and their putative origin was traced in Taiwan. The present paper reports evidence for its presence in Northern China. We reanalyzed recently published mitochondrial cytochrome oxidase C subunit I (COI) sequence data from presumed Dalianwan oysters (*C. talienwhanensis*) and compared them with those of *C. gigas* and *C. angulata*. Additionally, two new *C. angulata* haplotypes from Portugal were identified. The results clearly showed that some of the *C. talienwhanensis* sequences cluster with *C. angulata* sequences. The relative divergence between *C. gigas*, *C. angulata*, and *C. talienwhanensis* haplotypes indicated that *C. angulata*-like oysters are present in northern China. This opens new perspectives in terms of genetic resources and population genetics of *C. gigas* and *C. angulata*, two oyster species of aquacultural importance.

KEY WORDS: cupped oysters, *Crassostrea angulata*, *Crassostrea gigas*, *Crassostrea talienwhanensis*, cytochrome oxidase C subunit I gene, phylogeography

INTRODUCTION

The phenotypic plasticity of oysters and their wide range of distribution are known to have led to numerous taxonomic misclassification or redundancy in species names. However, during the last decade, molecular tools have contributed in highlighting and resolving several of these cases. For example, Anderson and Adlard (1994) proposed that *Saccostrea commercialis* and *S. glomerata* should be regarded as synonymous taxa based on rDNA internal transcribed spacer sequence data. More recently, Kenchington et al. (2002) suggested that *Ostrea edulis* and *O. angasi* are conspecific. In addition, O'Foighil et al. (1999) confirmed the transoceanic range (New Zealand and Chile) of *O. chilensis* using mitochondrial COI sequence data and proposed that dispersal by rafting was the most likely explanation for this distribution. Similarly, the mangrove oyster *Crassostrea gasar* was shown to be present not only along the coasts of Western Africa but also along the Atlantic coasts of South America where some specimens had been wrongly described as *C. rhizophorae* (Lapègue et al. 2002).

In this context, the relative taxonomic status of the Portuguese oyster, *C. angulata*, and the Pacific oyster, *C. gigas*, may be considered as a case study. *C. gigas* and *C. angulata* were classified as two different species by Thunberg in 1793 and Lamarck in 1819, respectively. This classification was chiefly due to apparently separated geographical distribution of the two species, because *C. angulata* was described in Europe and *C. gigas* in Asia. However, following morphologic comparison (Ranson 1948), experimental hybridization (reviewed by Gaffney & Allen 1993, Huvet et al. 2001, Huvet et al. 2002) and allozyme data (Mathers et al. 1974, Buroker et al. 1979, Mattiucci & Villani 1983), the authors concluded that there was only a single species, grouping Portuguese and Pacific oysters. Yet, significant phenotypic differences between the two taxa were observed, in terms of production yield (Bougrier et al. 1986, Héral 1986, Parache 1989, Soletchnik

et al. 2002), and eco-physiologic characteristics (His 1972, Goulletquer et al. 1999, Haure et al. 2003). Furthermore, differences have now been observed (1) from karyotype analyses (Leitão et al. 1999b) although these two taxa exhibited a close genetic similarity in comparison with other cupped oyster species (Leitão et al. 1999a); (2) in the genetics of the two taxa based on the mitochondrial COI gene (Boudry et al. 1998, O'Foighil et al. 1998) and nuclear microsatellites (Huvet et al. 2000).

The introduction of the Pacific oyster was relatively well documented because it was a recent voluntary introduction. Hence, the introduction of *C. gigas* from Japan into Europe was made in the early 1970s to replace the Portuguese oyster in the shellfish industry (Grizel & Héral 1991) that nearly disappeared probably due to an iridoviral disease (Comps 1969). As indicated by its common name, the Portuguese oyster, was believed to originate from Portugal or at least southern Europe. However, results from nuclear and mitochondrial DNA studies (Boudry et al. 1998, O'Foighil et al. 1998, Huvet et al. 2000) suggested an explanation for the separated geographical distribution of these genetically closely-related taxa by supporting the hypothesis of the introduction of *C. angulata* from Asia (and more precisely Taiwan) to the Portuguese coast by merchant ships during the 16th century. Until now, no *C. angulata* specimens were observed in any other Asian location (see Lam et al. 2003, Boudry et al. 2003).

China is the country with the largest *C. gigas* production (in 1997: Mainland China: 2.3 10⁶ metric tonnes, Taiwan: 24 10³ metric tonnes, Hong Kong: 66 metric tonnes, according to FAO, 1999). However, Guo et al. reported in 1999 that it is not *C. gigas*, but another species, *C. plicatula*, that accounts for the main 50% to 60% of the production, *C. ariakensis* for 20% to 30% and *C. gigas* for only 10% to 20%. Qi (1989) also cited the Dalianwan oyster (*C. talienwhanensis*) as of commercial importance after the Zhe oyster (*C. plicatula*) and the Suminoe oyster (*C. ariakensis*) (Qi 1989). Many other different taxa have been reported along Chinese coasts and species identification is often uncertain. In Northern China, *C. talienwhanensis*, *C. plicatula* and *C. gigas*

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were considered as sibling species (Liu & Dai 1998). In the East and South China, at least nine species have been described (Bernard et al. 1993). Recently, a new species was described in Hong Kong (Lam & Morton 2003, Boudry et al. 2003). Finally, a recent study based on mitochondrial DNA sequences showed that *C. talienwhanensis* specimens from Northern China were genetically close to *C. gigas* specimens which suggested that the Dalianwan and Pacific oysters belong to the same species (Yu et al. 2003). Similarly, their results suggested that *C. plicatula* and *C. ariakensis* are closely related.

In the present paper, we compared DNA sequence data of *C. gigas*, *C. talienwhanensis*, and *C. angulata* to establish their genetic relationships. To do so, we reanalyzed the mitochondrial COI sequence data of *C. talienwhanensis* specimens from Yu et al. (2003) and compared with those of known haplotypes from *C. gigas*, *C. angulata* (Boudry et al. 1998) and two newly identified *C. angulata* haplotypes.

MATERIALS AND METHODS

New *Crassostrea angulata* Sequence Data

A total of 218 cupped oysters from Portugal were sampled in October of 2002 from 2 locations, 109 in the Sado estuary (Monte da Pedra site: 38°25'N, 8°39'W) and 109 in the Mira estuary (Roncão Velho site: 37°42'N, 8°44'W). DNA extraction of ethanol-preserved gill fragments was performed by a phenol/chloroform method, as described by Moore (1993). A partial COI fragment was amplified using the primers and conditions detailed in Folmer et al. (1994). Polymorphism was first studied using restriction enzymes as described by Boudry et al. (1998). Some of these PCR products were sequenced as described by Boudry et al. (2003). All the novel sequences have been submitted to the EMBL nucleotide sequence database.

DNA Sequence Analysis

The COI sequences of the new haplotypes, together with some sequences already obtained for *Crassostrea gigas* and *C. angulata*

(Accessions AJ553901, AJ553902, AJ553903, AJ553904, AJ553905; Boudry et al. 2003), *C. virginica*, *C. ariakensis*, and *C. sikamea* (Accessions AF152566, AF152569, AF152568; O'Foighil et al. 1998) and *C. talienwhanensis* (Yu et al. 2003) were aligned with CLUSTALW (Thompson et al. 1994). Pairwise sequence divergences between species were estimated with the DNADIST program in PHYLIP (Felsenstein 1989) according to Kimura's two-parameter model (Kimura 1980). Phylogenetic analyses were conducted using the program FITCH. Bootstrap analysis with 100 replicates was performed with the SEQBOOT and CONSENSE programs.

RESULTS

PCR-amplified fragments from the COI gene were obtained for 218 individuals from the newly sampled Portuguese populations. The PCR-RFLP analysis detected 2 new haplotypes, which were called F and G here. Haplotype G lacked a *Sau3A* restriction site when compared with the others haplotypes of *Crassostrea gigas* and *C. angulata* described by Boudry et al. (1998). For haplotype F a new restriction site was observed using *MseI* when compared with the same haplotypes. These new haplotypes are rare, each one being present in one of the two populations: the frequency of haplotype F is 5% in Mira and the frequency of haplotype G is 2% in Sado. The COI sequences of haplotypes F and G, together with haplotype J—that had been described by Boudry et al. (2003), but not sequenced—were respectively registered as Accessions AY397685, AY397686, and AY455664. We compared the sequences of these haplotypes, F, G, and J, with those of haplotypes from *C. gigas* (haplotype C, D, and E; Boudry et al. 2003), *C. angulata* (haplotype A and B; Boudry et al. 2003), and *C. talienwhanensis* (haplotype talienw1, talienw2, talienw3, talienw4, and talienw5; Yu et al. 2003). The multiple alignment showed that haplotypes C and talien1 had exactly the same sequence. The distances computed after this alignment is presented in Table 1. The phylogenetic tree obtained from sequence divergence of the COI fragment according to Kimura's model (Kimura

TABLE 1.
Pairwise sequence divergences, for the mt COI DNA fragments (*C. angulata* haplotypes are in bold).

	Haplotype E	Haplotype C	Haplotype D	talienw2	talienw3	Haplotype A	Haplotype B	Haplotype G	Haplotype J	Haplotype F	talienw4	talienw5	<i>C. sikamea</i>	<i>C. ariakensis</i>	<i>C. virginica</i>
Haplotype E	0														
Haplotype C	0,0036	0													
Haplotype D	0,0055	0,0018	0												
talienw2	0,0055	0,0018	0,0036	0											
talienw3	0,0055	0,0018	0,0036	0,0036	0										
Haplotype A	0,0279	0,0241	0,0222	0,0260	0,0260	0									
Haplotype B	0,0337	0,0299	0,0279	0,0318	0,0318	0,0055	0								
Haplotype G	0,0317	0,0279	0,0260	0,0299	0,0299	0,0036	0,0055	0							
Haplotype J	0,0375	0,0337	0,0317	0,0356	0,0356	0,0091	0,0110	0,0091	0						
Haplotype F	0,0317	0,0279	0,0260	0,0298	0,0298	0,0110	0,0129	0,0110	0,0166	0					
talienw4	0,0298	0,0260	0,0279	0,0279	0,0279	0,0055	0,0073	0,0055	0,0073	0,0129	0				
talienw5	0,0279	0,0241	0,0260	0,0260	0,0260	0,0036	0,0055	0,0036	0,0091	0,0110	0,0018	0			
<i>C. sikamea</i>	0,1051	0,1053	0,1031	0,1075	0,1075	0,1007	0,0986	0,1007	0,0984	0,1050	0,1029	0,1007	0		
<i>C. ariakensis</i>	0,1623	0,1625	0,1650	0,1650	0,1601	0,1652	0,1725	0,1701	0,1723	0,1723	0,1676	0,1652	0,1679	0	
<i>C. virginica</i>	0,2590	0,2538	0,2512	0,2565	0,2512	0,2513	0,2567	0,2567	0,2569	0,2592	0,2594	0,2567	0,2627	0,2789	0

talien1 and haplotype C have the same COI sequence.

1980) is given in Figure 1. The 12 haplotypes clustered into 2 groups (100% bootstrap *P* value) with a divergence varying from 2.2% to 3.7% depending the pairs of haplotypes being compared. One group encompassed the *C. gigas* haplotypes (C, D, and E) and the *C. talienwhanensis* haplotypes (talienw1, talienw2, and talienw3). The second group included the *C. angulata* haplotypes (A, B, J, F, and G) and the *C. talienwhanensis* haplotypes (talienw4 and talienw5). The nucleotide divergence within the first group varies from 0.2% to 0.5% and in the second group from 0.2% to 1.7%. *C. sikamea* and *C. ariakensis*, two other Asian cupped oysters species, respectively exhibited about 10% and 16% divergence with the *C. gigas* and *C. angulata* haplotypes. The American oyster, *C. virginica*, showed about 26% divergence with the *C. gigas* and *C. angulata* haplotypes and was considered as an outgroup.

DISCUSSION

The geographic distribution of the closely related taxa *Crassostrea gigas* and *C. angulata* in southwestern Europe is now well documented, so is the genetic variability within the populations and the genetic differentiation between the populations. *C. gigas* was observed in northern Europe bordered by the headland of northern Spain (La Corogne) in the south. *C. angulata* was observed in southern Spain, Portugal, and Morocco (Boudry et al. 1998, Fabioux et al. 2002, Huvet et al. 2004). According to the

grouping of the haplotypes observed here, haplotype F and G, detected in the new samples from the Mira and Sado Portuguese populations, can be considered as two new *C. angulata* haplotypes. This is confirmed by the divergence values that are of the order of those reported by Boudry et al. (2003): less than 0.5% for the *C. gigas* group compared with 0.2% to 0.5% in this study, and less than 1.1% for the *C. angulata* group compared with 0.2% to 1.7% in this study. When observing the results for the *C. talienwhanensis* haplotypes from Yu et al. (2003), talienw1 (identical to haplotype C), talienw2 and talienw3 are grouped with the *C. gigas* haplotypes, and talienw4 and talienw5 with the *C. angulata* haplotypes. Consequently, oysters of talienw1, talienw2 and talienw3 can be considered as *C. gigas*, as proposed by Yu et al. (2003), and the others (oysters of talienw4 and talienw5) can be considered as *C. angulata*. This partly confirms that the Dalianwan oyster, described by Zhang and Lou (1956), is another name for the Pacific oyster in China (Li & Qi 1994), but also supports that *C. talienwhanensis* being considered as *C. angulata*. After the evidence of the presence of *C. angulata* in Taiwan (Boudry et al. 1998), this species is now found existing in the northern China (Dalian, Liaoning province and Rongcheng, Shandong province) suggesting a broader Asian geographical distribution. Additionally, it should be noted that the cupped oysters specimens found in Hong-Kong (Lam et al. 2003, Boudry et al. 2003) and considered as belonging to a putative new taxa, cannot be considered as *C. talienwhanensis*.

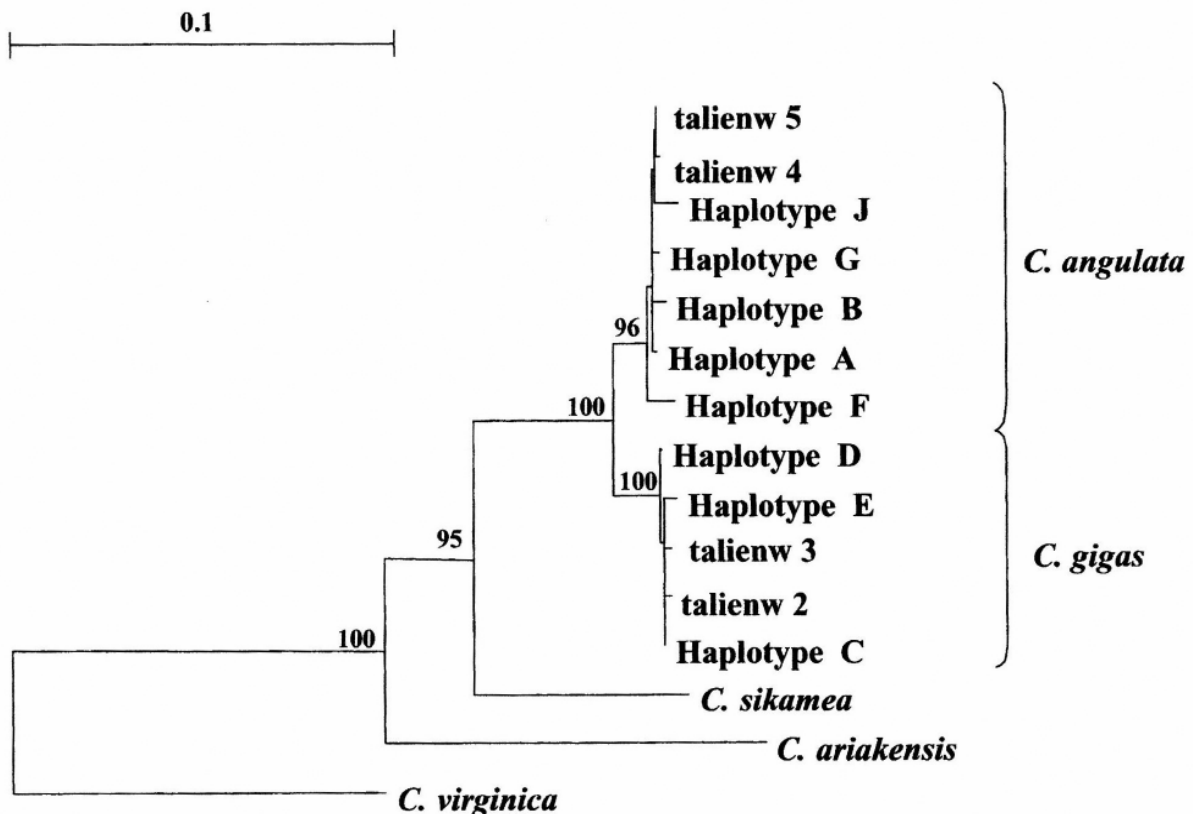


Figure 1. Phylogenetic trees obtained from sequence divergence of a 551 nucleotide mitochondrial COI DNA fragment according to Kimura's model (Kimura 1980) for *C. gigas*, *C. angulata*, and *C. talienwhanensis* haplotypes. talienw1 to 5 correspond to the haplotypes described by Yu et al. (2003). The sequences of haplotypes A, B, C, D, and were described in Boudry et al. (2003). The sequences of *C. ariakensis*, *C. sikamea* and *C. virginica* were obtained from O'Foighil et al. (1998). The sequences of haplotypes F, G, and J were described in this study. *C. virginica* was used as an outgroup. Numbers on the branches indicate bootstrap *P* values.

In total, 11 *C. angulata* COI haplotypes (A, B, and J from Boudry et al. 1998; angul1, angul2, angul3, and angul4 from O'Foighil et al. 1998; talienw4 and talienw5 reanalysed from Yu et al. 2003; F and G in this study) and 5 *C. gigas* COI haplotypes (C, D, and E from Boudry et al. 1998; talienw1 (identical to haplotype C), talienw2, and talienw3 reanalysed from Yu et al. 2003) have now been described. Interestingly, using relatively equivalent amount of research on both taxa, the level of variability of *C. gigas* appears to be lower than that of *C. angulata* for the COI sequence. Furthermore, studies based on allozyme data by Buroker et al. (1979) also showed a high genetic variability in *C. angulata*, from Portuguese populations, relative to other *Crassostrea* species. More data are needed from other mitochondrial and nuclear markers, but this high genetic variability observed in *C. angulata* opens interesting perspectives for the development of conservation programs for this taxa in Europe. Consequently this also underlines the importance of *C. angulata* as a potentially useful genetic resource for *C. gigas* aquaculture.

Although the two 16S haplotypes described for the 10 *C. talienwhanensis* individuals from Yu et al. (2003) study were each found in 2 different sampling locations (Dalian & Rongcheng), the 5 COI haplotypes (talienw1 to talienw5) distribution indicated that

C. gigas and *C. angulata* co-occur in the two sampled locations. Hence, one individual has the talienw1, one the talienw2, one the talienw3, four the talienw4, and three the talienw5 haplotypes. This result needs to be confirmed, because the number of samples (5 in each location) is low. It would be of particular interest to focus on the sympatric status of these two taxa in this region as it was done in Southern Europe. In Portugal, there was evidence for hybridization between *C. angulata* and *C. gigas* in the wild where the two taxa are in contact due to recent transportation of *C. gigas* stocks for aquacultural production (Huvet et al. 2004). An extensive sampling protocol is clearly needed in Northern China to investigate this hybridization in the native region of these two taxa, and, more generally, their relative evolutionary history.

ACKNOWLEDGMENT

The authors thank H. McCombie for help with the English. This work was made possible by an FCT grant (SFRH/BD/8972/2002) and a Marie Curie Training fellowship (PLUDAMOR, QLK5-CT-2000-60036) to F. M. Batista. This work was partly supported by the Région Poitou-Charentes (Convention N°2001-RPC-A-212).

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2.2. MITOCHONDRIAL DNA SEQUENCE VARIABILITY IN *CRASSOSTREA ANGULATA* AND *C. GIGAS* POPULATIONS

2.2.1. Background and objectives

The COI PCR-RFLP results obtained in section 2.1 suggests that the mitochondrial DNA (mtDNA) diversity was higher in the studied populations of *C. angulata* than in *C. gigas*. Indeed, O'Foighil *et al.* (1998) had previously observed no differences in the nucleotide sequence of a COI fragment in 20 specimens of *C. gigas* (from wild populations and research hatchery broodstock of Japanese origin) whereas 4 different haplotypes were observed in 5 individuals of *C. angulata* collected in Sado River.

The objective of this study was to compare the mtdna sequence variation in a population of *c. Angulata* from sado river (portugal) and a population of *c. Gigas* from seudre river (france).

2.2.2. Experimental procedure

Crassostrea gigas individuals were collected in 2005 from seudre river (france) and total dna was extracted from gill tissue using a modification of a phenol/chloroform method described by sambrook *et al.* (1989). For *c. Angulata*, individuals were collected in sado river (portugal) and total dna was extracted using the same methods as previously described. A 584 bp coi fragment was amplified using the primers coi3 (5'-gta ttt gga ttt tga gct gt-3') and coi4 (5'-gag gta tta aaa tga cga tc-3'). Reactions were performed in 50 µl volumes composed of 5.0 µl of 10x pcr buffer, 35.7 µl of distilled water, 5.0 µl of dntp (2 mm), 1.0 µl of each primer (20 µm), 0.3 µl of taq polymerase (5 u/ µl) and 2.0 µl of template dna (ca. 100 ng). Pcrs were performed for one cycle of 2 min at 94 °c and 30 cycles of 30 s at 94 °c, 30 s at 50 °c, and 45 s at 72 °c with a least extension of 7 min at 72 °c. Sequencing was performed directly on the pcr products using bigdye (applied biosystems) chemistry using an automatic sequencer (abi-prism, model 377, applied biosystems). Both forward and reverse strands were sequenced. Sequences analysis was performed using sequencing analysis 5.1 (applied biosystems) and single nucleotide polymorphism identified using seqscape 2.1 (applied biosystems). Statistical analyses of *c. Angulata* and *c. Gigas* mtdna sequence were performed using dnapsp version 4.10 (rozaz *et al.*, 2003). The program tcs version 1.12 (clément *et al.*, 2000) was used to generate a minimum spanning net work of haplotypes. Phylogenetic analyses were performed with the treecon package (van de peer and de watcher, 1994).

2.2.3. Results and discussion

A total of 11 mtDNA haplotypes distinguished by 11 segregating sites were identified among the 47 individuals (sequences) from the *C. angulata* population of Sado River (Figure 14).

```

CTCTTATTCGTTGGAGGCTTTATAACCCCGGAGCTAAGTTTTTAGACCCCGTGACTTATA 60
ATGCAGTTGTAAGTAGGCATGCGTTGGTTATGATTTTTTTCTTTGTTATACCCGTGATAA 120
TTGGGGGGTTTGGTAACTGGCTTATCCCTTTGATGCTTCAAGTAGCAGACATGCAATTC 180
CTCGATTAAATGCATTTAGATTTTGAGTTTGGCAGGGTCTCTTTATCTTATGCTTATGT 240
CTAACATCGTAGAAAACGGAGTTGGGGCAGGGTGAACAATTTACCCTCCTTTATCAACTT 300
ACTCTTATCATGGAGTTTGCATAGACCTTGCAATTTTAAGCCTTCACCTTGCCGGTATTA 360
GCTCTATTTTTCAGGTCAATTAATTTTATAGTAACGATTAGAAATATGCGATCTGTTGGGG 420
GCCATTACTAGCACTATTTCCCTGATCTATTAAGGTTACTTCATTCTTGCTTTTGACTA 480
CTCTCCCAGTGTTAGCTGGAGGTCTTACTATGCTTTTGACTGATCG 526

```

(A)

Haplotype	46	47	185	275	299	323	410	443	458	506	509	n
S1	A	C	A	A	T	A	A	C	T	T	T	(30)
S2	.	T	.	.	.	.	.	.	.	.	.	(2)
S3	G	.	.	.	.	.	.	.	.	.	.	(1)
S4	.	.	.	.	.	.	.	.	C	.	.	(2)
S5	.	.	.	G	.	.	.	.	.	.	.	(3)
S6	.	.	.	G	.	G	.	.	.	.	.	(1)
S7	.	.	.	G	.	G	.	.	.	.	C	(1)
S8	.	.	.	G	.	G	.	.	.	C	.	(1)
J	.	.	G	G	.	G	.	.	.	.	.	(2)
angul2	.	.	.	.	C	.	.	T	.	.	.	(3)
G	.	.	.	.	.	.	G	.	.	.	.	(1)

(B)

Figure 14. Nucleotide sequence of a 526 bp fragment of the COI gene that correspond to *C. angulata* haplotype S1 (A), nucleotide polymorphisms of *C. angulata* haplotypes observed in Sado River relatively to haplotype S1. Numbers in top refer to polymorphism position (bp) and the number of individuals per haplotype are indicated in parentheses at the end of each sequence.

For the *C. gigas* population (Seudre River), 4 mtDNA haplotypes were observed in the 44 individuals (sequences) analysed distinguished by 3 segregating sites (Figure 15).

```

CTCTTATTCGTTGGAGACTTTATAACCCTGGAGCTAAGTTTTTAGACCCCGTGACTTATA 60
ATGCAGTTGTAAGTAGGCATGCGTTGGTTATGATTTTTTTCTTTGTTATACCTGTAATAA 120
TTGGGGGGTTTGGTAACTGGCTTATCCCTTTGATGCTTCTAGTAGCAGACATGCAATTC 180
CTCGATTAAATGCATTTAGATTTTGAGTTTTGCCAGGGTCTCTTTATCTTATGCTTATGT 240
CTAACATTGTAGAAAACGGAGTTGGGGCAGGGTGAACAATTTACCCTCCTTTATCAACTT 300
ACTCTTATCATGGAGTTTGTATAGACCTTGCAATTCTAAGCCTTCACCTTGCTGGTATTA 360
GCTCTATTTTCAGGTCAATTAATTTCATAGTAACGATTAGAAATATGCGATCTGTTGGGG 420
GCCATTACTAGCACTATCCCTTGATCTATTAAGGTTACTTCATTCTTGCTTTTGACTA 480
CTCTCCCAGTGTTAGCTGGAGGTCTTACTATACTTTTGACTGATCG 526

```

(A)

Haplotype	83	144	371	n
C	G	A	C	(41)
C1	.	.	T	(1)
C2	T	.	.	(1)
C3	.	G	.	(1)

(B)

Figure 15. Nucleotide sequence of a 526 bp fragment of the COI gene that correspond to *C. gigas* haplotype C (A), nucleotide polymorphisms of *C. gigas* haplotypes observed in Sado River relatively to haplotype C. Numbers in top refer to polymorphism position (bp) and the number of individuals per haplotype are indicated in parentheses at the end of each sequence.

Three of the *C. angulata* haplotypes observed had been previously described namely, haplotype angul2 (O'Foighil *et al.*, 1998) and haplotypes G and J (in the previous section). Hence, 8 new *C. angulata* haplotypes (S1, S2, S3, S4, S5, S6, S7 and S8) were observed. For *C. gigas*, one of the observed haplotypes had been previously described by O'Foighil *et al.* (1998) and called haplotype C by Boudry *et al.* (2003). In addition, 3 new *C. gigas* haplotypes (C1, C2 and C3) were observed. Figure 16 shows the phylogenetic relationships of *C. angulata* and *C. gigas* haplotypes that clustered into 2 distinct groups.

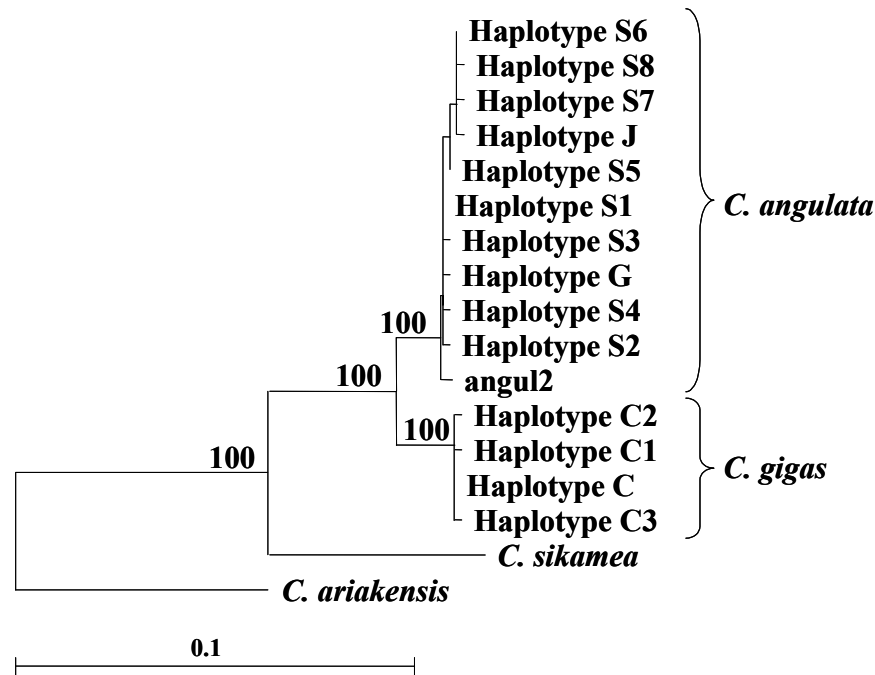


Figure 16. Phylogenetic tree based on neighbour-joining analyses of *Crassostrea* species of a partial COI sequence using Kimura two-parameter distances with 1000 bootstrap. *Crassostrea ariakensis* was used as an out-group. Numbers at specific nodes represent bootstrap values greater than 90 %.

The results obtained suggest that *C. angulata* and *C. gigas* are sister taxa, although the observed haplotypes of the two oysters differed by a minimum of 12 substitutions. Eleven of these differences are due to synonymous transitions and one results from a non-synonymous transversion. The mean sequence divergence between populations of the two taxa was 2.63 %. Within *C. angulata* haplotypes, sequence divergence varied between 0.19 and 0.96 % whereas within *C. gigas* haplotypes variation was between 0.19 and 0.38 %.

A higher haplotype diversity (H) and nucleotide diversity (π) was observed in *C. angulata* population ($H = 0.589$, $\pi = 0.0022$) in comparison with *C. gigas* population ($H = 0.133$, $\pi = 0.0003$). These findings are in agreement with known sequence data for the COI fragment studied, prefacing a total of 19 *C. angulata* haplotypes (A and B from Boudry *et al.*, 2003; angul1, angul2, angul3, and angul4 from O'Foighil *et al.*, 1998; S1, S2, S3, S4, S5, S6, S7, and S8 in this study; F, G and J from section 2.1; RF1 and RF2 observed in Ria Formosa, unpublished data) (see Figure 17).

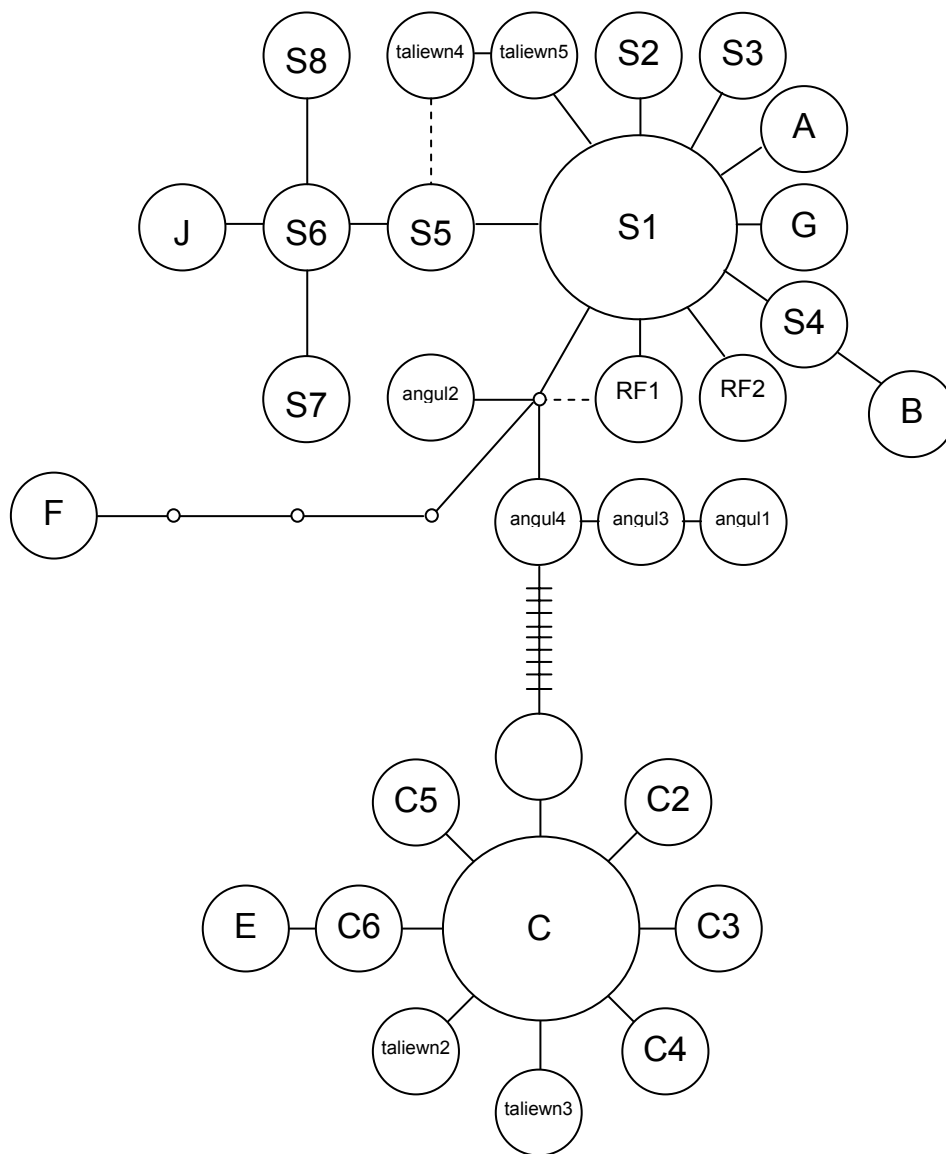


Figure 17. Reconstructed haplotype network for *C. angulata* (above) and *C. gigas* (below) of a 488 bp COI fragment based on published data and the one obtain in this Chapter and unpublished data (RF1 and RF2). The line with crosshatching between the two taxa haplotypes represents nine bp differences. Solid lines within each taxon represent single base pair differences among haplotypes and dashed lines indicate alternative connections. Big circles represent the two most common haplotypes for each taxon.

In addition, two haplotypes (talienw4 and talienw5) described by Yu *et al.* (2003), considered as being from *C. talienwhanensis*, clustered with *C. angulata* haplotypes suggesting the latter taxon is present in northern China (see Article 1). Hence, a total of 21 haplotypes were described for *C. angulata*. Surprisingly, the sequence haplotypes A

and B described by Boudry *et al.* (2003), that were thought to be the most common ones, were not observed in the present study. This may have been due to sequencing errors and haplotypes A and B are the same as S1 and S4, respectively. Since For *C. gigas*, 10 haplotypes were observed (C from O'Foighil *et al.*, 1998 and Boudry *et al.*, 2003; D and E from Boudry *et al.*, 2003; talienw2 and talienw3 from Yu *et al.*, 2003; C1, C2, and C3 from this study; C4 and C5 observed in Ria Formosa, unpublished data) using an equivalent amount of research as in *C. angulata* (see Figure 17). The results obtained in the present study are somehow surprising since previous studies showed no differences in genetic variability between *C. angulata* (Sado population) and *C. gigas* (Seudre population) using allozyme markers (Buroker *et al.* 1979; Moraga *et al.*, 1989). In addition, Huvet *et al.* (2000) using microsatellite markers observed similar levels of genetic variation in *C. angulata* (Mira population) and *C. gigas* (Seudre population). The high genetic variability revealed using both allozyme and microsatellite markers suggest that there where no major losses in genetic variability in relation with native populations. These results shows that observed genetic variability at the nuclear and mitochondrial levels appear to be different for the two studied taxa.

Population size changes leave particular footprints that may eventually be detected in mitochondrial DNA sequence data. The low genetic variability observed in the present study in *C. gigas* may have been due to demographic factors which is supported by Fu and Li's test of selective neutrality test ($D = -2.943$, $p < 0.05$) suggesting a reduction in population size. This could have been due to: (1) a founder effect, whereby a non representative group of *C. gigas* was introduced into France; (2) a small effective population size in native Japanese populations of *C. gigas* before the introduction; (3) a reduction in population size after the introduction of *C. gigas* in Europe; or (4) a combination of these factors. Since *C. gigas* individuals were introduced into Europe only from Miyagi Prefecture in Japan and from populations in Canada that had been also introduced from Miyagi prefecture supports the founder effect hypothesis. However, a study conducted by Boom *et al.* (1994) in introduced populations (from the Miyagi Prefecture, Japan) of *C. gigas* in Canada revealed considerable genetic mitochondrial diversity. However, Boom *et al.* (1994) sampled the entire mitochondrial genome by RFLP, including areas that may be experiencing higher mutations than the COI fragment analyzed in the present study, which make comparison difficult. Moreover, sequencing analysis of the same COI fragment from archival samples of *C. gigas* preserved few years after its introduction in France revealed two haplotypes that were never observed (see Chapter 6). On the other hand, O'Foighil *et al.* (1998) found no variation for the same COI fragment studied in different *C. gigas* populations. In addition, the haplotypes described for *C. gigas* in other studies (Boudry *et al.*, 2003; Yu *et al.*, 2003) only differed from the

most common haplotype by one or two nucleotide differences. These findings support the hypothesis of small effective population and a high rate of mitochondrial mutation in native *C. gigas* populations as suggested by Beckenbach (1994) (in O'Foighil *et al.*, 1998). Nevertheless, this doesn't exclude also a founder effect that may have also resulted from the introduction of *C. gigas* into Europe mainly from Miyagi populations. However, both hypothesis as well as the hypothesis of a reduction in population size after the introduction is contradictory with the high genetic variation observed at the nuclear level (Huvet *et al.*, 2000). In addition, no differences in genetic variation using allozymes markers was observed between introduced populations of *C. gigas* in France and populations of *C. gigas* in Miyagi (Moraga *et al.*, 1989). Selection at the mitochondrial level after the introduction of *C. gigas* is the most plausible explanation for low variation observed at the mtDNA level and hence the fixation of haplotype C that was observed in 93 % of the *C. gigas* individuals analyzed.

The higher mtDNA variability observed in *C. angulata* comparatively with *C. gigas* is surprising since it is presumed that the former taxon was involuntarily introduced into Europe. Indeed, Fu and Li's selective neutrality test ($D = -0.824$, $p > 0.10$) indicate that there is no reduction in effective population size in *C. angulata*. The absence of a reduction in effective population size in the European *C. angulata* population studied can be explained by continuous introduction throughout the years and not in a single event.

2.3. CONCLUSIONS

The high number of oysters examined using the COI PCR-RFLP approach clearly suggest that *C. gigas* is not present in Mira and Sado populations. Hence, the results of the present study indicate that these two populations can be regarded as being “pure” *C. angulata* populations.

A low genetic variability in Sado population of *C. angulata* was expected since: (1) it is presumed that *C. angulata* was introduced in Portugal from Asia; and (2) the Sado population of *C. angulata* suffered a considerable decrease in population size due to pollution (Ruano, 1997). However, the results of the present study together with the results of Buroker *et al.* (1979) using allozyme markers shows that there is no evidence for reduced genetic variation in *C. angulata* population in Sado. Hence, this population can be considered as an important genetic resource for the conservation of this taxon in Europe as well as a source of progenitors for genetic improvement programs for traits of aquacultural interest.

A higher genetic diversity revealed by mtDNA sequences was observed in the Sado population of *C. angulata* comparatively to the Seudre population of *C. gigas*. However, the differences observed at the mtDNA level may not reflect a lower nuclear DNA variability in the latter taxon.

CHAPTER 3. EXPERIMENTAL HYBRIDIZATION BETWEEN *CRASSOSTREA ANGULATA* AND *C. GIGAS*: GROWTH, SURVIVAL AND SOMATIC ANEUPLOIDY

3.1. GROWTH AND SURVIVAL OF *CRASSOSTREA ANGULATA*, *C. GIGAS* AND THEIR RECIPROCAL HYBRIDS AT DIFFERENT DEVELOPMENT STAGES

3.1.1. Background and objectives

The existence of heterosis for some traits in mollusc bivalves is supported by different studies, including: (1) positive correlation between growth and heterozygosity at allozyme loci (Singh and Zouros, 1978); (2) higher growth and survival of hybrids when inbred lines were crossed (Hedgecock *et al.*, 1995, 1996; Bayne *et al.*, 1999; Pace *et al.*, 2006; Hedgecock *et al.*, 2007); and (3) superior growth of hybrids in crosses between close related taxa (Beaumont *et al.*, 1993; Bierne *et al.*, 2002). These findings encourage evaluating heterosis in crosses between close related taxa, which can be seen as simple and efficient way to exploit heterotic effects (Beaumont and Hoare, 2003). Several studies have shown that there are no major barriers to hybridization between *C. gigas* and *C. angulata* under experimental conditions (Imai and Sakai, 1961; Menzel, 1974; Huvet *et al.*, 2001; Huvet *et al.*, 2002). Hence, these two genetically and phenotypically different taxa are good candidates to assess the existence of heterosis for traits of aquacultural interest. In addition, even if heterosis is not observed hybrids may combine favourable traits from both taxa, which should also be evaluated. Bougrier *et al.* (1986) and Soletchnik *et al.* (2002) performed crosses between *C. angulata* and *C. gigas* and compared the aquacultural performance of reciprocal hybrids and pure crosses. These two studies have focused on the grow-out stage and the performance of different progenies was evaluated under common culture conditions in the Atlantic coast of France. In both studies the reciprocal hybrids showed an intermediate performance in terms of growth and survival whereas *C. gigas* progeny showed always a superior performance comparatively to *C. angulata*. Hence, no heterosis for the traits analysed was observed. However, because dominance effects are not easily predictable, hybridization success must be assessed on the results of a number of crosses performed between animals of different origins and tested in different environments (Falconer and Mackay, 1996). In addition, experimental hybridization studies may also help to understand the genetic basis of some characters (e.g., somatic aneuploidy, shell morphology, susceptibility to pathogens).

Despite careful hatchery practices contamination has been systematically observed by several laboratories using genetic markers (Mallet *et al.*, 1985; Foltz, 1986; Allen and Gaffney, 1993; McGoldrick and Hedgecock, 1997). Contamination can take

place by inadvertent transfer of gametes (especially sperm) or animals (larvae, juveniles or adults) from one culture to another (Gaffney and Allen, 1993). The control of contamination in hybridization experiments is more critical since the viability of hybrids can be low and hence putative contaminants can be over represented (Allen and Gaffney, 1993; Gaffney and Allen, 1993).

The objectives of the present study were: (1) to compare the performance of *C. angulata*, *C. gigas* and their reciprocal hybrids progenies at larval, nursery and grow-out phases; and (2) to confirm the genetic identity of reciprocal hybrids and the presence of contaminants in all progenies.

3.1.2. Experimental procedure

Crosses and larval rearing

The production of the different progenies was performed in the Shellfish Hatchery of IFREMER Station in La Tremblade, France. Adults of *C. angulata* and *C. gigas* were collected from Sado River (Portugal) and Seudre River (France), respectively, in 2003. Experimental crosses were performed as shown in Figure 18, with 15 females and 5 males of *C. angulata* and 10 females and 8 males of *C. gigas*. Oocytes and spermatozoa were obtained by stripping the gonad, and fertilization was done at a ratio of 100 spermatozoa per oocyte. Fertilization was performed as described by Stephano and Gould (1988). Eggs were incubated with a density of 82 eggs/ml in filtered seawater with a salinity of approximately 35 ‰. Larvae were reared in cylindrical conical 50 l tanks (in triplicate) to evaluate the survival and development of the larvae of the different progenies. Larvae were also reared in cylindrical conical 150 l tanks (in duplicate) so periodic samples could be taken for pathological analysis. Samples for pathological analysis were also collected if high mortalities or a high percentage of moribund larvae were observed. Larvae were fed with a progressive diet of *Isochrysis* aff. *galbana* (clone T-ISO) and *Tetraselmis suecica*. Three times per week water was changed. During larval rearing, no larvae were discarded by selective sieving to reduce genetic variability losses (Taris *et al.*, 2006).

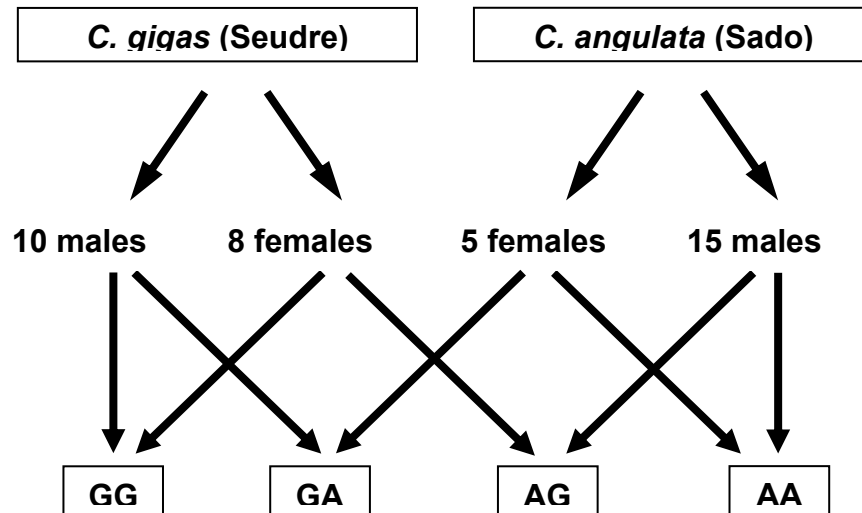


Figure 18. Experimental crosses made to produce the different progenies of *Crassostrea angulata* (AA), *C. gigas* (GG), hybrids derived from *C. angulata* females and *C. gigas* males (AG), and hybrids derived from *C. gigas* females and *C. angulata* males (GA).

During larvae rearing the temperature of the water was not constant (with a minimum and maximum values of 20 and 26 °C, respectively (Figure 19). Salinity was approximately 35 ‰ during larval rearing.

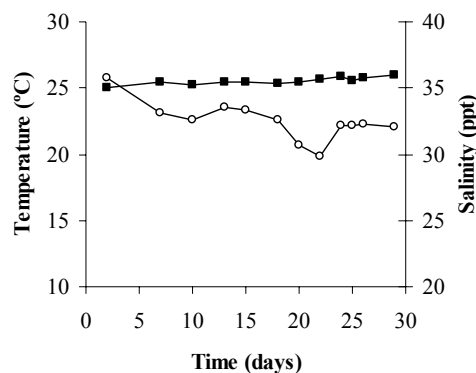


Figure 19. Temperature (open circles) and salinity (black squares) observed during the larval rearing of *C. angulata*, *C. gigas* and their reciprocal hybrids.

Yield of D larvae (number of D larvae/number of fertilized eggs), yield of ready-to-set larvae (number of larvae ready-to-set /number of D larvae) and metamorphosis rate (number of spat/number of larvae ready-to-set) were determined.

Nursery phase

After metamorphosis, spat was reared in a micro-nursery system and then transferred to Tavira Shellfish Hatchery Station of INIAP/IPIMAR at 49 days after fertilization (DAF). The oysters were reared for a period of 108 days in an indoor nursery closed system and each progeny was run in three replicate mesh-bottomed (150 μ m) containers. The density used was approximately 1 ind. cm^{-2} (810 oysters per experimental unit). The tank where the experimental units here held was supplied with filtered underground saltwater with a salinity of approximately 30 ‰ and a mean (SD) temperature of 17.3 (3.4) °C (Figure 20). During the nursery phase the animals were fed with a mixed diet of cultured *I. galbana*, *T. suecica* and *Skeletonema costatum*.

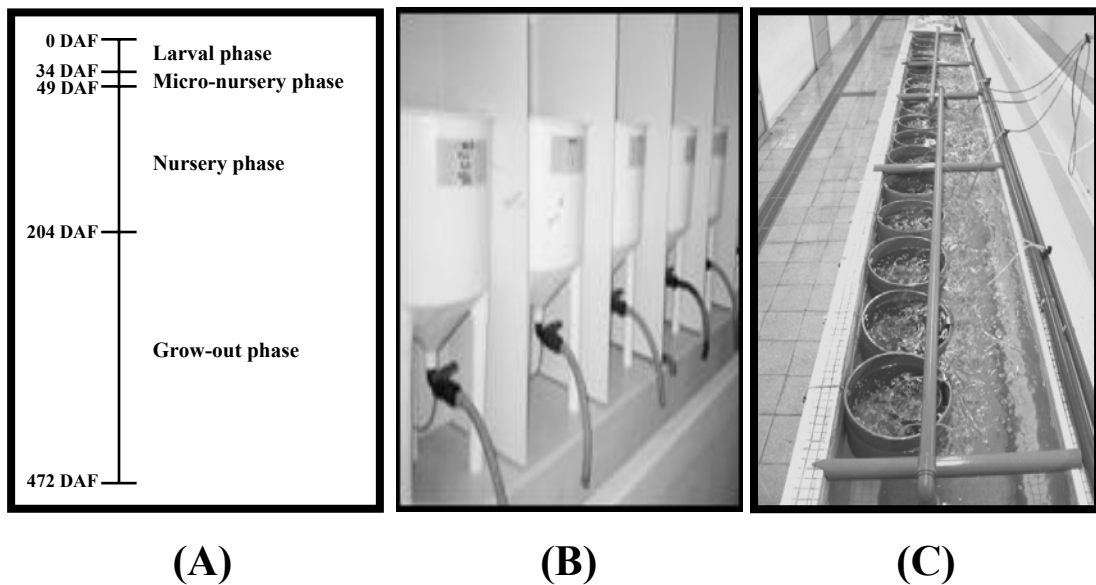


Figure 20. (A) Duration of the different rearing phases in days after fertilization (DAF); (B) cylindrical conical 50 L tanks used for larvae rearing; and (C) containers used during nursery phase.

At 204 DAF, the live weight and shell height of 50 animals from each experimental unit were determined. The live weight of oysters was determined after 10 min drainage on absorbent paper with a precision of 0.1 mg. Shell height was recorded with a digital caliper with an accuracy of 0.01 mm.

Grow-out phase

At day 204 after fertilization, 4 fine mesh spat bags with 50 oysters each were prepared for each of the four groups. Half of the animals in each bag were labelled with a non-toxic epoxy resin (see section 3.2). The oyster bags were then deployed in the experimental site at Cacela-Velha in Ria Formosa Lagoon (Portugal). The oysters were cultured off-bottom in iron frames tables on which the bags were attached. The position of the bags on the tables was changed every month. The sea floor of the study site was a mixture of sand and mud, and the emersion rate was approximately 50 %. The shell height and live weight of the labeled oysters from the different progenies was recorded every month during the grow-out phase (see section 3.2) at the beginning of the experiment on 3 March 2004, subsequently, on 6 April, 4 May, 02 June, 01 July, 29 July, 31 August, 30 September, 27 October and finally on 26 November of 2004. Mortality in each oyster bag was also recorded at each sampling period. At the end of the grow-out phase (472 DAF), shell height and live weigh of all oysters was determined after having removed any attached epifauna and wiped the water from the shell. The final yield for each genetic group was calculated using the formula: $\text{yield (\% day}^{-1}) = 100 \times (\text{final biomass} - \text{initial biomass}) / [(\text{initial biomass}) \times (\text{number of days})]$. Biomass is defined here as the sum of the live weight of all individuals in each oyster bag. The degree of precision of weight measurements was 0.1 g. Shell measurements were made using a digital caliper to a precision of 0.01 mm.

Genetic confirmation

Gill fragments from all parental oysters and from a sample of each group (ranged between 55 and 60 individuals per group) were sampled and preserved in absolute ethanol for subsequent genetic analysis. Animals were scored using mitochondrial and nuclear DNA markers. The methods for total DNA extraction and scoring with the mtDNA marker are described in Article 2. Animals were also scored using the nuclear marker CG44R developed by Huvet *et al.* (2004). The CG44 microsatellite was amplified by a denaturation cycle at 94 °C for 2 min, followed by 30 cycles of 1 minute at 94 °C, 1 minute at 53 °C, and 1 minute at 72 °C with a least extension of 7 min at 72 °C. Negative controls, in which distilled water was added to the PCR mix instead of extracted DNA, were used in all PCR reactions. Restriction enzyme digestions of CG44 PCR products were set up in a 20-µl volume containing 10.0 µl PCR products, 7.5 µl distilled water, 2.0 µl 10X reaction buffer, and 0.5 µl restriction enzyme (10 U/ µl). CG44 amplicons were digested with *BsrGI* (NewEngland BioLab) for 2 h at 37 °C. PCR products with the restriction site for *BsrGI* were used as controls. Digestion products were separated by electrophoresis in 2 % agarose gels with ethidium bromide staining. Visualization was carried out under UV light.

Statistical analyses

Statistical analyses of survival, shell height and yield in nursery phase and shell height, live weight and yield in grow-out phase were performed using one-way analysis of variance (ANOVA). Following the ANOVA, multiple comparisons between groups were performed using Tukey's honest significant (HSD) tests. The Mantel-Cox test (Mantel, 1966) was used to compare the survival trends between groups after correction for multiple testing using the sequential Bonferroni method (Rice, 1989). Chi-squared tests with Bonferroni corrections were used to compare genotypic ratios in progenies to Mendelian expectations.

3.1.3. Results and Discussion

Larval phase

Yield of D and ready-to-set larvae, and metamorphosis rate observed in the present study for the four groups are shown in Table 6. The high variation observed among tanks within each group, the lack of adequate experimental units (i.e., different culture volumes), and sampling for pathological analysis (see Chapter 7) did not allow the use of statistical analyses to determine if there were significant differences in the parameters analysed among groups. Nevertheless, some comparisons can be done with the results obtained by other authors. Robert and Gérard (1999) reported that between 1992 and 1996, in the same experimental hatchery, a mean yield of D larvae of 59.2 % was observed for *C. gigas*. Similar results were obtained in the present study (between 49.7 and 65.0 %), which suggests that the quality of gametes did affect fertilization success. The yield of ready-to-set larvae observed in all groups was lower than the values reported by Robert and Gérard (1999) (between 15 and 50 %). However, the results shown in Table 6 are under estimated since samples were collected for pathological analysis (see Chapter 7). Metamorphosis rate in all groups was low comparatively to the values usually observed for *C. gigas* under similar conditions (see Article 8 in Appendices).

Table 6. Mean (SD) yield of D and ready-to-set larvae, and metamorphosis rate of *C. angulata* progeny (AA), *C. gigas* progeny (GG), hybrids derived from *C. angulata* females and *C. gigas* males (AG), and hybrids derived from *C. gigas* females and *C. angulata* males (GA).

Trait	AA	AG	GA	GG
Yield of D larvae (%) ¹	49.7 (9.4)	53.5 (23.8)	65.0 (22.6)	56.1 (6.7)
Yield of ready-to-set larvae (%) ¹	1.0 (0.9)	1.1 (1.6)	1.7 (2.3)	1.7 (3.9)
Metamorphosis rate (%) ²	17.8	27.6	32.1	28.2

¹ mean from three 50 L and two 150 L tanks. ² no replicates

Nursery phase

Survival, shell height and yield of oysters of the four groups at 204 DAF are summarized in Table 7.

Table 7. Mean (SD) survival, shell height and yield (n = 3) of *C. angulata* progeny (AA), *C. gigas* progeny (GG), hybrids derived from *C. angulata* females and *C. gigas* males (AG), and hybrids derived from *C. gigas* females and *C. angulata* males (GA) at 204 DAF.

Trait	AA	AG	GA	GG
Survival (%)	34.7 (10.7) ^a	29.8 (10.3) ^a	44.2 (4.4) ^a	44.5 (1.9) ^a
Shell height (mm)	5.6 (0.3) ^c	11.5 (0.9) ^a	9.4 (0.4) ^b	11.6 (0.3) ^a
Yield (% day ⁻¹)	14.7 (7.3) ^c	63.2 (19.7) ^b	86.2 (17.1) ^b	125.3 (10.9) ^a

Different superscripts for each row denote statistically significant differences between groups (p < 0.05)

No significant differences in survival were observed among the four groups (ANOVA, p > 0.05), probably due to the high variation among replicates and their small number. However, the four groups differed significantly in shell height (ANOVA, p < 0.001), with the highest values observed in *C. gigas* progeny and AG hybrids in comparison with *C. angulata* progeny and GA hybrids (Tukey's test, p < 0.05). Moreover, shell height in GA hybrids was higher than *C. angulata* progeny (Tukey's test, p < 0.05). The apparently faster growth of AG hybrids comparatively with GA hybrids may be explained by the higher mortality observed in AG hybrids (although no significant differences were observed) and the resulting lower density. Huvet (2000) performed factorial crosses between *C. angulata* and *C. gigas* and followed the total weight of individuals of the four progenies with approximately the same age as in the present study during 224 days under controlled conditions. At the end of the experimental period, Huvet (2000) reported only minor differences in total weight between the four groups. However, he associated the

lack of differences in growth rate between *C. gigas* and *C. angulata* to differences in individual densities that were to time higher in the former taxon. In the present study, a faster growth in shell height was observed in *C. gigas* in comparison with *C. angulata* even though density was lower in latter taxon. In addition, *C. gigas* progeny showed a much higher yield than *C. angulata* progeny with reciprocal hybrids showing an intermediate performance. These differences may be due to not only the faster growth of *C. gigas* under the culture conditions tested, but also as a result of a higher mortality in *C. angulata* even though no statistical differences were observed between the two taxa.

Grow-out phase

A high mortality was observed during the first months of the experimental period in the four groups with the highest mortality rate observed between 2 and 29 of July (Figure 21). The lowest mortality rates were observed in the last three months of the experiment (*i.e.*, September, October and November). A significant higher survival was observed in *C. gigas* progeny in comparison with *C. angulata* progeny (Mantel-Cox, $p < 0.05$). However, no significant differences in survival were observed between reciprocal hybrids and the other groups. These results are in agreement with those obtained in France by Bougrier *et al.* (1986) and Soletchnik *et al.* (2002) who also observed a higher mortality in *C. angulata* when compared to *C. gigas*.

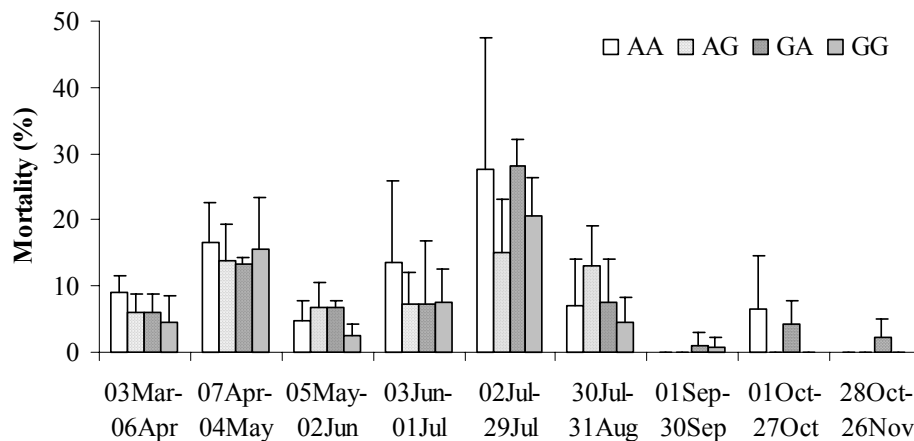


Figure 21. Mortality rate (SD) of *C. angulata* progeny (AA), *C. gigas* progeny (GG), hybrids derived from *C. angulata* females and *C. gigas* males (AG), and hybrids derived from *C. gigas* females and *C. angulata* males (GA) between 3 March and 26 November of 2004.

A lower mean shell height and live weight were observed in *C. angulata* progeny comparatively with both hybrids (AG and GA) and *C. gigas* progeny (ANOVA, $p < 0.01$; Table 8).

Table 8. Mean (SD) shell height, live weight and yield ($n = 4$) of *C. angulata* progeny (AA), *C. gigas* progeny (GG), hybrids derived from *C. angulata* females and *C. gigas* males (AG), and hybrids derived from *C. gigas* females and *C. angulata* males (GA) at the end of the grow out phase.

Trait	AA	AG	GA	GG
Shell height (mm)	45.5 (6.6) ^b	53.0 (6.6) ^a	53.2 (6.1) ^a	55.8 (6.5) ^a
Live weight (g)	17.5 (6.5) ^b	23.1 (7.3) ^a	24.5 (6.9) ^a	25.9 (6.7) ^a
Yield (% day ⁻¹)	4.3 (2.1) ^b	7.8 (1.9) ^a	6.5 (0.5) ^{ab}	8.7 (1.1) ^a

Different superscripts for each row denote statistically significant differences between groups ($p < 0.05$)

The faster growth of *C. gigas* than *C. angulata* observed in the present study support previous observations (His, 1972; Bougrier *et al.*, 1986; Soletchnik *et al.*, 2002). It is noteworthy that the present and previous studies have been conducted under similar culture conditions (*i.e.*, off-bottom in intertidal sites), although in different regions. Hence, differences in growth observed between *C. angulata* and *C. gigas* progenies may be to some extent due to the sub-optimal growing conditions for the former taxon or could have been even more accentuated under different culture conditions (*e.g.*, bottom culture, subtidal culture).

Significant differences in yield were detected among groups (ANOVA, $p < 0.01$), being the yield of *C. gigas* progeny and AG hybrids significantly higher than *C. angulata* progeny (Tukey's test; $p < 0.05$), with GA hybrids showing an intermediate performance (Table 8). These results are consistent with previous findings in which a superior yield was observed in *C. gigas* comparatively with *C. angulata* (Héral *et al.*, 1986; Parache, 1989; Soletchnik *et al.*, 2002). These differences have been attributed mainly to the faster growth of *C. gigas* but also to the lower survival generally observed in *C. angulata*. The results obtained in the present study are in agreement with these findings since a higher survival and a faster growth were observed in *C. gigas* in comparison to *C. angulata*. Consequently, at the end of the experimental period, the yield was 2 times higher in *C. gigas* progeny than in *C. angulata* progeny, with hybrids having an intermediate performance. Although Soletchnik *et al.* (2002) also observed a lower yield in *C. angulata* when compared to *C. gigas*, the reciprocal hybrids had similar or lower yield than *C. angulata*. These differences could have been due to genotype x environment interactions. This hypothesis is consistent with the instability of relative family performance across

environments that has been observed in other studies, namely in *C. virginica* by Mallet and Haley (1983) and in *C. gigas* by Langdon *et al.* (2003).

Genetic confirmation

Genetic confirmation in the four progenies was conducted at the end of the grow-out phase in order to increase the probability of detecting contamination that may have occurred during the different culture phases (*i.e.*, crosses, larval rearing, nursery phase and grow-out phase). All the progenitors and progenies produced had the expected mitochondrial DNA (mtDNA) haplotypes with exception of one individual (out of 226) in the *C. angulata* progeny, suggesting it was a contaminant or leakage of parental mtDNA (Table 9). Indeed, leakage of mtDNA has been observed in interspecific crosses (Kaneda *et al.*, 1995).

Table 9. Mitochondrial DNA haplotypes frequencies of *Crassostrea angulata* progeny (AA), *C. gigas* progeny (GG), hybrids derived from *C. angulata* females and *C. gigas* males (AG), and hybrids derived from *C. gigas* females and *C. angulata* males (GA).

	n	f (C)	f (NC)
<i>Broodstock</i>			
<i>C. angulata</i>	20	1.00	0.00
<i>C. gigas</i>	18	0.00	1.00
<i>Progenies</i>			
AA	55	0.98	0.02
AG	55	1.00	0.00
GA	56	0.00	1.00
GG	60	0.00	1.00

C, "cut" haplotype; NC, "non cut" haplotype

Assuming that contamination occurred, it may have been due to (1) contamination by individuals from *C. gigas* progeny or GA group, or (2) contamination during genetic analysis.

Although the bi-allelic nuclear marker used (CG44R) is not specific, the high allelic frequencies differences between *C. angulata* and *C. gigas* makes it an useful marker for genetic confirmation. For *C. angulata* progenitors, a high frequency of the "cut" (C) allele was observed (0.87, Table 10), which is very close to the frequency reported by Huvet *et al.* (2004) for the Sado population ($f[C] = 0.93$). A high frequency of the "non cut" (NC) allele (0.81) was observed in *C. gigas* progenitors, which is in agreement with Huvet *et al.* (2004) results for the Seudre population ($f[NC] = 0.89$). However, a high frequency of null

alleles was observed in *C. gigas* progenitors, with a estimated value of 0.40 using Brookfield's (1996) method. In contrast, a low frequency of null alleles ($f[\emptyset] = 0.03$) was observed in *C. angulata* progenitors using the same method. As expected, a high proportion of individuals with the genotype $\emptyset \emptyset$ (0.23, non amplification) was observed in *C. gigas* progeny. The high frequency of null alleles makes the genetic confirmation with CG44R difficult to examine.

Table 10. Allele and genotype frequencies for the nuclear marker (CG44R) in *Crassostrea angulata* progeny (AA), *C. gigas* progeny (GG), hybrids derived from *C. angulata* females and *C. gigas* males (AG), and hybrids derived from *C. gigas* females and *C. angulata* males (GA).

	n	Allele frequencies		Genotype frequencies			
		C	NC	C C	C NC	NC NC	Ø Ø
Broodstock							
C. angulata	20	0.87	0.13	0.70	0.25	0.05	0.00
C. gigas	18	0.19	0.81	0.11	0.11	0.66	0.11
Progenies							
AA	55	0.89	0.11	0.82	0.11	0.07	0.00
AG	55	0.53	0.47	0.51	0.00	0.45	0.04
GA	56	0.66	0.34	0.45	0.37	0.14	0.04
GG	60	0.15	0.85	0.07	0.10	0.60	0.23

C, "cut" allele; NC, "non cut" allele; $\emptyset$, null allele

The allelic frequencies of the four different progenies (AA, AG, GA, and GG) yield the expected results. However, although the observed genotype frequencies of GA hybrids, *C. angulata* and *C. gigas* progenies were not significantly different (Chi-square with Bonferroni correction, $p > 0.05$) from the expected ones (Table 10) the same was not observed in AG group (Chi-square with Bonferroni correction, $p < 0.001$). This was mainly due to the lack AG hybrids with the genotype C NC and an excess of individuals with the genotype NC NC. Since all AG hybrids showed the mitochondrial haplotype specific of *C. angulata*, these suggest that if contaminants were present they could have been only from *C. angulata* progeny. However, the genotype in excess was NC NC (*i.e.*, characteristic of *C. gigas*), which indicate that they were very unlikely to be *C. angulata*. In addition, in the AG group the mean size (both in shell height and weight) was higher in individuals with the C C genotype comparatively with individual with the NC NC genotype and also to *C. angulata* progeny. This suggests that the AG hybrids with C C genotype were not contaminants from the *C. angulata* progeny. Hence, although the genotype frequencies were not as expected in the AG group, there were no evidences of contamination in this group. Departures from expectations may have been due to high frequency of null alleles

observed in *C. gigas* male progenitors and/or zygotic viability selection against heterozygotes.

The mitochondrial and nuclear markers used in the present study allowed the genetic confirmation of hybridization between *C. angulata* and *C. gigas* and the assessment of absence of contaminants in the pure lines with exception of one individual. In addition, the phenotypic differences observed in growth rate as well as for other characters analysed (see below) among the four progenies suggest that if contamination occurred it was at a very low-level.

3.2. RELATIONSHIP BETWEEN GROWTH AND SOMATIC ANEUPLOIDY IN *CRASSOSTREA ANGULATA*, *C. GIGAS* AND THEIR RECIPROCAL HYBRIDS

3.2.1. Background and objectives

The results of section 3.1 agree with previous studies in which differences in growth rate between *C. angulata* and *C. gigas* were observed (His, 1972; Bougrier *et al.*, 1986; Soletchnik *et al.*, 2002). A negative correlation between the degree of somatic aneuploidy and growth rate within and among families of *C. gigas* has been reported (Thiriot-Quiévreux *et al.*, 1988; Zouros *et al.*, 1996; Leitão *et al.*, 2001a). It is presumed that in certain populations of *C. gigas* somatic aneuploidy can accounts for a significant proportion of the variance in body weight (Zouros *et al.*, 1996). Somatic aneuploidy had never been investigated in *C. angulata* and to what extent it may contribute to differences in growth rate between *C. angulata* and *C. gigas* is unknown.

The objectives of this study were: (1) to determine if the differences in growth rate between *C. angulata* and *C. gigas* are associated with differences in somatic aneuploid levels; (2) to establish a relationship between mortality and the level of somatic aneuploidy in reciprocal hybrids and pure crosses; and (3) to examine the genetic basis for somatic aneuploidy by scoring it in reciprocal hybrids.

3.2.2. Experimental procedure

Briefly, growth rate, survival and somatic aneuploidy were analysed in *C. angulata* progeny (AA), *C. gigas* progeny (GG), hybrids derived from *C. angulata* females and *C. gigas* males (AG), and hybrids derived from *C. gigas* females and *C. angulata* males (GA) grown in the natural environment. The detailed experimental procedure is described in Article 2.

3.2.3. Results and discussion

A significantly higher proportion of aneuploid cells (PAC) and number of missing chromosomes (PMC) was observed in *C. angulata* progeny comparatively with *C. gigas* progeny. In addition, reciprocal hybrids showed intermediate PAC and PMC values which support an additive genetic basis for these two parameters. Significant differences in survival were observed between *C. angulata* and *C. gigas* progenies, with the latter taxon showing a higher value and reciprocal hybrids intermediate ones. The detailed results and their discussion are present in Article 2.

Article 2 (submitted)

INDIVIDUAL RELATIONSHIP BETWEEN ANEUPLOIDY OF GILL CELLS AND GROWTH RATE IN THE CUPPED OYSTERS *CRASSOSTREA ANGULATA*, *C. GIGAS* AND THEIR RECIPROCAL HYBRIDS.

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Summary

The Portuguese oyster, *Crassostrea angulata*, is taxonomically close to the Pacific oyster, *C. gigas*, but there are clear genetic and phenotypic differences between these taxa. Among those differences, the faster growth of *C. gigas* compared with *C. angulata* has often been observed in the field and was found related to physiological parameters such as feeding and respiratory time activities. Factorial crosses between *C. angulata* and *C. gigas* were performed to investigate the relationship between growth variation and somatic aneuploidy at the individual level in the two taxa and their reciprocal hybrids. The different progenies were reared in Ria Formosa (Portugal) under standard farming conditions. Growth rate and survival were significantly higher in *C. gigas* than in *C. angulata*, and the reciprocal hybrids showed intermediate performances. Significant differences were also observed in the proportion of aneuploid cells (PAC) and number of missing chromosomes (PMC) between the two taxa, *C. angulata* showing the highest values. Intermediate values of PAC and PMC were observed in the reciprocal hybrids, supporting additive genetic bases of these parameters. Our results also confirm the negative correlation between somatic aneuploidy and growth rate at the individual level, as previously reported in *C. gigas*.

Key words: *Crassostrea angulata*; *Crassostrea gigas*; hybrids; aneuploidy; growth variation

Introduction

Somatic aneuploidy of an animal can be defined as an alteration in the number of chromosomes in a proportion of the somatic cells due to abnormalities that arise during mitosis. This phenomenon has been documented in several species and some of the mechanisms responsible for the appearance and cellular surveillance of aneuploidy have been demonstrated (Thomas, 1995; Cimini and Degraasi, 2005). In bivalve molluscs, aneuploid gill cells have been observed in natural populations of the Pacific oyster *Crassostrea gigas* (Thiriot-Quiévreux, 1986), the Antipodean flat oyster *Ostrea angasi* (Li and Havenhand, 1997) and mussels of the genus *Mytilus* (Martínez-Expósito *et al.*, 1992). In *C. gigas*, which has a normal diploid chromosome number of $2n = 20$, a high proportion of hypodiploid cells, with $2n = 19$, 18 or 17 , has been observed in several natural and hatchery produced populations (Leitão *et al.*, 2001a). Further studies in this species showed that monosomies were observed only in 4 of the 10 chromosome pairs (i.e. chromosome from pairs 1, 5, 9 and 10) and no nullisomy has ever been observed (Leitão *et al.*, 2001b; Bouilly *et al.*, 2005). These findings clearly demonstrate that chromosome loss in *C. gigas* was not random or an artefact of the air drying technique (Thiriot-Quiévreux and Ayraud, 1982), as investigated by Gong *et al.* (2004). A similar phenomenon can be manifested in tetraploid *C. gigas*, at higher frequency and level than in diploids (McCombie *et al.*, 2005).

The causal factors of somatic aneuploidy remain unknown in oysters. However, negative correlation between the degree of somatic aneuploidy (i.e., the proportion of somatic cells with one or more chromosome missing) and growth rate within and among families of *C. gigas* has been reported in several previous studies (Thiriot-Quiévreux *et al.*, 1988; Zouros *et al.*, 1996; Leitão *et al.*, 2001a). Additionally, pollutants such as herbicides (atrazine: Bouilly *et al.*, 2003 ; diuron: Bouilly *et al.*, 2007), aromatic hydrocarbons (Dixon, 1982) and heavy metals (cadmium: Bouilly *et al.*, 2006) significantly increase aneuploidy and affect the same chromosome pairs (Bouilly *et al.*, 2005). In oysters, however, most studies were performed on *C. gigas* and the relevance of this phenomenon in other oyster taxa remained to be investigated (Landau and Guo, 2005).

The Portuguese oyster *C. angulata* is a species of commercial value that sustained European oyster production for almost one century until early 1970s. Between 1967 and 1973 however *C. angulata* was almost wiped out from Europe by a viral disease (Renault, 1996). Since then, *C. gigas* has been introduced to different European countries to replace *C. angulata* and to sustain the oyster industry (Grizel and Héral, 1991). Based on larval shell morphology (Ranson, 1960), experimental hybridization (Menzel, 1974; Huvet *et al.*, 2001, 2002) and electrophoretic studies of enzyme polymorphism (Buroker *et al.*, 1979) several authors have considered *C. angulata* and *C. gigas* to be the same species.

Studies on the mitochondrial cytochrome oxidase C subunit I (COI) gene (Boudry *et al.*, 1998; O'Foighil *et al.*, 1998) have shown that these two taxa are of Asian origin and closely related, although there are clear genetic (Huvet *et al.*, 2000) and cytogenetic (Leitão *et al.*, 1999a; 1999b; 2004) differences between them. The respective natural geographic distribution of the 2 taxa is still unclear (Fabioux *et al.*, 2002; Lapègue *et al.*, 2004) but they can hybridize in areas where they are in contact (Huvet *et al.* 2004). In addition, significant phenotypic differences between the two taxa have also been reported, including differences in production yield thought to be mainly due to the fast growth rate of *C. gigas* (His, 1972; Bougrier *et al.*, 1986; Soletchnik *et al.*, 2002). Some ecophysiological parameters (valve activity: His, 1972; oxygen consumption: Gouilletquer *et al.*, 1999; feeding and respiratory time activities: Haure *et al.*, 2003) were found to account for the differences in growth between these taxa. However, aneuploidy had never been investigated in *C. angulata* or compared with the levels observed in *C. gigas*.

The known negative relationship between aneuploidy and growth in *C. gigas* and the growth difference between *C. gigas* and *C. angulata* led us to examine survival, growth and somatic aneuploidy in these two taxa and their reciprocal hybrids. The main objective of this work was to establish if the individual relationship between growth and aneuploidy, as previously observed within *C. gigas*, could also be observed in these two taxa, both at the individual and the taxon levels.

Material and methods

Parental populations

Ripe *Crassostrea angulata* were collected from a wild population in Monte-da-Pedra in Sado estuary (Portugal). *C. gigas* adults were collected from a naturalized population in Seudre estuary in the Marennes-Oléron Sound (France). These populations were chosen since they are presumed to be pure populations of *C. gigas* and *C. angulata* based on population genetic studies by Boudry *et al.* (1998) and Lapègue *et al.* (2004).

Crosses and larval rearing

Crosses and larval rearing of the different progenies were performed in the Shellfish Hatchery of IFREMER Station in La Tremblade (France). Gametes were stripped directly from gonads as described by Stephano and Gould (1988). The number of oocytes and spermatozooids was estimated using Thoma and Mallassez slides coupled to an image processing system (Samba technologies, Marcoussis, France). Oocytes from 15 *C. angulata* and 10 *C. gigas* females were pooled for each taxon and distributed in beakers with filtered seawater. The same procedure was used with the spermatozoa of 5 *C. angulata* and 8 *C. gigas* males. Crosses between *C. angulata* and *C. gigas* were

produced by a 2 X 2 factorial mating to obtain the following four groups: *C. angulata* (AA); *C. gigas* (GG); hybrids derived from *C. angulata* females and *C. gigas* males (AG); and hybrids derived from *C. gigas* females and *C. angulata* males (GA).

Fertilization was performed at a ratio of 100 spermatozoa per oocyte. The eggs were then incubated with a density of 82 eggs/ml in filtered seawater with a salinity of approximately 35 ‰. Larvae were reared in cylindrical conical tanks and fed with a progressive diet of *Isochrysis* aff. *galbana* (clone T-ISO) and *Tetraselmis suecica*. Three times per week the water was changed, but no larvae were discarded by selective sieving in order to reduce the loss genetic variability known to be associated with this practice (Taris *et al.*, 2006). During larvae rearing water temperature ranged between 20 to 26 °C and the salinity was approximately 35 ‰. Eyed larvae were settled on cultch in raceways as described by Walne (1974).

Juvenile rearing and grow-out phase

Spat with 49 days post-fertilization (DPF) was transferred to the Tavira Shellfish Hatchery Station of the Portuguese Institute for Fisheries and Sea Research (INIAP/IPIMAR). The animals were reared in an indoor nursery system supplied with filtered underground saltwater with a salinity of approximately 30 ‰ and a mean (SD) temperature of 17.3 (3.4) °C. During the nursery phase the animals were fed with a mixed diet of cultured *I. galbana*, *T. suecica* and *Skeletonema costatum*. At day 204 after fertilization, 4 fine mesh spat bags with 50 oysters each were prepared for each of the four groups. Twenty five individuals in each bag were tagged with a non-toxic epoxy resin. Hence, 100 individuals for each group were individually labelled. The oyster bags were then deployed in the experimental site at Cacela-Velha in Ria Formosa Lagoon (Portugal). The oysters were cultured off-bottom in iron frames tables on which the bags were attached. The position of the bags on the tables was changed every month. The sea floor of the study site was a mixture of sand and mud, and the emersion rate was approximately 50 %.

Growth and survival

The live weight of each tagged individual from the different groups was recorded every month during the grow-out phase. The animals were first measured at the beginning of the experiment on 3 March 2004, subsequently, on 6 April, 4 May, 2 June, 1 July, 29 July, 31 August, 30 September, 27 October and finally on 26 November of 2004. Individual growth rates (GR) were calculated using the slope of the linear regression of live weight against time as described by Boudry *et al.* (2003). Mean Pearson R^2 correlation of live weight against time for each group (n=30) ranged from 0.935 to 0.959, indicating that it is a very

good estimator of individual growth in the present experiment (the lowest individual value being 0.839). The degree of precision of weight measurements was 0.001 g.

Aneuploidy scoring

At the end of the grow-out phase, 30 animals of each group (total of 120 individuals) were incubated for 10 h in seawater containing 0.005 % colchicine. Their gills were then dissected in seawater, treated for 30 min in 0.9 % sodium citrate and fixed in a freshly prepared solution of absolute ethanol-acetic acid (3:1) with three 20 min changes. The air drying technique developed by Thiriot-Quévèreux and Ayraud (1982) was used to prepare individual slides of gill tissue from each animal. The gill preparations were then stained for 10 min with Giemsa (4%, pH 6.8) and scored by microscope observation (Olympus microscope). Chromosome counts were made directly on 30 well-spread metaphases per individual. Two different measures of somatic aneuploidy described by Thiriot-Quévèreux *et al.* (1992) were used: (1) the proportion of aneuploid gill cells (out of 30) that had at least one chromosome missing (PAC) and (2) the proportion of missing chromosomes from a random sample of 30 cells (PMC).

DNA isolation, amplification and PCR-RFLP analysis

Gill fragments from parental oysters and studied progenies analysed (*i.e.* scored for somatic aneuploid and growth rate) were preserved in absolute ethanol. Total DNA was extracted from gill tissue using a modification of a phenol/chloroform method described by Sambrook *et al.* (1989). The primers COI3 (5'-GTATTTGGATTTTGAGCTGT-3') and COI4 (5'-GAGGTATTAAAATGACGATC-3') were used to amplify a 584 bp fragment of the mitochondrial gene cytochrome oxidase C subunit I (COI). The primers COI3 and COI4 were designed based on the sequence of the mitochondrial genome of *C. gigas* available on GenBank. Reactions were performed in 50 µl volumes composed of 5.0 µl of 10X PCR buffer, 35.7 µl of distilled water, 5.0 µl of dNTP (2 mM), 1.0 µl of each primer (20 µM), 0.3 µl of Taq polymerase (5 U/ µl) and 2.0 µl of template DNA (ca. 100 ng). PCRs were performed for one cycle of 2 min at 94 °C and 30 cycles of 30 s at 94 °C, 30 s at 50 °C, and 45 s at 72 °C with a least extension of 7 min at 72 °C. Negative controls, in which distilled water was added to the PCR mix instead of extracted DNA, was used in all PCR reactions. Restriction enzyme digestions of COI PCR products were set up in a 20 µl volume containing 10.0 µl PCR products, 7.5 µl distilled water, 2.0 µl 10X reaction buffer, and 0.5 µl restriction enzyme (10 U/ µl). COI amplicons were digested with Msp I (Promega) restriction enzyme for 2 h at 37 °C. PCRs products with the restriction site for the Msp I were systematically used as controls. Digestion products were separated by electrophoresis in 2 % agarose gels with ethidium bromide staining.

Statistical analyses

Survival of oysters of the different groups was examined using the Kaplan-Meier method (Kaplan and Meier, 1958). The Mantel-Cox test was used to compare the survival trends between groups (Mantel, 1966) at the 0.05 level after correction for multiple testing using the sequential Bonferroni method (Rice, 1989). Since no significant difference (Kruskal-Wallis test; $p > 0.05$) in live weight was observed among bags within each group, the effect of bags was not considered for analysis of individual growth rate. The suitability of data for parametric analysis was evaluated prior to examination using the Kolmogorov-Smirnov test for goodness of fit (normality) and the Cochran test (heteroscedasticity). Growth rate (GR) differences among groups were analysed by one-way analysis of variance (ANOVA). Following the ANOVA, multiple comparisons between groups were performed using Tukey honest significant (HSD) tests. The Kruskal-Wallis test (non-parametric data) was used to identify differences among groups in the proportion of aneuploid cells (PAC) and proportion of missing chromosome (PMC). When significant differences ($p < 0.05$) were identified with Kruskal-Wallis test, multiple comparisons were performed using Nemenyi tests as described by Zar (1996). In order to determine if PAC and live weight or GR of the individuals analysed were correlated Kendall's non-parametric rank correlation coefficient was used. Data analysis was carried out using Statistica version 5.1.

Results

All oysters progenitors ($n = 38$) and progenies ($n = 120$) analysed with the mitochondrial marker showed the expected RFLP patterns. This allowed the confirmation of the taxa of the sampled parental oysters and the partial genetic confirmation of their progenies (i.e., maternal origin). In addition, significant phenotypic differences (see point 3.2) were observed between the parental lines and the hybrids, which to a certain degree also confirm their specific or hybrid status.

Survival and growth

A low, but not unusual in the study site, survival (40.0 to 54.5 %) was observed over the study period. Survival rates of *C. angulata* and *C. gigas* progenies as well as their reciprocal hybrids, using the Kaplan-Meier method, are presented in Figure 1. The highest and lowest survival rates were observed for *C. gigas* and *C. angulata* progenies, respectively, with significant differences between the two groups (Mantel-Cox = 7.09; $P <$

0.05). No significant differences in survival were observed among the other groups (Mantel-Cox test; $P > 0.05$).

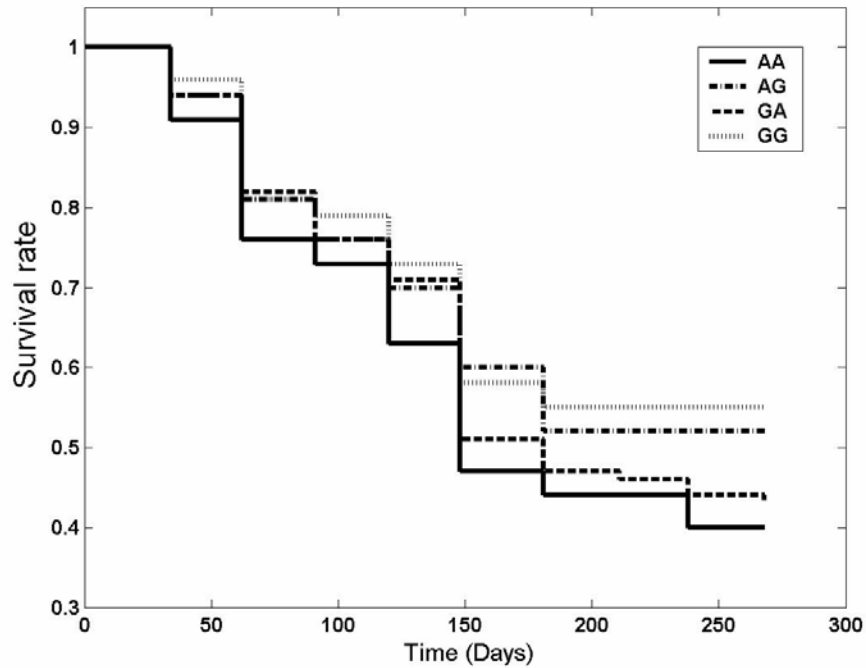


Figure 1. Survival rate of *C. angulata* (AA), *C. gigas* (GG) and the reciprocal hybrids AG and GA using the Kaplan-Meier method. The female origin in the crosses is listed first.

Only the data from oysters that were alive at end of the experimental period and that were scored for chromosome loss were used for growth rate analysis (i.e. 120 individuals). Live weight of labelled oysters from the four groups increased through time (Figure 2). Growth rate (GR) were significantly different between groups (ANOVA, $p < 0.01$). The GR of GG group (mean of 0.098 and standard deviation of 0.031) was significantly higher (Tukey's HSD, $p < 0.01$) than AA group (0.071 ± 0.029), but not from AG (0.089 ± 0.027) and GA (0.097 ± 0.031) groups. No significant differences were observed between AG group and any of the other groups (Tukey's HSD, $p > 0.05$). The GR of GA group was only significantly higher than the AA group (Tukey's HSD, $p < 0.01$).

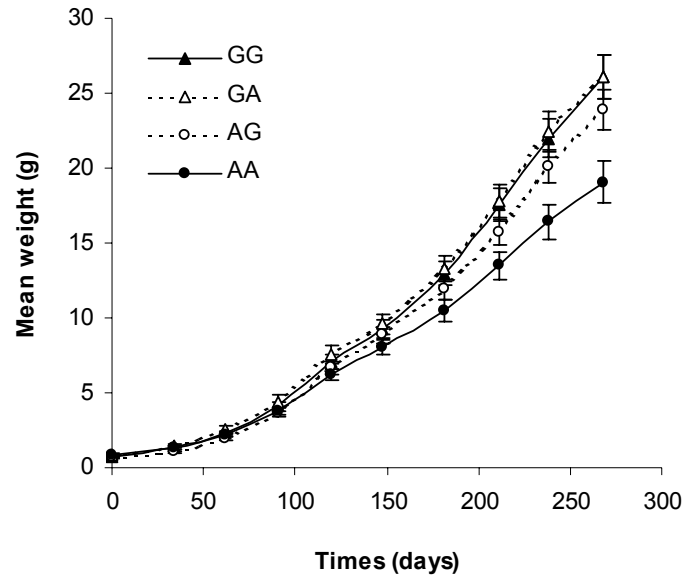


Figure 2. Mean (the error bars represent standard error) live weigh of *C. angulata* (AA), *C. gigas* (GG) and the reciprocal hybrids AG and GA (30 individuals per group) during the experimental period. The female origin in the crosses is listed first.

Somatic aneuploidy

The proportion of aneuploid cells (PAC) and missing chromosomes (PMC) for the four groups is presented in Table 1. These two parameters are well correlated (Kendall's τ within each group ranging from 0.63 and 0.85, $p < 0.001$) and consequently provide rather similar results. A significant difference in PAC was observed among the four groups (Kruskal-Wallis test; $H = 9.34$; $p = 0.02$). An a posteriori test (Nemenyi test) failed to distinguish hybrids from both parental lines, but revealed significant differences ($p < 0.05$) between *C. angulata* (PAC = 20.0) and *C. gigas* (PAC = 16.7) progenies. Similarly, PMC also differed significantly among the four groups (Kruskal-Wallis test; $H = 8.55$; $p = 0.04$), with significant differences observed only between *C. angulata* (PMC = 1.67) and *C. gigas* (PMC = 0.83) progenies (Nemenyi Test; $p < 0.05$).

Table 1. Proportion of aneuploid cells (PAC) and missing chromosomes (PMC) in adult oysters of *C. angulata* (AA), *C. gigas* (GG) and the reciprocal hybrids AG and GA at the end of the experimental period (n = 120). The female origin in the crosses is listed first.

Aneuploidy		AA	AG	GA	GG
PAC (%)	Median	20.0	18.3	18.3	16.7
	Mean	20.1	18.0	18.2	16.1
	SD	6.3	4.8	5.4	5.3
PMC (%)	Median	1.67	1.50	1.42	0.83
	Mean	1.54	1.44	1.36	1.11
	SD	0.59	0.55	0.56	0.62

Kendall's correlation coefficients between PAC and final live weight and RG within each 4 group are presented in table 2. Kendall's correlation coefficient between PAC and GR was -0.236 (p = 0.067) for the AA group, -0.229 (p = 0.076) for AG hybrids, -0.357 (p < 0.01) for GA hybrids and -0.265 (p = 0.040) for the GG group.

Table 2. Kendall's non-parametric correlation coefficient between the proportion of aneuploid cells (PAC) and final live weight as well as growth rate for *C. angulata* (AA), *C. gigas* (GG) and the reciprocal hybrids AG and GA. The female origin in the crosses is listed first.

	Final live weight	Growth rate
AA	-0.266**	-0.236*
AG	-0.252*	-0.229*
GA	-0.370***	-0.357***
GG	-0.265**	-0.265**

* p < 0.1; ** p < 0.05; *** p < 0.01

Relationships between final live weight and somatic aneuploidy are illustrated in Figures 3 (at the individual level within each group) and 4 (between groups).

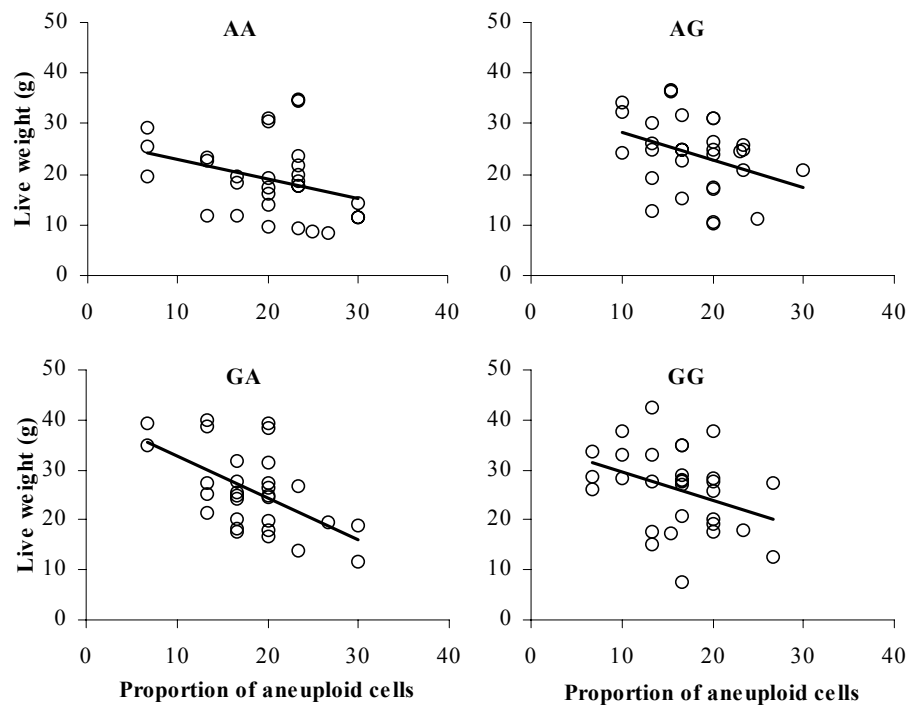


Figure 3. Linear regression lines of live weight (at the end of the experimental period) against the percentage of aneuploid cells (PAC) in *C. angulata* (AA), *C. gigas* (GG) and the reciprocal hybrids AG and GA (30 individuals per group). The female origin in the crosses is listed first.

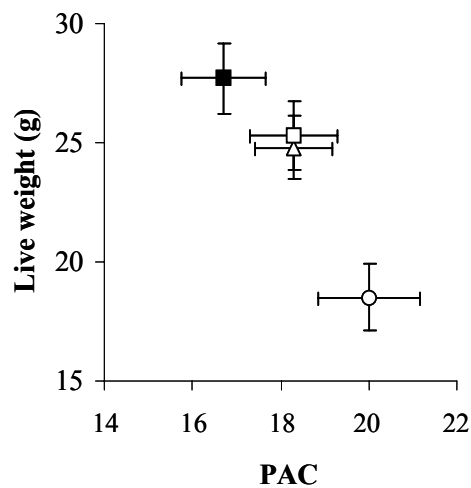


Figure 4. Relationships between the median live weight (at the end of the experimental period) and the median percentage of aneuploid cells (PAC) in *C. angulata*

(AA), *C. gigas* (GG) and the reciprocal hybrids AG and GA (30 individuals per group). The female origin in the crosses is listed first. Error bars represents SE.

Discussion

Survival

The mortality of *C. gigas* was lower than that of *C. angulata* supporting results previously obtained by other authors (Bougrier *et al.*, 1986; Soletchnik *et al.*, 2002). The differences observed at the survival level between the two taxa may have been due to different environmental requirements (e.g., emersion rate, temperature, salinity) but, to our knowledge, the available ecophysiological studies do not provide evidence for such different requirements. The high mortality observed in *C. angulata* could have been also due to higher disease susceptibility. In the early 1970's in France, irido-like viruses infections were detected in *C. angulata* and *C. gigas* (Comps and Bonami, 1977; Comps and Duthoit, 1976; Renault, 1996), but no major losses were observed in the latter taxon while *C. angulata* suffered mass mortalities (Comps, 1988). However, despite numerous examinations using various techniques (histopathology, transmission electron microscopy, and PCR-based techniques), irido-like viruses have not been observed in France or Portugal for decades (Renault, com. pers) and it is unlikely that the mortality observed during our experiment is due to such viruses. Further studies are needed to document the relative susceptibility of *C. gigas* and *C. angulata* to pathogens.

The reciprocal hybrids showed an intermediate survival between *C. gigas* and *C. angulata* progenies and no significant differences were obtained between them and the other groups. This is consistent with the results obtained by Bougrier *et al.* (1986) and Soletchnik *et al.* (2002) and supported by the additive genetic basis of the observed variation for survival in *C. gigas* (Ernande *et al.*, 2004, Degremont *et al.*, 2007).

Growth rate of C. angulata and C. gigas

The growth rate results obtained in the present study support previous findings that showed that *C. gigas* grow faster than *C. angulata*. His (1972) observed that the total weight of *C. gigas* individuals was 2 times higher than *C. angulata* individuals with the same age and reared under similar conditions after approximately 3 years. Similar results were reported by Héral *et al.* (1986) and Parache (1989) by analysing historic series from the Marennes-Oléron Sound and Archachon Bay, respectively, two important oyster farming areas located on the French Atlantic coast. The faster growth of *C. gigas* relatively to *C. angulata* was also observed by Bougrier *et al.* (1986) and Soletchnik *et al.* (2002) on

comparative field studies where hatchery-produced animals were reared in different sites on the coast of France.

In order to understand the physiological mechanisms that underlie the differences in growth rate between *C. angulata* and *C. gigas* some studies have compared different eco-physiological parameters between the two taxa. On a first approach, His (1972) observed that *C. gigas* had a higher clearance rate and valve activity than *C. angulata*. Later, Goulletquer *et al.* (1999) observed that the oxygen consumption rate of *C. gigas* juveniles was higher than in *C. angulata* juveniles. More recently, Haure *et al.* (2003) observed that *C. gigas* adults had a significant higher feeding time activity than *C. angulata* adults, but they did not observe differences in clearance and oxygen consumption rates as previously reported.

Growth rate of the reciprocal hybrids

The reciprocal hybrids showed an intermediate growth rate between *C. angulata* and *C. gigas* progenies and hence no heterosis for growth was observed. This observation is consistent with the results obtained by Bougrier *et al.* (1986) and Soletchnik *et al.* (2002) that did not also observe hybrid vigour nor out-breeding depression in crosses between the two taxa. However, these findings do not exclude the possibility of a superior growth of *C. angulata* x *C. gigas* hybrids under different environmental conditions and/or for other traits.

Differences in growth rate between reciprocal hybrids produced by crosses have been reported amongst inbred lines of *C. gigas* (Hedgecock *et al.*, 1995; Bayne *et al.*, 1999), as well as in inter-specific hybrids between *Mytilus edulis* and *M. galloprovincialis* (Beaumont *et al.*, 2004). Maternal effects can usually be detected at early stages and are expected to dissipate with age of the offspring and are therefore often neglected (Roff and Sokolovska, 2004). They can also be caused by mitochondrial genes. Although a maternal effect for growth rate has been observed in reciprocal hybrids of *C. angulata* and *C. gigas* (Bougrier *et al.*, 1986; Soletchnik *et al.*, 2002), this was not detected in our study nor in the eco-physiological study by Haure *et al.* (2003).

Somatic aneuploidy

In the present study, a significant higher proportion of aneuploid cells (PAC) and missing chromosomes (PMC) were observed in the *C. angulata* progeny comparatively to the *C. gigas* progeny. These results suggest the existence of a different susceptibility to chromosome loss between the two taxa and therefore support the hypothesis of a genetic basis for somatic aneuploidy (Leitão *et al.*, 2001c; McCombie *et al.*, 2005). An intermediate level of somatic aneuploidy, although not significantly different from parental

lines, was observed in the reciprocal hybrids, suggesting the additive nature of such genetic variation. Bouilly (2004) also did not find any heterotic effect for somatic aneuploidy in individuals produced by controlled crosses amongst inbred lines of *C. gigas*, although the hybrids showed considerable heterosis for growth rate (Hedgecock *et al.*, 1995).

Our results also support the existence of a negative correlation between the degree of somatic aneuploidy and growth rate in oysters, as previously observed in *C. gigas* (see for review Leitão *et al.*, 2001a), but not in *C. virginica* (Landau and Guo, 2005). Most previous studies compared “slow” and “fast” growing oysters (e.g. Thiriot-Quiévreux *et al.*, 1988; 1992, Zouros *et al.*, 1996) and only one study examined the individual relationship between growth rate during a 8 month period in 36 individual of a hatchery bred progeny (Leitão *et al.*, 2001a). In this last case, highly significant linear negative correlations were observed between aneuploidy and total weight in 12-, 15- and 20-month-old oysters $R^2 = 0.40, 0.53$ and 0.58 ; $P < 0.001$., respectively. In our study, such correlations are lower, but still highly significant. This might be due to the fact that, in our study, rearing of oysters was performed in a more natural and consequently less controlled (and therefore less homogeneous) environment than in the study of Leitão *et al.* (2001a). Interestingly, this relationship is also observed between taxa, the slower growing taxon showing the highest level of aneuploidy. So the present study confirms that a negative correlation between the degree of somatic aneuploidy and growth rate exists in oysters, both at the individual (Figure 3) and the taxon levels (Figure 4).

It would be of great interest to determine if this is also the case in other oyster taxa showing slow growth performance such as *C. sikamea* and *C. plicatula* and if the missing chromosomes belong to the same pairs in these oyster species. The application of banding techniques that allow the individual identification of all chromosomes pairs (and thus the identification of the missing chromosomes in the aneuploid cells) would permit to determine if the phenomenon of aneuploidy affects, in a similar way, all *Crassostrea* species and, more precisely, if the same chromosome pairs are affected. This would be particularly interesting to better understand the role of aneuploidy, if the negative relationship with growth rate is a general phenomenon among cupped oysters.

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3.3. CONCLUSIONS

The insufficient number of replicate and high variation observed among replicates did not allowed a formal comparison of the different zootechnique parameters measured among *C. angulata*, *C. gigas* and their reciprocal hybrids during the larval phase. Nevertheless, the variation among groups in yield of D and ready-to-set larvae, and metamorphosis rate was low. However, the higher difficulty observed in the larval rearing of *C. angulata* comparatively to *C. gigas* (data not shown), indicate that the larvae of these taxa may have different requirements (e.g., temperature, salinity).

During both nursery and grow-out phases, a higher growth and survival was observed in *C. gigas* progeny relatively to *C. angulata* progeny. For most traits, reciprocal hybrids showed a significant higher performance than *C. angulata* progeny, but similar or inferior to *C. gigas* progeny. Therefore, no hybrid vigour was observed for any trait since hybrid performance was not superior to the best parental line. The combination of a superior growth and survival (although not statistically different in nursery phase) in *C. gigas* progeny relatively to *C. angulata* progeny resulted in a yield approximately 8.5 and 2.0 times higher at nursery and grow-out phases, respectively. The differences between the two phases may have been due to the fact that the culture conditions at the nursery were more favourable to *C. gigas*.

The use of mitochondrial and nuclear markers allowed the genetic confirmation of AG and GA hybrids. In addition, the use of these two markers together with the phenotypic differences observed among progenies confirmed that if contamination occurred it was at a very low level.

A higher proportion of aneuploid cells and missing chromosomes were observed in *C. angulata* progeny than in *C. gigas* progeny, with hybrids showing intermediate levels which support an additive genetic basis for these parameters. In addition, a negative correlation between the proportion of aneuploid cells and growth was also observed within each group.

CHAPTER 4. MORPHOLOGICAL AND PIGMENTATION DIFFERENCES BETWEEN *CRASSOSTREA ANGULATA* AND *C. GIGAS*

4.1. SHELL MORPHOLOGY AND PIGMENTATION IN *CRASSOSTREA ANGULATA*, *C. GIGAS* AND THEIR RECIPROCAL HYBRIDS

4.1.1. Background and objectives

Cupped oyster such as *Crassostrea angulata* and *C. gigas* are delicatessen food items in some countries and thus product quality (e.g., shell shape and colour, shell hardness for robustness on handling, flavour of meat) can be of paramount importance (Mahon, 1983; Ward *et al.*, 2005). For example, shell shape is recognized as an important marketing aspect for the Canadian, French, Irish, North American and Australian oyster industries (Brake *et al.*, 2003; Ward *et al.*, 2005). In addition, the existence of distinctive characters, specific to certain lines of oysters, associated with quality by the consumer can also be important for the oyster industry (Mahon, 1983).

The shape of an oyster shell is highly influenced by the environment, especially in the genus *Crassostrea*, and can be manipulated throughout appropriate husbandry techniques (e.g., frequency in turning bags, density,). Nevertheless, a genetic basis for shell shape and external pigmentation has been reported in oyster species by several authors (Imai and Sakai, 1961; Wada, 1994; Brake *et al.*, 2004; Ward *et al.*, 2005). These two traits may become increasingly important as production increases and consequently consumer's exigency for product quality also augments. Differences in shell morphology can be also used as a tool to differentiate close genetic taxa or stocks of mollusc bivalves in common garden experiments, which allows reducing variation due to age and environment (Wilbur and Gaffney, 1997).

The objective of this study was to compare the shell shape, pigmentation of the adductor muscle scar, external pigmentation of right and left valve, and external yellow coloration of left valve of *C. angulata*, *C. gigas* and their reciprocal hybrids.

4.1.2. Experimental procedure

Shell shape and pigmentation of the adductor muscle scar of *C. angulata* progeny (AA), *C. gigas* progeny (GG), hybrids derived from *C. angulata* females and *C. gigas* males (AG), and hybrids derived from *C. gigas* females and *C. angulata* males (GA) were compared using univariate and multivariate analyses. The oysters were produced as describe in section 3.1 and the detailed experimental procedure is described in Article 3. In addition, external shell coloration was also examined in 72 individuals from each group.

External shell pigmentation of left and right valves was described based on Imai and Sakai (1961) and Brake *et al.* (2004) into the following categories: (1) very light (whole surface having no or very little pigmentation); (2) light (a small part having pigmentation); (3) dark (most part having pigmentation); and (4) very dark (whole or almost surface having pigmentation). The categories used to describe the yellow coloration of the left valve were: (1) no yellow coloration; (2) a small part of the shell having yellow coloration; (3) most part of the shell having yellow coloration; and (4) almost all surface having yellow coloration (Figure 22).

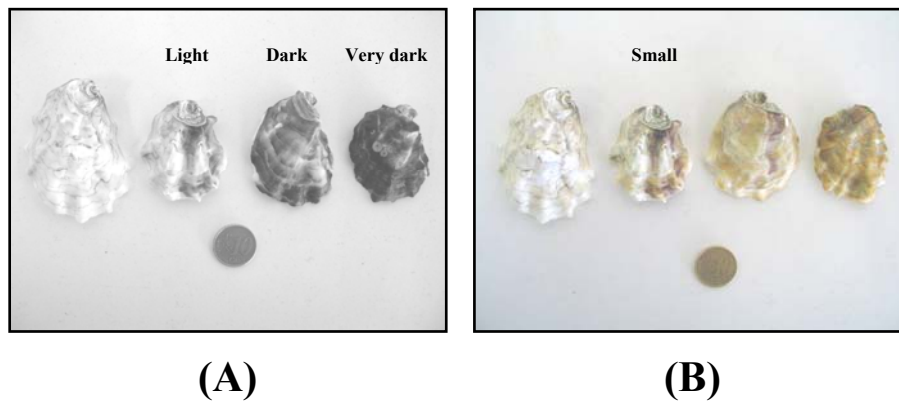


Figure 22. Shell representatives of the four categories used to classify the degree of (A) external pigmentation of the left valve (very light, light, dark, very dark) and (B) external yellow coloration of the left valve (none, small part, half part, most part).

Chi-squared tests were used to compare pigmentation of left and right valves between groups after correction for multiple testing using the sequential Bonferroni method (Rice, 1989). Due to the low number and even absence of individuals in category 4 in certain groups, counts from categories 3 and 4 were pooled for chi-squared analyses.

4.1.3. Results and Discussion

Significant morphometric differences in shell shape were observed between *C. angulata* and *C. gigas* progenies, and reciprocal hybrids showed intermediate morphometric characters. These results indicate the existence of a genetic basis for the morphometric characters examined. The *C. angulata* progeny had deeper shells with shorter adductor muscle scars in length and height as well as smaller ligamental areas comparatively with our *C. gigas* progeny. The detailed results of morphometric characters and their discussion are presented in Article 3.

Significant differences in pigmentation of adductor muscle scar were observed among the four groups. Pairwise comparisons showed that *C. gigas* progeny had a significant lower pigmentation of the adductor muscle scar of both left (MSL) and right (MSR) valves than all the other groups. No significant differences were observed between *C. angulata* progeny, AG and GA hybrids for MSL and MSR pigmentation (see Article 3).

The frequency of individuals with a darker external pigmentation of the right valve was significantly higher in *C. angulata* progeny than in *C. gigas* progeny (Chi-squared, $p < 0.05$), but no differences were observed between AG and GA hybrids as well as between hybrids and pure crosses (Figure 23A).

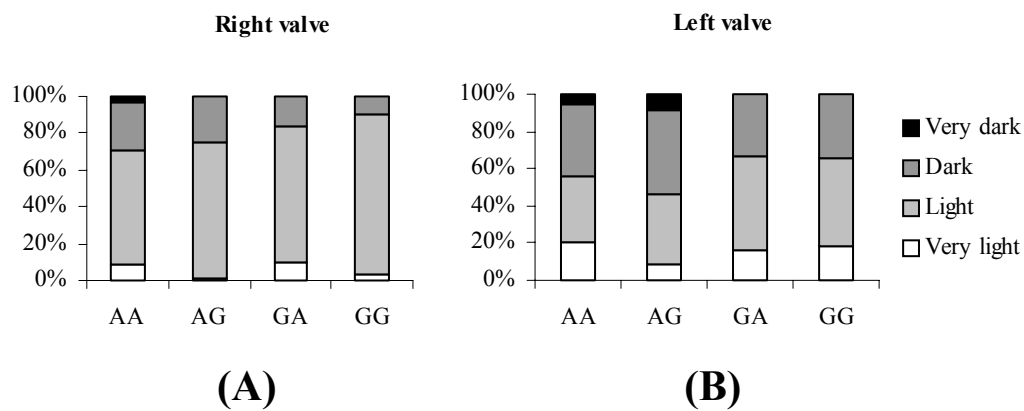


Figure 23. The percentage frequency of external pigmentation classified into 4 categories (very light, light, dark, very dark) of the (A) left and (B) right valves of *C. angulata* progeny (AA), *C. gigas* progeny (GG), hybrids derived from *C. angulata* females and *C. gigas* males (AG), and hybrids derived from *C. gigas* females and *C. angulata* males (GA).

On the other hand, no significant differences were observed in external pigmentation of the left valve among groups (Chi-squared, $p > 0.05$, Figure 23B).

External pigmentation of the left and right valves was positively correlated in all groups with Kendall's correlation coefficient of 0.389, 0.434, 0.513 and 0.232 in *C. angulata* progeny, AG hybrids, GA hybrids and *C. gigas* progeny, respectively. However, it is noteworthy that the correlation observed was weak especially in *C. gigas* progeny. The results of the present study are consistent with the findings of Imai and Sakai (1961). These authors describe differences in shell pigmentation in races of *C. gigas* that persisted for generations. More recently, Brake *et al.* (2004) suggested that a large fraction of variation in external shell pigmentation in *C. gigas* was due to genetic causes based on the analysis of experimental cohorts of pedigreed oysters.

A yellow coloration of the surface of the left valve was observed in a higher frequency and intensity in *C. angulata* progeny comparatively to the other groups (Figure 24). The absence of this coloration in *C. gigas* progeny and the intermediate frequency in AG and GA hybrids suggests a genetic basis for these traits. However, the presence of this type of coloration in *C. gigas* cultured in Tasmania (Australia), called “golden oyster” (Ward *et al.*, 2005), suggest that this coloration is not specific to *C. angulata*. Indeed, individuals with this coloration from population of *C. gigas* on the Atlantic coast of France have been also observed.

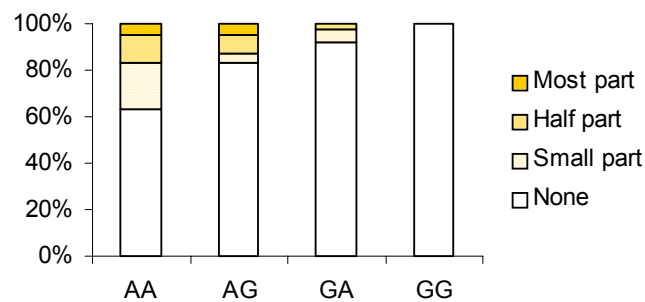


Figure 24. The percentage frequency of external yellow coloration of the left valve classified into 4 categories (none, small part, half part, most part) of *C. angulata* progeny (AA), *C. gigas* progeny (GG), hybrids derived from *C. angulata* females and *C. gigas* males (AG), and hybrids derived from *C. gigas* females and *C. angulata* males (GA).

Article 3 (submitted)

**COMPARATIVE STUDY OF SHELL SHAPE AND MUSCLE SCAR PIGMENTATION IN
THE CLOSELY RELATED CUPPED OYSTER TAXA *CRASSOSTREA ANGULATA*, *C.*
GIGAS AND THEIR RECIPROCAL HYBRIDS**

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Abstract

The taxonomic status of the cupped oysters *Crassostrea angulata* and *C. gigas* has received considerable attention in the last decades. Based on larval shell morphology, experimental hybridization, allozymes and nuclear DNA studies several authors have considered these two taxa as being synonymous. However, mitochondrial data showed clear genetic differences between the two taxa. In addition, microsatellite-based studies and cytogenetic studies have also provided evidences that support their differentiation. Considerable differences have also been observed at the phenotypic level in terms of growth rate and ecophysiological parameters. In the present study, *C. angulata* from Sado estuary (Portugal) and *C. gigas* from Seudre estuary (France) were collected and factorial crosses were performed. Juveniles of the different progenies were reared in Ria Formosa (Portugal) under common conditions to determine if they exhibited differences in shell shape and in pigmentation of the adductor muscle scar. Significant morphometric differences between *C. angulata* and *C. gigas* progenies were indicated by univariate and multivariate analyses. The shell characters that were more important in separating the two taxa were shell depth and muscle scar size. Both reciprocal hybrids showed intermediate morphometric characters between parental lines. In addition, significant differences were also observed between *C. angulata* and *C. gigas* progenies in terms of pigmentation of adductor muscle scar. *C. angulata* and both reciprocal hybrid progenies showed highly pigmented adductor muscle scars whereas in *C. gigas* progeny the pigmentation was lighter. The differences in shell shape and muscle scar pigmentation observed in the present study supports the distinction of the two taxa.

1. Introduction

The Portuguese oyster *Crassostrea angulata* (Lamarck, 1819) and the Pacific oyster *C. gigas* (Thunberg, 1793) are both important commercial species. According to FAO (2006), *C. gigas* is one of the most important aquatic animals farmed in the world, with around 4.4 million tonnes in 2004. *C. angulata* sustained the European oyster production for almost one century, with an annual production that reached 100 000 tons. Major mortalities between 1967 and 1973 almost wiped out *C. angulata* from Europe (Comps, 1988). Nowadays, there is only a small production in Portugal and Spain, but *C. angulata* is presumed to be the main oyster species produced in Taiwan (Boudry et al., 1998) and probably in other regions in Asia (Lapègue et al., 2004; Wang et al., 2007).

The taxonomic status of *C. angulata* and *C. gigas* has received considerable attention in the last decades. Ranson (1960) based on the morphological similarity of late larval shells suggested that the two taxa should be classified as the same species. It was also observed that *C. angulata* and *C. gigas* interbreed readily in the laboratory (Imai and Sakai, 1961) and F1 hybrids show normal meiosis and mitosis (Menzel, 1974). In addition, Huvet et al. (2002) observed that the progeny of the hybrids is viable and fertile and it can be successfully backcrossed to either parental taxa. Studies using allozyme markers also showed that *C. angulata* and *C. gigas* are closely related, with genetic differences between populations at the species level (Mathers et al., 1974; Buroker et al., 1979; Biocca and Matta, 1982; Mattiucci and Villani, 1983). Further analysis based on centromeric satellite DNA confirmed the high genetic similarity between them (López-Flores et al., 2004). However, studies on the mitochondrial cytochrome oxidase subunit I (COI) gene demonstrated clear genetic differences between the two taxa (Boudry et al., 1998; O'Foighil et al., 1998; Boudry et al., 2003). An average of 2.3% differences in COI nucleotide sequence suggests that the two taxa may have diverged several hundred thousand years ago (Hedgecock et al., 2004). Huvet et al. (2000) observed low but significant differences between *C. angulata* and *C. gigas* populations using microsatellites markers. Cytogenetic studies have also provided evidences that support their genetic differentiation (Leitão et al., 1999a; 1999b; 2004). More recently, Leitão et al. (2007) provided cytogenetic evidence of both parental genomes in the interspecific hybrids. At the phenotypic level, considerable differences have also been observed. A higher production yield has been reported for *C. gigas* when compared with *C. angulata*, due to the faster growth of *C. gigas* and higher mortality in *C. angulata* (His, 1972; Bougrier et al., 1986; Soletchnik et al., 2002). Differences have also been revealed in terms of their ecophysiological characteristics, such as clearance rate (His, 1972), oxygen consumption (Goulletquer et al., 1999) and feeding time activity (Haure et al., 2003).

Shell morphology is an extremely plastic trait in oysters, but certain shell characters can be used in taxonomic studies (Stenzel, 1971; Harry, 1985; Lawrence, 1995). Several factors influence the morphology of oyster shells such as the type of substrate on which they grow degree of crowding and various physico-chemical parameters (Galtsoff, 1964; Quayle, 1988). Hence, oyster identification based only on morphology has produced confusion in many taxa, especially in close related ones. At the within species level, evidences of a genetic basis for variation in shell shape and pigmentation has been reported (Imai and Sakai, 1961; Wada, 1994; Brake et al., 2004; Ward et al., 2005).

It has been observed that adult shells of *C. angulata* and *C. gigas* are similar (Menzel, 1974; Biocca and Matta, 1982), albeit the criteria's used to compare them were not specified. The aim of this study was to re-examine differences in shell shape and pigmentation between *C. angulata* and *C. gigas* throughout the comparison of pure and hybrids progenies cultured under common conditions.

2. Material and methods

2.1. Biological material and shell measurements

Adults of *C. angulata* and *C. gigas* were collected from Sado River (Portugal) and Seudre Estuary (France), respectively, in 2003. Oyster were identified according to their geographical origin (see Boudry et al., 1998; Huvet et al., 2000; Fabioux et al., 2002; Lapègue et al., 2004) and this was also confirmed by polymerase chain reactions with restriction fragment length polymorphism (PCR-RFLP) analysis of a fragment of the mitochondrial gene cytochrome oxidase C subunit I (Boudry et al., 1998). Gametes were collected by stripping the gonads from ripe adults of *C. angulata* (15 females and 5 males) and *C. gigas* (10 females and 8 males) as described by Stephano and Gould (1988). The gametes for each sex and taxon were pooled and crosses were performed in order to obtain the following groups: *C. angulata* progeny (AA); *C. gigas* progeny (GG); hybrids derived from *C. angulata* females and *C. gigas* males (AG); and hybrids derived from *C. gigas* females and *C. angulata* males (GA). Larvae from the four groups were reared using standard culture techniques (Loosanoff and Davies, 1963) in the IFREMER facilities in La Tremblade (France). After settlement, the animals of the different groups were transferred to an indoor nursery system in the INIAP/IPIMAR facilities in Tavira (Portugal) and grown until 204 days after fertilization (DAF). The animals were finally transferred to the grow-out site in Ria Formosa (Portugal) and cultured off-bottom in oyster bags attached in iron frames tables until the age of 472 DAF. The sea floor of the study site was a mixture of sand and mud, and the emersion rate was approximately 50 %.

At the end of the grow-out phase a total of 260 oysters shells (65 individuals per group) were measured (Figure 1). The following measurements were made from the oyster shells with both valves fitted together: (i) height (H); (ii) length (L); and (iii) depth (D). The measurements taken only on the right shell valve were: (i) length of ligamental area (LLA); (ii) height of the adductor muscle scar (MSH); (iii) length of the adductor muscle scar (MSL). The terminology used for the shell measurements was adapted from Stenzel (1971) and the shells were measured using a digital caliper to a precision of 0.01 mm.

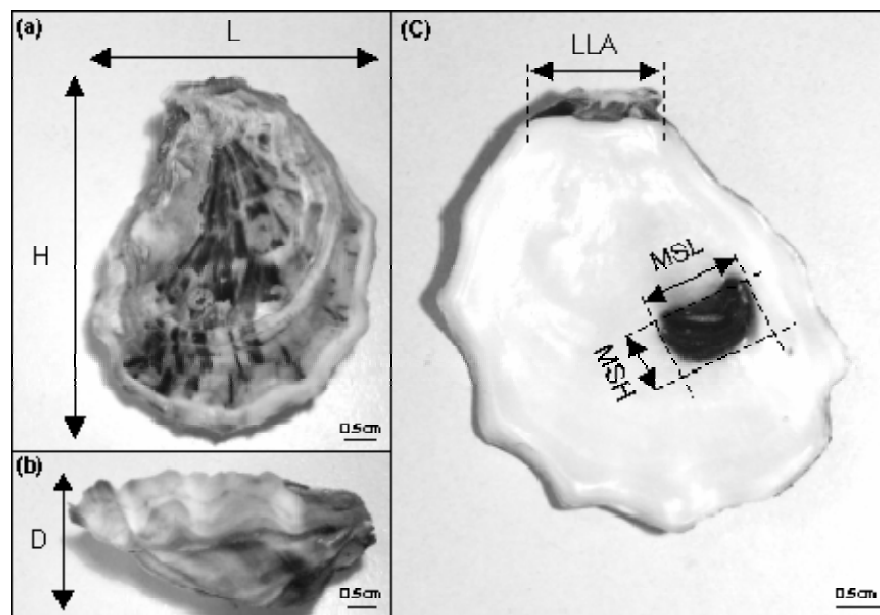


Figure 1. Shell characters measured. (a) Shell height (H) and length (L) with both valves fitted together; (b) shell depth (D) with both valves fitted together; (c) length of ligamental area (LLA), height (MSH) and length (MSL) of the adductor muscle scar of the right valve.

In order to characterize the pigmentation of the adductor muscle scar (MS) of the left and right valves two different methods were used: (i) the degree of pigmentation of the MS was estimated by a densitometric method using Quantity One Software (Bio-Rad, Hercules, CA), where the surface of the MS of the same oyster shell (showing no pigmentation) was used as background; (ii) a system with four MS pigmentation categories was used, based on the degree of darkness (semi-quantitative method), as previously employed by Imai and Sakai (1961) and Brake et al. (2004). The categories were described as: (1) very light (whole surface having no or very little pigmentation); (2) light (a small part having pigmentation); (3) dark (most part having pigmentation); and (4)

very dark (whole or almost surface having pigmentation). As described by Brake et al. (2004), a representative oyster shell for each category was selected based on shell pigmentation and chosen as a standard for which all shells were compared against.

2.2. Statistical analysis

Univariate analyses were performed to determine if the groups differed from one another for the different shell measurements (H, L, D, MSH, MSL and LLA). Data were tested for univariate normality and homogeneity of variance before comparing means using one-way ANOVA. The Kolmogorov-Smirnov test for goodness of fit was used to investigate normality and the Cochran test was used to assess heteroscedasticity. Following ANOVA, multiple comparisons between groups were performed using Tukey honest significant (HSD) tests.

Differences among groups in form (size and shape) were examined using principal components analysis (PCA) based on the covariance matrix of the untransformed six measurements variables over all 260 oysters. Formal comparisons of principal components (PC's) scores were conducted using the non-parametric Kruskal-Wallis test since variance among groups for PC2 was not homogeneous. The Nemenyi test was used for multiple comparisons when significant differences were identified with the Kruskal-Wallis test as described by Zar (1996).

In order to separate size and shape variation, another approach was used in which the raw data were transformed using three different methods: (i) ratios of the logarithms of measurements (LGRAT); (ii) within-groups allometric method (WGALL); and (iii) common-within groups regression line method (CWRES).

In the first method (LGRAT), each shell character was log-transformed (using base-10 logarithms) and divided by shell height:

$$Y_{adj} = \log Y / \log X \quad (1)$$

where Y_{adj} is the adjusted measurement, Y is the individual shell measurement and X is the shell height.

In the second method (WGALL), shell characters were log transformed and adjusted to a common size (a shell height of 51.5 mm) using the groups-specific slopes as described by Wilbur and Gaffney (1997) with the following formula:

$$Y_{adj} = \log Y - \beta (\log X - \log X_{gm}) \quad (2)$$

where Y_{adj} , Y and X are as described in (1), β is the allometric coefficient and X_{gm} is the mean shell height across all groups. Relationships between shell height and the other variables were linear and examination of the residuals for the relationships with low fit showed no obvious structure in the residuals.

In the third method (CWRES), residual values were computed using the common-within group regression line method as described by Reist (1985; 1986). Briefly, an analysis of covariance (ANCOVA) was performed for each shell character among the four genetic groups. From the ANCOVA, the lines from the different groups are characterized by the same slope, but different intercepts. Differences in elevation between the lines were determined by subtracting the adjusted group mean from the overall mean. The efficiency of the three methods to remove size effects was assessed by testing the significance of correlations between transformed variables and shell height within each group using Pearson correlation coefficient (r).

The size-adjusted morphometric data were then analysed using PCA. Univariate analyses were used to compare the variation among groups for the transformed data as well as for the components scores as described above. Finally, a discriminant function analysis (DFA) was run (using transformed data) to determine what proportion of *C. angulata* and *C. gigas* progenies could be correctly assigned to their group of origin. Multivariate normality and homogeneity of variance of the transformed data used for the DFA were assessed using Mardia and Box's M tests, respectively. The individual transformed shell characters (using the WGALL and CWRES) of AG and GA hybrids were inserted in the respective function in order to determine their discriminant scores. A summary of the overall analysis of shell morphometry is shown in Figure 2.

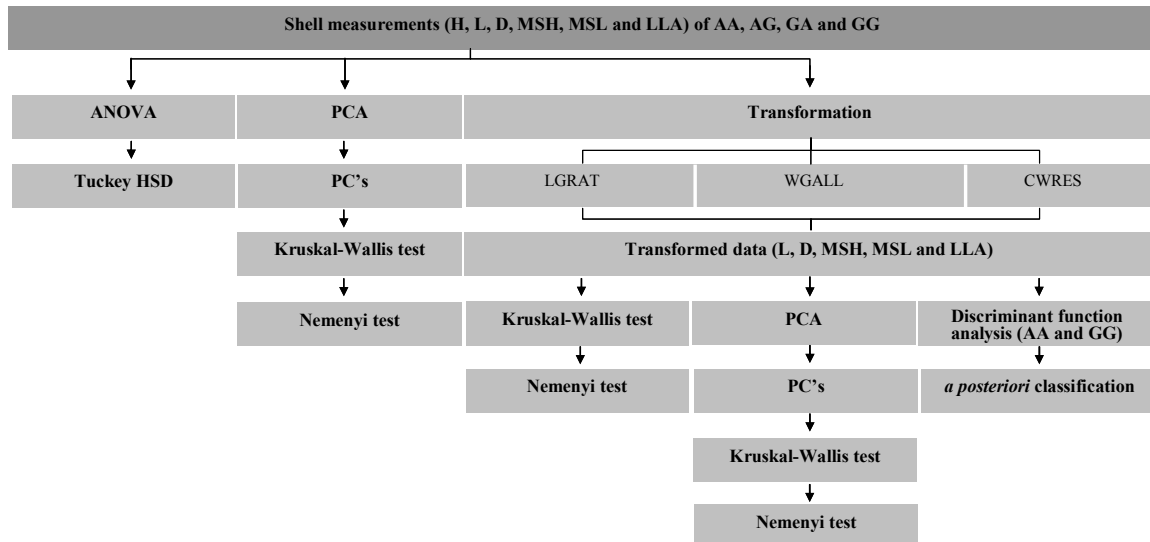


Figure 2. Summary of the statistical analysis used to compare the raw data and shell morphology of *C. angulata* (AA), *C. gigas* (GG) and their reciprocal hybrids (AG and GA) progenies.

The degree of pigmentation of the adductor muscle scar of the four genetic groups, estimated with densitometric method, was compared by ANOVA (40 individuals per group), and where significant, pairwise differences were analysed by Tukey HSD test. In order to compare the proportion of individuals assigned to the different pigmentation categories (semi-quantitative method) between groups (the same 40 individuals per group used for the previous analysis), chi-square tests with Bonferroni corrections were used (i.e., 2x4 analyses). Data analysis was carried out using SPSS (version 11.5), MATLAB (version 6.5; MathWorks Inc., 2002) and Statistica (version 6.1) softwares.

3. Results

3.1. Untransformed morphometric data

The summary statistics for the untransformed shell characters are presented in Table 1.

Table 1. Shell characters of *C. angulata* (AA), *C. gigas* (GG) and their reciprocal hybrids (AG and GA) progenies. Means and 95 % confidence interval calculated from raw data are based on sample size of 65 individuals per group.

	AA	AG	GA	GG
Shell height	44.7 ± 1.4 ^c	53.2 ± 1.3 ^b	53.9 ± 1.3 ^{ab}	56.0 ± 1.3 ^a
Shell length	36.3 ± 1.0 ^b	39.4 ± 1.0 ^a	40.5 ± 1.2 ^a	41.3 ± 1.0 ^a
Shell depth	20.5 ± 0.6 ^b	20.7 ± 0.7 ^{ab}	21.7 ± 0.6 ^a	20.2 ± 0.5 ^b
Muscle scar height	5.9 ± 0.3 ^c	7.7 ± 0.3 ^b	7.6 ± 0.2 ^b	9.0 ± 0.3 ^a
Muscle scar length	8.9 ± 0.4 ^c	10.1 ± 0.4 ^b	10.4 ± 0.4 ^b	11.7 ± 0.4 ^a
Length ligamental area	13.6 ± 0.4 ^c	15.0 ± 0.5 ^b	14.9 ± 0.4 ^b	17.1 ± 0.5 ^a

The data was analysed using ANOVA and multiple comparisons were done by Tukey's HSD test. Mean values in rows with different superscripts are significantly different ($p < 0.05$)

The distributions of all variables in the 4 groups were observed to be approximately normal and variance was homogeneous. Shell height, shell length, muscle scar height, muscle scar length and length of ligamental area were greater in *Crassostrea gigas* progeny when compared with *C. angulata* progeny. Shell depth was not significantly different ($p < 0.05$) between the two taxa. No significant differences were observed between the hybrids AG and GA for all the shell characters analyzed. Mean values of shell characters of both hybrid progenies were intermediate between, or similar, to those of the pure progenies for all variables except shell depth that was significantly higher for GA hybrids.

Three principal components (PC1, PC2 and PC3) that explain over 83% of the variation among individuals were extracted from the covariance matrix of the untransformed shell characters (Figure 3).

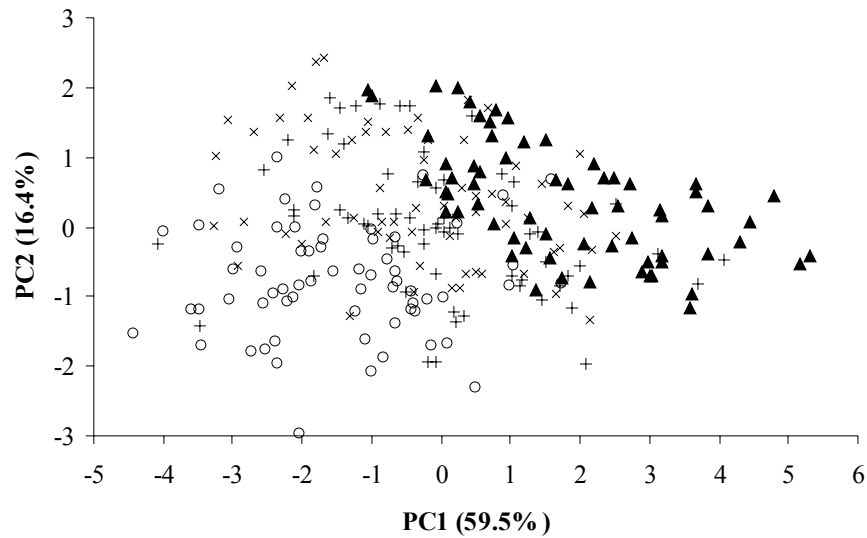


Figure 3. Scatterplots of principal components 1 and 2 for *C. angulata* ($\blacktriangle$), *C. gigas* (O), AG hybrid (+) and GA hybrid (x) progenies from the analysis of untransformed shell characters (H, L, D, MSH, MSL and LLA).

The first principal component (PC1) from the analysis of all 260 individuals had negative and high loadings on all six variables (Table 2).

Table 2. Loadings of the shell measurements of *C. angulata* (AA), *C. gigas* (GG) and their reciprocal hybrids (AG and GA) progenies on the principal components axes from the raw data.

	Eigenvectors					
	PC1	PC2	PC3	PC4	PC5	PC6
Shell height	-0.87	0.05	0.05	0.29	0.32	0.23
Shell length	-0.85	0.21	-0.33	0.22	0.03	-0.27
Shell depth	-0.45	0.83	0.18	-0.27	-0.02	0.02
Muscle scar height	-0.77	-0.35	0.50	-0.03	0.02	-0.17
Muscle scar length	-0.85	-0.09	-0.04	0.08	-0.50	0.14
Length ligamental area	-0.76	-0.34	-0.26	-0.47	0.14	0.05
Eigenvalue	3.57	0.98	0.47	0.44	0.37	0.18
% of total variation	59.48	16.36	7.77	7.26	6.18	2.95

However, the loadings were not uniform since a value of -0.45 was observed for shell depth whereas for the other variables the loadings ranged between -0.76 and -0.87. Nevertheless, variance on this axis can be mainly attributed to size differences among groups. PC2 was strongly influenced by shell depth that accounted for 70.4% of the

variance explained. Median component scores on PC2 differed significantly among groups (Kruskal-Wallis test, $p < 0.001$) and pairwise comparisons revealed significant differences between *C. angulata* and *C. gigas* progenies (Nemenyi's test). For all principal components, no significant differences were observed between the AG and GA hybrids (table 3).

Table 3. Results of the Kruskal-Wallis test and pairwise comparisons (Nemenyi's test) among principal components scores from the untransformed shell characters of *C. angulata* (AA), *C. gigas* (GG) and their reciprocal hybrid (AG and GA) progenies.

Principal component	Kruskal-Wallis test	Differences
PC1	***	AA > (AG=GA) > GG
PC2	***	(AA = AG=GA) > GG
PC3	*	AA < (AG=GA=GG)

*** $p < 0.001$, * $p < 0.05$

3.2. Transformed morphometric data

Most transformed shell characters using the ratio method (LGRAT) showed a significant correlation with shell height ($r = -0.44$ to 0.37 , with $p < 0.05$) within each group. Hence, the data transformed with LGRAT was not use for further analysis. The transformed shell characters using both the common-within group regression line (CWRES) and within-groups allometric methods (WGALL) showed no significant correlation ($p > 0.05$) with shell height within each group. When the data obtained with WGALL for the 4 groups was pooled, a significant correlation ($r = -0.24$ to 0.31 , $p < 0.05$) was obtained between shell height and three (D, MSH, LLA) of the five shell characters. Similar results were obtained for the CWRES with a correlation coefficient for the same shell characters ranging between -0.25 and 0.31 . However, the significant correlation observed was due to the lack of homogeneity in the pooled samples from which the correlation was calculated.

Data transformed using the CWRES and WGALL methods yielded very similar results, and hence, we will only show the univariate and multivariate results obtained with the WGALL method. Significant differences (Kruskal-Wallis test, $p < 0.01$) were observed among the 4 groups for all transformed characters. Pairwise comparisons indicated significant differences (Nemenyi's test) between *C. angulata* and *C. gigas* progenies for all transformed characters with exception of shell length (table 4).

Table 4. Median (standard deviation) of the transformed shell characters using the within-groups allometric method for *C. angulata* (AA), *C. gigas* (GG) and hybrids (AG and GA) progenies.

Shell characters	AA	AG	GA	GG
Shell length	39.9 (3.0) ^a	38.3 (2.5) ^b	38.6 (3.8) ^{ab}	38.8 (3.0) ^{ab}
Shell depth	22.5 (2.1) ^a	20.1 (2.4) ^c	21.2 (2.2) ^b	19.6 (1.9) ^c
Muscle scar height	6.7 (1.1) ^c	7.4 (1.2) ^b	7.4 (0.9) ^b	8.9 (1.2) ^a
Muscle scar length	10.2 (1.4) ^b	9.8 (1.5) ^b	10.0 (1.5) ^b	11.2 (1.2) ^a
Length ligamental area	14.6 (1.5) ^b	14.5 (1.7) ^b	14.5 (1.8) ^b	16.4 (1.7) ^a

The data was analysed using Kruskal-Wallis test and multiple comparisons were done with the Nemenyi's test. Medians values in rows with different superscripts are significantly different ($p < 0.05$).

Crassostrea gigas progeny showed a significant higher MSH, MSL and LLA than *C. angulata* progeny. On the other hand, shell depth was significantly higher in *C. angulata* progeny when compared with the other groups. Median values of transformed shell characters of both hybrids progenies were intermediate between, or similar, to those of the parental lines. All pairwise comparisons between the reciprocal hybrids showed no significant differences for all shell characters with the exception of shell depth that was significantly higher for GA hybrids (Nemenyi's test).

Two principal components (PC1 and PC2) that explain over 65% of the variation among individuals were extracted from the covariance matrix of the transformed shell characters using WGALL (Figure 4).

Both PC1 and PC2 showed no significant correlation with shell height ($p > 0.05$). PC1 accounted for 38.7% of the variance, and loadings of individuals along this axis was strongly influenced by MSL and LLA that accounted for 35.0 and 27.9% of the variance, respectively. Median components scores on PC1 differed significantly (Nemenyi's test) among groups (table 5). Pairwise comparisons showed no significant differences among *C. angulata*, AG and GA progenies, however, these three groups differed significantly from *C. gigas* progeny (Nemenyi's test). PC2 explained 27.0% of the total variance and this axis was strongly associated with the MSH contrasted with shell length and depth. These three characters accounted for 23.8, 26.1 and 45.9%, respectively, of the variance explained by PC2. Groups differed significantly in median component scores along PC2 (Kruskal-Wallis, $p < 0.001$) reflecting the significantly deepest shell and smaller muscle scar height of *C. angulata* progeny relative to *C. gigas* progeny (Nemenyi's test). This analysis also revealed that AG and GA hybrids showed an intermediate median scores between the *C. angulata* and *C. gigas* progenies along PC2. No significant differences (Kruskal-Wallis test, $p > 0.05$) were observed among the four groups for the other PC's.

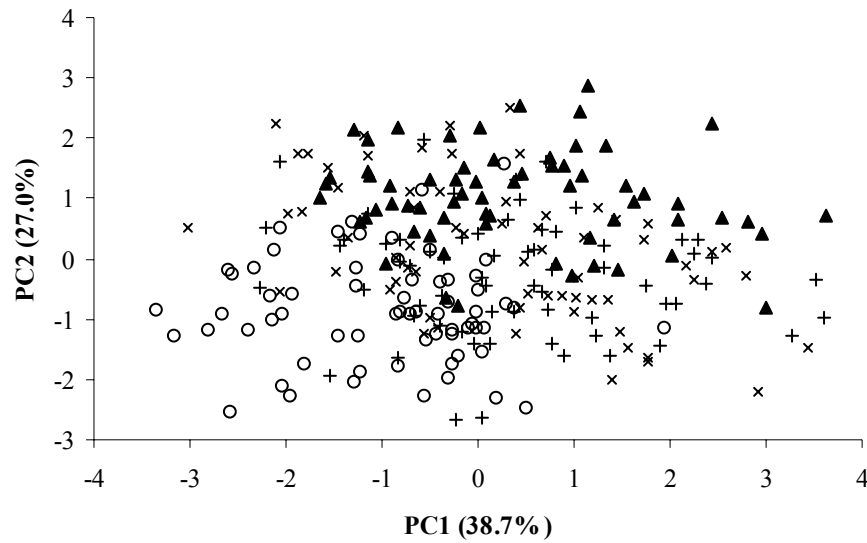


Figure 4. Scatterplot of principal components 1 and 2 for *C. angulata* (▲), *C. gigas* (O), AG hybrids (+) and GA hybrids (x) progenies from the analysis of the transformed shell characters (L, D, MSH, MSL and LLA) using the within-groups allometric method.

Table 5. Eigen values and percentage of total variance explained by each principal component (PC) extracted from the data transformed using the within-groups allometric method. Results of the Kruskal-Wallis test (KWT) and pairwise comparisons (Nemenyi's test) among *C. angulata* (AA), *C. gigas* (GG) and their reciprocal hybrids (AG and GA) progenies.

Principal component	Eigenvalue	% of total variation	KWT	Differences
PC1	1.93	38.7	***	(AA=AG=GA) > GG
PC2	1.35	27.0	***	AA > (AG=GA) > GG
PC3	0.71	14.1	NS	
PC4	0.60	12.0	NS	
PC5	0.41	8.2	NS	

*** $p < 0.001$, NS = not significant

The discriminant analysis demonstrated significant morphometric differences between *C. angulata* and *C. gigas* progenies. The accuracy of the *a posteriori* classification using data transformed with the WGALL method was 93.1 % overall, with 61 of 65 (93.8%) *C. angulata* and 60 of 65 (92.3%) *C. gigas* were correctly classified. The shell characters that were more important in the separation of the two groups were shell depth and muscle scar height. Hybrid individuals classified using the discriminant function

derived from *C. angulata* and *C. gigas* progenies were grouped about midway between parental lines (Figure 5).

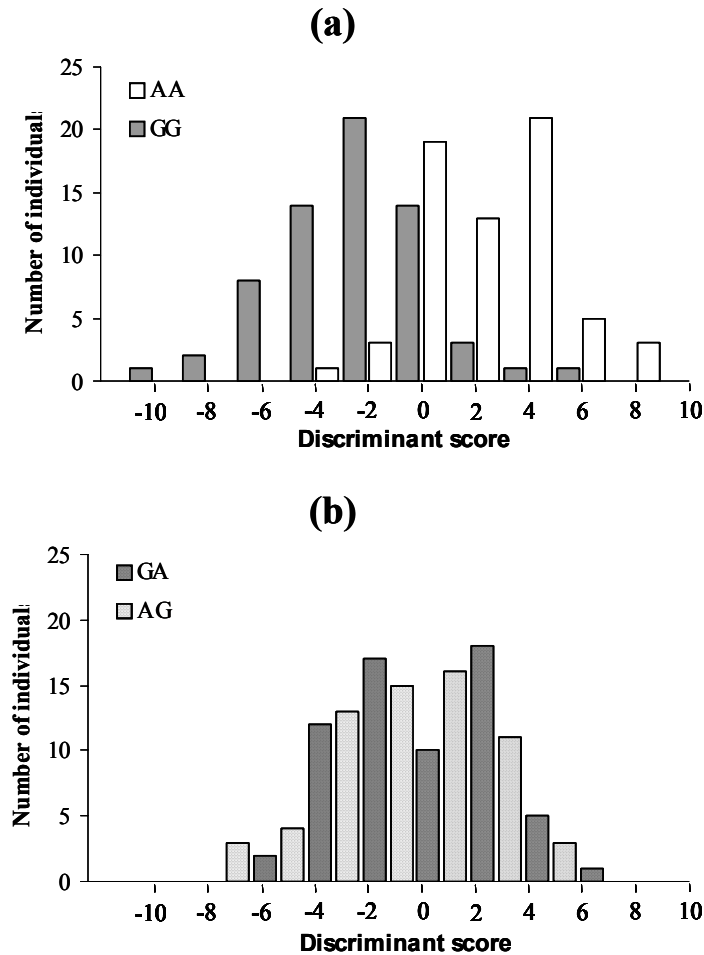


Figure 5. Histograms of discriminant scores for *C. angulata* (AA), *C. gigas* (GG) and hybrid (AG and GA) progenies using the data transformed data with the within-groups allometric method. (a) Distribution of the scores for AA and GG used to construct the discriminant function. (b) Distribution of scores of AG and GA individuals using the discriminant function constructed from the parental lines.

3.3. Pigmentation of the adductor muscle scar

The distribution of the degree of pigmentation, estimated using the densitometric method, in each group were observed to be normal and the variance among groups homogeneous. The pigmentation of the adductor muscle scar (MS) of the left (MSL) and right (MSR) valves were significantly correlated ($r = 0.61$, $p < 0.001$). Shell height and the degree of pigmentation of either the MSR or MSL, within each group, were not correlated

($p > 0.05$). ANOVA showed significant differences ($p < 0.01$) among the four groups for the degree of pigmentation of MSL and MSR. Pairwise comparisons revealed that *C. gigas* progeny had a significant lower pigmentation of MSL and MSR than all the other groups (Tukey's HSD). No significant differences were observed between *C. angulata*, AG and GA progenies for MSL and MSR pigmentation (Tukey's HSD). Similar results were obtained when individuals from the four groups were assigned to the different pigmentation categories (Figure 6).

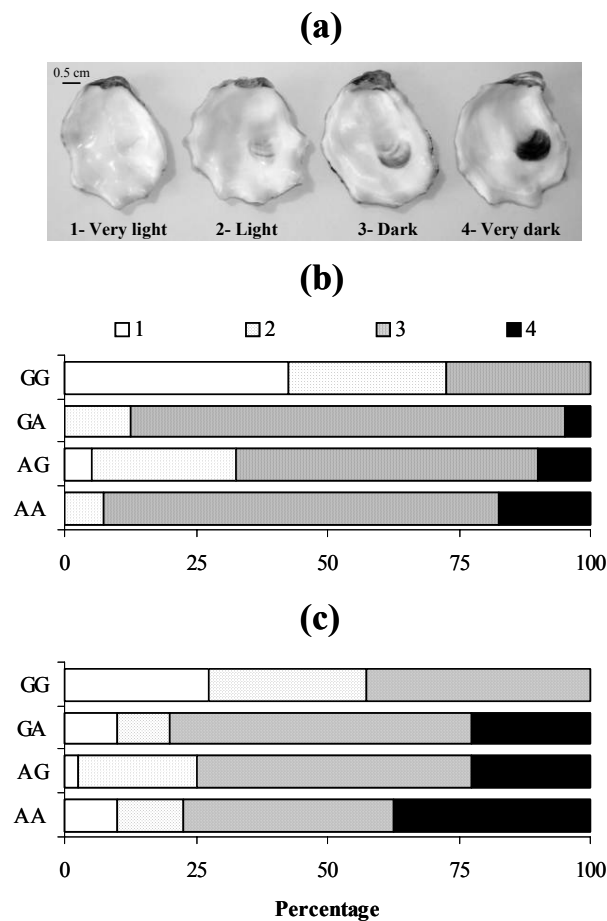


Figure 6. Adductor muscle scar pigmentation (MS). (a) Shells representatives of the four categories used to classify the degree of pigmentation of the MS (1-very light, 2-light, 3- dark, 4-very dark). (b) Frequency of *C. angulata* (AA), *C. gigas* (GG) and hybrids (AG and GA) individuals for each category for the left valve. (c) Frequency of AA, GG, AG and GA individuals for each category for the right valve.

Chi-squared analysis revealed significant differences between *C. gigas* progeny and all the other groups, whereas no differences were observed between *C. angulata*, AG and

GA progenies. The results of the chi-squared analyses were mainly due to *C. gigas* progeny being distributed in categories representative of lower levels of pigmentation.

4. Discussion

The analysis of our raw data showed that there were significant differences among the four genetic groups for all the studied shell characters. The univariate and multivariate analyses of the untransformed data suggest that a large proportion of the variation observed was due to differences in growth rate between *C. angulata* and *C. gigas*. Indeed, previous studies showed that *C. gigas* grows faster than *C. angulata*, under culture conditions similar to the ones used in the present study, and that hybrids have an intermediate performance (Bougrier et al., 1986; Soletchnik *et al.*, 2002). Since much of the variation observed in shell characters among groups was due to differences in size, it was necessary to remove this effect from the data in order to allow comparison of shell morphology. Principal component analysis (PCA) was used to remove size effect by disregarding the first principal component (PC1) derived from the original data matrix, which usually equates with size variation. Shape variation is assumed to be contained in the other principal components. In the present study, the first principal component obtained from the raw data matrix can be interpreted as explaining size variation, given that all variables loaded heavily and in the same direction. PC2 and PC3 were not significantly correlated with shell height and significant differences among groups were observed for these axes. However, PC1 may contain shape variation and the other axes may enclose size-related information, which can obscure shape interpretation since each axis may not have a unique meaning (Reist, 1985). The use of the ratio between raw measurements and some standard size measure is one of the most widespread techniques used to standardize for size. However, it has been observed that generally ratios do not completely remove the influence of size and have some undesirable statistical properties (Reist, 1985). In the present study, the LGRAT did not only remove efficiently size effect, but also yield data for *C. angulata* and *C. gigas* that did not have a normal multivariate distribution (Mardia test, $p < 0.05$) and variance was no homogeneous (Box's M test, $p < 0.05$). Small departures from multivariate normality and heterogeneity of variance do not affect the results of principal component analysis (especially with reasonably large samples), but can violate the assumptions of other multivariate analysis such as discriminant function analysis. The residual (CWRES) and allometric (WGALL) methods used in an attempt to separate size and shape were effective in removing size variation. In addition, data for *C. angulata* and *C. gigas* had an approximately multinormal distribution (Mardia test, $p > 0.05$) and variance was homogeneous between the two groups (Box's M test, $p > 0.05$). Significant differences were observed among the four

groups for all transformed characters and similar results were obtained with data transformed using both CWRES and WGALL. On the other hand, the interpretation of PC2 and remaining axes derived from the raw matrix also yielded similar differences among groups as the ones obtained with transformed data using CWRES and WGALL. Hence, the different methodological approaches used suggest that the morphological differences observed are not due to an inefficient removal of size effects.

The univariate (using size adjusted data) and the multivariate results (using both untransformed and transformed characters) showed significant morphological shell differences between *C. angulata* and *C. gigas* progenies. The same analysis also revealed that both reciprocal hybrids had intermediate shell characters between parental lines, although slightly skewed toward *C. angulata* progeny. The development of distinct morphologies in *C. angulata* and *C. gigas* progenies together with intermediate position of hybrids under the same environmental conditions indicates a genetic basis for this variation. *C. angulata* progeny had deeper shells with shorter adductor muscle scars in length and height as well as smaller ligamental areas comparatively with *C. gigas* progeny. The differences observed between the two taxa progenies for shell length were small although statistically significant. A genetic basis for variation in shell flatness in *C. gigas* was first suggested by Imai and Sakai (1961), based on differences in relative shell depth observed among geographical races of this species. They reported that such differences persisted for several generations, which indicated its hereditary nature. Recently, Ward et al. (2005) estimated that heritability for depth index (shell depth to height) in *C. gigas* was moderate to high. The results obtained in the present study support the finding of Imai and Sakai (1961) and Ward et al. (2005) of a genetic basis for relative shell depth to height. In addition, the differences observed between *C. angulata* and *C. gigas* progenies in the present study for adductor muscle scar height and length as well as for ligamental area length suggest that these characters may also be genetically based. A genetic basis for hinge length, as well as for other shell characters, was also suggested in other mollusc bivalve species namely, in the scallops *Pecten maximus* (Wilding et al., 1998) and *Argopecten irradians* (Wilbur and Gaffney, 1997). Multivariate statistics showed that there were no significant morphological differences between AG and GA hybrids and they showed intermediate values between parental lines. However, univariate analyses using size-adjusted data (WGALL) revealed a tendency of the hybrids to be skewed toward *C. angulata* for muscle scar length and ligamental area. This indicates that dominance for some shell may exist, whereas for others such as shell depth codominance is more likely.

Morphological divergence between *C. angulata* and *C. gigas* may have resulted from local adaptation by natural selection, genetic drift in absence of gene flow or a

combination of these two evolutionary forces. The morphological differences observed may influence their fitness under certain environmental conditions. The size of the muscle scar reflects the size of the adductor muscle that controls the opening and closing of the valves and opposes the action of hinge ligament (Quayle, 1988). The ability of oysters to keep the shells closed avoids desiccation during the time of exposure in the tidal zone and allows them to remain protected against unfavourable conditions or attacks from predators (Galtsoff, 1964). The relative smaller muscle scar and ligamental area observed in *C. angulata* comparatively with *C. gigas* may result in a differential ability to keep the shells closed and hence might influence their fitness under some environmental conditions. Nevertheless, the adaptive significance of the other morphological differences observed between the two taxa remains unknown.

Other cases have been reported of closely related bivalve molluscs that interbreed and sexually viable offspring, but that are genetically distinct and show morphometric differences. One of those well documented cases is the *Mytilus* complex composed by the mussels *M. edulis*, *M. galloprovincialis* and *M. trossulus* for which morphometric differences were observed among the three taxa, although varying amounts of overlap between them have been reported (McDonald et al., 1991; Mallet and Carver, 1995; Innes and Bate, 1999; Gardner, 2004). Another case is the clams *Mercenaria mercenaria* and *M. campechiensis* that also show significant morphometric differences, but overlap for same characters (Dillon and Manzi, 1989). In the present study, the *a posteriori* cross-validation analysis showed that 92.3 and 93.1% of the individuals were categorized correctly (as either *C. angulata* or *C. gigas*) using data transformed with the CWRES and WGALL methods, respectively. The low degree of overlapping between *C. angulata* and *C. gigas* was surprising due to the high variability of oyster shells and the small number of useful landmarks for taking measurements, which greatly complicate morphometric analyses. The AG and GA hybrids were generally morphologically intermediate, but showed a large overlap with each taxon.

For the oyster industry, shell morphology is considered an important trait since it can dramatically influence product value when animals are commercialised alive or half shell (Mahon, 1983; Ward et al., 2005). Brake et al. (2003) showed that length and depth indexes as well as the index of shell depth (which combines the information of the other two indexes) were useful to describe oyster shell as having a desirable shape. The same authors observed that length index was not as important as depth index to describe a desirable shell shape. Although it is known that the relative length and depth of oyster shells is extremely influenced by environmental conditions and husbandry procedures (Galtsoff, 1964; Quayle, 1988), genetic variation has been observed for these traits (Wada, 1994; Ward et al., 2005). However, Ward et al. (2005) observed that for *C. gigas*

the genetic gains for length and depth indexes were low, and hence genetic selection was not a powerful tool to manipulate shape. The results obtained in the present study, not only support the genetic basis for relative shell depth to height, but also suggest that cross-breeding between *C. angulata* and *C. gigas* can be seen as a possible strategy to manipulate shell shape. Moreover, the intermediate growth rate of their hybrids, skewed toward *C. gigas* (Bougrier *et al.*, 1986; Soletchnik *et al.*, 2002), indicate that depth index can be improved using this strategy with a limited decrease in growth performance.

The approximate normal distribution of MS pigmentation (estimated using the densitometric method) and the diversity of pigmentation colour/pattern observed suggests that pigmentation of MS in *C. angulata* and *C. gigas* can be considered a quantitative trait. This is in agreement with Brake *et al.* (2004), which hypothesised that the pigmentation of the shell surface in *C. gigas* is controlled by many genes. The results of the present study suggest that there are genetic differences between *C. angulata* and *C. gigas* for the degree of pigmentation of the MS. These results support earlier observations of Galtsoff (1964) that described the MS of *C. angulata* as being highly pigmented whereas in *C. gigas* pigmentation was either absent or very light. Furthermore, both AG and GA hybrids showed a pigmentation of the MS similar to *C. angulata*, which indicate that darker pigmentation is dominant over lighter one.

The differences in shell shape and MS pigmentation observed in the present study supports the distinctness of the two taxa as pointed out by other authors (Héral and Deslous-Paoli, 1991; Boudry *et al.*, 1998; Foighil *et al.*, 1998; Leitão *et al.*, 1999a; 1999b; 2004). However, similar works should be performed using *C. angulata* and *C. gigas* progenitors from several populations to confirm that the differences observed are at the taxon level and not only at the population level.

Acknowledgements

We are grateful to Dr. C. Monteiro for facilitating part of this work at the INIAP/IPIMAR station in Tavira (Portugal). We thank Maurício Teixeira for his excellent assistance in maintaining spat in the nursery and progeny groups in the field. This work was made possible by a FCT grant (SFRH/BD/8972/2002) and a Marie Curie Training fellowship (PLUDAMOR, QLK5-CT-2000-60036) to F. M. B.

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4.2. CONCLUSIONS

The results presented in the present chapter suggest that there is a genetic basis for the following traits: relative shell width, relative shell length, ligament area, adductor muscle size, pigmentation of the adductor muscle scar, and pigmentation of the surface of right valve. In addition, a yellow coloration on the surface of the left valve was observed in higher frequency in *C. angulata* progeny than in *C. gigas* progeny, which suggest that the gene(s) responsible for this trait may be in a higher frequency in the former taxon. The differences in shell shape and pigmentation observed supports the taxonomic distinctness of the two taxa. Moreover, the differences observed may be used in genetic improvement programs to produce oyster with a more favourable shell shape as well as lines of oyster with characteristics (e.g., yellow coloration) that provide product label identification

CHAPTER 5. RECRUITMENT OF OYSTERS IN RIA FORMOSA AND NATURAL HYBRIDIZATION BETWEEN *CRASSOSTREA ANGULATA* AND *C. GIGAS*

5.1. RECRUITMENT OF CUPPED AND FLAT OYSTERS IN RIA FORMOSA

5.1.1. Background and objectives

Oyster recruitment can be defined as the process of successful colonization of a substratum after some specified period of time. It depends on many factors which can result in a high spatial and temporal variability of recruitment. Among other factors, environmental conditions (e.g., temperature, salinity, food availability) have a major influence on the different phases of the life cycle of oysters (i.e., gametogenesis, spawning, larval development, and settlement). In addition, competition and predation during the period between metamorphosis and the moment when recruitment is assessed may also affect its estimation. Other factors that can also negatively affect recruitment are unfavorable water currents, lack of suitable substratum, water depth, diseases and pollutants (Quayle, 1988). Collection of oyster spat is often of great importance to sustain the oyster production. For example, farming of the Pacific oyster *Crassostrea gigas* in France relies almost exclusively on juveniles collected from the natural environment despite an increasing supply of spat produced by hatcheries (Robert and Gérard, 1999).

The Ria Formosa is a tidal lagoon system located on the southern coast of Portugal. It stretches about 60 Km from Ancão to Cacela and has several entrances which permits the interchange of water with the sea. The lagoon cover an area of about 20 000 ha with a mean depth of 4 m. The lagoon does not receive permanent fresh water streams. Nonetheless, it may receive important fresh water flows during storm events. Apart from these periods the lagoon is highly dependent of the coastal water conditions. Ria Formosa is the main region in Portugal for the production of grooved shell clam *Ruditapes decussatus* (Ruano, 1997). Three different oyster species have been described in Ria Formosa, namely *C. angulata*, the dwarf oyster *Ostreola stentina* and the European flat oyster *Ostrea edulis* (Leal, 1984). Additionally *C. gigas* was unofficially introduced in Ria Formosa since the 1980's (F. Ruano, personal communication). *O. edulis* occurs naturally in the eastern Atlantic in euryhaline waters and is usually found at several meters depth, but also at low tidal levels (Harry, 1985). It usually grows to a larger size (10-12 cm) and is an important economical marine resource. *O. edulis* is protandric hermaphrodite that incubate their larvae (brooding) after which they are released and spend about 10 days as a pelagic stage before settlement. *O. stentina* also occurs naturally also in the eastern Atlantic in euryhaline waters and is found chiefly in shallow

subtidal waters to a few meters depth (Harry, 1985). It attains a smaller size than *O. edulis* and consequently has no commercial importance. It is presumed that *O. stentina* like other species of the genus *Ostreola* incubate their larvae, but little is known about the breeding of this species.

Crassostrea sp. specimens can be distinguished from *O. edulis* and *O. stentina* specimens based on the presence of the chomata (Harry, 1984). However, differentiation between these species based only on external morphology, especially at younger stages, can be very difficult. Although *O. edulis* and *O. stentina* are morphologically very similar, they can be differentiated at older stages based on shell shape, type of chomata, presence/absence of lamellae and mostly due to the higher size attained by *O. edulis* (Harry, 1985). However, at younger stages these two species are very difficult to differentiate based on morphological criteria easily leading to misclassification. DNA-based techniques have been used to differentiate morphologically similar oyster taxa (O'Foighil *et al.*, 1995; Boudry *et al.*, 1998; Klinbunga *et al.*, 2003) and can be seen as an alternate diagnostic method for species identification.

Nowadays, it is presumed that recruitment of *Crassostrea* sp. in Ria Formosa is a mixture of *C. angulata*, *C. gigas* and their hybrids (Huvet *et al.*, 2000; 2004), but it is not known if the two taxa and their hybrids recruit at the same periods. Moreover, the recruitment of *O. stentina* can be a problem for spat collection of *Crassostrea* sp. and *O. edulis* since it also settles on collectors set out for the latter taxa (Pascual, 1972). The knowledge on the recruitment pattern of these four oyster species in the Ria Formosa can not only shed some light on their breeding at this region, but also provide information for local farmers to know when and where collectors should be set out.

The objectives of the present study were: (1) to identify and evaluate the abundance of oyster species and other mollusc bivalves that settle in Tavira (Ria Formosa); (2) to develop a mtDNA PCR/RFLP method to distinguish *O. edulis* from *O. stentina*; and (3) to characterise the temporal pattern of *Crassostrea* sp., *O. edulis* and *O. stentina* recruitment in two sites Tavira and Cacela-Velha (Ria Formosa).

5.1.2. Experimental procedure

Settlement of oysters and other mollusc bivalves in Tavira (Ria Formosa)

Collectors were set out in Tavira site (Ria Formosa) on 13 March 2002 and collected in 20 January 2004. At the end of this period, the bivalve molluscs in the collectors were identified, counted and total biomass measured.

Development of a new mtDNA PCR-RFLP method to distinguish O. edulis from O. stentina

Primers were designed based on available sequences of *O. edulis* (accession numbers AF120651) and *O. stentina* sequence data from a homologous fragment sequenced from individuals collected in Mira River (data not shown; see Article 7 in Appendices). The primers were called COI-S1/COI-S2 and COI-E1/COI-E2 for *O. stentina* and *O. edulis*, respectively, and their nucleotide sequence is shown in Table 11. The restriction enzyme *RsaI* that recognise the sequence 5' - GTAC - 3' was chosen for the RFLP assay since it cuts twice in the amplified *O. edulis* COI fragment and only once in a different site in *O. stentina*. In order to confirm the specificity of the PCR/RFLP strategy total genomic DNA was extracted from ethanol-preserved gill fragments using a modification of a phenol/chloroform method described by Sambrook *et al.* (1989) from 32 individuals of *O. edulis* and 67 individuals of *O. stentina* collected in Ria Formosa. A 584 bp COI fragment was amplified using the primers described above. PCR reactions were performed in 50 µl volumes composed of 5.0 µl of 10X PCR buffer, 35.7 µl of distilled water, 5.0 µl of dNTP (2 mM), 1.0 µl of each primer (20 µM), 0.3 µl of Taq polymerase (5 U/ µl) and 2.0 µl of template DNA (ca. 100 ng). The digestion reaction was performed at 37 °C during 120 min with 10 µl of PCR product, 7.5 µl of distilled water, 2 µl of 10X *RsaI* buffer and 0.5 µl of *RsaI* enzyme. In order to confirm the specificity of the restriction pattern, part of the COI fragment amplified was sequenced in all *O. stentina* and *O. edulis* individuals analysed.

Temporal and spatial pattern of oysters recruitment in Ria Formosa

Collectors were set out in two different sites in Ria Formosa (4 collectors per site) named hereafter “Tavira” and “Cacela-Velha” (Figure 25) from 15 January 2004 to 17 January 2005.

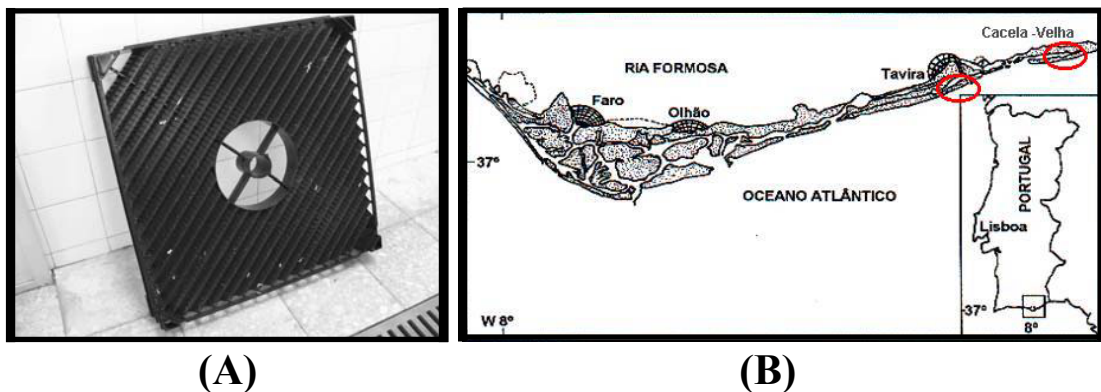


Figure 25. (A) Collector (60x60x8 cm); and (B) sites where the collectors were set out.

Immersion rate was approximately 50 and 90 % in Cacela and Tavira sites, respectively. Every two weeks the 6 collectors from each site were collected and replaced by new ones during one year. Water temperature and salinity were measured in Cacela-Velha site at each sampling (Figure 26).

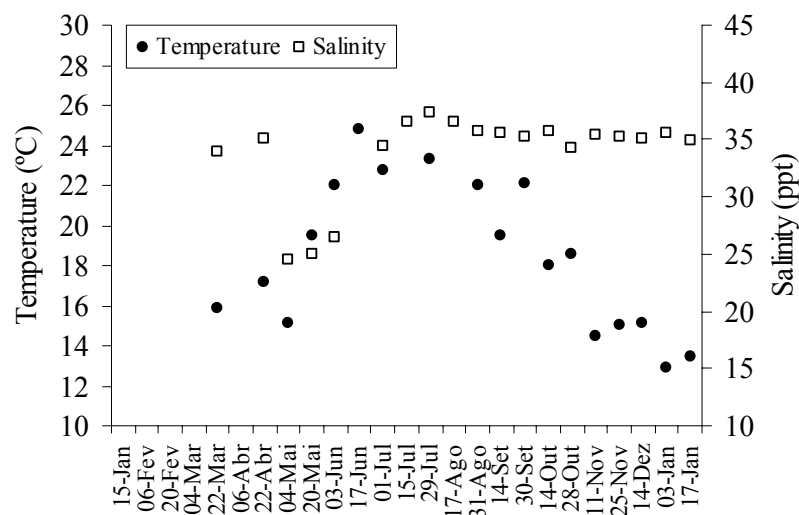


Figure 26. Surface water temperature (black circles) and salinity (white squares) at Cacela-Velha site.

Collectors were renewed approximately every 15 days. After this period, they were taken from both sites in Ria Formosa and placed in a indoor tank with filtered sea water enriched with *Isochrysis* aff. *galbana* (clone T-ISO), *Skeletonema costatum* and *Tetraselmis suecica* to allow putative settled organisms to grow. The water was changed every other day. Approximately 3-4 months after the collectors were put in the tanks, oysters fixed in each collector were counted, and gill tissue was sampled and kept in absolute ethanol for genetic analysis.

The oysters were identified as belonging to the genus *Ostrea/Ostreola* or *Crassostrea* based on the morphological criteria (*i.e.*, pigmentation of the adductor muscle scar and presence/absence of chomata) as described by Harry (1984). Total genomic DNA was extracted from *Ostrea/Ostreola* sp. individuals sampled from the collectors at the different sampling periods (148 and 45 individuals from Tavira and Cavela-Velha sites, respectively) as described above. Since *O. stentina* is thought to be more abundant than *O. edulis* based on the results of the recruitment of oysters during 2002 and 2004 in Tavira (see below), PCRs was performed using the primer pair COI-S1/COI-S2 (designed for *O. stentina*) to amplify a 584 bp COI fragment as described above. In samples in which

no amplification was observed, a second PCR was performed with the primer pair COI-E1/COI-E2 (designed for *O. edulis*) using the same PCR conditions. PCR products obtained with both primer pairs were digested with *RsaI* as described above.

Table 11. Primers used to amplify a 584 bp fragment of the *coi* gene.

Taxon	Name	F/R	Nucleotide sequence
<i>O. stentina</i>	COI-S1	F	5' - GTGTTTGGATTTTGGGCAGT - 3'
	COI-S2	R	5' - GATGTATTAAAATGACGATCTGT - 3'
	COI-E1	F	5' - GTTTTTGGTTTTTGATCTGT - 3'
<i>O. edulis</i>	COI-E2	R	5' - GATGTGTTAAAATGACGATC - 3'

F, forward primers; R, reverse primers

5.1.3. RESULTS AND DISCUSSION

Settlement of oysters and other mollusc bivalves in Tavira

The majority of the individuals in number and biomass observed belonged to the family *Ostreidae* (Figure 27A). Within this family, the largest part of the individuals were identified based on the morphological criteria described by Harry's (1984) as belonging to the genus *Ostrea/Ostreola* (Figure 27B). Most of these individuals were apparently from the species *O. stentina* and only a small number from the species *O. edulis*. Although the identification of older individuals of these two species is possible based on shell size and shape, type of chomata, and presence of lamellae, it was not possible in the present study to be unambiguously distinguish the specimens examined due to inconspicuous morphological features because of their small size (ranged between 7 and 29 mm).

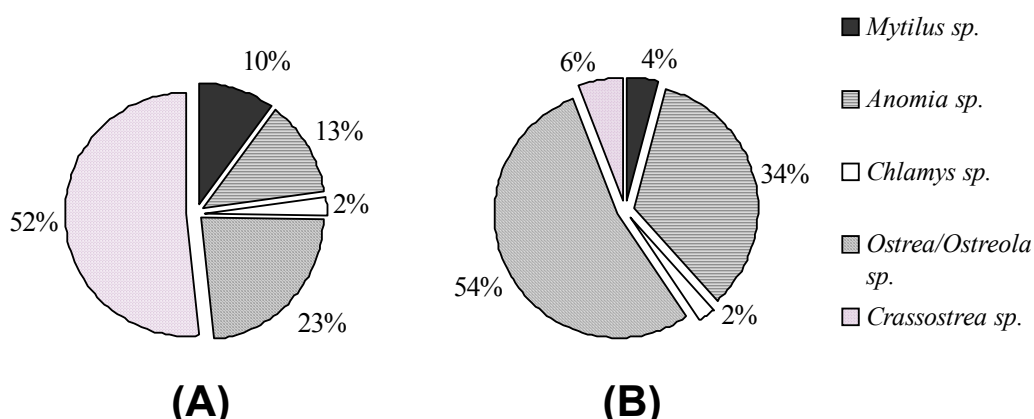


Figure 27. Percentage in biomass (A) and number (B) of bivalve molluscs observed on the collectors in Tavira.

All the *Crassostrea* sp. individuals observed had more than 7 cm in shell height and a mean weight of 55 g, which suggests that a large number of the individuals have settled in the first year of the experiment. Sediment deposition and colonization of the collectors by other organisms may have reduced recruitment during the second year of the experiment, which can explain the absence of small *Crassostrea* sp. specimens at the end of experimental period.

Development of a new mtDNA PCR-RFLP method to distinguish O. edulis from O. stentina

A representation of the restriction profile of the 584 bp COI fragment of *O. edulis* and *O. stentina* using the restriction enzyme *RsaI* is shown in Figure 28. *RsaI* cuts in two sites yielding a three banded pattern for *O. edulis*. The smallest band is not easily identified via electrophoresis in agarose gels and hence only two bands are clearly visualized. For *O. stentina*, *RsaI* cuts the amplified COI fragments in one site yielding a two banded pattern that is clearly distinguished via electrophoresis from the pattern obtain for *O. edulis*. All *O. edulis* (n = 32) and *O. stentina* (n = 67) individuals analysed showed the expected species-specific pattern. Sequence analysis confirmed the specificity of *O. edulis* and *O. stentina* PCR-RFLP haplotypes.

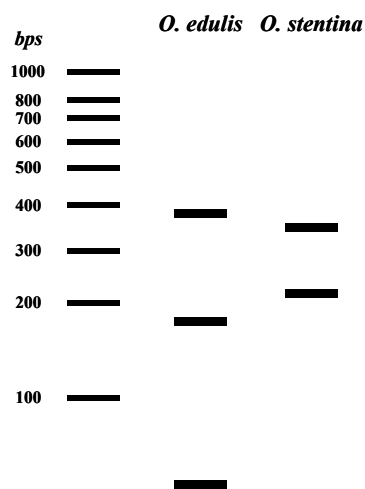


Figure 28. Representation of restriction patterns of a 584 bp COI gene fragment of *O. stentina* and *O. edulis* digested with *RsaI*.

Temporal and spatial recruitment of oysters in Ria Formosa

The abundance of *Crassostrea* sp. individuals was more than 4 times higher in Cacela-Velha (a total of 613 oyster) than in Tavira (a total of 141 oysters). This difference may

have been due to the effect of tidal height, since immersion rate in Cacela-Velha and Tavira were approximately 50 and 90 %, respectively. Cacela-Velha can be described as up-mid intertidal and Tavira as low intertidal. These results are in agreement with the observations of Diederich (2005), in which a significantly higher abundance of *C. gigas* spat was observed at higher tidal levels. Indeed, *C. gigas* has been described as an intertidal species that is only rarely observed in subtidal locations (Buroker, 1985). Different factors may explain this vertical distribution, such as predation, competition, sediment deposition on settlement surfaces, water temperatures, and current velocity (Quayle, 1988; Diederich, 2005). The temporal pattern of *Crassostrea* sp. recruitment was approximately the same in Cacela-Velha and Tavira (Figure 29). A first peak was observed in April followed by a second one between July and August, and finally a last one between September and November (Figure 29). Hence, these results suggest that recruitment of *Crassostrea* sp. oysters in Ria Formosa in 2004 occurred between April and November.

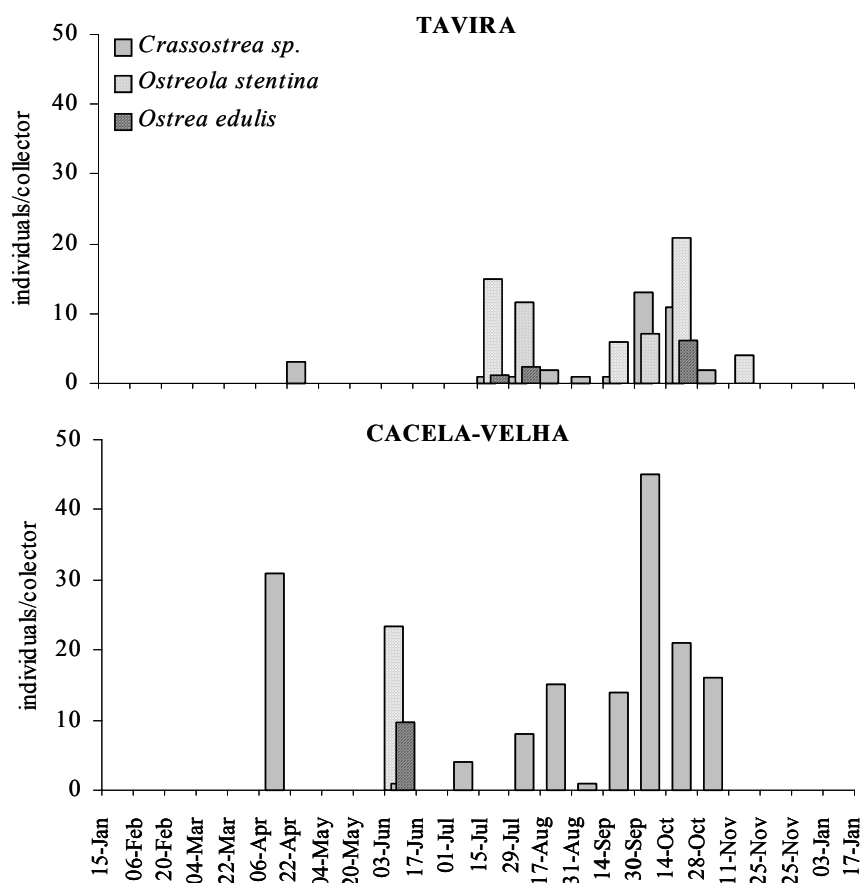


Figure 29. Recruitment of *Crassostrea* sp., *Ostreola stentina* and *Ostrea edulis* based on an ca. 15-day sampling interval at Tavira and Cacela-Velha sites.

On the other hand, the abundance of both *O. stentina* and *O. edulis* was higher in Tavira than in Cacela-Velha. This can be explained by the different submersion time of the collectors in the two sites since *O. stentina* and *O. edulis* are found predominantly on the subtidal (low depths) zone (Harry, 1985). Hence, the absence of *O. stentina* and *O. edulis* individuals in Cacela-Velha between July and October may have been due to mortality caused by desiccation. However, recruitment of *O. stentina* and *O. edulis* was observed in Cacela-Velha between 3 and 17 June but not in Tavira. On one hand, recruitment of these two species at a high intertidal level such as that one observed in Cacela-Velha may be explain by settlement immediately before sampling, which allow their collection before a lethal period of air exposure. On the other hand, the non-observation of recruitment of *O. stentina* and *O. edulis* in Tavira in the same period is more difficult to explain, but could have been due to: (1) a higher predation in Tavira between 3 and 17 June; (2) adverse environmental condition in Tavira during this period (e.g., low salinity due to the proximity in this site of Gilão River); or (3) a higher settlement in Cacela-Velha comparatively to Tavira, which was not observed for the other periods due to mortality of *O. stentina* and *O. edulis* caused by desiccation. In Tavira, between July and October, three recruitment events of *O. stentina* were observed namely, a first one between 15 July and 17 August, a second one between 14 September and 28 October, and a last one between 11 and 25 November. Surprisingly, some *O. stentina* females that have settled between 3 and 17 June and were kept under laboratory conditions were observed to be brooding larvae on 23 September. This findings indicate that *O. stentina* have a short generation time (between 98 and 112 days) and suggest that at least two generations might be produced in this area each year. This contrast with maturation in *O. edulis* in which individuals may reproduce under favourable conditions only after one year (Cole, 1941). The short generation time of *O. stentina* observed in the laboratory suggests that individuals settling between 3 and 17 June might have contributed to the setting observed between 14 September and 28 October. Moreover, taken into account the presumed short generation time of *O. stentina* it is also possible the individuals settling between 15 July and 17 August might have contributed to the setting observed between 11 and 25 November. For *O. edulis*, two setting periods were observed namely, between 15 July and 17 August, and 14 and 28 October at Tavira site. In Ireland and Brittany, *O. edulis* females brooding larvae were also observed at two different periods, namely between June and August, and in early September, but are more commonly found only between July and August (Cole, 1941; Wilson and Simons, 1985). According to Wilson and Simons (1985) spawning occurred with a water temperature between approximately 12 and 17 °C. Taking into account both Tavira and Cacela-Velha the results obtained in the present study suggest

that the breeding season of *O. edulis* in Ria Formosa in 2004 goes from June to October and seems longer than at higher latitudes in Europe.

Recruitment of *Crassostrea* sp., *O. stentina* and *O. edulis* overlapped for the majority of sampling periods studied, which may lead to the simultaneous collection of these taxa for culture purposes. The *Crassostrea* sp. individuals observed are thought to be a mixture of *C. angulata*, *C. gigas* and hybrids based on the study by Huvet *et al.* (2004). The occurrence of hybridization between *C. gigas* and *C. angulata* in both sites and at the different sampling periods is shown in section 5.2.

5.2. NATURAL HYBRIDIZATION BETWEEN *CRASSOSTREA ANGULATA* AND *C. GIGAS* IN RIA FORMOSA: SPATIAL AND TEMPORAL ANALYSES

5.2.1. Background and objectives

Imai and Sakai (1961) were the first to report that *C. angulata* and *C. gigas* can be successfully hybridized in the laboratory. Later, Menzel (1974) observed that meiosis in the F1 hybrids and mitosis in the F2 hybrid embryos appeared normal. In addition, molecular analysis of six-hour old embryos revealed no evidence of preferential fertilization between gametes from the same taxon when spermatic competition was allowed between taxa (Huvet *et al.*, 2001). Although Numachi (1966, in Gaffney and Allen, 1993) also reported that F1 hybrids of both taxa display normal viability and fertility, he observed that F2 hybrids did not survive to settlement. However, Huvet *et al.* (2002) obtained different results in which F2 hybrids showed normal developmental yields and settlement rates. Hence, these studies, with exception of Numachi's work, suggest that there no major reproductive barriers between *C. angulata* and *C. gigas* under laboratory conditions.

Some studies also suggest that *C. angulata* and *C. gigas* hybridize in the natural environment. Boudry *et al.* (1998) and Fabioux *et al.* (2002) observed haplotypes of both taxa in Tavira region (Ria Formosa), displaying the highest mix of specific mtDNA haplotypes from all known cupped oyster populations in Europe. Huvet *et al.* (2000) suggested that hybridization between the two taxa may have occurred in Tavira based on the observation of individuals carrying the PCR-RFLP haplotype C (specific of *C. gigas*) and showing specific *C. angulata* microsatellite alleles at the CG44 locus. Moreover, Huvet *et al.* (2004) using nuclear (CG44R) and mitochondrial markers provided more evidences of natural hybridization between *C. angulata* and *C. gigas* in the same region based on the absence of linkage disequilibrium and heterozygosity deficiency. Although these studies indicate that the two taxa hybridise in Tavira region it remains unknown if there are partial reproductive barriers and what is the nature of the hybridization process (e.g., random-mating, hybridization confined to the F1 generation, unidirectional hybridization with respect to gender). Variation in the timing of gonadal development and spawning can lead to the reproductive isolation of close related taxa, under certain environmental conditions, even within the same species as observed for the American oyster *C. virginica* (Loosanoff and Nomeijko, 1951; Barber *et al.*, 1991). The analysis of chronological series on the occurrence of *C. angulata* and *C. gigas* larvae on the Atlantic coast of France in the Marennes-Oléron basin (P. Soletchnik, personal communication) suggest that the two taxa may have different spawning periods. This hypothesis is supported by differences observed in the gametogenesis pattern of the two taxa grown

under the same conditions (Soletchnik *et al.*, 2002). Hence, differences in spawning time may occur in areas where *C. angulata* and *C. gigas* co-exist, which may act as partial reproductive barrier.

The objectives of the present study were: (1) to develop a PCR-RFLP method to distinguish individuals carrying mtDNA from *C. angulata* and *C. gigas* using a single restriction enzyme; (2) to document hybridization between the two taxa throughout the analysis of wild cupped oyster populations in Ria Formosa; and (3) to describe the annual pattern of *C. angulata*, *C. gigas* and putative hybrids recruitment in Ria Formosa.

5.2.2. Experimental procedure

Development of a PCR-RFLP method to distinguish mtDNA of C. angulata from C. gigas

Based on published data of homologous fragments of the COI gene of *C. angulata* and *C. gigas* (O'Foighil *et al.*, 1998; Boudry *et al.*, 2003; Yu *et al.*, 2003), taxon-specific restriction sites were identified for PCR-RFLP analyses. All *C. gigas* haplotypes shared the MvaI (5' - CCTGG - 3') restriction site and both taxa shared the MvaI (5' - CCAGG - 3') restriction site. Moreover, this enzyme was chosen for PCR/RFLP assays since it apparently allows the differentiation of the two taxa from *O. edulis* and *O. stentina*. In order to confirm the specificity of the method, samples of *C. angulata* (20 individuals from Sado River, Portugal), *C. gigas* (18 individuals from Seudre River, France), *O. edulis* (16 individuals from Ria Formosa, Portugal, and 16 individuals from Brittany, France) and *O. stentina* (23 individuals from Mira River, Portugal) were analysed. Total DNA was extracted using a modification of a phenol/chloroform method described by Sambrook *et al.* (1989). A 584 bp COI fragment was amplified using the primers COI3 (5' - GTA TTT GGA TTT TGA GCT GT - 3') and COI4 (5' - GAG GTA TTA AAA TGA CGA TC - 3'). PCR reactions were performed in 50 µl volumes composed of 5.0 µl of 10X PCR buffer, 35.7 µl of distilled water, 5.0 µl of dNTP (2 mM), 1.0 µl of each primer (20 µM), 0.3 µl of Taq polymerase (5 U/ µl) and 2.0 µl of template DNA (ca. 100 ng). The digestion reaction was performed at 37 °C during 120 min with 10 µl of PCR product, 7.5 µl of distilled water, 2 µl of 10X MvaI buffer and 0.5 µl of MvaI enzyme. Digestion products were separated by electrophoresis in 2 % agarose gels with ethidium bromide staining. Visualization was carried out under UV light. In order to further confirm the specificity of the method, part of the COI fragment was sequenced in 42 and 44 individuals of *C. angulata* and *C. gigas*, respectively, that showed the expected restriction pattern. This marker was called COIR.

Recruitment of C. angulata, C. gigas and putative hybrids in Tavira: a preliminary study

Total genomic DNA was extracted as described above from ethanol-preserved gills tissue of the *Crassostrea* sp. individuals that settled between 13 March 2002 and 20 January

2004 on plastic collectors as described in. section 5.1. Individuals were scored using the CG44R marker (Huvet *et al.*, 2004) and the new mitochondrial marker COIR (Figure 30).

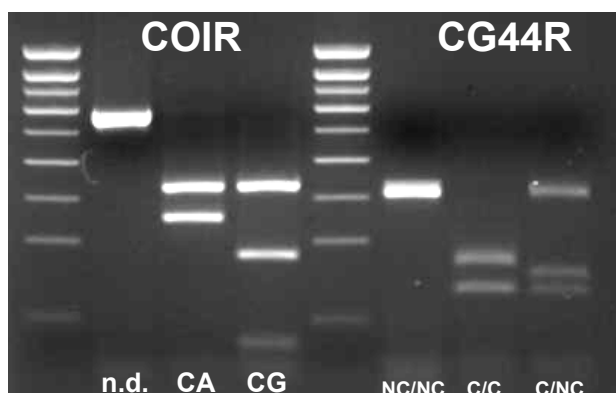


Figure 30. Electrophoresis digestion products of the COIR and CG44R markers from *C. angulata* and *C. gigas*. n.d., COI gene fragment (584 bp); CA, *C. angulata* restriction endonucleases pattern using *Mva*I; CG, *C. gigas* restriction endonucleases pattern using *Mva*I; NC/NC, non-cut/non-cut genotype; C/C, cut/cut genotype; C/NC, cut/non-cut genotype.

The CG44 microsatellite was amplified by a denaturation cycle at 94 °C for 2 min, followed by 30 cycles of 1 minute at 94 °C, 1 minute at 57 °C, and 1 minute at 72 °C with a least extension of 7 min at 72 °C. Negative controls, in which distilled water was added to the PCR mix instead of extracted DNA, was used in all PCR reactions. Restriction enzyme digestions of CG44 PCR products were set up in a 20- μ l volume containing 10.0 μ l PCR products, 7.5 μ l distilled water, 2.0 μ l 10X reaction buffer, and 0.5 μ l restriction enzyme (10 U/ μ l). CG44 amplicons were digested with *Bsr*GI (NewEngland BioLab) for 2 h at 37 °C. PCR products with the restriction site for *Bsr*GI were used as controls. Digestion products were separated by electrophoresis in 2 % agarose gels with ethidium bromide staining. Visualization was carried out under UV light.

Genetic characterization of Crassostrea sp. populations in Tavira and Cacela-Velha

Cupped oysters were collected from Tavira and Cacela-Velha sites in Ria Formosa, in July 2005. Samples of gills were dissected and preserved in absolute ethanol. Total genomic DNA was extracted from all individuals and subsequently scored using the CG44R and COIR markers as described above.

Annual recruitment pattern of C. angulata, C. gigas and putative hybrids in Tavira and Cacela-Velha

Total genomic DNA was extracted from the *Crassostrea* sp. individuals that settle on the collectors set out in Tavira (a total of 50 individuals) and Cacela-Velha (a total of 280 individuals) at the different sampling periods. All the individuals were scored using the COIR and CG44R markers as described above.

Data analyses

Pairwise tests for homogeneity of haplotype frequencies between samples were performed using chi-square analysis with Yate's correction. Multiple test significance levels were calculated using Bonferroni method (Rice, 1989). The use of the diagnostic mitochondrial marker COIR and the pseudospecific nuclear marker CG44R does not allow to determine whether an individual is *C. angulata*, *C. gigas*, a first generation (F1) hybrid, second generation (F2) hybrid, a backcross to one of the parental taxa, or a latter generation hybrid. Although parental types and F1 hybrids can be reliably identified if several diagnostic nuclear markers are used the distinction between the other classes is still very difficult. However, the CG44R alleles are close to being taxon-specific, with allelic frequencies of 0.1/0.9 (NC/C) for pure *C. angulata* populations and 0.9/0.1 (NC/C) for *C. gigas* populations (Huvet *et al.*, 2004). Moreover, the use of the diagnostic mitochondrial marker (COIR) together with pseudospecific nuclear marker (CG44R) allows the study of hybridization between *C. angulata* and *C. gigas* throughout analysis of heterozygote deficiency and cytonuclear disequilibria (Huvet *et al.*, 2004). F_{is} values were calculated according to Weir and Cockerham (1984) to estimate deviations from the Hardy-Weinberg model. Deviations were tested in each sample by generating 10 000 permutations. These computations and tests were done using the package GENETIX version 4.05. The frequency of null alleles ($\emptyset$ freq) was estimated using the method of Brookfield (1996), which assumes panmixia and that all of the heterozygote deficiency relative to Hardy-Weinberg proportions is due to null alleles. In addition, the Brookfield's (1996) method also takes into account the individuals in which no amplification was observed, which are analyzed as homozygotes for the null allele. Cytonuclear disequilibria were analyzed using the metropolis algorithm (Raymond and Rousset, 1995) with the R x C program. Due to the presence of null alleles (see below), 2 x 4 contingency tables were used to analyze cytonuclear disequilibria using two haplotypes groups (CA and CG for the COIR marker) and four genotypes (for the CG44R marker), namely: (1) "CC" genotype composed by CC and C $\emptyset$ (heterozygotes that are scored as CC); (2) C NC genotype; (3) "NC NC" genotype composed by NC NC and NC $\emptyset$ (heterozygotes that are scored as NC NC); and (4) $\emptyset\emptyset$ genotype (null homozygotes that appear as missing data). An example is

shown is Table 12 in which two different situations are simulated: (1) absence of hybridization (association between haplotype and genotype); and (2) random-mating (no association between haplotype and genotype). In the first example it is assumed that there is no hybridization between *C. angulata* and *C. gigas* ($n = 100$), with allelic frequencies of 0.1/0.9 (NC/C) for individuals with *C. angulata* haplotype (CA) and 0.9/0.1 (NC/C) for individuals with *C. gigas* haplotype (CG), and a frequency of null alleles ($\emptyset$) of 0.30. In the second example, random mating between the two taxa is assumed with allelic frequencies of 0.5/0.5 (NC/C) and the same frequency of null alleles.

Table 12. Examples of 2 x 4 contingency tables analyzed using the metropolis algorithm.

		CG44R				p-value (SE)
		CC + CØ	C NC	NC + ØØ	ØØ	
1. No hybridization						
	CA	39	4	2	5	0.00 (0.00)
	CG	2	4	39	5	
2. Random mating						
	CA	17	12	17	5	1.00 (0.00)
	CG	17	12	17	5	

5.2.3. Results and discussion

Development of a new PCR-RFLP method to distinguish mtDNA of C. angulata from C. gigas

A new PCR-RFLP method (COIR) was developed to identify individuals that carry mtDNA specific of *C. angulata* and *C. gigas* using a single restriction enzyme. All individuals analysed showed the expected restriction profile using *Mva*I, with exception of 3 individuals of *O. edulis* (Figure 31). Nevertheless, these individuals showed a restriction profile that could be unambiguously distinguished from the other ones. The two *O. edulis* haplotypes were called E1 and E2. Haplotype E2 was observed only in Ria Formosa population with a frequency of 0.19. The *C. angulata*, *C. gigas* and *O. stentina* PCR-RFLP haplotypes were called CA, CG and OS, respectively.

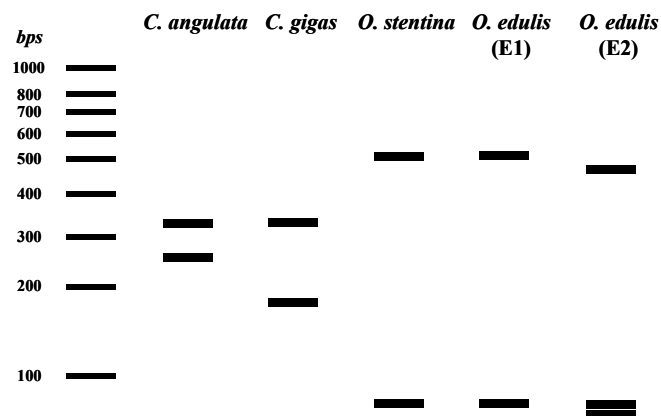


Figure 31. Representation of restriction fragment patterns of a 584 bp COI gene fragment digested with *MvaI* for *C. angulata*, *C. gigas*, *O. stentina* and *O. edulis*. For *O. edulis* two restriction profiles were observed (E1 and E2).

Recruitment of *C. angulata*, *C. gigas* and putative hybrids in Tavira in 2002/2004 and genetic characterization of wild cupped oyster populations in Tavira and Cacela-Velha. The analysis of 49 cupped oysters that settled in the collectors set out in Tavira between 13 March 2002 and 20 January 2004 revealed that most of the cupped oyster had the *C. angulata* haplotype ($f = 0.94$) whereas only a small number had the *C. gigas* haplotype ($f = 0.06$). The analysis of a wild population of cupped oysters in the same location in 2005 ($n = 47$) revealed that 87 % of the oysters carried the *C. angulata* haplotype (Table 13).

Table 13. Haplotype frequencies for the COIR marker (CA freq for *C. angulata* and CG freq for *C. gigas*), allelic frequencies (cut allele, C freq; non-cut allele, NC freq), observed (H_0) and expected (H_e) heterozygosity, and F_{is} values for the CG44R locus in Cacela-Velha and Tavira wild populations.

	COIR			CG44R					
	N	CA freq	CG freq	N	C freq	NC freq	H_0	H_e	F_{is}
Cacela-Velha	50	0.98	0.02	50	0.87	0.13	0.18	0.23	0.21 ^{ns}
Tavira	47	0.87	0.13	46	0.78	0.22	0.13	0.34	0.62**

Similar results were also obtained by Huvet *et al.* (2004) where 82 % of oysters collected in the same location (samples collected before 2000) had *C. angulata* haplotypes. Non-significant differences in the haplotypes frequencies were observed among these 3 studies (χ^2 tests, $p > 0.05$). On one hand, these results suggest that the type of collectors used and their location did not influence settlement of individuals with *C. angulata* and *C. gigas* haplotypes. On the other hand, the results also suggest that no major modifications

occurred on the proportion of oysters carrying the two types of mitochondrial DNA since Huvet *et al.* (2004) study. For the nuclear marker CG44R, a high frequency ($f = 0.76$) of the C allele was observed in the oysters from the collectors set out in Tavira between 13 March 2002 and 20 January 2004, which is in agreement with the frequency ($f = 0.78$) observed in the wild population on the same location. A similar frequency of the C allele ($f = 0.78$) was observed by Huvet *et al.* (2004) in the same site. The observed heterozygosity at the CG44R locus was 0.117 whereas the expected heterozygosity was 0.368, resulting in a highly significant ($p < 0.001$) F_{is} value of 0.69. In the case of a single population that is actually composed by two populations with limited gene flow, a deficit of heterozygotes for alleles that differentiate the two populations is observed (Wahlund effect). However, the heterozygosity deficiency observed in the present study may be explain by the high frequency of null alleles ($f = 0.35$ using Brookfield's method) and not due to the absence of hybridization between *C. angulata* and *C. gigas*. Indeed, no cytonuclear disequilibria was observed in the oysters from the collectors set out in 2002/2004 ($p = 0.757$) neither from those collected in the wild cupped oyster population in Tavira ($p = 0.358$). Hence, the results of the present study support the hypothesis of the occurrence of natural hybridization between *C. angulata* and *C. gigas* in Tavira region (Huvet *et al.*, 2004). Moreover, the analysis of a wild cupped oyster population in Cacela-Velha in which a low level of null alleles ($f = 0.04$) was observed, showed no evidence of heterozygotes deficiencies (Table 13). In addition, no cytonuclear disequilibria ($p = 1.000$) was observed in this population, which support the hypothesis of hybridization between *C. angulata* and *C. gigas*. Hence, these findings suggest that hybridization between the two taxa is probably widespread in Ria Formosa and not restricted to a single area.

Annual recruitment pattern of specimens of C. angulata, C. gigas and hybrids at Tavira and Cacela-Velha

The number of individuals carrying the *C. angulata* haplotype was significantly higher than that carrying *C. gigas* haplotype both in Cacela-Velha ($\chi^2 = 29.44$, $p < 0.001$) and in Tavira ($\chi^2 = 17.69$, $p < 0.001$) (Table 14). A higher number of individuals carrying *C. angulata* haplotypes was also observed by Boudry *et al.* (1998) and Huvet *et al.* (2004) in Ria Formosa, which suggest that despite the continuous introduction of *C. gigas* for aquacultural purposes (Ruano, personal communication) the number of pure *C. angulata* or hybrids carrying *C. angulata* haplotypes is still high. A significantly ($\chi^2 = 9.53$, $p < 0.01$) higher proportion of oysters carrying *C. angulata* haplotypes was observed in Tavira ($f = 0.76$) comparatively to Cacela-Velha ($f = 0.61$) as shown in Table 14. These findings contrast with the haplotype frequencies of *C. angulata* and *C. gigas* observed in wild cupped oyster populations located at the same sites (Table 13) that did not differ

significantly between them ($\chi^2 = 2.74$, $p = 0.10$). These can be due to a higher survival of *C. gigas* spat at higher tidal levels comparatively with *C. angulata* spat, since between the moment that larvae settled and that collectors were brought in to the laboratory a maximum of 15 days may have passed. This hypothesis also implies that survival of juveniles and adults carrying the *C. gigas* haplotype is lower than those carrying the *C. angulata* haplotype. The findings obtained in Chapter 3 do not support this hypothesis since a significantly higher survival was observed in *C. gigas* adults comparatively with *C. angulata*. However, it is noteworthy that the progenitors of the *C. angulata* specimens used in the experiment reported in Chapter 3 were from Sado River and hence they may be less adapted to Ria Formosa conditions comparatively to local populations of *C. angulata*.

Table 14. Number of *Crassostrea* sp. specimens scored with the COIR marker in each sampling period in Cacela-Velha and Tavira sites (N), and frequency of individuals with *C. angulata* (CA freq) and *C. gigas* (CG freq) haplotypes.

Settlement period	Cacela-Velha			Tavira		
	N	CA freq	CG freq	N	CA freq	CG freq
6 – 22Apr	48	0.10	0.90			
22Apr - 4May				8	0.00	1.00
1 – 15 Jul	13	0.31	0.69			
29Jul - 17Aug	27	0.52	0.48			
17 - 31Aug	48	0.85	0.15	8	0.88	0.12
14 - 30 Sep	44	0.80	0.20			
30Sep - 14Oct	47	0.70	0.30	26	0.81	0.19
14 - 28Oct	45	0.71	0.29	15	0.87	0.13
28Oct - 11Nov	40	0.90	0.10	5	0.80	0.20
Total	312	0.61*	0.39*	61	0.76*	0.24*

* calculated based on the total oysters that settled at each period and on the relative frequency of *C. angulata* and *C. gigas* haplotypes for each settlement period.

A similar temporal pattern of recruitment was observed in Cacela-Velha and Tavira in terms of haplotype frequencies (Figure 32). The first recruitment peak was observed at Cacela-Velha between 6 and 22 of April and was mostly constituted by individuals carrying the *C. gigas* haplotype (90 %, Table 14). In Tavira, oyster setting was only observed for the first time on the next sampling period (from 22 April to 4 May) and was composed by specimens only carrying the *C. gigas* haplotype. The non-overlap between the previous settlement periods may have been due to a delay of a few days or even

hours, but the 15-day sampling interval does not allow determining more precisely the extent of the discrepancy. Hence, this suggests that spatial variation in settlement time may occur, which may be caused by different settlement cues that can delay or anticipate the induction of the settlement behaviour (Coon *et al.*, 1990). The non observation of the *C. angulata* haplotype in Tavira site may have been due to the small sample size ($n = 8$) comparatively to that of Cacela-Velha ($n = 48$), and not due to the absence the *C. angulata* haplotype.

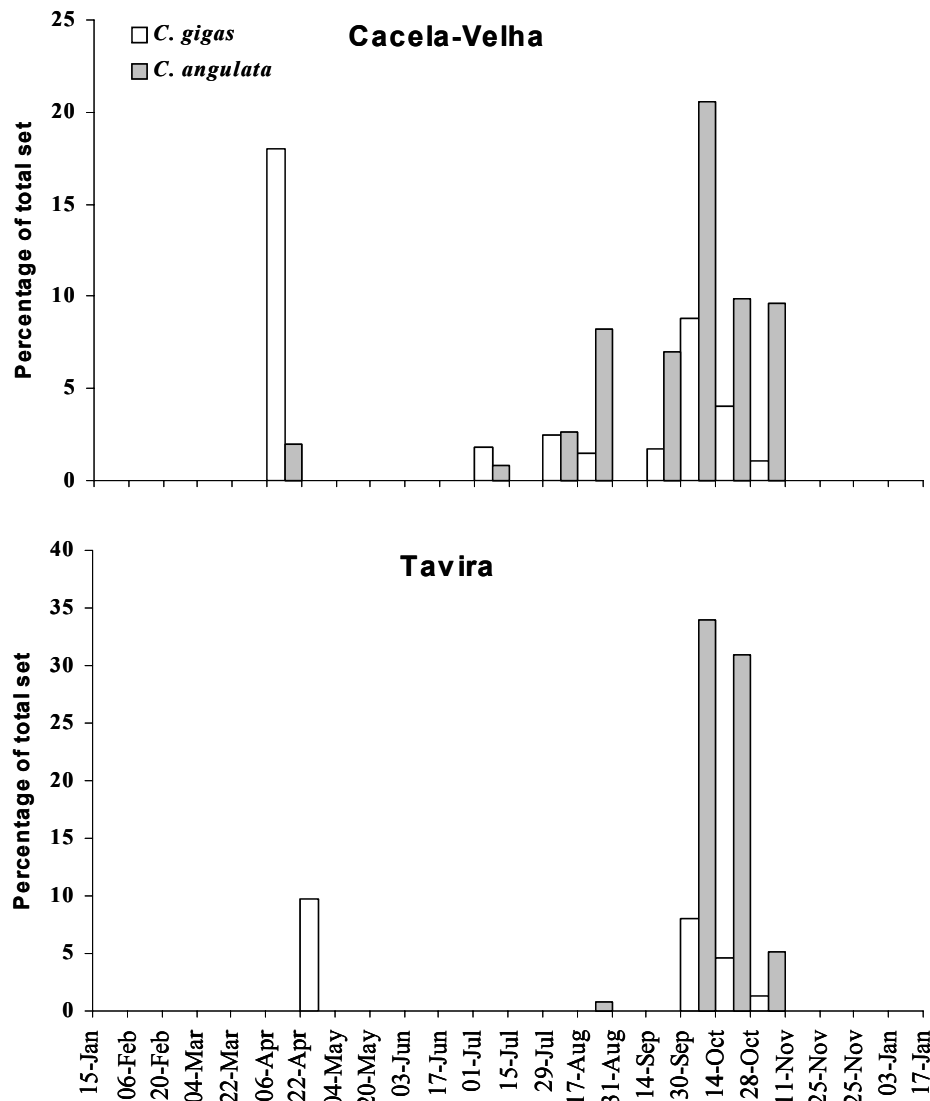


Figure 32. Percentage of total set of *Crassostrea* sp. individuals carrying *C. angulata* (gray bars) and *C. gigas* (white bars) haplotypes based on the total number of individuals that settled on approximately 15-day sampling intervals at the Cacela-Velha and Tavira sites.

The frequency of oysters carrying the *C. gigas* haplotype decreased in the following recruitment periods and by the end of the setting season the *C. angulata* haplotype was dominant (80-90 %). Indeed, significant differences on haplotype frequencies were observed among settlement periods both in Cacela-Velha (Table 15) and Tavira (Table 16). The analysis of pairwise tests revealed that the first settlement period in Cacela-Velha (from 6 to 22 April) was distinct from the setting that started between 29 July and continued until the end of the setting season with exception of an intermediate period from 1 to 15 July.

Table 15. Pairwise tests for homogeneity of haplotype frequencies between settlement periods at Cacela-Velha.

	1	2	3	4	5	6	7	8
1 6 – 22Apr	-							
2 1 – 15 Jul	1.9 ^{ns}	-						
3 29Jul - 17Aug	13.6**	0.8 ^{ns}	-					
4 17 - 31Aug	51.1**	13.1**	8.3 ^{ns}	-				
5 14 - 30 Sep	41.9**	8.9 ^{ns}	4.8 ^{ns}	0.2 ^{ns}	-			
6 30Sep - 14Oct	32.9**	5.1 ^{ns}	1.8 ^{ns}	2.4 ^{ns}	0.6 ^{ns}	-		
7 14 - 28Oct	33.2**	5.4 ^{ns}	1.9 ^{ns}	2.0 ^{ns}	0.5 ^{ns}	0.0 ^{ns}	-	
8 28Oct - 11Nov	52.4 **	15.5 **	10.5 *	0.1 ^{ns}	1.0 ^{ns}	4.0 ^{ns}	3.6 ^{ns}	-

^{ns} not significant, * p < 0.05, ** p < 0.01

Table 16. Pairwise tests for homogeneity of haplotype frequencies between settlement periods at Tavira.

	1	2	3	4	5
1 22Apr – 4May	-				
2 17 - 31Aug	9.14 *	-			
3 30Sep - 14Oct	13.7 **	0.0 ^{ns}	-		
4 14 - 28Oct	12.6 **	0.3 ^{ns}	0.0 ^{ns}	-	
5 28Oct - 11Nov	5.9 ^{ns}	0.2 ^{ns}	0.3 ^{ns}	0.1 ^{ns}	-

^{ns} not significant, * p < 0.05, ** p < 0.01

However, for homologous settlement periods no significant differences were observed between the two sites, namely from 17 to 31 August ($\chi^2 = 0.152$, p = 0.697), 30 September to 14 October ($\chi^2 = 0.498$, p = 0.480), 14 to 28 October ($\chi^2 = 0.741$, p = 0.389), and 28 October to 11 November ($\chi^2 = 0.007$, p = 0.933).

Heterozygosity deficiency was observed in the majority of the settlement periods analyzed in Cacela-Velha site with exception from 6 to 22 April. (Table 17). At Tavira site, no significant *F_i* values were observed for the majority of settlement periods analyzed.

However, the differences in observed and expected heterozygosity were high, which suggest that no significant differences were observed due to small sample sizes.

Table 17. Non-cut allele frequency (NC freq), observed (H_o) and expected (H_e) heterozygosity, and F_{is} values for each settlement period in Cacela-Velha and Tavira sites for the CG44R locus without taking into account null alleles.

Settlement period	N	C freq	NC freq	H_o	H_e	F_{is}
Cacela-Velha						
6 – 22Apr	44	0.01	0.99	0.02	0.02	0.00
1 – 15 Jul	12	0.63	0.37	0.08	0.49	0.84**
29Jul - 17Aug	25	0.50	0.50	0.20	0.51	0.61**
17 - 31Aug	48	0.72	0.28	0.15	0.41	0.65***
14 - 30 Sep	33	0.62	0.38	0.27	0.48	0.43*
30Sep - 14Oct	41	0.65	0.35	0.17	0.46	0.63***
14 - 28Oct	39	0.56	0.44	0.21	0.50	0.59***
28Oct - 11Nov	38	0.72	0.28	0.18	0.40	0.55**
Tavira						
22Apr – 4May	7	0.00	1.00	0.00	0.00	-
17 - 31Aug	7	0.50	0.50	0.14	0.54	0.75 ^{ns}
30Sep - 14Oct	19	0.63	0.37	0.42	0.48	0.12 ^{ns}
14 - 28Oct	12	0.58	0.42	0.00	0.51	1.00 ***
28Oct - 11Nov	5	0.80	0.20	0.40	0.36	-0.14 ^{ns}

Heterozygosity deficiency can be interpreted as an absence of hybridization between *C. angulata* and *C. gigas*. However, the non amplification of the CG44 marker was observed in several individuals from the different settlement periods in both sites, which clearly indicates the presence of null alleles (Table 18). Moreover, the hypothesis of non-amplification due to bad quality DNA is undermined by amplification of the COI fragment in all individuals using the same DNA source. Null alleles can arise when mutations prevent primers from binding and have been observed in high number in *C. gigas* (Hedgecock *et al.*, 2004). Huvet *et al.* (2004) observed significant positive F_{is} values in 48 % of the populations analyzed using 8 different microsatellites, and suggested null alleles was the best explanation for most of the effect. For the CG44R marker, Huvet *et al.* (2004) observed significant positive F_{is} in 5 out of 15 populations.

Table 18. Total number of individuals scored for CG44R marker (N), frequency of individuals collected for which no amplification was observed ($\emptyset \emptyset$ freq) and frequency of null alleles (Null freq) estimated using Brookfield's (1996) method.

Settlement period	Cacela-Velha			Tavira		
	N	$\emptyset \emptyset$ freq	Null freq	N	$\emptyset \emptyset$ freq	Null freq
6 – 22Apr	48	0.09	0.28			
22Apr – 4May				8	0.13	0.35
1 – 15 Jul	13	0.09	0.37			
29Jul - 17Aug	27	0.07	0.31			
17 - 31Aug	48	0.01	0.18	8	0.13	0.39
14 - 30 Sep	44	0.25	0.43			
30Sep - 14Oct	47	0.13	0.37	26	0.27	0.35
14 - 28Oct	45	0.13	0.36	15	0.20	0.53
28Oct - 11Nov	40	0.05	0.26	5	0.00	0.00

The estimated frequency of null alleles ranged between 0.00 and 0.53, which may explain the significantly F_{is} values observed for most of the samples from Cacela-Velha (Table 17). However, as mentioned above, null allele frequency was estimated assuming random mating which may not be true for all settlement periods. However, Huvet *et al.* (2004) observed for the CG44R marker a non significant F_{is} value of 0.12 in Tavira population contrarily to the highly significant F_{is} value of 0.62 observed in the present study (Table 13). The high frequency of null alleles observed greatly complicate the analysis of hybridization between *C. angulata* and *C. gigas* based on F_{is} values.

Several lines of evidences suggest that settlement of *C. angulata* should have occurred first than *C. gigas*. According to Lubet (1994), the minimum temperature for gametes release is around 17-18 °C for *C. angulata* and 20-21 °C for *C. gigas*. Marteil (1976 in Héral *et al.*, 1986) indicated a minimum value of 16 °C for spawning to occur in *C. angulata* whereas minimum value of 18 °C was reported by Mann (1979) and Steele and Mulcahy (1999) for *C. gigas*. However, spawning in *C. angulata* has been observed at different water temperatures (between 17 and 23 °C) depending on the year (Le Dantec, 1968). A broader range of temperatures to induce spawning (between 19 to 24 °C) as also been suggested for *C. gigas* (Buroker, 1985). Nevertheless, despite the different minimum temperature values reported by these studies all indicate that a higher temperature is required to induce spawning in *C. gigas* comparatively to *C. angulata*. Second, based on the observation of young larvae (between 1 and 4 days old) in the Marennes-Oléron bay between 1949 and 1970 for *C. angulata* and between 1970 and 1998 for *C. gigas*, it is though that spawning occurs early in the former taxon (P. Soletchnik, personal communication). Third, there are evidence that gametogenesis rate is faster in *C. angulata* based on Soletchnik *et al.* (2002) study on the Atlantic coast of France. The

previous authors suggested that the reproductive season of *C. angulata* goes from mid-June to mid-August whereas reproductive season of *C. gigas* goes from mid-July to mid-August. Fourth, Marteil (1960, in Héral *et al.*, 1986) indicated that the optimal temperature for the development of *C. angulata* larvae is 22 °C whereas for *C. gigas* larvae is 23 °C, which support the hypothesis of early spawning by *C. angulata*.

The high frequency of the CG haplotype (0.90) together with the high frequency of the NC allele (0.99) observed at Cacela-Velha between 6 and 22 April suggests that the majority of the oyster that settle first the year “pure” *C. gigas* and hybrids. Five out 48 individuals analyzed had the CA haplotype, which indicate that hybrids or pure *C. angulata* individuals also settled during this period. However, the absence of association between mtDNA and the nuclear marker, and the fact that all individuals with CA haplotype had the “NC NC” genotype suggest these specimens were hybrids. Hence, these results suggest that during this period there where no settlement of *C. angulata* and the oysters that settled first were a mixture of *C. gigas* and hybrids. The absence of *C. angulata* settlement between 6 and 22 April is in agreement with the results of Pascual (1974) who reported that settlement season of *C. angulata* at Guadalquivir estuary (located approximately 50 km away from Cacela-Velha site) starts only in July. For the other settlement periods in both sites, with exception of the period between 1 and 15 July at Cacela-Velha, a higher proportion of individuals possessing *C. angulata* mtDNA was observed. The analysis of cytonuclear disequilibrium suggests that hybrids settled during the majority of the settlement periods analyzed (Table 19). However, this does not exclude the possibility that during these periods pure *C. angulata* and *C. gigas* individuals have also settled. Indeed, at Cacela-Velha site between 14 and 28 October cytonuclear disequilibrium was observed (Table 19), mainly due to an excess of individuals with CG haplotype/NC NC genotype, which indicate some pre or post-zygotic barriers may reduce gene flow between *C. angulata* and *C. gigas*.

Table 19. Analysis of cytonuclear disequilibrium using the metropolis algorithm for 2x4 contingency table.

Settlement period	Cacela-Velha		Tavira	
	N	p-value (SE)	N	p-value (SE)
6 – 22Apr	48	1.000 (0.000)	8	1.000 (0.000)
22Apr – 4May				
1 – 15 Jul	13	1.000 (0.000)		
29Jul - 17Aug	27	0.109 (0.004)	8	1.000 (0.000)
17 - 31Aug	48	0.278 (0.005)		
14 - 30 Sep	44	1.000 (0.000)		
30Sep - 14Oct	47	0.963 (0.001)	26	0.625 (0.004)
14 - 28Oct	45	0.039 (0.003)	15	1.000 (0.000)
28Oct - 11Nov	40	0.303 (0.007)	5	0.401 (0.007)

3.3. CONCLUSIONS

Recruitment of *Crassostrea* sp., *O. stentina* and *O. edulis* was observed both at Cacela-Velha and Tavira sites. The setting season of these taxa partially overlap. In addition, differences in the number of *Crassostrea* sp., *O. stentina* and *O. edulis* individuals were observed between the two sites, which were probably due to differences in tidal level between sites. The results of the present study show that *Crassostrea* sp. can be selectively collected if collectors are set out at certain periods and especially at high tidal levels. Nevertheless, contamination with *O. stentina* will be very difficult to avoid although its number can be significantly reduce if collectors are kept at high tidal levels, since *O. stentina* has a lower resistance to air exposure comparatively to *C. angulata* and *C. gigas* (data not shown).

The analysis of haplotype frequencies and cytonuclear disequilibrium at each settlement period in both sites suggest that hybrids settled during almost all setting season. Hence, this indicates that the two taxa cannot be selectively collected from the wild and that hybridization is widespread in time. Nevertheless, different haplotypes frequencies were observed for almost all setting periods. At the beginning of the setting season the *C. gigas* haplotype was present at a higher frequency. In the following recruitment periods the proportion of the *C. gigas* haplotype decrease and by the end of the setting season the *C. angulata* haplotype was dominant. These findings suggest that genetic variation in the timing of gonadal development and/or spawning exists between the two taxa, which can lead to partial temporal reproductive isolation.

CHAPTER 6. DEVELOPMENT OF MOLECULAR TECHNIQUES FOR DETECTION OF DNA FROM VIRUSES INFECTING *CRASSOSTREA ANGULATA* AND *C. GIGAS*

6.1. DEVELOPMENT OF A DNA EXTRACTION METHOD FOR THE DETECTION OF OSTREID HERPESVIRUS 1 DNA BY PCR

6.1.1. Background and objectives

Herpes-like viral infections have been identified in various bivalve mollusc species throughout the world (Farley *et al.*, 1972; Nicolas *et al.*, 1992; Hine *et al.*, 1992; Vásquez-Yeomans *et al.*, 2004). Infections are often associated with high mortality rates among hatchery reared larvae as well as cultured juveniles (Renault *et al.*, 1994a; Renault and Arzul, 2001). Although this type of viral infections was also observed in adult bivalves, they are apparently less susceptible to such infections (Arzul *et al.*, 2002). Infected larvae show a reduction in feeding and swimming activity, until they stop swim and settle at the bottom of the tanks (Le Deuff *et al.*, 1994). They exhibit velar and mantle lesions, particularly velum cells are hypertrophied and detached from the tissue. For both larvae and juveniles, sudden and high mortality outbreaks are observed in a short period of time (Renault *et al.*, 1994a, b). Cytopathological changes are confined to connective tissues. Fibroblastic-like cells present enlarged nuclei with chromatin marginalization and cells interpreted as hemocytes show highly condensed nuclei (Hine *et al.*, 1992; Renault *et al.*, 1994b). The genome characterization and the analysis of the capsid structure of a herpes-like virus infecting *Crassostrea gigas* larvae in France allowed it to be included in the Herpesviridae family and named Ostreid Herpesvirus 1 (OsHV-1) (Le Deuff and Renault, 1999; Davidson *et al.*, 2005). The ability of OsHV-1 to infect different bivalve species may indicate that it has emerged as a consequence of selection under intensive farming conditions (Arzul *et al.*, 2001a).

Nucleic acid-based techniques have been developed to detect OsHV-1 DNA, such as the polymerase chain reaction (PCR). A technique to prepare samples for PCR was developed by Renault *et al.* (2000a), which allowed the detection of OsHV1-DNA in *C. gigas* larvae by PCR. However, this technique requires large amounts of larvae (50 mg), which can be seen as limitation when only a small quantity of sample is available.

The objective of the present study was to develop a method of DNA extraction for small size samples that allows the detection of OsHV-1 DNA using PCR.

6.1.2. Experimental procedure

The experimental procedure is described in detail in Article 4. A summary of the DNA extraction method developed in the present study is shown in Table 20.

Table 20. DNA extraction from oyster larvae.

Step	Procedure
1. Sampling	Transfer approximately 3 mg of fresh-frozen (preserved at - 20 °C, - 80 °C or in liquid nitrogen) larvae to a 0.2 ml microtube (PCR tube)
2. Washing	Wash the larvae by re-suspending them in double distilled water and removing the supernatant by aspiration. Repeat the procedure three times to remove any trace of seawater.
3. Grinding	Ground the larvae in 50 µl of extraction buffer solution (5 µl of Goldstar taq DNA polymerase buffer, 45 µl of double distilled water and 0.5 % of tween-20) using a single-use curved (at the tip) steel needle.
4. Digestion and lysis	Add 5 µl of proteinase K (10 mg ml^{-1}) to each tube. Incubate the tubes at 55 °C for 60 min followed by a second incubation step at 100 °C for 20 min using a thermal cycle (PTC-100™, MJ Research).
5. Conservation	Mix the samples by vortexing (5 s) and centrifuge at 10 000 rpm (1200 x g) for 5 min at 4° C. Transfer the supernatants to 0.5 ml microtubes and dilute (1:10) them using double distilled water. Frozen the samples at - 20 °C immediately.

6.1.3. Results and discussion

OsHV-1 DNA was detected in one of the 8 samples analysed by nested PCR using the primer pairs C5/C13 and C2/C4 (Figure 33). No inhibitory effect was observed in any of the samples analysed (see Figure 1 of Article 4). Moreover, the amplification of oyster RNA16S fragments in all samples analysed indicates that the method allowed the extraction of good quality DNA. Cells with nuclear abnormalities were reported in the sample in which OsHV-1 DNA was detected by nested PCR (Figure 34). These results demonstrate that the DNA extraction method developed in the present study allows the detection of OsHV-1 DNA by PCR using small amounts of larvae (3 mg).

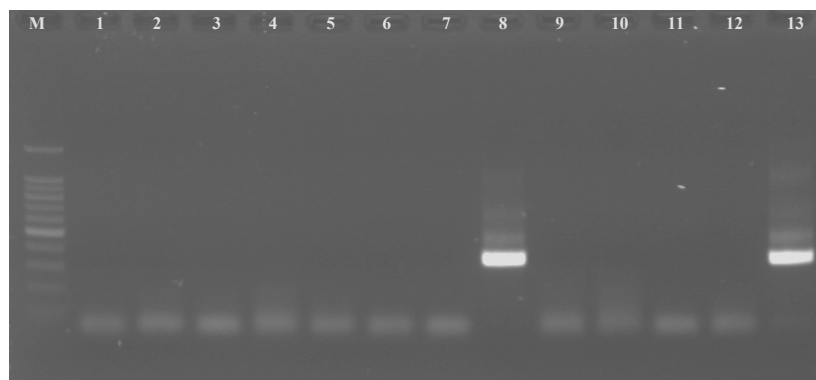


Figure 33. Detection of OsHV-1 DNA by nested PCR using the primer pairs C5/C13-C2/C4. Lanes 1, 6 and 11, negative controls of the second PCR. Lanes, 2, 3, 4 and 5, 10-dayold larvae from tank 1, 2, 3 and 4, respectively. Lanes, 7, 8, 9 and 10, 15-day old larvae from tank 1, 2, 3 and 4, respectively. Lane 12, negative control of the first PCR. Lane 13, positive control (OsHV-1 DNA). Lane M, size markers (100 bp ladder, Promega).



Figure 34. Nuclear abnormalities in a *C. angulata* larva from the batch in which OsHV-1 DNA was detected by PCR. The white arrow indicate a condensed nuclei and the black arrow a nuclei presenting marginalization of chromatin.

Article 4 (published in Diseases of Aquatic Organisms)

Vol. 64: 1–4, 2005	DISEASES OF AQUATIC ORGANISMS Dis Aquat Org	Published April 6

Detection of ostreid herpesvirus-1 (OsHV-1) by PCR using a rapid and simple method of DNA extraction from oyster larvae

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ABSTRACT: A DNA extraction procedure was developed for the detection of ostreid herpesvirus-1 (OsHV-1) using the polymerase chain reaction (PCR) in oyster larvae. The DNA extraction procedure developed was tested on 8 larval samples. Abnormal nuclei with characteristic features associated with OsHV-1 infections were only observed in samples in which the viral DNA was detected by PCR. A previously described competitive PCR method was applied to detect inhibition during PCR reactions. The results show that the method can be used on small amounts of oyster larvae (3 mg) for the detection of OsHV-1 DNA by PCR.

KEY WORDS: Herpesvirus · OsHV-1 · Oyster · Larvae · Detection · DNA extraction

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INTRODUCTION

Herpes-like virus infections of bivalve molluscs were first reported in *Crassostrea virginica* by Farley et al. (1972). Viruses presumed to belong to the *Herpesviridae* family have subsequently been associated with mortalities in larvae and juveniles of various bivalve species around the world (Hine et al. 1992, Nicolas et al. 1992, Renault et al. 1994, Renault & Arzul 2001). The pathogenicity of the herpes-like virus infecting *Crassostrea gigas* larvae was demonstrated by experimental transmission of the disease to axenic healthy larvae (Le Deuff et al. 1994). The virus was isolated from moribund *Crassostrea gigas* larvae and its genome characterized (GenBank number AY509253), which allowed it to be included in the *Herpesviridae* family. The virus was named ostreid herpesvirus-1 (OsHV-1) (Minson et al. 2000, Arzul & Renault 2002, Davison 2002). The characterization of the virus genome has facilitated the development of molecular tools including detection of viral DNA using the polymerase chain reaction (PCR) (Renault et al. 2000, Arzul et al. 2001, 2002, Lipart & Renault 2002). PCR is a suit-

able tool for the diagnosis of OsHV-1 infections due to specificity, high sensitivity, time and cost efficiency. The method used to prepare samples for DNA detection using the PCR is known to be critical for the success of the amplification reaction. The procedure currently used for preparing samples for OsHV-1 detection using the PCR (Renault et al. 2000) requires large amounts of larvae (50 mg). The aim of the present study was to develop an alternative rapid and simple procedure of DNA extraction for small samples of oyster larvae (3 mg) that allows the detection of OsHV-1 DNA using PCR.

MATERIALS AND METHODS

Oyster samples. Larvae from a hybridization study between *Crassostrea angulata* and *C. gigas* were reared in 4 independent 150 l tanks. Larvae were collected in September 2003 after 10 and 15 d of development, and fresh-frozen at -80°C.

Sample preparation. Two procedures for total nucleic acid extraction were carried out: (1) a DNA

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sample preparation procedure previously described (Renault et al. 2000), (2) a variation of this procedure using a pre-treatment with Proteinase K/Tween-20. In the first extraction procedure (1), 50 mg of frozen larvae were measured in 1.5 ml microfuge tubes and ground in 50 µl of distilled water. Samples were vortexed and boiled in a water bath for 10 min, followed by quick chilling in ice (Renault et al. 2000). In the second extraction procedure (2), 3 mg of fresh-frozen larvae were measured in 0.2 ml microtube and washed in double distilled water to remove any traces of seawater. Larvae were then ground in 50 µl of extraction buffer solution (5 µl of Goldstar Taq DNA Polymerase buffer, 45 µl of double distilled water and 0.5 % of Tween-20) using a single-use curved steel needle. Five microlitres of Proteinase K (10 mg ml⁻¹) were added to each tube. The samples were then incubated at 55°C for 60 min followed by a second incubation step at 100°C for 20 min in a thermal cycler (PTC-100™, MJ Research). Samples were then mixed and centrifuged at 10 000 rpm (1200 × *g*) for 5 min at 4°C. Supernatants obtained by both procedures were recovered in 0.5 ml microfuge tubes, diluted 10-fold in double distilled water and immediately frozen at -20°C.

Detection of OsHV-1 by nested PCR. Two primer-pair combinations were used for the detection of OsHV-1 DNA, the external C5 (5' CCG TGA CTT CTA TGG GTA TGT CAG 3')/C13 (5' CCT CGA GGT AGC TTT TGT CAA G 3') combination which generated a 765 bp product, followed by the internal C2 (5' CTC TTT ACC ATG AAG ATA CCC ACC 3')/C4 (5' GCA GTT GTG GTA TAC TCG AGA TTG 3') pair which yielded a 352 bp product (Arzul et al. 2001). All PCR reactions were performed on a Crocodile III thermal cycler (Appligene Oncor). Fifty microlitre PCR reactions were prepared using 2.5 U of Goldstar Taq DNA Polymerase (Eurogentec), 0.05 mM of each dNTP, 100 ng of each primer, 2.5 mM MgCl₂ and 1 µl of extracted DNA. After heating samples for 2 min at 94°C, 35 cycles consisting of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min were carried out, followed by a final elongation step of 5 min at 72°C. Nested PCR was carried out using 0.5 µl of the primary reaction as template following identical reaction conditions. Negative controls were incorporated in all PCR runs containing all the PCR reagents and double distilled water instead of template DNA. Genomic OsHV-1 DNA extracted from purified virions infecting *Crassostrea gigas* larvae (Le Deuff & Renault 1999) were used as positive control.

PCR inhibition controls. A competitive PCR method previously developed (Arzul et al. 2002, Renault et al. 2004) was used to detect inhibition during PCR reactions using the primer pair C2 (5'-CTC TTT ACC ATG AAG ATA CCC ACC-3')/C6 (5'-GTG CAC GGC TTA

CCA TTT TT-3'). These primers amplify an OsHV-1 DNA fragment of 710 bp as well as an internal standard competitor that differs from the target viral DNA by a 76 base pair deletion. One microliter of both extracted and competitor DNA was added to the reaction mixture, while the conditions for the PCR reaction were the same as described in the previous paragraph. For the internal standard control, 1 µl of double distilled water was added instead of extracted DNA.

Detection of oyster DNA. A 16S mitochondrial fragment (16SrDNA: the large subunit rRNA-coding gene) was amplified from the oyster larvae following the protocol of Boudry et al. (1998) using the primers described by Banks et al. (1993).

Electrophoresis of PCR products. All PCR products were visualized by electrophoresis in 1.5 % agarose gels containing ethidium bromide (0.5 mg l⁻¹) in 1X Tris-acetate-EDTA (TAE) buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). Molecular mass markers (100 bp DNA ladder, Promega) were included in each gel to determine the approximate size of the PCR products, and DNA bands were photographed under UV transillumination.

Microscopical examination. Larvae were fixed in 2.5 % glutaraldehyde in 0.2 M cacodylate buffer at pH 7.2 at 4°C. After 2 washes in 0.2 M cacodylate buffer, samples were then post-fixed in 1 % osmium tetroxide in the same buffer at 4°C. Larval samples were dehydrated by serial ethanol baths (70 to 100 %), cleared twice for 15 min in propylene oxide and infiltrated for 1 h in 50:50 propylene oxide:Epon resin. After 1 h infiltration in pure Epon resin, they were embedded in resin and cured for 48 h at 60°C. One micrometer sections were stained with 0.5 % toluidine blue in 1 % sodium borate solution for light microscopic examination.

RESULTS AND DISCUSSION

PCR products with the expected size were observed after the first (C5/C13) and second (C2/C4) reactions in one (15 d old *Crassostrea gigas* larvae) of the 8 analysed samples using both DNA extraction methods. Cells with enlarged nuclei of abnormal shape and chromatin pattern characteristic of herpes-like viral infections in mollusc bivalves (Hine et al. 1992, Renault et al. 1994) were observed in semi-thin sections in the 15 d old larvae in which the virus was detected by PCR. No nuclear abnormalities were observed in the 7 samples that did not yield PCR products. These results suggest that: (1) only 1 of the larval batches was infected with OsHV-1 and (2) that the DNA extraction method used was sensitive enough to allow the detection of OsHV-1 DNA from a small amount of larvae. This conclusion is further supported by the mortality

rate of 100 % (3 d after the detection of OsHV-1 using PCR) in the infected larval batch, while no subsequent abnormal mortality was observed (data not shown) in any other batch in which the virus was not detected by PCR. The absence of OsHV-1 detection in other batches does not necessarily demonstrate the complete absence of the virus, as low levels of viral DNA in larval tissues may be below the threshold of our methods.

Factors that inhibit the PCR reaction can interfere by nucleic acid degradation or capture and may inhibit polymerase activity for amplification of the target DNA (Wilson 1997). No inhibitory effect was detected in the samples obtained by both DNA extraction methods (Fig. 1). Amplification of the internal standard (634 bp) was observed in all samples in which the nested PCR did not detect the viral DNA. In the positive sample a band of expected size (710 bp) corresponding to viral DNA was observed, as well as a lighter band corresponding to the internal standard (634 bp) (Fig. 1, Lane 7).

False-negative results can also result from inadequate cell lysis and subsequently low levels of DNA for amplification (Wilson 1997). Extraction by boiling alone has been noted to reduce PCR sensitivity due to poor lysis efficiency and insufficient separation of DNA from structural proteins (Todd et al. 1992). Moreover, the high concentration of salt in fresh frozen larvae can inhibit cell lysis and negatively affect PCR. The combined use of Proteinase K/Tween-20 and larval wash with distilled water may thus improve cell lysis and hence increase the efficiency of PCR (Estoup et al.

1996). On the other hand, alternative methods of DNA extraction from *Crassostrea gigas* larvae including Chelex-100 and phenol/chloroform extractions occasionally yield false-negative results (Renault et al. 2000). We successfully amplified the oyster 16S fragments in all our samples (data not shown), hence supporting our extraction methods for good quality DNA.

The DNA extraction procedure described is simple and rapid, and allows the detection of OsHV-1 DNA from small amounts of oyster larvae (3 mg). Moreover, the number of required steps is low when compared to other extraction protocols, hence reducing the risk of sample contamination. This method can be useful for disease diagnosis and studies concerning the mechanisms involved in infections by OsHV-1 when only small quantities of larvae are available. Furthermore, this method may also be used for detection of the virus by PCR in individual larva since it was successfully used in extracting DNA from single *Crassostrea gigas* larva for microsatellite multiplexing (Taris unpubl. data). Therefore, this method can open new perspectives in studies concerning OsHV-1 transmission and putative resistance in early life stages.

Acknowledgements. We are grateful to Dr. P. Goulletquer for facilitating this work at the IFREMER station in La Tremblade (Charente Maritime, France). We also thank Dr. T. Sharbel for critical comments on the manuscript. This work was made possible by a FCT grant (SFRH/BD/8972/2002) and a Marie Curie Training fellowship (PLUDAMOR, QLK5-CT-2000-60036) to F.M.B.

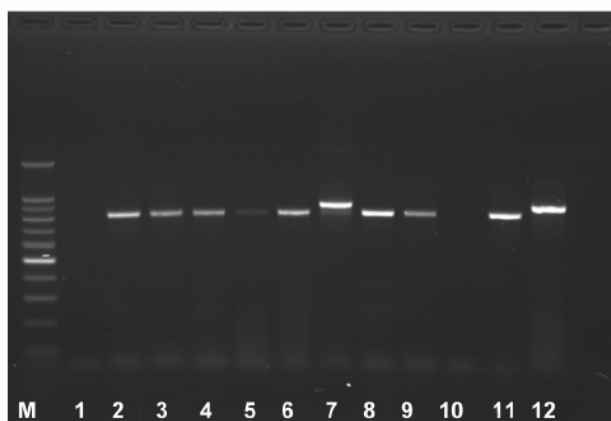


Fig. 1. Ostreid herpesvirus-1. Inhibition controls using a competitive PCR method. Lanes 1 and 10: negative controls; Lanes 2 and 6: 10 and 15 d old larvae (dol) from Tank 1, respectively; Lanes 3 and 7: 10 and 15 dol from Tank 2, respectively; Lanes 4 and 8: 10 and 15 dol from Tank 3, respectively; Lanes 5 and 9: 10 and 15 dol from Tank 4, respectively; Lane 11: internal standard; Lane 12: genomic viral DNA; Lane M: size markers (100 bp DNA ladder, Promega)

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Editorial responsibility: Albert Sparks,
Seattle, Washington, USA

Submitted: June 12, 2004; Accepted: September 22, 2004
Proofs received from author(s): February 25, 2005

6.2. TRIALS TO DETECT IRIDO-LIKE VIRUSES IN *CRASSOSTREA ANGULATA* AND *C. GIGAS* USING A MOLECULAR APPROACH

6.2.1. Background and objectives

From 1966 to 1973, mass mortality episodes almost led to the disappearance of *C. angulata* from Europe (Renault, 1996). During this period two distinct mortality events were described. Gill disease (“maladies des branchies”) was identified as the cause of the first mortality episode that started around 1966 in France (Marteil, 1969). This disease was later described in other European countries, namely in Great Britain (Alderman and Gras, 1969), Portugal (Ferreira and Dias, 1973) and Spain (Comps and Masso, 1978). In some years, gill disease affected 70-80 % of the population, with resultant mortalities reaching up to 40 % (Marteil, 1969). The first symptoms of the gill disease are the appearance of one or several yellow spots on the gills and labial palps (Alderman and Gras, 1969). In the gills, these spots increase in size and the tissues in the center die become brown and disintegrate resulting in larger and deeper ulcerations (Figure 35). In advanced stages of the disease, it may lead to total degeneration of the entire gills. Yellow or green pustules may also develop on the adductor muscle and mantle, and in the latter tissue perforations may occur as on the gills. The disease is characterized by tissue necrosis and massive hemocytic cellular infiltration around the lesions. Apparently some oysters managed to recover from the disease, showing cicatrisation of the old lesions (Comps, 1970). Although the gill disease was also observed in *C. gigas*, this taxon proved to be much more resistance to the disease than *C. angulata*. At present, *C. angulata* oysters showing gill lesions characteristic of gill disease persists in natural beds in Sado River, Portugal (F. Ruano, personal communication). Although different microorganisms were described associated with gill disease in different bivalve species, it is now generally assumed that the disease affecting *C. angulata* and *C. gigas* was caused by an irido-like virus that was called Gill Necrosis Virus (GNV). However, GNV was not isolated and experimental transmission studies need to demonstrate its pathogenicity were not undertaken (Comps, 1988). In oysters infected with GNV, giant polymorphic cells (ca. 30 µm in size) were observed with a hypertrophied nucleus (Comps, 1988). The nucleus of the infected cells has usually an oval shape and the chromatin disappear. The cytoplasm is also affected containing large inclusion bodies (5-15 µm), numerous vacuoles and finer grains (ca. 0.4 µm). Electron microscopy revealed that inclusions are viroplasm and the fine granules are virions with a icosahedral shape (Comps, 1988).

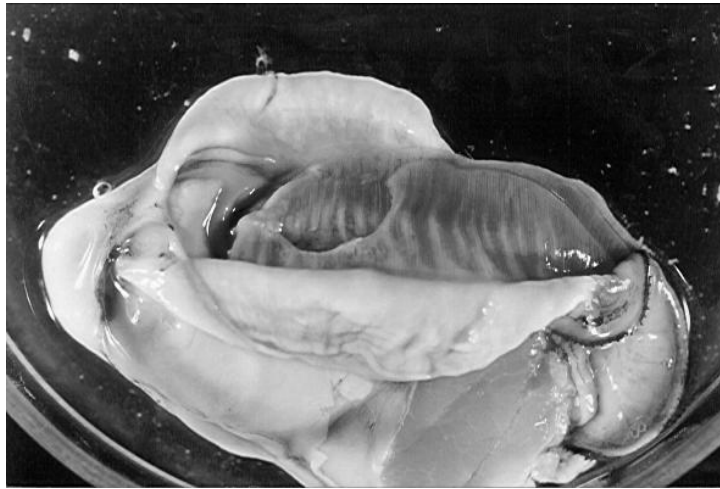


Figure 35. *Crassostrea angulata* specimen from Sado River (Portugal) showing advance indentation and necrosis on several gill filaments (photo provided by Dr. Francisco Ruano).

A second period of mass mortalities occurred between 1970 and 1973 in France. Although some oysters exhibit atrophy and weakness of the adductor muscle, no distinctive clinical signs were noticed. An irido-like virus was also associated with this mortality and was called Hemocyte Infection Virus (HIV). The most characteristic histological lesion of HIV infections is an acute inflammatory response associated with the presence of atypical hemocytes in the connective tissue and a increase number of brown cells. These atypical hemocytes showed pycnotic nuclei and intracytoplasmic inclusions bodies, often with attached groups of fine granules. Although GNV and HIV are very similar in terms of morphology and morphogenesis, they differ in their pathogenic effects as described above. Mature particles have around 380 nm in diameter and are icosahedral in shape. Morphogenesis takes place in the cytoplasm of oyster cells and Feulgen-positive staining reaction suggest the presence of DNA (Comps, 1988).

In 1977, a similar type of HIV infection was observed in *C. gigas* in the Bay of Arcachon (France) associated with 15 % mortality (Comps and Bonami, 1977). An irido-like virus similar to HIV was observed by the last time in *C. angulata* spat in France in 1984 (Bougrier *et al.*, 1986). Another irido-like virus was also described in *C. gigas* larvae reared in the United States of America associated with high mortalities and was named Oyster Velar Virus (OVV) (Elston and Wilkinson, 1985). OVV infections affected *C. gigas* larvae greater than 150 µm in shell height. Larvae exhibiting clinical lesions of the disease were less active than normal larvae and have a tendency to keep the visceral mass retracted. Virus infected cells of the velum become deciliated, detach, and form

characteristic “blisters”. Cytoplasmic inclusion bodies are observed in ciliated velar epithelium. Virus particles showed a mean diameter of 228 nm, which is considerably smaller than the diameter of GNV and HIV (Comps, 1988).

It must be emphasized that the pathogenicity of GNV and HIV was not demonstrated, although these viruses have been identified as the most probable cause of recurrent mass mortalities of *C. angulata* observed in Europe since 1966. If these irido-like viruses are interpreted as the causative agents of these mortalities, they must be considered the major threat to *C. angulata* production in Europe. Moreover, they will be among the most destructive known pathogens of bivalve molluscs.

Several characteristics of bivalve molluscs make the diagnosis of viral diseases difficult since they do not produce antibodies and, despite several attempts, mollusc cell lines are still not available. The grow-out of bivalve molluscs is generally done in open coastal zones, which seriously restrict the range of measures that can be implemented to fight and prevent the occurrence of infectious diseases. In this context, the development of nucleic acid-based techniques can be of great importance for a rapid diagnosis of viral diseases, which is not possible using traditional methods like light microscopy and transmission electron microscopy (TEM). Moreover, the sensitivity of these techniques (e.g., PCR) is very high allowing the detection of virus DNA even when present in low amount.

The objectives of the present work were: (1) to develop a method to extract DNA from archival formalin-fixed, paraffin-embedded oyster tissues; and (2) to detect irido-like virus DNA from archival and/or recent *C. angulata* samples.

6.2.2. Experimental procedure

Oyster samples

Four different type of samples were analysed:

- (1) A paraffin block containing tissues from an infected *C. angulata* specimen was kindly provided by Dr. Michel Comps from IFREMER. The tissues from this oyster specimen were fixed using Bouin's fixative and paraffin-embedded in the early 1970s. Light microscopy examination revealed cellular abnormalities associated with HIV (Figure 36). The presence of HIV was confirmed by TEM.
- (2) In addition, other *C. angulata* specimens from the same period conserved in a similar way were also obtained from the same source. These samples were analysed in order to confirm the repeatability of the DNA extraction method.

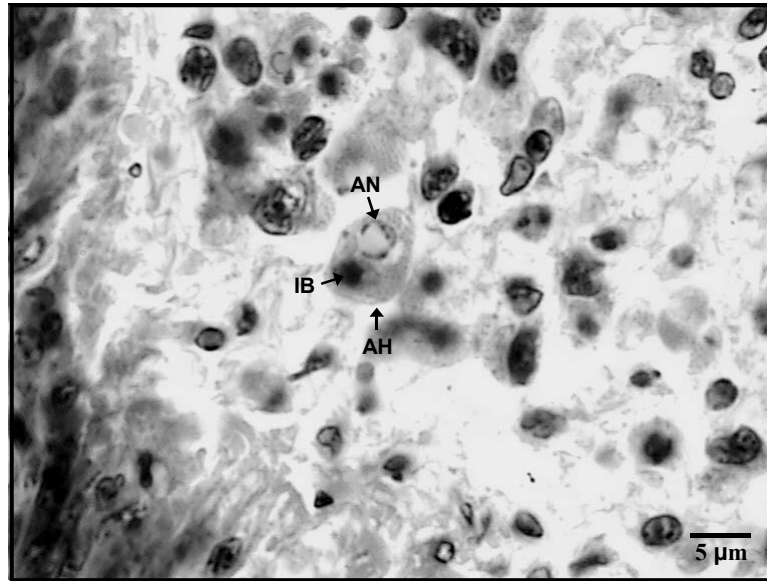


Figure 36. Atypical hemocyte (AH) of *C. angulata* infected with HIV showing an abnormal nucleus (AN) and an inclusion body (IB). Hemotoxylin and Eosin staining (block provided by Dr. Michel Comps from IFREMER).

(3) Recently fixed (less than 1 year) *C. angulata* (Ria Formosa, Portugal) specimens using Davidson's fixative for approximately 24 hours and subsequently transferred to 70 % ethanol and conserved at 4 °C before paraffin-inclusion was also analysed. These samples were used as a control since the conditions in which the tissues were conserved are considered suited for DNA conservation and subsequent PCR amplification.

(4) Samples consisting of recently (less than 1 year) ethanol preserved gill tissues from two *C. angulata* specimens (Ria Formosa, Portugal) showing lesions associated with GNV infections (figure 37) were also used. In addition, gills from three apparently healthy *C. angulata* specimens preserved in ethanol were also analysed. These five specimens had been previously analysed using conventional histologic procedures as described in Chapter 7.

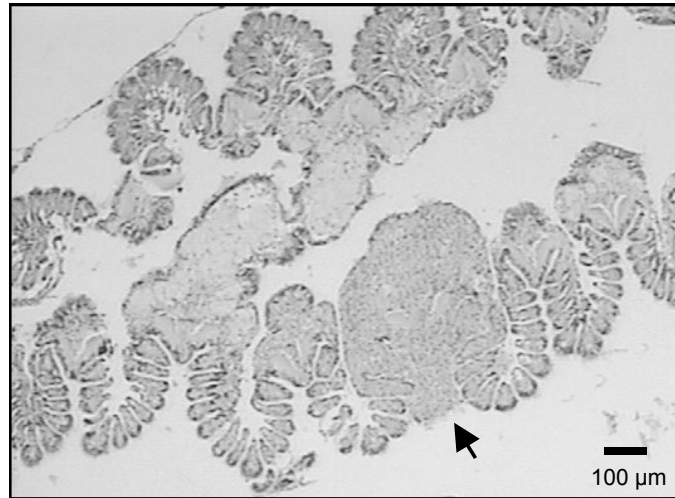


Figure 37. Histopathological changes in the gills of a *C. angulata* specimen showing a group of filaments completely destroyed and an intense infiltration around the lesion. Hemotoxylin and Eosin staining.

DNA extraction from paraffin-embedded samples

DNA was extracted from paraffin-embedded samples using two methods: (1) a protocol based on the use of a commercially available kit (DNA mini Kit, Qiagen) for fixed paraffin-embedded tissues; and (2) a DNA extraction procedure based on the methods described by Longy *et al.* (1997) and Shedlock *et al.* (1996), shown in detail in Table 21. The amount of DNA extracted was estimated using a photometer (Eppendorf BioPhotometer).

DNA extraction from ethanol preserved gill samples

DNA was extracted from ethanol preserved gill using a modification of a phenol/chloroform method described by Sambrook *et al.* (1989).

Amplification of oyster DNA, PCR-RFLP analysis and sequencing

The primer pair COI3/COI4 was used to amplify a 584 bp COI fragment as described in Section 2.2 of Chapter 2. Restriction enzyme analysis of PCR products was done using the enzyme *Mva*I as described in Section 5.2 of Chapter 5. In addition, sequencing was performed directly on the PCR products and single nucleotide polymorphism identified as described in Section 2.2 of Chapter 2.

Table 21. DNA extraction from archival oyster tissues fixed using Bouin's fixative and paraffin-embedded.

Step	Procedure
1. Deparaffinization	1.1. Cut five sections of 15 µm and put in a 2 ml microtube (3 microtubes per paraffin block) 1.2. Add 1 ml of xylene to each microtube and incubate for 55 °C for 15 min 1.3. Centrifuge at 20 000 x g for 10 min 1.4. Discard the supernatant and repeat steps 1.2 and 1.3 1.5. Discard the supernatant and add 1 ml of 1:1 xylene:absolute ethanol 1.6. Vortex each microtube for 10 sec 1.7. Centrifuge at 20 000 x g for 10 min 1.8. Repeat steps 1.5, 1.6 and 1.7 1.9. Discard the supernatant and add 1 ml of absolute ethanol 1.10. Vortex each tube for 10 sec 1.11. Centrifuge at 20 000 x g for 10 min 1.12. Discard the supernatant and dry the pellet at 55 °C for approximately 10 min
2. Decolourisation	2.1. Add 1 ml of an aqueous solution of 27 mM lithium carbonate and incubate at room temperature for 30 min 2.2. Centrifuge at 20 000 x g for 10 min and discard the supernatant
3. Washing	3.1. Transfer the pellet of the 3 microtubes (of the same paraffin block) to a 15 ml tube and add 10 ml of a fresh solution of 1XGTE (100 mM glycine; 10 mM Tris-HCl, pH 8; 1 mM EDTA) 3.2. Put the tube in a rotary shaker on low rpm for 24 hours at room temperature 3.3. Centrifuge at maximum speed and discard the supernatant 3.4. Add 10 ml of a fresh solution of 1XGTE 3.5. Put the tube in a rotary shaker on low rpm for 24 hours at room temperature 3.5. Repeat steps 3.3., 3.4 and 3.5 3.6. Centrifuge at maximum speed and discard the supernatant
4. Digestion	4.1. Transfer the tissues to a 2 ml microtube 4.2. Centrifuge briefly and remove the excess of the solution with a micropipette 4.3. Allow the pellet to dry at room temperature 4.4. Add 500 µl of extraction buffer (1 % SDS; 25 mM TrisHCl, pH 7.5; 100 mM EDTA), 20 µl of 1 M DTT (dissolve 3.09 g of dithiothreitol in 20 ml of 0.01 M sodium acetate (pH 5.2) and sterilize by filtration. Dispense into 1 ml aliquots and store at -20°C) and 100 µl of proteinase K (10 mg/ml) 4.5. Incubate at 65 °C for 8 hours 4.6. Add more 50 µl of proteinase K (10 mg/ml) and 10 µl of Dnase-free Rnase (10 mg/ml) and incubate at 65 °C for more 16 hours

Table 21 (continuation) DNA extraction from archival oysters tissues fixed using Bouin's fixative and paraffin-embedded.

Step	Procedure
5. Extraction	5.1. Add 500 µl of 25:24:1 solution of phenol:chloroform:isoamyl alcohol and mix gently by inversion 5.2. Centrifuge at 14 000 x g for 10 min at room temperature 5.3. Transfer the supernatant to new 2 ml microtube 5.4. Repeat steps 5.1, 5.2 and 5.3 5.5. Add 500 µl of 24:1 solution of chloroform:isoamyl alcohol and mix gently by inversion 5.6. Centrifuge at 14 000 x g for 10 min at room temperature 5.7. Transfer the supernatant to new 2 ml microtube 5.8. Repeat steps 5.5, 5.6 and 5.7
6. Purification	6.1. Mix the supernatant with the resin "Wizard DNA clean-up system" (PROMEGA) by inversion 6.2. Attach syringe barrel to minicolumn and pipet the resin/DNA into syringe barrel 6.3. Insert syringe plunger and push slurry into minicolumn 6.4. Wash three times with 2 ml of 80 % isopropanol using the syringe 6.5. Centrifuge at 20 000 x g for 2 min to dry the minicolumn 6.6. Transfer the minicolumn to a new microtube and apply 50 µl of pre-warmed water (65 – 70 °C) and wait 10 min 6.7. Centrifuge the minicolumn for 1 min at 20 000 x g

Amplification of irido-like virus DNA

Due to the lack of information concerning the genome of irido-like viruses infecting bivalve molluscs, two sets of primers (IR1/IR2 and IR3/IR6) previously designed to amplify DNA by PCR from conserved areas of the major capsid protein of iridovirus infecting insects and amphibians (T. Renault, personal communication) were used (Table 22). The primer pair IR1/IR2 was designed to amplify a fragment with 143 bp and the primer pair IR3/IR6 a fragment with 199 bp.

Table 22. Specific oligonucleotides used to amplify iridovirus DNA.

Name	F/R	Sequence
IR1	F	5' - AGAACGTCACACACCCTTCC - 3'
IR2	R	5' - GAGCCTTGTGGTGTTCGT - 3'
IR3	F	5' - CAAACCAGCTGGGAGACAAT - 3'
IR6	R	5' - AGATCGCTGGTGTTCCTAT - 3'

F, forward primers; R, reverse primers

PCR reactions were performed in a 50 µl volume composed of 5 µl of 10X PCR buffer, 35.7 µl of distilled water, 5 µl of dNTP (2 mM), 1 µl of each primer (20 µM), 0.3 µl of Taq polymerase (5 U/ µl) and 2 µl of template DNA solution. Touchdown (TD) PCR was used since this technique compensates for suboptimal reagent concentrations as well as putative mismatches among primers and template (Hecker and Roux, 1996). This approach relies on incremental annealing temperature decreases in progressive cycles. The first primer-template hybridizations and consequently amplifications are those with the highest specificity. Even if the annealing temperature drops to levels that could lead to spurious amplification, the target fragment will have begun its geometric amplification. Therefore, the target fragment is in a position to out compete any nonspecific product. Hence, TD PCR can increase the yield and specificity of the amplification of the target fragment. Cycling conditions consisted of:

- 1 cycle of 2 min at 94 °C
- 2 cycles of 30 s at 94 °C, 30 s at 58 °C, and 30 s at 72 °C
- 2 cycles of 30 s at 94 °C, 30 s at 56 °C, and 30 s at 72 °C
- 2 cycles of 30 s at 94 °C, 30 s at 54 °C, and 30 s at 72 °C
- 2 cycles of 30 s at 94 °C, 30 s at 52 °C, and 30 s at 72 °C
- 2 cycles of 30 s at 94 °C, 30 s at 50 °C, and 30 s at 72 °C
- 30 cycles of 30 s at 94 °C, 30 s at 48 °C, and 30 s at 72 °C
- 1 last cycle 7 min at 72 °C

The positive control consisted of Frog virus 3 (FV3) DNA that was kindly provided by Dr. Sandra Essbauer (Munich University, Germany). The PCR products were analysed on 2 % agarose gels stained with ethidium bromide and visualized using a 302-nm UV transilluminator.

6.2.3. Results and discussion

DNA extraction from archival samples

A large number of bivalve samples have been routinely fixed and paraffin-embedded for histological examination. Extraction of nucleic acids from these types of samples can allow retrospective analyses such as detection of infectious agents (e.g., Barbosa-Solomieu *et al.*, 2004). PCR can be used to amplify DNA even if only a low number of target molecules is present. However, extraction of nucleic acids from fixed material typically yields low quantity of DNA that is generally degraded (Coates *et al.*, 1991). The type of fixative used can also influence the success of the DNA extraction procedure (Jackson *et al.*, 1990). Among fixatives, Bouin's fluid is considered one of the less suited to preserve the integrity of DNA (Longy *et al.*, 1997).

No DNA was observed by spectrophotometry when the commercial kit was used to extract DNA from all archival samples. However, DNA was successfully extracted using the commercial kit from oysters tissues fixed in Davidson's fixative and paraffin-embedded. These results confirm the difficulty in extracting DNA from archival Bouin's fixed paraffin-embedded samples. However, the use of the new extraction method that combines the protocols described by Longy *et al.* (1997) and Shedlock *et al.* (1996) allowed DNA extraction from the archival samples (from all different types of sample). The extraction yield was approximately 4 times lower for the archival samples comparatively to the tissues fixed in Davidson's fixative and paraffin-embedded (control). Nevertheless, these results suggest that DNA can be successfully extracted from archival Bouin's fixed paraffin-embedded samples. Other factors besides the type fixative used may have also influenced the DNA extraction yield namely, the age of the samples, fixation time and suboptimal storage conditions of the archival samples.

The 584 bp COI fragment was successfully amplified from all archival samples using DNA extracted using the new extraction method (Figure 38). The results of the present study suggest that considerably large fragments (584 bp) can be amplified from archival samples fixed in Bouin's fixative. This is generally not the case for this type of material due to degradation of the DNA and extensive cross-linking with surrounding proteins (Longy *et al.*, 1997; Barbosa-Solomieu *et al.*, 2004).

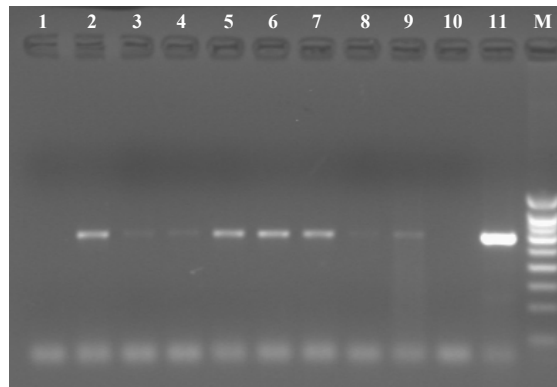


Figure 38. Amplification of an oyster 584 bp COI fragment by PCR. Lanes 1 and 10, negative controls. Lanes, 2, 3, 4, 5, 6 and 7, DNA extracted from archival Bouin-fixed paraffin-embedded tissue samples. Lanes, 8 and 9, DNA extracted from recent Davidson-fixed paraffin-embedded tissue samples. Lane 11, positive control (*C. angulata* DNA). Lane M, size markers (100 bp ladder, Promega).

Surprisingly, restriction enzyme analysis of all PCR products revealed that all specimens analysed (archival samples presumed to be *C. angulata*) carried *C. gigas* mtDNA (Figure 39), which suggest that they were *C. gigas* or hybrids and not *C. angulata*.

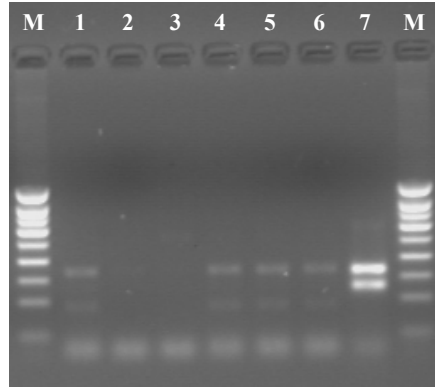


Figure 39. PCR-RFLP analysis of the COI gene fragments from archival samples digested with *Mva*I. Lane 1, 2, 3, 4 and 5, samples in which HIV was not observed. Lane 6, sample in which HIV was observed by TEM. Lane 7, control (amplification products obtained from *C. angulata* DNA).

In order to confirm that the COI fragments amplified from the archival samples were from *C. gigas*, part of the fragment amplified from 3 individuals were partially sequenced. The haplotype sequence confirmed the PCR-RFLP, indicating the oysters from the archival samples carried *C. gigas* mtDNA (Table 23).

Table 23. Nucleotide polymorphisms of a COI fragment (353bp) sequenced from archival Bouin-fixed paraffin-embedded oyster samples. Numbers above columns refer to polymorphism position (bp) relatively to *C. angulata* haplotype S1. The polymorphic sites in grey are diagnostic for *C. angulata* and *C. gigas* haplotypes.

	17	20	29	37	65	113	116	160	200	223	248	320	336	353
Haplotype S1	G	T	C	A	A	C	G	A	A	T	C	C	T	C
B-Positive	A	.	T	.	.	T	A	T	.	.	T	T	C	T
CM15	A	C	T	G	G	T	A	T	.	C	T	T	C	T
CM40	A	.	T	.	.	T	A	T	G	.	T	T	C	T

B-positive, archival sample in which HIV was observed by TEM; CM15 and CM40, archival samples from the same period and fixed using a similar procedure as for B-positive sample.

This indicate that specimens were misclassified as *C. angulata* or that samples were incorrectly identified, highlighting the importance of exact identification of host when making a diagnosis of pathogens. In addition, these results confirmed that the individual infected with HIV was in fact *C. gigas* or a hybrid. Indeed, Comps and Bonami (1977) reported the presence of an irido-like virus in *C. gigas* similar to HIV previously described in *C. angulata* in 1970-1973. Furthermore, two of the *C. gigas* haplotypes (CM15 and CM40; see table 23) observed had never been described before. This may have been due to selection acting directly on mtDNA genes or merely by neutral-drift stochasticity since the introduction of *C. gigas* in Europe. It is also possible that the new haplotypes observed are artifacts that have resulted from the fixative used. Indeed, Longy *et al.* (1997) reported similar results and suggested caution in interpreting data obtained from material fixed with Bouin's fixative and stored under non optimum conditions. For haploype CM40, a single transition was observed in the third position of the codon resulting in the same amino-acid. For haplotype CM15, four transitions were observed, but two of them occurred in the second position of the codon resulting in amino-acid changes. The analysis of the type of mutations and consequent changes in the amino-acid sequence support the first explanation of selection acting directly on mtDNA genes assuming that the haplotype CM15 is not an artifact. However, the stochastic hypothesis cannot be discarded based only on these findings.

Detection of iridolike-virus DNA

No amplicons were obtained using the primer pairs IR1/IR2 and IR3/IR6 using the DNA extracted from the archival sample known to be infected with HIV neither from the gill of the individuals recently collected showing gill lesions (Figure 40). A 584 bp COI fragment was amplified from the ethanol preserved gill samples, indicating that DNA was successfully extracted such as previously reported for the archival samples. For the archival sample known to be infected with HIV, the non amplification of viral DNA may have been due to low yield of the DNA extraction method and not due to inadequacy of primer pairs used and/or PCR conditions. Another possible explanation is that although the new method extraction method is suitable for oysters DNA the same may not be true for viral DNA. It also possible that the primer pairs used were not suitable for amplifying DNA from the irido-like virus present in the archival sample. For oysters recently collected showing gill lesions, although the lesions observed are generally associated with gill disease, no cytopathological changes were observed characteristic of GNV infections. Similar gill lesions have also been reported by other authors in *C. gigas* that observed giant polymorphic cells associated with GNV infections, but no virus particles were observed by TEM (Cáceres-Martínez and Vásquez-Yeomans, 2003).

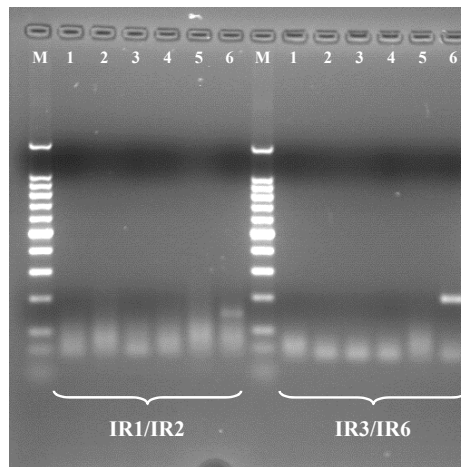


Figure 40. Amplification of iridovirus DNA using the primer pairs IR1/IR2 and IR3/IR6 by PCR. Lanes 1 and 5, negative controls. Lanes, 2, archival Bouin-fixed paraffin-embedded tissue sample in which HIV was observed. Lane 3 and 4, ethanol preserved gill samples from oysters showing gill lesions. Lane 6, positive control (FV3 DNA). Lanes M, size markers (100 bp ladder, Promega).

6.3. CONCLUSIONS

The method developed to extract DNA from small sample size (3 mg) allowed not only the detection of oyster mtDNA but also OshV-1 DNA. In addition, no inhibitory effect of PCR was observed in all samples analysed. Hence, this method can be useful for detection of OshV-1 when only small amounts of larvae are available. A similar extraction method with minor modifications was successfully used to extract DNA from a single larva (Taris *et al.*, 2006). This suggests that the method developed in the present study can be used to detect OshV-1 DNA in a single larva, which can open new perspective for epidemiological studies concerning this virus.

Oyster mtDNA suitable for amplification by PCR was extracted from archival Bouin-fixed paraffin-embedded samples using another method developed in the present study. However, no iridovirus DNA was detected by PCR from archival samples known to be infected by HIV as well as from recent samples of *C. angulata* individuals showing gill lesions. However, the DNA extraction method from archival samples may allow further retrospective analyses using other primer pairs that can be more specific and sensitive to detect irido-like virus DNA. Indeed, in a recent study iridovirus DNA was successfully detected by PCR and in situ hybridization from the mollusc *Nautilus* sp. (Gregory *et al.*, 2006). The sequence data reported by the previous authors as well as the experimental approach (*e.g.*, primer pairs, PCR conditions) can be useful to study oysters infected by irido-like viruses. In addition, the ability to extract DNA suitable for PCR from archival Bouin-fixed paraffin-embedded samples provides an additional tool for retrospective studies concerning other pathogens as well as population genetics of oysters.

CHAPTER 7. PATHOGENS, SYMBIONTS AND PATHOLOGICAL CONDITION OF *CRASSOSTREA ANGULATA*, *C. GIGAS* AND THEIR RECIPROCAL HYBRIDS

7.1. AN OUTBREAK OF OSTREID HERPESVIRUS 1 DURING LARVAL REARING OF *CRASSOSTREA ANGULATA*, *C. GIGAS* AND THEIR RECIPROCAL HYBRIDS

7.1.1. Background and objectives

Herpes and herpes-like virus infections associated with high mortality rates have been reported around the world in hatchery-reared larvae from various bivalve species, including *Crassostrea gigas* (Nicolas *et al.*, 1992; Hine *et al.*, 1992; Renault *et al.*, 1994a), *Tiostrea chilensis* (Hine *et al.*, 1998), *Ostrea edulis* (Renault *et al.*, 2000b), *Ruditapes philipinarum* (Renault *et al.*, 2001), *R. decussatus* (Renault and Arzul, 2001) and *Pecten maximus* (Arzul *et al.*, 2001b). The pathogenicity of Ostreid herpesvirus 1 (OsHV-1) for *C. gigas* larvae was demonstrated by experimental transmission of the virus to apparently healthy larvae (Le Deuff *et al.*, 1994; Arzul *et al.*, 2001a). In these assays, *C. gigas* axenic larvae were exposed to a viral suspension obtained from infected larvae, and moribund larvae were observed few days after exposure. In addition, interspecific transmission of OsHV-1 was also reported using viral suspensions and throughout cohabitations assays (Arzul *et al.*, 2001a). These results, strongly suggest that OsHV-1 can be transmitted horizontally from infected larvae to healthy larvae within and between different species. Based on the importance of parental origins on herpes-like infections in larvae, a possible vertical transmission of the virus was suggested (Le Deuff *et al.*, 1996). The detection of OsHV-1 in asymptomatic *C. gigas* adults using molecular and immunological techniques suggested that adults may play the role of carriers and reservoirs of the virus (Arzul *et al.*, 2002). More recently, Barbosa-Solomieu *et al.* (2005) detected OsHV-1 DNA in two-day old larvae, indicating that infection may take place at very early stages. These results support the hypothesis of a vertical transmission of OsHV-1. Moreover, it was also reported that the infective status of the parents appeared to have an influence on the presence of OsHV-1 DNA and survival rates of the progeny (Barbosa-Solomieu *et al.*, 2005). However, Barbosa-Solomieu *et al.* (2005) also reported that for some families OsHV-1 was detected in larvae but not in their parents, and infected parents do not always produce infected larvae.

The susceptibility of *C. angulata* larvae to OsHV-1 infections have been demonstrated throughout transmission assays using a viral suspension obtained from infected *C. gigas* larvae (Arzul *et al.*, 2001a). Setting of *C. angulata* larvae occurred 6 days after exposure to the viral suspension. Transmission electron microscopy

examination revealed herpes-like viral particles and viral DNA was detected by PCR (Arzul *et al.*, 2001a). Recurrent high mortalities of hatchery reared *C. angulata* larvae have been observed (unpublished data). However, it remains unknown to what extent herpesvirus infections are associated with these mortalities. Moreover, the susceptibility of *C. angulata* x *C. gigas* hybrid larvae to herpes-virus is also not known.

The main objective of the present study was to determine if mortalities observed in hatchery-reared larvae of *C. angulata*, *C. gigas* and their reciprocal hybrids at different development stages were associated with the detection of OsVH-1 DNA. In order to determine the putative origin of the virus and its transmission mode, assays were also conducted to detect viral DNA in the broodstock.

7.1.2. Experimental procedure

Crosses, larval rearing and sampling

Crassostrea gigas broodstock (18 animals) originated from Seudre river (Charente Maritie, France) and *C. angulata* broodstock (20 animals) from Sado river (Portugal). Gametes were recovered by strip spawning and fertilization was performed as described by Stephano and Gould (1988). After collecting gametes, a longitudinal section through the body passing through the digestive gland, gonads, mantle, gills and adductor muscle was fixed in Davidson's fixative and later transferred to 70 % ethanol, and stored at 4 °C. In addition, gill samples from all parents were collected and preserved in absolute ethanol. Experimental crosses were made to produce the following groups: *C. angulata* (AA); *C. gigas* (GG), hybrids derived from *C. angulata* females and *C. gigas* males (AG); and hybrids derived from *C. gigas* females and *C. angulata* males (GA) (see Chapter 3). Larvae were reared in 150 l tanks (in duplicate) as described in Section 3.1 of Chapter 3. Every 48 or 72 h, larvae were collected by sieving and their density estimated by counting larvae in four samples per tank. Samples of larvae were collected from the same tank (one tank per group) at 1, 10 and 15 days after fertilization (DAF) for pathological analyses (AA2, AG6, GA1 and GG4 tanks). After these sampling periods, larval samples were also collected whenever abnormal high mortality rates were observed. The other replicate tanks (AA5, AG6, GA3 and GG7) were used to estimate survival for the entire larval period since no sampling was made in these tanks unless when 100 % mortality was observed. Larvae samples were fresh frozen at – 80 °C.

DNA extraction

DNA was extracted from larvae using the procedure described by Renault *et al.*, (2000a). Fifty milligrams of fresh frozen larvae were weight out in a 2 ml Eppendorf tube and ground in 50 µl of distilled water with a single-use tissue homogeniser. Samples were

mixed using a vortex and denatured in a boiling water bath for 10 min, followed by quick chilling in ice. Samples were mixed again and centrifuged at 10 000 rpm for 5 min. Supernatant were recovered and diluted (1:10) in double distilled water and frozen at – 20 °C.

For the broodstock, two methods were used to extract DNA from tissues previously fixed in Davidson's fixative and preserved in 70 % ethanol, namely: (1) a DNA extraction procedure previously described by Barbosa-Solomieu *et al.* (2005); and (2) a variation of this procedure using a phenol/chloroform extraction. In the first DNA extraction procedure, sections were cut by hand and put into 2 ml Eppendorf tubes. One millilitre of absolute ethanol was added to each tube prior to vortexing and horizontally agitated for 30 min. The supernatant was discarded and the previous procedure repeated twice. The supernatant was discarded and pellets were then dried for 60 min at 55 °C prior to overnight digestion with 200 µg/ml of proteinase K in 0.5 ml of 50 mM Tris, 1mM EDTA, 0.5 % Tween 20 buffer at 55 °C. Samples were then centrifuged at 10 000 rpm for 1 min before being denatured at 95 °C in a water bath for 10 min. Finally, samples were centrifuged at 12 000 rpm for 15 min and supernatants were recovered, and frozen at – 20 °C. In the second extraction method, the same procedure was carried out as previously described, but prior to overnight digestion with proteinase K the tissues were grounded. After overnight digestion, DNA was extracted from gill tissue using a modification of a phenol/chloroform method described by Sambrook *et al.* (1989). DNA was also extracted from ethanol-preserved gill fragments of *C. angulata* and *C. gigas* broodstock using a modification of a phenol/chloroform method as described above. For these samples, the amount of DNA extracted was estimated using a photometer (Eppendorf BioPhotometer).

PCR analysis

The experimental procedure scheme and primer pairs used to detect OsHV-1 DNA in larval samples are shown in Figure 2 of Article 5. For the broodstock samples, the experimental procedure scheme and primer pairs used to detect OsHV-1 DNA are shown in Figure 41. The sequence of the primers used is shown in Table 2 of Article 5. For the samples fixed in Davidson's fixative, a competitive PCR method using an internal standard (PCR-IS) was used to detect inhibition during PCR reactions (Renault *et al.*, 2004). For the ethanol-preserved gill samples, the COI3/COI4 primer pair was used to amplify a 584 bp COI fragment from oyster mtDNA, as described in Section 2.2 of Chapter 2. This was done to assess the quality of the DNA extracted and the presence of inhibitors of PCR.

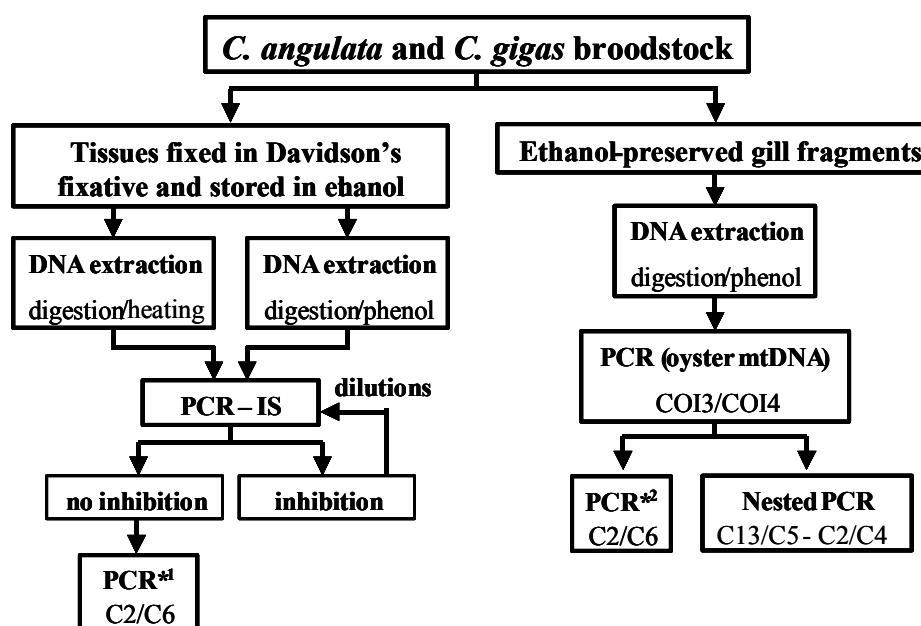


Figure 41. Experimental procedure scheme used for the detection of OshV-1 DNA in *C. angulata* and *C. gigas* broodstock samples. *¹, using an annealing temperature of 50 °C; *², using an annealing temperature of 60 °C; IS, internal standard.

PCR reactions were conducted in 50 µl volumes composed of 5 µl of 10X appropriate reaction buffer, 31.5 µl of distilled water, 5 µl of dNTP (5 mM), 5 µl MgCl₂ (25 mM) 1 µl of each primer (100 µg/ml), 0.5 µl of Taq polymerase (5 U/ µl, Goldstar, Eurogentec, Belgium) and 1 µl of template DNA (Renault *et al.*, 2000a). PCR reactions were performed for one cycle of 2 min at 94 °C and 35 cycles of 1 min at 94 °C, 1 min at 50 °C, and 1 min at 72 °C with a least extension of 5 min at 72 °C. For nested PCR, the reaction conditions of the second reaction were the same as for the first reaction (previously described), except that 0.5 µl of the first PCR reaction products were used as a template. Negative controls consisting of distilled water were included during each PCR experiment. Reference OshV-1 DNA (100µg/µl) extracted from purified virus particles constituted the positive control. Ten microliters of each PCR products were size-selected on 1.5 % agarose gels stained with ethidium bromide and visualized using a 302-nm UV transilluminator. Due to the high sensitivity of PCR special precautions were adopted to avoid contamination, namely: (1) separated working areas were used for sample preparation, preparation of amplification mixture components, and PCR amplification; (2) all pipetting procedures were performed using filter tips; (3) preparation of amplification mixture components was carried out in a hood equipped with UV lights; and (4)

disposable gloves were frequently changed and gloves changed always before entering a DNA/PCR-products free area.

Optimization of OsHV-1 DNA amplification

In order to increase the specificity of PCR using the C2/C6 primer pair, an annealing temperature of 60 °C was tested instead of 50 °C as previously described by Renault and Arzul (2001). The limit of detection, which correspond to the last dilution of viral DNA allowing detection of expected amplicons, was determined using triplicate reactions. PCR reactions were performed using dilutions of genomic viral DNA (100 pg/μl to 0.1 fg/μl) as template. The PCR conditions were the same as described by Renault *et al.* (2000a) except for the annealing temperature. PCR assays with or without addition of non-infected *C. gigas* DNA (ca. 500 ng) were performed.

. The limit of detection was also defined for the C13/C5 and C2/C4 primer pairs using a annealing temperature of 60 °C as described above, but only without adding non-infected *C. gigas* DNA. The specificity of the nested PCR with an annealing temperature of 60 °C was assessed using oyster DNA from different individuals.

Cloning and sequencing

Selected C2-C4 and Gp17-Gp18 PCR products obtained from DNA extracted from larvae (from the four groups) were inserted into pCR®II-TOPO® vector (Invitrogen, USA) and resulting plasmids were transformed into *Escherichia coli* competent cells. Selected clones were sequenced using primers from the vector. Sequencing was performed using BigDye (Applied Biosystems) chemistry and an automatic sequencer (ABI-PRISM, model 377, Applied Biosystems). Sequences analysis was performed using Sequencing Analysis 5.1 (Applied Biosystems) and single nucleotide polymorphism identified using SeqScape 2.1 (Applied Biosystems).

7.1.3. Results and discussion

Optimization of OsHV-1 DNA amplification

The use of an annealing temperature of 60 °C with C2/C6 primer pair allowed systematically detection of 10 fg of viral DNA. A similar limit of detection was observed with or without the addition of 500 ng of DNA from non-infected *C. gigas* oyster. Renault *et al.* (2004) reported a lower detection threshold (1 fg) using the same primer pair with an annealing temperature of 50 °C. Moreover, the previous authors did not observe extraneous bands when genomic DNA from *C. gigas* oyster was used as template. This suggests that the extraneous PCR products observed in the present study may have been due to the amplification of DNA from organisms present in gills of the oysters analysed

and not from the oyster DNA. Although the use of an annealing temperature of 60 °C apparently reduced the PCR sensitivity comparatively to an annealing temperature of 50 °C, a higher specificity was apparently gained.

The nested PCR using C13/C5 and C2/C4 primer pairs yielded amplicons with the expected size when OsHV-1 DNA was used, but extraneous bands were also observed. These bands presented a higher size than the expected amplicons, which may correspond to PCR amplicons from the first PCR reaction or non-specific amplification. The minimum template quantity needed to detect amplicons using C13/C5 and C2/C4 primer pairs was 1 fg in 2 out of 3 assays when only viral DNA was used (Figure 42). Renault *et al.* (2004) observed that C13/C5 primer pair failed to produce detectable amplicons when 500 fg of OsHV-1 DNA were used as template. Hence, a considerably higher sensitivity was obtained by nested PCR using C13/C5 and C2/C4 primer pairs as expected. No extraneous bands were observed (with an annealing temperature of 60 °C) when oyster DNA was used as template for the nested PCR (data not shown).

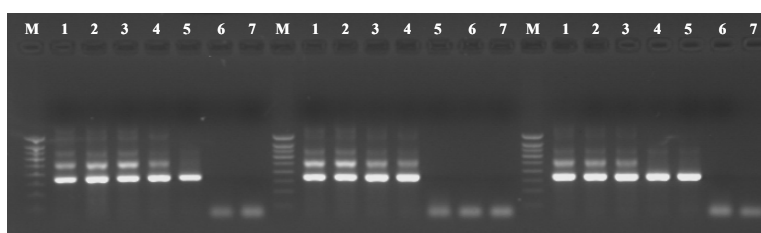


Figure 42. Limit of detection of OsHV-1 DNA by nested PCR using C13/C5 and C2/C4 primer pairs (triplicate reactions). Results were obtained on a serial dilution (1:10) of purified viral DNA. Lane 1, 10 pg of viral DNA; Lane 2, 1 pg of viral DNA; Lane 3, 100 fg of viral DNA; Lane 4, 10 fg of viral DNA; Lane 5, 1 fg of viral DNA; Lane 6, 0.1 fg of viral DNA; Lane 7, negative control; Lane M, size markers (100 bp DNA ladder, Promega).

Larval mortality and OsHV-1 detection by PCR

OsHV-1 DNA was detected by PCR in larvae of *C. angulata* (AA), *C. gigas* (GG) and in their hybrids (AG and GA) at different development stages (from 15 to 29 days after hatching). Almost all primer pairs used to detect OsHV-1 gave similar PCR results (Figure 43). However, ambiguous results were obtained using some primer pairs, namely C13/C5, B3/B4 and C9/C10. PCR products approximately 200 bp smaller than the amplicons of the expected size for OsHV-1 DNA were obtained with C13/C5 primer pair (Figure 44). Based on their size, these amplicons could be interpreted as the amplification of a variant of OsHV-1 (OsHV-1var; Arzul *et al.*, 2001b, c). However, when PCR analyses were performed using other primer pairs (i.e., A3/A4, B3/B4 and Gp3/Gp4), that have been

successfully used to amplify OsHV-1var DNA (Arzul *et al.*, 2001b, c), no amplicons were observed.

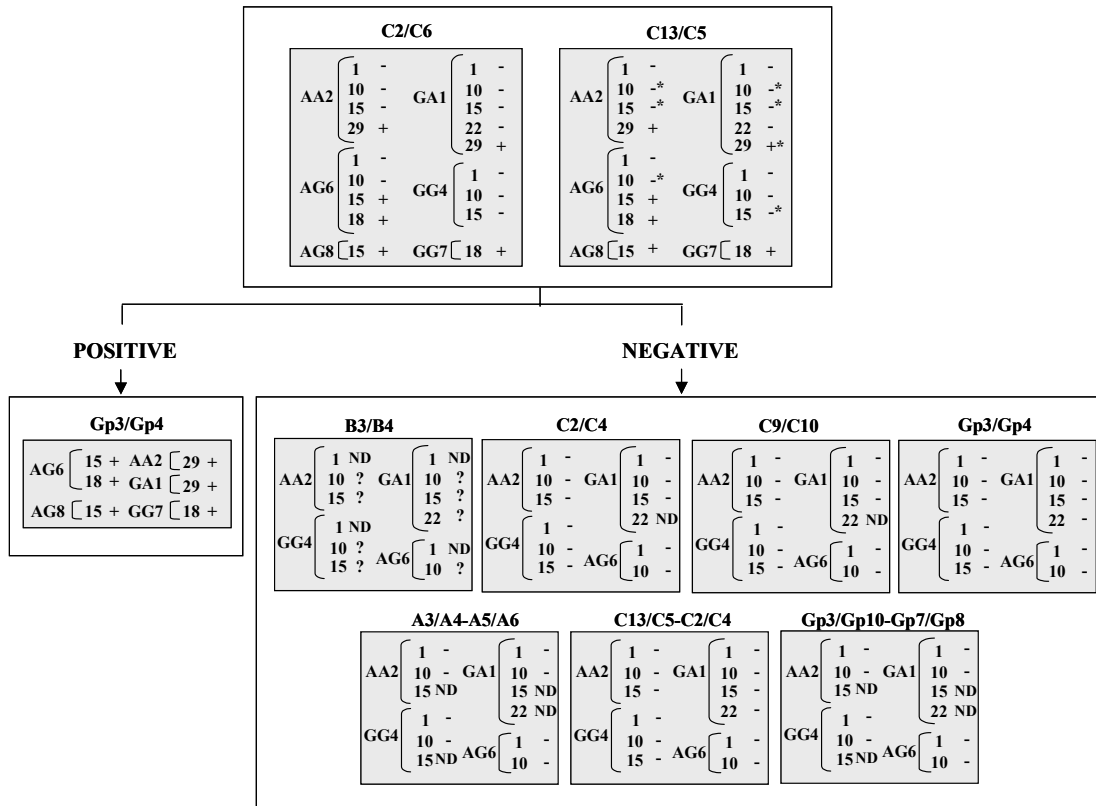


Figure 43. Detection of OsHV-1 DNA by PCR in larvae of *C. angulata* (AA), *C. gigas* (GG), hybrids derived from *C. angulata* females and *C. gigas* males (AG), and hybrids derived from *C. gigas* females and *C. angulata* males (GA) at different days after hatching (after the bracket). The number of the tank is indicated in front of the group code. +, positive PCR result; -, negative PCR result; ?, doubtful result; ND, sample not analyzed; *, extraneous bands observed.

For C9/C10 primer pair, no amplification artefacts were reported when DNA extracted from larvae using the method of Renault *et al.* (2000a) was used. However, when DNA was extracted from the same samples using the method described in Section 6.2 of Chapter 6, a considerably high number of extraneous bands were observed. Some of these amplicons had a similar size to the amplicons expected for OsHV-1 (data not shown).

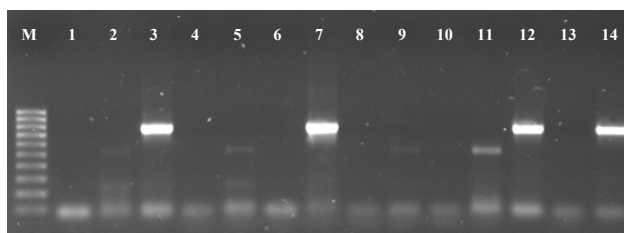


Figure 44. OsHV-1 DNA detection by PCR using C13/C5 primer pair. Lanes 1, 6 and 13, negative controls; Lanes 3, 7 and 12, larval samples that yield bands with the expected size for OsHV-1; Lanes 2, 5, 9 and 11, larval samples that yield bands with the expected size for OsHV-1var; Lanes 4, 8 and 10, larval samples in which no amplicons were observed. Lane 14, positive control (OsHV-1 DNA); Lane M, size markers (100 bp DNA ladder, Promega).

PCR products slightly smaller than the expected size for OsHV-1 DNA were obtained with B3/B4 primer pair for some larval samples. However, the presence of viral DNA in these samples was not confirmed by PCR using other primer pairs. For all the other primer pairs used amplicons with the expected size were obtained (*e.g.*, Gp3/Gp4 primer pair; Figure 45).

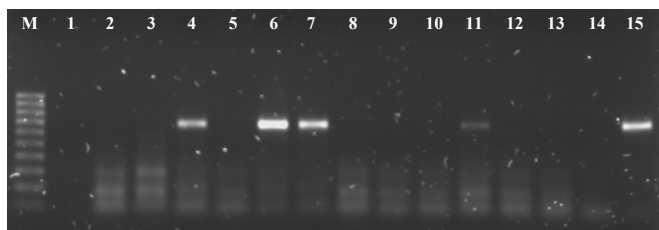
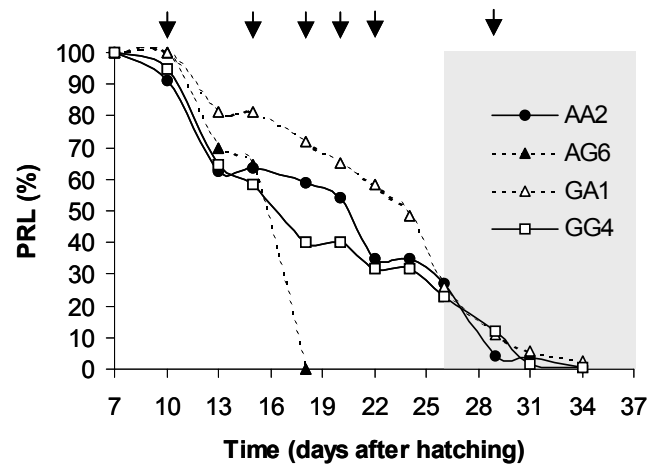
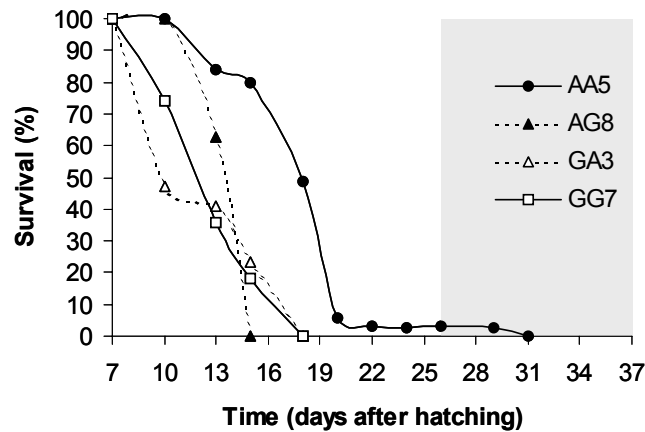


Figure 45. OsHV-1 DNA detection by PCR using Gp3/Gp4 primer pair. Lanes 1 and 14, negative controls; Lanes 2, 3, 5, 8, 9, 10, 12 and 13, larval samples in which no amplicons were observed with the expected size for OsHV-1. Lanes 4, 6, 7 and 11, larval samples that yield bands with the expected size for OsHV-1; Lane 15, positive control (OsHV-1 DNA); Lane M, size markers (100 bp DNA ladder, Promega).

Cumulative larval mortality reached 100 % in tanks AG6, AG8, GA3 and GG7 between Day 15 and Day 19 after hatching (Figure 46).



(A)



(B)

Figure 46. Percentage of *C. angulata* (AA), *C. gigas* (GG), GA hybrids, and AG larvae observed during rearing relatively to day 7 after hatching. (A) Proportion of remaining larvae (PRL) in tanks AA2, AG6, GA1 and GG4; Arrows indicate periods where larvae were collected for pathological analyses. (B) Larval survival in tanks AA5, AG9, GA3 and GG7. The areas in grey correspond to the settlement period. Note: abnormal larvae were observed in tank GA3 since the beginning of larval rearing.

Five out of 6 positive PCR results were associated with high mortality rates observed in the last 48-72 h before sampling (Figure 47).

	GA1	AA2	GA3* ³	GG4	AA5	AG6	GG7	AG8
DAY 10* ²	0.0 (-)	8.9 (-)	53.0 (ND)	5.4 (-)	0.0 (ND)	0.0 (-)	25.8 (ND)	0.0 (ND)
DAY 15* ¹	0.0 (-)	0.0 (-)	43.4 (ND)	9.7 (-)	5.2 (ND)	8.0 (+)	48.6 (ND)	100 (+)
DAY 18* ²	ND (ND)	ND (ND)	100 (ND)	ND (ND)	ND (ND)	100 (+)	100 (+)	
DAY 22* ¹	11.0 (-)	35.3 (ND)		25.7 (ND)	80.7 (ND)			
DAY 29* ²	56.4 (+)	84.4 (+)		43.3 (ND)	33.3 (ND)	SETTLEMENT		

Figure 47. Schematic representation of the position of the tanks, mortality observed at different periods in the last 48 – 72 h, and overall result of the detection of OsHV-1 DNA by PCR (in brackets). Mortalities superior to 40 % in the last 48-72 h before sampling is signalised in grey. At day 29, settlement had already started. +, positive PCR result; -, negative PCR result; ND, mortality or OsVH-1 detection not analysed; *¹, mortality observed in the last 48 h; *², mortality observed in the last 72 h; *³, abnormal larvae observed since the beginning of larval rearing.

The results of the present study support previous studies in which the detection of herpesvirus DNA was associated with high mortality rates among hatchery reared larvae of different bivalve species (Renault and Arzul, 2001; Arzul *et al.*, 2001a, b, c; Barbosa-Solomieu *et al.*, 2005). Three PCR positive samples corresponded to larval batches in which 100 % mortality was observed. The other 2 positive PCR results corresponded to larval batches experiencing high mortality rates (56.4 and 84.4 % in the last 72 h) in which a proportion of the larvae were pediveliger (ca. 17 % in one of the batches). Since individual larvae were not examined, it is not possible to know if the virus was present in pediveliger or in veliger larvae. However, based on experimental transmission assays (Le Deuff *et al.*, 1994; Arzul *et al.*, 2001a) it is likely that all larvae were infected (veliger and pediveliger larvae). A high proportion of the pediveliger larvae from these batches managed to settle, metamorphose and reach sexual maturity (data not shown). These results suggest that infected larvae with OsHV-1 from batches experiencing high mortality

rates may survive and become putative carriers and reservoirs of the virus. The single PCR positive result that was not associated with high mortality rates before sampling corresponded to apparently healthy larvae at day 15 after hatching (i.e., swimming normally). However, 3 days after sampling (18 DAH) 100 % mortality was observed in this larval batch and viral DNA was again detected. These results indicate that OsHV-1 DNA may be detected in apparently healthy larvae few days before a mortality outbreak. OsHV-1 DNA was not detected in one-day old larvae in any of the four groups (AA, AG, GA and GG). All other negative PCR results were obtained in batches of larvae not experiencing mortality (4 samples) or only presenting low mortality rates in the last 48-72 h before sampling (between 5.4 and 11.1 %).

Confirmation of the presence of OsHV-1 DNA in larvae

Four C2-C6 PCR products were sequenced (one per group: AA, AG, GA and GG). Sequencing was also carried out for Gp17-Gp18 PCR products. The sequence obtained for the majority of the clones analysed in the four groups (AA, AG, GA and GG) was exactly the same as the sequence of the reference virus (OsHV-1). However, some clones presented single nucleotide polymorphism (SNP; see Figure 3 of Article 5). However, polymorphism observed could be due to DNA polymerase errors and not true SNPs. These results confirm the authenticity of amplicons and hence the presence of OsHV-1 DNA in larval samples. These results also indicate that a similar virus was present in the four different oyster groups.

Detection of OsHV-1 DNA in the broodstock

DNA was extracted from all *C. angulata* (n = 20) and *C. gigas* (n = 18) parents using 3 different approaches: (1) from tissues fixed in Davidson's fixative and preserved in ethanol by a combination of proteinase K/Tween 20 digestion followed by heating (DF/DH); (2) from tissues fixed in Davidson's fixative and preserved in ethanol by a combination of proteinase K/Tween 20 digestion followed by phenol/chloroform extraction (DF/DP); and (3) from ethanol-preserved gill samples by a combination of proteinase K/Tween 20 digestion followed by phenol/chloroform extraction (EP/DP). The presence of potential PCR inhibitors contained in broodstock samples was investigated using an internal standard (PCR-IS). Amplification was carried out using DNA extracted using the DF/DH and DF/DP methods as template and adding 100 fg of internal standard in each PCR tube. Using DNA extracted from *C. angulata* broodstock by DF/DH, no bands were detected in 2 animals and in 2 other animals very weak bands were observed (Figure 48A). However, when DNA extracted by DF/DP was used as template, no inhibitory effect was detected in *C. angulata* broodstock samples (Figure 48B).

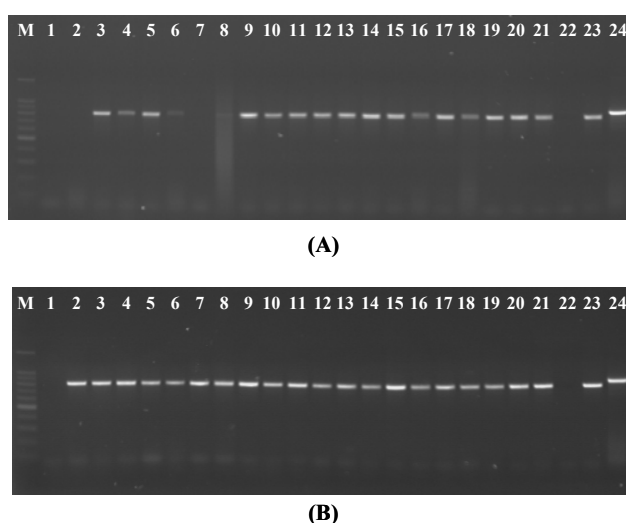


Figure 48. Detection of PCR inhibition using an internal standard from *C. angulata* broodstock samples obtained using two different methods of DNA extraction. (A) DNA extracted using the digestion/heating method. (B) DNA extracted using the digestion/phenol method. Lanes 1 and 22, negative controls; Lanes 2 to 21, *C. angulata* broodstock samples; Lane 23, internal standard; Lane 24, positive control (OshV-1 DNA); Lane M, size markers (100 bp DNA ladder, Promega).

For *C. gigas* broodstock samples, in 5 out of 18 animals no amplicons were detected and in 2 animals very weak bands were observed by PCR-IS when using DNA extracted by DF/DH. Inhibition was also observed using DNA extracted by DF/DP as template from the *C. gigas* broodstock samples, but only in 2 out of 18 animals. Hence, the use of an internal standard revealed inhibition in 29 and 5 % of the case when DNA was extracted by DF/DH and DF/DP, respectively. The greater inhibition observed with DF/DH could be due to the presence of cell components and DNA degrading enzymes that may have been removed with phenol/chloroform step of the DF/DP protocol. Indeed, the removal of inhibitory effects by phenol/chloroform extraction has been reported (An and Fleming, 1991). No inhibitory effect was observed in *C. angulata* and *C. gigas* broodstock samples when DNA obtained by EP/DP was used as template to amplify oyster mtDNA using COI3/COI4 primer pair. Samples where inhibition was initially detected were diluted until no inhibitory effects were detected by PCR-IS.

No amplicons with the expected size for OshV-1 DNA were observed using C2/C6 primer pair in all samples (*C. angulata* and *C. gigas*) obtained by the 3 different approaches (DF/DH, DF/DP and EP/DP). The samples obtained by EP/DP were also

analysed by nested PCR using C13/C5 and C2/C4 primer pairs with an annealing temperature of 60 °C. Amplicons of expected size were apparently detected in one of 20 *C. angulata* animals analysed (Lane 5, Figure 49). However, the band observed in the agarose gel was not very clear and sign was weak.

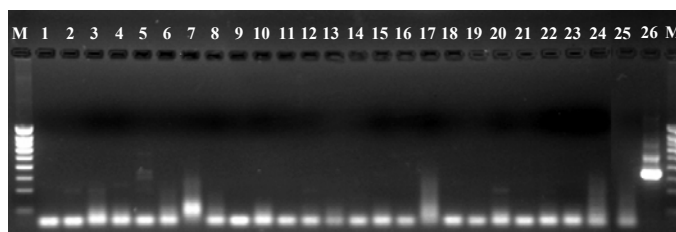


Figure 49. OsHV-1 DNA detection by nested PCR using C13/C5 and C2/C4 primer pairs. Lanes 1, 7, 13, 19 and 25, negative controls; Lanes 2, 3, 4, 5, 6, 8, 9, 10, 11, 12, 14, 15, 16, 17, 18, 20, 21, 22, 23 and 24, *C. angulata* broodstock samples; Lane 26, positive control (OsHV-1 DNA); Lanes M, size markers (100 bp DNA ladder, Promega).

Arzul *et al.* (2002) detected OsHV-1 DNA in 19 out of 30 *C. gigas* adults by PCR using the C2/C6 primer pair. The DNA used was extracted from gills (fresh frozen at –20 °C) by a combination of proteinase K/SDS digestion followed by phenol/chloroform extraction. The oysters analysed by Arzul *et al.* (2002) were collected in the same region as the *C. gigas* animals analysed in the present study. Hence, the main methodological difference between the present study and the previous work was the method used to conserve gill tissues. It is noteworthy that the ethanol-preserved gill samples analysed in the present study had been preserved for approximately two years before analysis and were conserved at room temperature. This may have reduced the quality and quantity of putative viral DNA present in the samples. Indeed, Vigneron *et al.* (2004) observed that storage temperature had a significant influence on detection of viral DNA extracted from purified particles or virions present in infected oyster larvae storage in different media (Tris EDTA buffer, distilled water and three types of seawater). However, to what extent storage of gill tissues in ethanol may influence the detection of OsHV-1 DNA by PCR is not known. A reduction in viral DNA quantity and quality can greatly decrease the likelihood of detecting OsHV-1 since the amount of viral DNA in adults is presumed to be low varying between 3 and 650 pg/mg oyster tissue (Arzul *et al.*, 2002; Renault *et al.*, 2004).

Article 5 (Published in Journal of Virological Methods)

Available online at www.sciencedirect.com

Journal of Virological Methods 139 (2007) 1–11

www.elsevier.com/locate/jviromet

Review

Detection of ostreid herpesvirus 1 DNA by PCR in bivalve molluscs: A critical review

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Received 29 May 2006; received in revised form 4 September 2006; accepted 13 September 2006

Available online 31 October 2006

Abstract

Herpes-like viral infections have been reported in different bivalve mollusc species throughout the world. High mortalities among hatchery-reared larvae and juveniles of different bivalve species have been associated often with such infections. The diagnosis of herpes-like viruses in bivalve molluscs has been performed traditionally by light and transmission electron microscopy. The genome sequencing of one of these viruses, oyster herpesvirus 1 (OsHV-1), allowed the development of DNA-based diagnostic techniques. The polymerase chain reaction (PCR) has been used for the detection of OsHV-1 DNA in bivalve molluscs at different development stages. In addition, the PCR used for detection of OsHV-1 has also allowed the amplification of DNA from an OsHV-1 variant. The literature on DNA extraction methods, primers, PCR strategies, and confirmatory procedures used for the detection and identification of herpesviruses that infect bivalve molluscs are reviewed.

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Keywords: Herpesvirus; OsHV-1; Detection; PCR; Bivalve molluscs; Oyster

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1. Introduction

Herpes-like viral infections have been identified in various marine bivalve mollusc species throughout the world, including the USA (Farley et al., 1972; Friedman et al., 2005), New Zealand (Hine et al., 1992), France (Nicolas et al., 1992; Arzul et al., 2001b; Renault and Arzul, 2001; Renault et al., 2000b, 2001b), Australia (Hine and Thorne, 1997), and Mexico (Vásquez-Yeomans et al., 2004). The first description of a virus morphologically similar to members of the *Herpesviridae* family in a bivalve mollusc was made by Farley et al. (1972) in the eastern oyster, *Crassostrea virginica*. Since then, a wide host range has been reported for herpes and herpes-like viruses infecting bivalve species, including the Pacific oyster *C. gigas* (Hine et al., 1992), the European oyster *Ostrea edulis* (Nicolas et al., 1992), the Antipodean flat oyster *O. angasi* (Hine and Thorne, 1997), the Chilean oyster *Tiostrea chilensis* (Hine et al., 1998), the Manila clam *Ruditapes philippinarum* (Renault et al., 2001a), the carpet shell clam *R. decussatus* (Renault and Arzul, 2001), the Portuguese oyster *C. angulata* (Arzul et al., 2001a), the Suminoe oyster *C. rivularis* (Arzul et al., 2001a) and the French scallop *Pecten maximus* (Arzul et al., 2001b). High mortalities among hatchery-reared larvae (Hine et al., 1992; Nicolas et al., 1992; Renault et al., 1994; Renault and Arzul, 2001) and juveniles (Comps and Cochenne, 1993; Renault et al., 1994, 2000b; Friedman et al., 2005) of different bivalve species have often been associated with herpes and herpes-like virus infections. Observations by transmission electron microscopy (TEM) revealed that larvae exhibit generalized infections, whereas focal infections were generally observed in spat (Renault et al., 1994). Viral infections were also observed in adult bivalves (Hine and Thorne, 1997; Arzul et al., 2001b, 2002; Vásquez-Yeomans et al., 2004), but adults are apparently less sensitive to such infections as compared to younger stages (Arzul et al., 2002; Renault and Novoa, 2004). The pathogenicity of herpes-like viruses was demonstrated by experimental transmission assays either for larval stages of *C. gigas* (Le Deuff et al., 1994) and other bivalve species (Arzul et al., 2001a,b). Similar experiments were conducted with juveniles and adults of *C. gigas*, but the results were inconclusive (Renault and Novoa, 2004). It is noteworthy that a highly pathogenic herpes-like virus was observed recently by TEM in the gastropod mollusc *Haliotis diversicolor supertexta* in Taiwan associated with high mortality rates (Chang et al., 2005).

The development of a method for purifying herpes-like virus particles from infected *C. gigas* larvae facilitated the extraction of viral DNA and a partial genome characterization (Le Deuff and Renault, 1999). The genome was subsequently completely sequenced (GenBank accession no. AY509253) revealing a tenuous relationship with other herpesviruses. This oyster virus was classified as a member of the *Herpesviridae* family under the name oyster herpesvirus 1 (OsHV-1) (Minson et al., 2000) and was considered the only member of a new major class of herpesvirus (Davison, 2002; Davison et al., 2005). A variant of OsHV-1 (OsHV-1var) was also described in larvae of different bivalve species (Arzul et al., 2001b,c) and OsHV-1 and OsHV-1var are considered representatives of a single viral species. In

contrast with vertebrate herpesviruses, which are generally confined to a single host, OsHV-1 has been identified in several bivalve species and interspecies viral transmission was demonstrated (Arzul et al., 2001a; Friedman et al., unpublished data).

Detection of viruses in mollusc bivalves cannot be done by classic serological methods because molluscs do not produce antibodies. In addition, viral replication in cell culture to facilitate the diagnosis is not possible due to the absence of mollusc cell lines. The diagnosis of herpes-like virus infections has traditionally been performed by light microscopy, as a first approach in order to detect cytological abnormalities, followed by transmission electron microscopy to complete the diagnosis (Hine and Thorne, 1997; Renault et al., 2000b). These procedures are time consuming, impractical for epidemiological surveys, and some viruses can be difficult to detect and identify when present in low amounts. As a result of these limitations, other diagnostic methods have been developed such as immunochemistry and nucleic acid-based techniques. A protocol using polyclonal antibodies produced in BalbC mice immunized with viral particles purified from infected *C. gigas* larvae has been used to detect OsHV-1 proteins (Le Deuff, 1995; Arzul et al., 2002). Another technique that has also been developed is *in situ* hybridization, which allows the detection of viral DNA (Renault and Lipart, 1998; Lipart and Renault, 2002; Barbosa-Solomieu et al., 2004). Both techniques have high sensitivity and specificity, and allow the visualization of viral proteins or DNA, respectively. Polymerase chain reaction (PCR) diagnostic methods have also been developed for detection of OsHV-1 DNA (Renault et al., 2000a; Renault and Arzul, 2001; Arzul et al., 2001a,b,c). PCR is considered a suitable tool for the diagnosis of OsHV-1 infections owing its specificity, high sensitivity relative to other methods (e.g., TEM), ease of sample processing, availability of reagents, and time and cost efficiency. However, as this test only detects viral nucleic acid, other tests such as histology, ISH or antigen-based tests must confirm PCR tests, particularly in a new species or new geographic location. In areas where the pathogen is endemic, PCR alone may be sufficient for diagnosis of its presence. A competitive PCR method was developed that can be used to demonstrate the presence of PCR inhibitors or to quantify OsHV-1 DNA (Renault et al., 2004). Different methods of DNA extraction (Renault et al., 2000a; Arzul et al., 2002; Batista et al., 2005; Friedman et al., 2005) as well as various primer pairs (Renault et al., 2000a; Arzul et al., 2001a,b,c; Renault and Arzul, 2001; Barbosa-Solomieu et al., 2004, 2005) have been designed and used to detect viral DNA using one-round or nested PCR. In addition, the PCR conditions used for detection of OsHV-1 also allow amplification of DNA from other closely related herpesviruses in different bivalve species, as was the case of OsHV-1var (Arzul et al., 2001b,c). In order to confirm the authenticity of PCR products, different techniques have been used, including digestion of PCR products with restriction enzymes (PCR-RFLP) (Arzul et al., 2001c; Renault et al., 2004; Barbosa-Solomieu et al., 2004, 2005) and DNA sequencing. The objectives of the present work are: (1) to provide a review of the different DNA extraction methods, PCR protocols, primers and confirmatory procedures that have been used in the detection of OsHV-1 DNA by PCR; (2) to discuss the adequacy of the

Table 1
Techniques used to extract nucleic acids from bivalve molluscs samples for detection of herpesvirus DNA by PCR

Development stage	Sampling conditions	Method	Reference
Larvae and spat	Frozen at -20°C	Grinding, boiling	Renault et al. (2000a)
Larvae	Frozen at -80°C	Washing, grinding, digestion with proteinase K, boiling	Batista et al. (2005)
Spat	Fresh material and samples fixed in 95% ethanol	Commercial kit (lysis, digestion with proteinase K, DNA binding to silica gel)	Friedman et al. (2005) and Burge et al. (2006)
Adults	Frozen at -80°C	Grinding, digestion with proteinase K, phenol–chloroform purification	Le Deuff (1995)
Adults and spat	Fixed in Davidson's and Carson's solutions, paraffin-embedded	Deparaffination, digestion with proteinase K, heating	Barbosa-Solomieu et al. (2004)
Adults	Fixed in Davidson's solution	Digestion with proteinase K, heating	Barbosa-Solomieu et al. (2005)
Adults	Fixed in Davidson's solution	Digestion with proteinase K, phenol–chloroform purification	Unpublished data
Adults	95% ethanol (gill)	Digestion with proteinase K, phenol–chloroform purification	Unpublished data

different methods based on published and unpublished data; (3) to address different aspects that should be considered in order to avoid false PCR results.

2. Nucleic acid extraction techniques

The exquisite sensitivity of the PCR reaction can allow the amplification of DNA even when a very small number of target molecules are present. Nevertheless, the type of samples analysed as well as the procedure used for nucleic acid extraction can greatly affect the success of the amplification reaction. If samples cannot be processed immediately after harvesting they should be conserved and stored under conditions that preserve the integrity of nucleic acids. Extraction of nucleic acids from fresh frozen samples (e.g., larval or adult tissues), stored at -20 or -80°C , generally yields good quality DNA for OsHV-1 detection by PCR (Renault et al., 2000a; Arzul et al., 2002; Batista et al., 2005). Samples fixed and stored using non-denatured ethanol have also been used for amplification of OsHV-1 DNA (Friedman et al., 2005; Burge et al., 2006; Renault et al., unpublished data). Other trials using formalin fixed samples (e.g., Davidson's and Carson's solutions) have also allowed amplification of herpesvirus DNA from bivalve mollusc samples (Barbosa-Solomieu et al., 2005). However, nucleic acid extraction from formalin-fixed tissues usually yields DNA that is degraded and can affect subsequent nucleic acid-based techniques (Jackson et al., 1990; An and Fleming, 1991), especially those involving larger amplified fragments such as that produced by the nested A primer test of OsHV-1 at 917 bp (Renault et al., 2000a).

Several techniques have been used for the extraction of nucleic acids from different types of bivalve mollusc samples in order to detect herpesvirus DNA by PCR (Table 1). Renault et al. (2000a) used a simple method of nucleic acid extraction from frozen oyster specimens by grinding and boiling the tissues. This method allowed the extraction of nucleic acids and subsequently amplification of viral DNA from larvae and spat of *C. gigas* infected with a herpes-like virus as confirmed by TEM. However, this technique does not appear suitable to amplify OsHV-1

DNA from adults. Other techniques of DNA extraction were also examined by Renault et al. (2000a) namely, Chelex-100 and a combination of proteinase K digestion with phenol/chloroform purification, but both techniques yielded false-negative PCR results. It was also noticed that extraction by boiling ground oyster tissues in some cases produced compounds that inhibited the amplification reaction (Renault et al., 2000a). A method of DNA extraction from oyster larvae was reported by Batista et al. (2005) that combines the use of proteinase K, Tween-20 and boiling. This method allowed the amplification of OsHV-1 DNA by PCR from a small amount of larvae (3 mg). Commercial DNA extraction kits (e.g. Qiagen's QIAamp DNA mini kit using the tissue protocol) have also been used to extract amplifiable herpesvirus DNA from *C. gigas* spat (Friedman et al., 2005; Burge et al., 2006). These authors obtained good quality DNA but variable PCR results depending on the amount of template DNA used. Herpesvirus DNA has also been extracted from *C. gigas* adults by grinding, digestion with proteinase K and phenol/chloroform purification from fresh frozen tissues (Le Deuff, 1995).

For decades, specimens of bivalve molluscs have been routinely fixed in formalin and embedded in paraffin for histological analyses. Although extraction of sufficient quantity of amplifiable DNA from these tissues can be difficult (Jackson et al., 1990; An and Fleming, 1991), these samples constitute invaluable resources for pathological analysis using DNA-based techniques. Barbosa-Solomieu et al. (2004) could extract nucleic acids from fixed paraffin-embedded archival samples by deparaffination, digestion with proteinase K and heating. The method allowed the amplification of small fragments (196 and 207 bp) of OsHV-1 DNA, and the absence of PCR inhibitors was demonstrated by amplification of an internal standard. A similar protocol was successfully used by Barbosa-Solomieu et al. (2005), who extracted nucleic acids from oyster tissues preserved in Davidson's solution, which allowed the detection of OsHV-1 by PCR. A study in which the previous technique and a variation of this procedure, using phenol/chloroform purification and ethanol precipitation instead of the heating step, revealed PCR inhibition in 29 and 5% of the cases, respectively

(Batista, personal communication). The samples used in that study had been fixed in Davidson's solution and later transferred to 70% ethanol. The greater inhibition observed with the boiling protocol could be due to the presence of cell components and DNA degrading enzymes that may have been removed with the phenol/chloroform purification step. Indeed, the removal of inhibitory effects by phenol/chloroform purification has been reported (An and Fleming, 1991). The effect of inhibitors may also be reduced during the PCR reaction with the use of bovine serum albumin (BSA) as outlined below (Friedman et al., 2005). However, methods of nucleic acid extraction with fewer steps should be used preferentially in order to reduce the risk of sample contamination.

3. PCR analysis

Different PCR strategies can be used to detect viral DNA. Some methods utilize one-round PCR (or single PCR) in which the DNA sequence is amplified using one primer pair. Other methods use nested PCR in which first round amplification products are subjected to a second round of amplification and hence two primer pairs are used.

3.1. PCR primers

Based on OsHV-1 sequence data several oligonucleotide primers have been designed for the detection of herpesvirus DNA in bivalve molluscs (Table 2). Different primer pair combinations (Table 3) have been used to amplify DNA by PCR from regions A, B, C and Gp of the virus genome (Fig. 1). The A region encodes a protein of unknown function; the B region encodes a putative inhibitor of apoptosis belonging to the IAP family; the C region encodes parts of two proteins of unknown functions and is present twice in the genome (being located in the inverted repeats TR_L and IR_L) (Davison et al., 2005); and the Gp region encodes a putative glycoprotein (Arzul et al., 2001b,c).

3.2. Diagnostic specificity and sensitivity

Specificity is defined as the ability of an assay to amplify DNA only from the target agent. Sensitivity (or limit of detection) is defined as the smallest quantity of viral DNA that can be systematically detected.

Two pairs of primers (A3/A4 and A5/A6) corresponding to the A region of the OsHV-1 genome have been used to detect

Table 2
Oligonucleotide primers used for OsHV-1 detection by PCR

Name	Primer sequence (5'–3')	Forward/reverse	Reference
Region A			
A3	GCCAACCGTTGGAACCATACAGCG	Forward	Renault et al. (2000a)
<u>A3</u>	GCCAACCGTTGGAACCATACAGGCG	Forward	*
A4	GGGAATGAGGTGAACGAACTATAGACC	Reverse	Renault et al. (2000a)
A5	CGCCCCAACCCACGATTTTCACTGACCC	Forward	Renault et al. (2000a)
A6	CCCGCTAGATATAGGATGAGATTG	Reverse	Renault et al. (2000a)
<u>A6</u>	CCCGTCTAGATATAGGATGAGATTG	Reverse	*
Region B			
B1	ATGTAATGGGTGGTGGTGCT	Forward	Arzul et al. (2001c)
B2	CAACAGCTTTGGAGGTTGGT	Reverse	Arzul et al. (2001c)
B3	GTGGAGGTGGCTGTTGAAAT	Forward	Arzul et al. (2001b)
B4	ACTGGGATCCGACTGACAAC	Reverse	Arzul et al. (2001b)
Region C			
C1	TTCCCTCGAGGTAGCTTTT	Forward/reverse	Arzul et al. (2001c)
C2	CTCTTTACCATGAAGATACCCACC	Forward/reverse	Arzul et al. (2001c)
C4	GCAGTTGTGGTATACTCGAGATTG	Forward/reverse	Arzul et al. (2001c)
C5	CCGTGACTTCTATGGGTATGTCAG	Forward/reverse	Arzul et al. (2001c)
C6	GTGCACGGCTTACCATTTT	Forward/reverse	Arzul et al. (2001c)
C9	GAGGGAAATTGCGAGAGAA	Forward/reverse	Barbosa-Solomieu et al. (2004)
C10	ATCACCGGCAGACGTAGG	Forward/reverse	Barbosa-Solomieu et al. (2004)
C11	GAGGGAAATTGCGAGAGAG	Forward/reverse	Barbosa-Solomieu et al. (2005)
C13	CCTCGAGGTAGCTTTTGTCAG	Forward/reverse	Renault et al. (2004)
C14	CCGTGACTTCTATGGGTATG	Forward/reverse	Renault et al. (2004)
C15	GATTACCCAGATTCCCCTC	Forward/reverse	Renault et al. (2004)
Region Gp			
Gp3	GGTTGTGGGTTTGGAATGT	Forward	Arzul et al. (2001b)
Gp4	GGCGTCCAACTCGATTAAA	Reverse	Arzul et al. (2001b)
Gp7	TTACACCTTTGCCGGTGAAT	Forward	Unpublished data
Gp8	TCACATCACTTGGTGGCAAT	Reverse	Unpublished data
Gp10	AAGCAAATGACACGACACCA	Reverse	Unpublished data
Gp17	AACCACCACAAAGCTCCTC	Forward	Unpublished data
Gp18	ACATCTGGTGGGTAGG	Reverse	Unpublished data

* The sequence of primer A3 and A6 based on OsHV-1 genome is presented in the table with a G instead of A for primer A3 in the underline position and with the insertion of a T in the underline position for primer A6.

Table 3

Primer pairs, product size and restriction enzymes analysis of PCR products used for the detection of OsHV-1 (OsHV-1var) DNA

Primer pair	Product size (bp)	Restriction enzyme analysis		
		Enzyme	Fragments size (bp)	Reference
Region A				
A3/A4	1001 (ca. 1001)	<i>Bst</i> I	765/236	Renault et al. (2001b)
A5/A6	917 (UPS)			
Region B				
B1/B2	464 (ca. 464)	<i>Tta</i> 9I	121/85	Barbosa-Solomieu et al. (2004)
B3/B2	332 (ca. 332)			
B3/B4	207 (ca. 207)			
Region C				
C1/C4	538 (NA)	<i>Mbo</i> I	747/94/55	Renault et al. (2004)
C1/C6	896 (NA)			
C2/C4	352 (180)	<i>Xho</i> I	375/335 (375/155)	Arzul et al. (2001c)
C2/C6	709 (ca. 529)			
C13/C5	765 (UPS)	<i>Sau</i> 3AI	118/78	Barbosa-Solomieu et al. (2004)
C9/C10	197 (UPS)			
C11/C10	197 (UPS)			
C15/C14	780 (UPS)			
Region Gp				
Gp3/Gp4	698 (698)			
Gp3/Gp10	1735 (UPS)			
Gp7/Gp8	699 (UPS)			
Gp17/Gp18	306 (UPS)			

UPS: unknown product size; NA: no amplification.

herpesvirus DNA in larvae and spat via nested PCR (Renault et al., 2000a). The specificity of these primer pairs was evaluated using DNA from *C. gigas* as well as DNA from vertebrate herpesviruses. The detection procedure was found to be specific, only amplifying OsHV-1 DNA and not leading to false-positives. The sensitivity of nested PCR using on A3/A4 and A5/A6 primer pairs was also tested. As little as 500 fg of virus DNA extracted from purified particles were routinely detected. The amplification sensitivity decreased with increasing amounts of non-infected oyster spat tissues (Renault et al., 2000a). The one-round PCR assay with the A3/A4 primer pair not only allowed

amplification of OsHV-1 DNA but also the detection of a variant of this virus (OsHV-1var) in *C. gigas* and *R. philippinarum* larvae (Arzul et al., 2001c).

The combination of primer pairs A3/A4 and A5/A6 allowed less PCR amplification than C2/C6 (21.4% versus 32.4%) when the same larval samples were analysed (Renault and Arzul, 2001). The differences observed in herpesvirus DNA detection suggested that the one-round PCR with C2/C6 was more useful for epidemiological surveys than the nested PCR using A3/A4 and A5/A6 (Renault and Arzul, 2001). Moreover, Renault et al. (2004) observed that the C2/C6 primer pair systematically

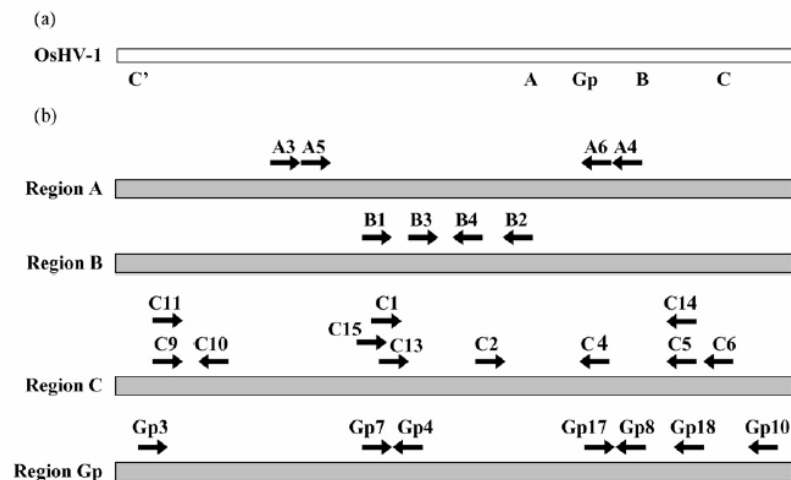


Fig. 1. General scheme of OsHV-1 genome. (a) Position of the regions A, B, C and Gp. C' represent the position of the inverted repeat of the C region. (b) Diagrams with primers (arrows) annealing sites on the four regions.

Table 4

Sensitivity and adequacy of some of the primer pairs that have been mostly used to detect herpesvirus DNA from bivalve mollusc samples by PCR

Primer pair	Sensitivity	Positive points	Limitations
One-round PCR			
A3/A4	ND	Also allows the amplification of OsHV-1var and the OsHV detected in USA ^a	
C1/C6	1 fg ^b	Target fragment present twice	Failed to amplify OsHV-1var ^c
C2/C6	1 fg ^b	Target fragment present twice; allows the differentiation of OsHV-1 from OsHV-1var ^c	Failed to amplify OsHV detected in USA ^a
C13/C5	10 fg ^d	Target fragment present twice	Extraneous bands observed
Gp3/Gp4	10 fg ^d	Target region apparently more conserved; also allows the amplification of OsHV-1var ^c	
Nested PCR			
A3/A4–A5/A6	500 fg ^e	PCR conditions optimized; also allows the amplification of OsHV detected in USA ^a	Low sensitivity
C13/C5–C2/C4	1 fg ^f	Target fragment present twice; allows the differentiation of OsHV-1 from OsHV-1var ^c	Extraneous bands observed

ND: not determined.

^a Friedman et al. (2005).^b Renault et al. (2004).^c Arzul et al. (2001b,c).^d Vigneron et al. (2004).^e Renault et al. (2000a).^f Unpublished data.

allowed the detection of 1 fg of purified viral DNA, and was more sensitive than the nested PCR with primer pairs A3/A4 and A5/A6 (detection limit of ca. 500 fg of purified viral DNA). The higher sensitivity of the one-round PCR with the primer pair C2/C6 can be partially explained by the fact that the targeted fragment is present twice in the OsHV-1 genome (Arzul et al., 2001b,c). However, other primer pairs designed in the C region (C13/C5 and C15/C14) failed to produce detectable amplicons with amounts of purified viral DNA detected with the C2/C6 primer pair (Renault et al., 2004). Vigneron et al. (2004) observed a detection limit of 10 fg of purified viral DNA for both primer pairs C13/C5 and Gp3/Gp4 (Table 4), the later target region being only present in one copy in the OsHV-1 genome. On the other hand, Friedman et al. (2005) detected herpesvirus DNA in *C. gigas* juveniles with A3/A4 and A5/A6 whereas no PCR products were obtained with primer pair C2/C6. In addition, partial sequencing of the amplified A region revealed a single nucleotide difference compared to the reference sequence of OsHV-1 (Arzul et al., 2001c). The herpesvirus described by Friedman et al. (2005) is very closely related to OsHV-1 based on the sequence of the A region. Non-amplification with C2/C6 primer pair could be due to nucleotide substitutions and/or insertions/deletions in the C region. The variant of OsHV-1 described by Arzul et al. (2001b,c) presented a major deletion of 2.8 kbp in the C region as well as other nucleotide deletions, substitutions and insertions. No amplification of OsHV-1var DNA was observed when primer pairs C1/C4 and C1/C6 were used. Whereas amplicons were produced with primer pairs C2/C4 and C2/C6; however, these amplicons were smaller than those obtained with reference viral DNA (Arzul et al., 2001c). In contrast, the PCR products obtained from samples infected with OsHV-1 and OsHV-1var using pair primers A3/A4, B1/B2, B3/B4 and Gp3/Gp4 were the same size (Arzul et al., 2001b,c). Nevertheless, some nucleotide differences (substitu-

tions) in the sequence of the Gp3/Gp4 fragment were observed between the two types of OsHV-1 (Arzul et al., 2001c). It is noteworthy that primers used to detect bivalve herpesviruses were designed based on specific OsHV-1 sequences. Hence, putative polymorphisms in primer-binding sites can result in non-amplification or lower sensitivity of the PCR analysis to detect other related herpesviruses such as OsHV-1var.

OsHV-1 was also detected by PCR in asymptomatic *C. gigas* adults using the primer pairs B3/B2 and C2/C6 (Arzul et al., 2002). Both primer pairs gave concordant PCR results. Although other techniques, *in situ* hybridization and immunohistochemistry, were also used by Arzul et al. (2002) to detect herpesvirus DNA and proteins, respectively PCR was the most sensitive method. Mantle and gills appear as organs of choice for OsHV-1 detection in adult oysters (Arzul et al., 2002).

Different PCR strategies and primer pairs were used to analyse hatchery-reared oyster larvae (Fig. 2) (Batista, personal communication). The larvae were collected at different stages of development and immediately frozen at -80°C . Samples for PCR analysis were prepared according to the method used by Renault et al. (2000a). No differences were observed in the number of virus DNA positive samples with the different primer pairs. Amplification artifacts were observed with the C9/C10 primer pair yielding PCR products of various sizes. However, no positive PCR results were obtained for the samples showing extraneous bands when other primer pairs were used. Amplicons approximately 200 bp smaller than the expected size were obtained with the C13/C5 primer pair. Based on their size, these PCR products could be interpreted as the amplification of OsHV-1var DNA. However, when PCR analyses were performed with others primer pairs (i.e., A3/A4, B3/B4 and Gp3/Gp4), that have been used to amplify DNA of OsHV-1var in previous studies (Arzul et al., 2001b,c), no PCR products were obtained (data not shown). These results suggest that the PCR condi-

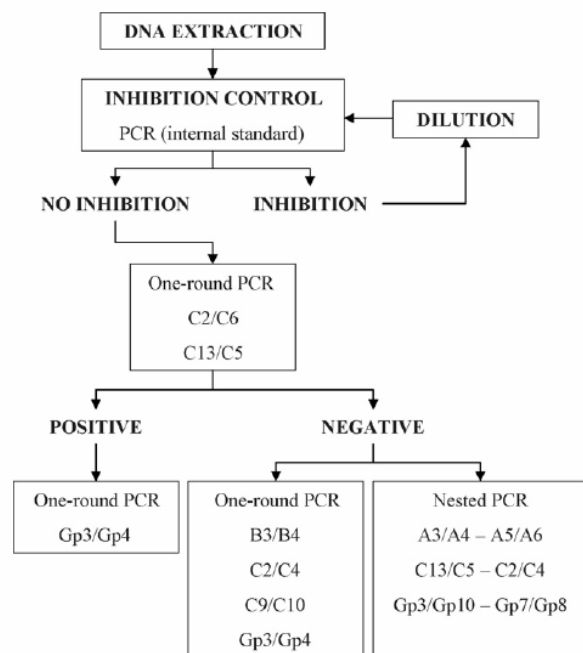


Fig. 2. Experimental procedure scheme used for the detection of OsHV-1 DNA.

tions used with the primer pairs C9/C10 and C13/C5 should be optimized in order to eliminate the non-specific amplification observed.

3.3. Amplification from formalin fixed samples

Samples for histopathological examination are usually formalin fixed using different types of fixatives (e.g., 10% formalin, Davidson's solution, Carson's solution). The quality and quantity of the DNA extracted from these types of samples is generally low due to degradation and leaching of nucleic acids during the fixation procedure, particularly under prolonged fixation durations (more than 24–48 h). Primer pairs that amplify small fragments can be used to increase the efficiency of the amplification reaction, in particular, when the template DNA is highly fragmented (Coates et al., 1991). Barbosa-Solomieu et al. (2004) used primer pairs C9/C10 and B3/B4 successfully to amplify OsHV-1 fragments of 196 and 207 bp, respectively, from formalin-fixed paraffin-embedded archival samples. Despite the similar size of the amplified fragments, a higher number of PCR positive results were obtained with primer pair C9/C10 compared to B3/B4 (Barbosa-Solomieu et al., 2004). In contrast, no detectable PCR products were obtained in previous assays using primers that generate larger fragments (600–1000 bp) when the same type of samples were analysed (Renault, personal communication).

3.4. Competitive PCR

A competitive PCR method was developed in order to detect and quantify herpesvirus DNA (Renault et al., 2004).

The method was based on co-amplification of OsHV-1 DNA and a mimic molecule, which differs from the target DNA by a deletion of 76 bp, using the same set of primers (C1/C6 or C2/C6). Production of the mimic molecule was done by PCR using the C1 primer and a modified primer (C116). The size differences of amplicons obtained from viral DNA and the mimic molecule can easily be observed by electrophoresis on agarose gel. This method has been successfully used to quantify herpesvirus DNA (Arzul et al., 2002) and to detect the presence or absence of PCR inhibitory factors (Barbosa-Solomieu et al., 2004; Renault et al., 2004; Batista et al., 2005).

4. Analysis of amplification products

Assessing the specificity of a PCR only on the basis of amplicon size determined by agarose gel electrophoresis can result in false-positive results, especially in PCR assays, that have not been properly validated. Different techniques have been used to verify the specificity of PCR products from bivalve herpesviruses, including Southern blot analysis, restriction digestion and sequencing. Arzul et al. (2001c) used a digoxigenin-labelled probe in order to verify the specificity of amplification products by Southern blot hybridization. Digestion of PCR products with restriction enzymes has been the technique most frequently used to confirm the specificity of the amplification of herpesvirus DNA (Table 3). The method is simple and the results can be obtained in a few hours. Nevertheless, insufficient or absent digestion caused by the presence of certain inhibitory compounds or low enzyme activity due to sub-optimal storage conditions can lead to misinterpretations of the results. Consequently, restriction digestion of PCR products obtained from reference OsHV-1 DNA (e.g., the positive control) should be used in every assay as an external control of the digestion reaction. Sequencing of PCR products is the most accurate method for determining the authenticity of the amplicons. Despite the accuracy of sequencing, this technique it is not very practical for amplicon confirmation and the cost is high specially when analysing a large number of samples. Nevertheless, it can provide unique information about the virus detected and hence allow the identification of new herpesviruses or variants of OsHV-1. If the previous confirmatory methods are not available, different primer pairs targeting different areas of the viral genome can also be used to confirm the presence of the target viral DNA.

During an outbreak of OsHV-1 in hatchery-reared larvae, PCR products obtained with the primer pairs C2/C4 and Gp17/Gp18 were cloned and sequenced (Batista, personal communication). Although no differences were observed in the sequence of the majority of clones when compared with the reference virus (OsHV-1) some clones presented single nucleotide polymorphism (Fig. 3). The polymorphism observed could be due to polymerase errors and not because of the presence of more than one type of viruses. In order to eliminate these uncertainties, high-fidelity polymerases (e.g., Goldstar Polymerase, Eurogentec, Belgium) should be used. Sequencing can also be performed directly from the PCR

Reference	ctcttttaccatgaagataccaccAAATGTGGTAAAGACGGAACAATCTTTTCTAGGATA
Variant-A	
Reference	TGGAGCTGCGGCGCTATGGATTTAACGAGTGCCACCAAAAGTTGGGATAATGATTTTAGA
Variant-A	
Reference	ATAGATGTGATGTGCGGCAAGATGAATGGCAAGATACACAATGAGCTATTGCCCGACCAC
Variant-A	
Reference	AAACCTAACGTTGTATTTCGATTACGGATTAAGAAAAATGGGTTCACAAATCTAAAAATAAA
Variant-A	
Reference	AAAACCACATGGGGGCCAAGGAATTTAAACCCCGGGGAAAAAGTATAAATAGCGCGGATT
Variant-A	
Reference	TGTCAGTTTAGAATCATACCCACACACTcaatctcgagtataaccacaactgc
Variant-A	..C.....
(a)	
Reference	aaccaccacacaagctcctcCTCCACCTATCGTTATTAATACCACAGCAGCACCTTTGGC
Variant-B	
Reference	GCCCATTACCAATGCCACATTGCCACCAAGTGATGTGATCACACCGGAGGCTGTGAATTT
Variant-B	
Reference	AACAGATGATACCCCTGTTGTAATGAACCGGTAAATTCTACATTATCAATGATACGGA
Variant-B	
Reference	TGTATTAGATGATTCTCCACCCCTCTGCTCCACAAGCGCCTGGTATAGTTGGTATAAT
Variant-B	A.....
Reference	TGTAAATAAGATTACAACAACACCTGCACCATCCATCGGTAGGGCGcctatcccaccacc
Variant-B	
Reference	agatgt
Variant-B	
(b)	

Fig. 3. Comparison of the nucleotide sequence of (a) the C2–C4 fragment (variant-A) and (b) the Gp17–Gp18 fragment (variant-B) from oyster larvae with reference OsHV-1 DNA. Primer sequences are in lower cases.

products since putative polymerase errors will be underrepresented.

5. PCR results and infection levels

Amplification of nucleic acid by PCR has revolutionized the diagnosis of viral infections, in particular when it is difficult or impossible to cultivate the infectious agent due to a lack of continuous cell lines. However, caution should be applied when assigning a virus to a disease based only on the detection of its genomic material. The presence of virus nucleic acid could be due to latent, low-grade persistent or active infections (Kleinschmidt-DeMasters et al., 2001), or to the presence of virions in the gastrointestinal tract and not due to actual infection. In the case of latent infections, even if highly sensitive PCR methods are used in association with well-established nucleic acid extraction procedures, viral DNA may not be detected (e.g., Khadijah et al., 2003). Consequently,

negative PCR results always have to be interpreted with some caution.

Detection of herpesvirus DNA by PCR has been reported in larvae of bivalve species in which intracellular viral particles were observed by TEM (Arzul et al., 2001a; Renault et al., 2001b). Moreover, positive PCR results have been associated with high mortality rates during larval rearing (Renault et al., 2000a; Arzul et al., 2001a,b,c; Renault and Arzul, 2001). The failure to detect viral DNA by PCR does not necessarily mean that the animals are not infected, as low levels of viral DNA in tissues may be below the threshold of the detection method. It has been observed that the number of positive PCR results in moribund *C. gigas* juveniles is higher than in apparently healthy individuals (C. Garcia, IFREMER LGP La Tremblade, personal communication; Burge et al., 2006). OsHV-1 has been detected by PCR in apparently healthy *C. gigas* adults, which suggest that the virus is able to persist in adult oysters without inducing disease and mortality (Arzul et al., 2002).

6. Avoiding false PCR results

6.1. False-positive results

The high sensitivity that makes PCR a very powerful technique for the amplification of DNA can also be seen as a negative aspect because of inadvertent contamination by nucleic acids. Even very low levels of contamination can result in false-positive results. There are basically three major sources of contamination: (1) target DNA previously extracted (e.g., in reagents used for the PCR reaction); (2) PCR products obtained from previous assays; and (3) cloned target molecules in plasmids (Rys and Persing, 1993). Contamination of PCR reaction by previously amplified sequences is the most likely form of contamination and occurs especially in environments where the same amplification is run repeatedly (e.g., diagnostic laboratories).

In order to avoid contamination, separate work areas should be used for: (1) preparation and storage of reagents; (2) sample preparation; (3) preparation of amplification mixture components; (4) PCR amplification; and (5) analysis of amplicons. Equipment (e.g., pipetting devices) and reagents should not be exchanged between the separate work areas. Preparation of amplification mixture components is best carried out in a hood equipped with UV lights (Fox et al., 1991). These lights should be turned on before and after preparation of the PCR mixture. To limit contamination, all pipetting procedures should be performed using filter tips, and disposable gloves should always be worn and changed frequently. Cross-contamination can also be minimized throughout the elimination of contaminants from surfaces and equipments with sodium hypochlorite (e.g., decontamination of laboratory bench with 10% bleach). For each PCR analysis, at least one negative control (containing distilled water instead of template DNA) per five diagnostic samples should be used. Due to the high sensitivity of PCR (i.e., less than 1 fg of purified viral DNA can be detected), the nature of samples, the environment (e.g., seawater, hatchery) and the time of sampling (e.g., mortality outbreaks), particular care should be taken during sample collection and preparation. Although, this type of contamination is most frequently encountered, plasmid contamination may also be problematic due to the ease of plasmid aerosol formation. To avoid plasmid contamination, in addition to the precautions listed above, a dedicated room where plasmids are handled is recommended.

6.2. False-negative results

There are several factors that can cause false-negative PCR results such as: (1) pipetting errors; (2) inadequate sample preparation; (3) nucleic acid degradation and capture; and (4) polymerase inhibition. Inadequate sample preparation can yield insufficient amounts of target molecules as well as poor quality DNA (e.g., poor lysis efficiency). The method of nucleic acid extraction may also yield compounds that can inhibit the PCR reaction by degradation and sequestration of target DNA and/or inhibiting polymerase (Wilson, 1997). The presence of inhibitor factors can also depend on the development stage of the animal. For example, severe PCR inhibition has been observed

in oyster larvae 1 day after fertilization, but not in older larvae from the same larval batch (Batista, personal communication). PCR inhibition was also observed in adult oyster samples using ground tissues, but not in spat samples (Renault and Novoa, 2004). Therefore, it is advisable to employ standard molecules as indicators of the efficacy of the reaction (Ballagi-Pordány and Belák, 1996), such as the internal standard developed by Renault et al. (2004). To assure that the extraction method used yields good quality DNA and also to evaluate the presence of inhibitory factors, universal primers that amplify DNA of the hosts can be used (Le Roux et al., 1999; Batista et al., 2005). The universal primers developed by Le Roux et al. (1999) have been successfully used to amplify DNA from several species including the oysters *C. gigas* and *O. edulis* (Audemard et al., 2002; Friedman et al., 2005). The dilution of inhibited samples provides a rapid and simple way to overcome this problem (Renault et al., 2000a). However, sample dilution can reduce the number of target molecules below the limit of the detection method. Commercial DNA purification kits procedures can also be used to remove inhibitors and at the same time concentrate the total DNA extracted (An and Fleming, 1991; Wiedbrauk et al., 1995). The addition of BSA can effectively reduce PCR inhibition (Friedman et al., 2005).

7. Final considerations

PCR has been used successfully to detect the DNA of herpesvirus infecting several species of bivalve molluscs. Although PCR specificity has been assessed for some of the primer pairs used to detect herpesvirus DNA (i.e., A3/A4, A5/A6, C1/C6,

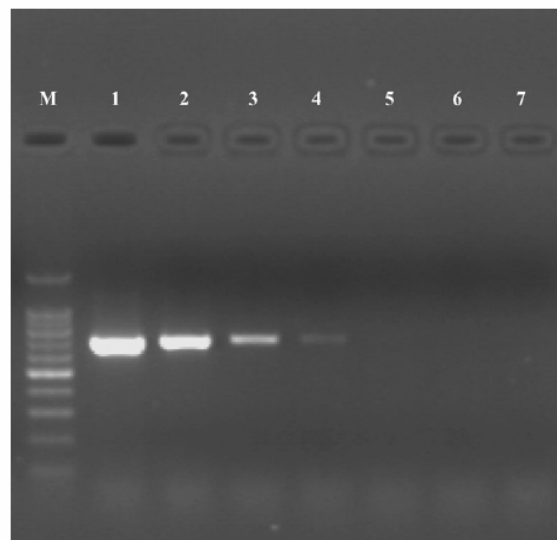


Fig. 4. Sensitivity of OsHV-1 DNA detection by PCR using the C2/C6 primer pair when 500 ng of non-infected *C. gigas* DNA was added. Results were obtained on a serial dilution (1:10) of purified viral DNA. Lane 1: 10 pg of purified viral DNA; lane 2: 1 pg of purified viral DNA; lane 3: 100 fg of purified viral DNA; lane 4: 10 fg of purified viral DNA; lane 5: 1 fg of purified viral DNA; lane 6: 100 ag of purified viral DNA; lane 7: negative control; lane M: size markers (100 bp DNA ladder, Promega).

Table 5
Recommendations for the detection of herpesvirus DNA by PCR in bivalve mollusc samples

Steps	Recommendations
Sampling	Samples should be preferably fresh frozen (-20°C , -80°C or liquid nitrogen), if not possible they can be fixed in 70% ethanol and stored at 4°C If samples are formalin-fixed (e.g., Davidson's and Carson's solutions), over-fixation should be avoided (over 24–48 h) and subsequently transferred to 70% ethanol and stored at 4°C Among the different formalin-based fixatives Davison's solution should be preferentially used in order to better preserve nucleic acids integrity
DNA extraction	Sample preparation should be done in a specific area and protocols used should be adequate to the type of material Extraction by boiling ground tissues can be used for larval samples or as below DNA extraction from spat and adult animals can be performed by proteinase K digestion followed by phenol/chloroform purification and ethanol precipitation or Qiagen extraction kits The presence of PCR inhibitors should always be checked (e.g., internal control) The quality and quantity of the nucleic acids extracted as well as the presence of inhibitory factors can be assessed throughout the use of universal primers To eliminate PCR inhibitors, dilution of the DNA preparation can be done. However, this procedure will also reduce the putative number of target molecules
PCR and primers	Positive (i.e., viral DNA) and negative (e.g., distilled water) controls should be always used. At least one negative control per five diagnostic samples should be used BSA should be added to the PCR master mix to reduce inhibition The selected primers should detect both OsVH-1 and OsHV-1var DNA The use of more than one primer pair is advantageous, such as C2/C6 and Gp3/Gp4 (several primer pairs targeting different areas of the viral genome) Since no gain in sensitivity has been documented by nested PCR procedures, its use should be avoided in order to reduce the risk of contamination
Analysis of amplicons	Confirmation of positive PCR results can be done by restriction enzyme analysis or Southern blot hybridization In each restriction enzyme analysis, amplicons obtained from reference OsHV-1 DNA should also be digested (positive control) Sequencing of PCR products can also be used to confirm the authenticity of amplicons and will provide additional information about the identity of the fragments amplified

C2/C6, C13/C5 and C14/C15), this has not been done for all primer pairs used routinely. Moreover, the amplification conditions that have been used in PCR assays using different primer pairs were based on the conditions optimized for A3/A4 and A5/A6 (Renault et al., 2000a). The sensitivity (between 1 and 10 fg of purified viral DNA) observed for the majority of the primer pairs (e.g., C2/C6, Gp3/Gp4) suggests that the PCR conditions did not have a significant negative influence on the detection limit. However, it should be noted that non-specific amplification has been reported, which may be due to sub-optimal PCR conditions (e.g., annealing temperature and Mg^{2+} concentration). For example, detection of bands with unexpected sizes was observed in PCR assays with the C2/C6 primer pair. The template DNA used for these assays was extracted from the gills (preserved in 95% ethanol) of adult oysters by digestion with proteinase K followed by phenol/chloroform purification and ethanol precipitation. However, when using an annealing temperature of 60°C (an increase of 10°C) these extraneous bands were not observed (unpublished data). Under these conditions, the minimum template quantity needed systematically to detect amplicons was 10 fg of purified viral DNA extracted from purified particles. The same sensitivity was also observed when 500 ng of DNA from non-infected *C. gigas* was added (Fig. 4). Consequently, validation and standardization of the PCR conditions for the primer pairs that are apparently more useful for epidemiological surveys should be performed. The adequacy of some of the primer pairs often used is presented

in Table 4, and practical recommendations for the detection of herpesvirus DNA by PCR in bivalve mollusc samples are given in Table 5. Development of a quantitative real-time PCR test is needed for simultaneous identification and quantification of virus copies (viral load) in hosts or environmental samples as the conventional end-point PCR tested developed to date provide qualitative data (presence or absence).

Acknowledgements

We are grateful to Dr. P. Goulletquer for facilitating part of this work at the IFREMER station in La Tremblade (Charente Maritime, France). We also thank Florence Rivet for helping in compiling some of the papers cited and two anonymous referees for their suggestions. This work was made possible by a FCT grant (SFRH/BD/8972/2002) and a Marie Curie Training fellowship (PLUDAMOR, QLK5-CT-2000-60036) to F.M.B.

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7.2. PATHOGENS, SYMBIONTS AND PATHOLOGICAL CONDITION OF *CRASSOSTREA ANGULATA*, *C. GIGAS* AND THEIR RECIPROCAL HYBRIDS GROWN IN RIA FORMOSA

7.2.1. Background and objectives

Bivalve mollusc aquaculture has adversely been affected by mass mortality outbreaks caused by diseases (Bower *et al.*, 1994). For example, the protozoan *Perkinsus marinus* has been found to be responsible for recurrent devastating mortality of *Crassostrea virginica* in the east coast of USA. Another example is the production of *Ostrea edulis* in Europe that has severely been affected by two parasitic diseases caused by *Bonamia ostreae* and *Marteilia refringens* (Renault, 1996). The uncontrolled transfers and introduction of exotic bivalve species for aquaculture purposes greatly increases the risk of introducing new pathogens (Bower *et al.*, 1994). In addition, under certain environmental conditions some organisms can become virulent and cause considerable damage to their host. Another factor that can lead to the appearance of diseases is pollution and generalized human disturbance since it can affect the defence mechanisms of bivalves (Pipe and Coles, 1995).

Ria Formosa lagoon is located in the south of Portugal and it is an important area for bivalve molluscs production. The anthropogenic impact in the lagoon is not high, but it is subjected to some pressure mainly from urban discharges as well as contamination from other sources such as tributyltin (TBT) from fishing vessels (Newton *et al.*, 2003). The main farmed species is *Ruditapes decussatus* but there is also a small production of *C. angulata*. In the 1980s, *C. gigas* was introduced in Ria Formosa and since then spat was regularly introduced from Spain and France for aquaculture purpose (F. Ruano, personal communication).

There is little information about pathogens of *C. angulata* despite the commercial importance of this taxon. Most pathological studies on *C. angulata* were conducted in France during the mass mortality outbreaks that occurred between 1967 and 1973 in order to identify causative agents (Comps, 1988). Some pathological studies have also been conducted on natural beds of *C. angulata* in Sado river (Portugal) (F. Ruano, personal communication). There is much more information available for *C. gigas* mainly due it worldwide production. This taxon is known for not having been mainly affected by epizootics of infectious diseases (Elston, 1993). However, there is no information about the zoosanitary status of *C. angulata* and *C. gigas* oysters farmed in Ria Formosa. There is also no information about the susceptibility of *C. angulata* x *C. gigas* hybrids to pathogens.

The objectives of the present study were to search and to identify pathogens, symbionts and pathological condition of *C. angulata*, *C. gigas* and their reciprocal hybrids grown in Ria Formosa (Portugal).

7.2.2. Experimental procedure

Samples

Oyster samples from *Crassostrea angulata* (AA), *C. gigas* (GG), hybrids derived from *C. angulata* females and *C. gigas* males (AG), and hybrids derived from *C. gigas* females and *C. angulata* males (GA) were collected at the end of the grow-out phase of the experiment described in Section 3.1 of Chapter 3. The number of oysters examined is shown in Table 24.

Table 24. Number of analysed oysters.

	AA	AG	GA	GG	Total
Macroscopic observation	72	78	75	75	300
Histological examination	75	76	72	65	288

Macroscopic observation

The soft tissues were examined by macroscopic observation and the following characters were monitored: watery cysts, gill lesions, colour of the digestive gland, presence and number of parasitic copepods attached to gills, and focal lesions (e.g., abnormal coloration, abscesses).

Histological examination

The oysters were opened and a transverse section (ca. 5 mm thick) through the body, containing mantle, gonad, digestive gland and gills from each specimen was fixed in Davidson's fixative and embedded in paraffin. Three to 4 µm sections were cut and stained with hematoxylin-eosin (H & E). In addition, for some specimens sections were also stained with Feulgen and Rossenbeck's stain. A third staining technique was used to determine if bacteria-like organisms observed were gram-positive or gram-negative. The histological methods used were adapted from Howard and Smith (1983).

Statistical analysis

Association between infestation with parasitic copepods and groups was analysed using G-test (2x4). Series of 2x2 G-tests were used to reveal significant differences between groups after correction for multiple testing using the sequential Bonferroni method (Rice,

1989). The non-parametric Kruskal-Wallis test was used to determine if number of copepods attached in gills (infestation intensity) differed significantly among groups. The Nemenyi test was used for multiple comparisons when significant differences were identified with the Kruskal-Wallis test as described by Zar (1996).

7.2.3. Results and discussion

The macroscopic observation revealed gill lesions in 8.3, 15.4, 8.0 and 18.7 % oysters of the AA, AG, GA and GG groups, respectively. Watery cysts were observed at low frequency (between 0 and 6.1 %). The digestive gland of the majority of the oysters examined presented a normal coloration.

The histological examination revealed the occurrence of hemocytic infiltrations, gill lesions, metazoan parasites, ciliates, bacterial and virus-like lesions. A higher parasite (ciliates and copepods) load was observed in *C. angulata* and hybrids oysters in comparison with *C. gigas* oysters (Table 25).

Table 25. Prevalence of pathogens, symbionts and pathological condition of oysters from the AA, AG, GA and GG groups grown in Ria Formosa (Portugal).

	AA	AG	GA	GG
Hemocytic infiltration	1/75 (1.3 %)	0/76 (0.0 %)	2/72 (2.8 %)	1/65 (1.5 %)
Gill lesion	2/75 (2.7 %)	0/76 (0.0 %)	4/72 (5.5 %)	0/65 (0.0 %)
Copepods in gill	7/75 (9.3 %)	5/76 (6.6 %)	6/72 (8.3 %)	0/65 (0.0 %)
Copepods in intestine	3/75 (4.0 %)	2/76 (2.6 %)	1/72 (1.4 %)	0/65 (0.0 %)
Ciliates	14/75 (18.7 %)	12/76 (15.8 %)	6/72 (8.3 %)	0/65 (0.0 %)
Bacterial infections	0/75 (0.0 %)	0/76 (0.0 %)	1/72 (1.4 %)	0/65 (0.0 %)
Virus-like lesions	0/75 (0.0 %)	1/76 (1.3 %)	0/72 (0.0 %)	0/65 (0.0 %)

Virus-like lesions

Basophilic inclusions with perinuclear condensed material were observed in the gonad tubules of one GA hybrid specimen. The centre of the inclusions was moderately Feulgen-positive and the perinuclear material was strongly Feulgen-positive, indicating the presence of DNA (Figure 50). The hypertrophied cells observed in the present study varied in size and shape, and it was not possible to distinguish their origin as either residual gametes or germinal epithelia.



Figure 50. Intranuclear inclusions in the gonoducts of a GA hybrid specimen. Feulgen-stained histological section. Arrow had indicate perinuclear material strongly Feulgen-positive.

The cytopathological changes previously described are generally associated with papova-like virus infections and are known by viral gametocytic hypertrophy (VGH). VGH has been described in several bivalve species, including *C. virginica*, *Ruditapes phillipinarum*, *Mya arenaria*, *Pinctada maxima*, *Ostrea edulis*, *Saccostrea glomerata*, *C. rhizophorae*, *O. conchaphila* and *C. gigas* (Elston, 1997; Montes *et al.*, 2001; Choi *et al.*, 2004). However, the presence of virus particles has only been confirmed by transmission electronic microscopy (TEM) in some of these species. In the case of *C. gigas*, two studies reported the presence of papova-like virus particles associated with lesions similar to the ones observed in the present work (Choi *et al.*, 2004; Garcia *et al.*, 2006). In the present study, VGH was only detected in one out of 76 AG hybrids analyzed and the infection intensity was less than ten infected cells per histological section. This is in agreement with Garcia *et al.* (2006) that observed a low VGH prevalence (between 3.3 and 13.3 %) and a low infection intensity (an average of 16 infected cells per histological section) in *C. gigas*. Choi *et al.* (2004) also observed a low prevalence of VGH in *C. gigas* (between 3.3. and 7.1 %).

It is thought that VGH has no lethal impact on oysters and the host response to infection is negligible (Bower *et al.*, 1994). Nevertheless, VGH may have a negative impact on oyster reproduction and its oncongenic impact (like in some members of the *Papovaviridae* family) remains unknown. The results of the present study suggest that VGH is present in southern Portugal and it can also affect *C. angulata* x *C. gigas* hybrids.

Bacterial organisms

An abnormal strong heart beating as well as white abscesses on the tissue surface were observed in a single AG hybrid. The heart of this oyster was grossly enlarged. During a survey of *C. angulata* from Sado river (samples collected in November 2005 from which the heart of 30 specimens were macroscopically and microscopically analysed), an oyster with similar gross signs was observed. An image of its heart is shown in Figure 51 to illustrate the abscesses observed in the AG hybrid specimen.

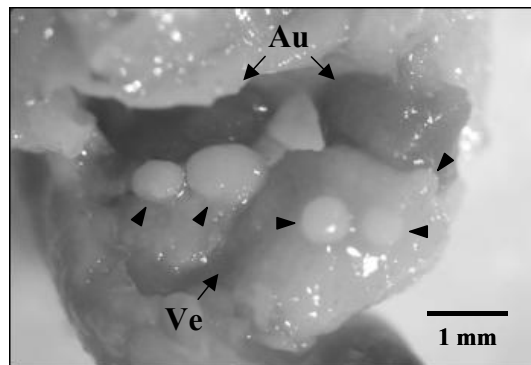


Figure 51. Heart of an adult *C. angulata* specimen captured in Sado river in December 2005 with bacterial heart infection (BHI). Arrowheads show white abscesses on the ventricle (Ve) whereas no abscesses were observed in the auricles (Au).

Colonies of bacteria with a diameter between 14 and 153 μm were observed histologically in the heart, gills, digestive gland and connective tissues of the AG hybrid specimen (Figure 52). Bacteria around 1-3 μm in size, were very lightly Feulgen-positive in the center and stained Gram-negative. This infection was called “bacterial heart infection” (BHI) and the “poor” condition (*i.e.*, grossly enlarged heart and weakness of the adductor muscle) of the infected oysters suggest that it may be lethal.

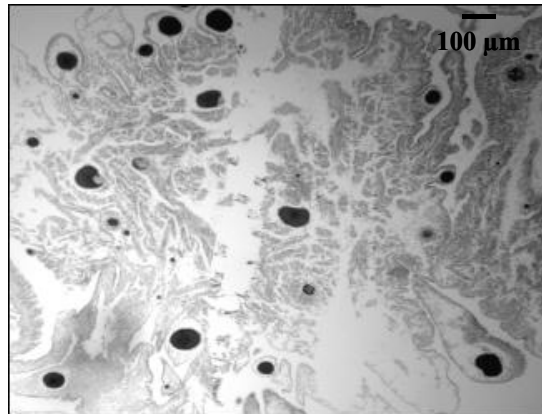


Figure 52. Colonies of bacteria observed in heart of an AG hybrid specimen. H & E staining.

More than 80 % of the colonies were found in the heart and within this organ the large majority of the colonies were observed in the auricles (Figure 53).

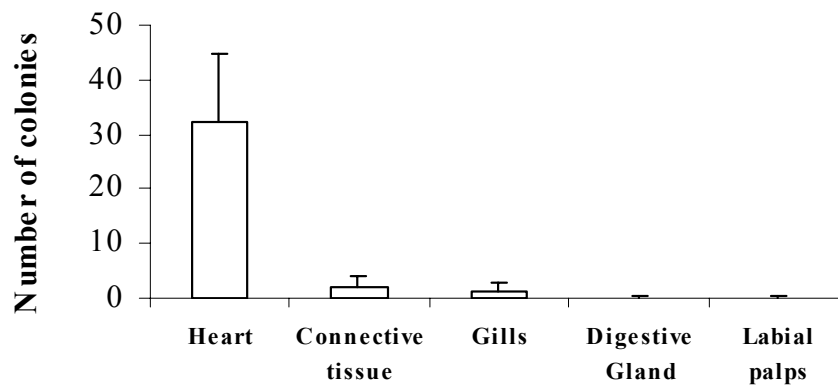


Figure 53. Mean number of colonies of bacterium observed in different tissues per histological section (n = 6). The error bars represents the standard deviation.

There was a moderate to strong inflammatory response and in some colonies it was possible to observe an intense phagocytic activity (Figure 54).

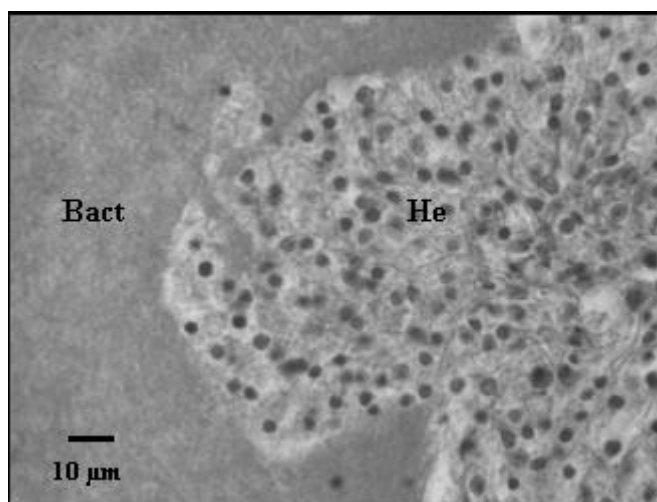


Figure 54. Colony of bacteria being engulfed and destroyed by hemocytes. H & E staining. Bact, bacteria; He, hemocytes.

Tubiash *et al.* (1973) observed *C. virginica* specimens with enlarged and edematous pericardia associated with Gram-negative bacteria identical to *Vibrio anguillarum*. However, no abscesses were reported and histology examination revealed no abnormalities other than the cardiac involvement. This syndrome was named “cardiac edema” by Tubiash *et al.* (1973). Although apparently both types of bacterial infections (BHI and “cardiac edema”) affect the heart of their host, they differ in their pathogenic effects as described above. Friedman *et al.* (1991) reported a bacteriosis caused by *Nocardia crassostreae* in which abscesses were also observed in the tissue surface. However, the abscesses caused by *N. crassostreae* had a yellow coloration and the bacterium stained Gram-positive. The prevalence of BHI observed in the present study was low (1.3 %). However, it is noteworthy that the heart of the majority of the oysters was not analysed, which may have resulted in a under estimation of BHI.

Protozoans

Ciliates (*Ancistrocoma*-like, *Sphenophrya*-like and *Rynchodid*-like ciliates) were observed in the gills, mantle and digestive gland of oysters of the AA, AG and GA groups, but not in GG group. *Ancistrocoma*-like ciliates were observed in the lumen of the digestive gland tubules and ducts of the digestive gland. These protozoans had large, granular and polymorphic nuclei. *Sphenophrya*-like ciliates were found attached to the mantle. These ciliates had a pear-shape with a large and densely basophilic nucleus (Figure 55).

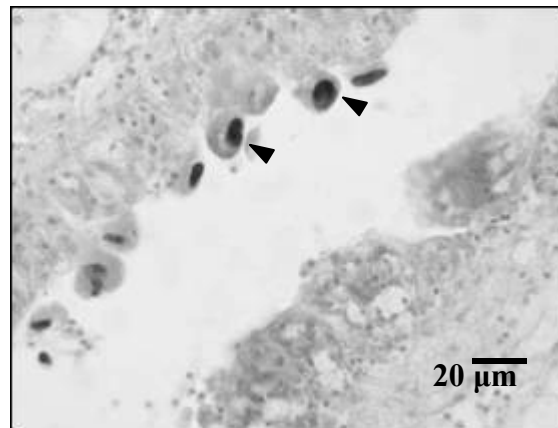


Figure 55. *Sphenophrya*-like ciliates attached to the mantle epithelium of *C. angulata* (arrowheads). H & E staining.

The third type of ciliates observed was *Rynchodid*-like and were observed in gills. They had a spindle-shape with a polymorphic basophilic macronucleus and a spherical micronucleus that stain less than the macronucleus (Figure 56).

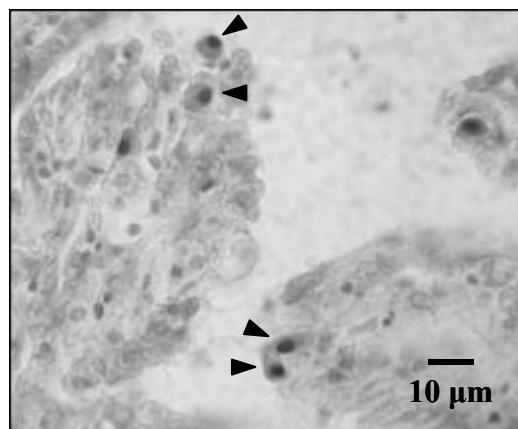


Figure 56. *Rynchodid*-like ciliates attached to the gills of *C. angulata* (arrowheads). H & E staining.

The three types of ciliates observed in the present study did not cause apparently any pathological damage and infections were light (less than 30 ciliates per histological section).

Metazoans

The parasitic copepods attached to the gills of the oysters from the four groups were identified as belonging to the species *Myicola ostreae* based on the description by Hoshina and Sugiura (1953) (Figures 57 and 58).

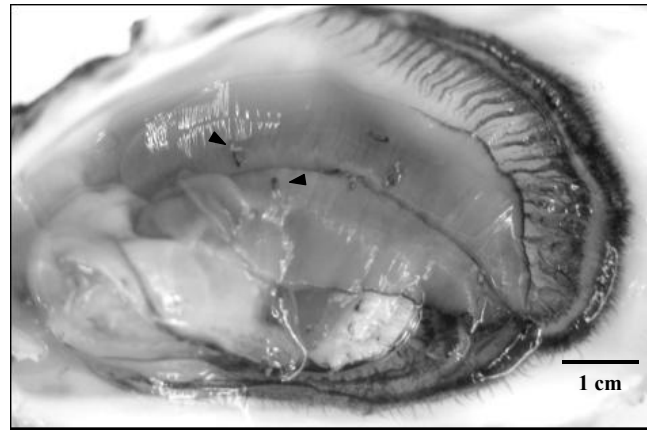


Figure 57. Adult females of *Myicola ostreae* (arrowheads) attached to the gills of *C. angulata*.

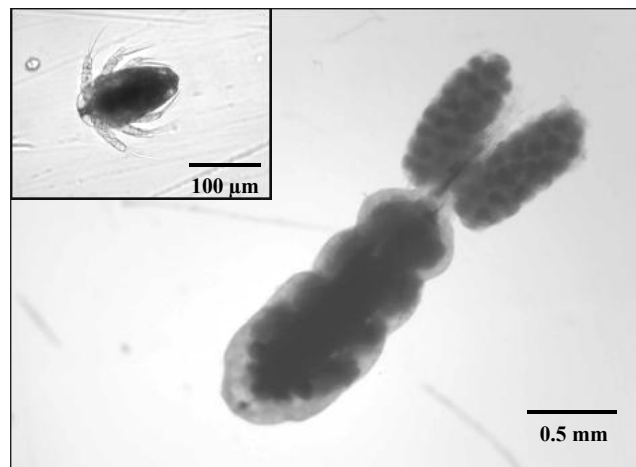


Figure 58. Adult female of *M. ostreae*. A free-swimming *Nauplius* is shown in the top left corner.

No significant relationship was observed between the presence of *M. ostreae* and gill lesions observed by macroscopic examination (G-test, $p > 0.05$). However, the site in the gills where *M. ostreae* was attached had a yellow/green colouration which could be associated with a haemocyte infiltration. The histological examination also revealed copepods in the gills that were interpreted as being *M. ostreae* based on the

macroscopic identification (Figure 59). Moreover, hemocytic infiltrations were observed near the region where the copepods were attached (Figure 59).



Figure 59. A section of the gills of *C. angulata* showing a *M. ostreae* attached to gills (arrowhead) and haemocyte infiltration near that region (arrow). H & E staining.

Some *M. ostreae* attached to gills of *C. gigas* specimens where engulfed by cells (Figure 60). The haemocyte-like cells accumulation appeared to be a defence mechanism of the oyster. This reaction was not observed in oysters from the other three groups (AA, AG and GA).

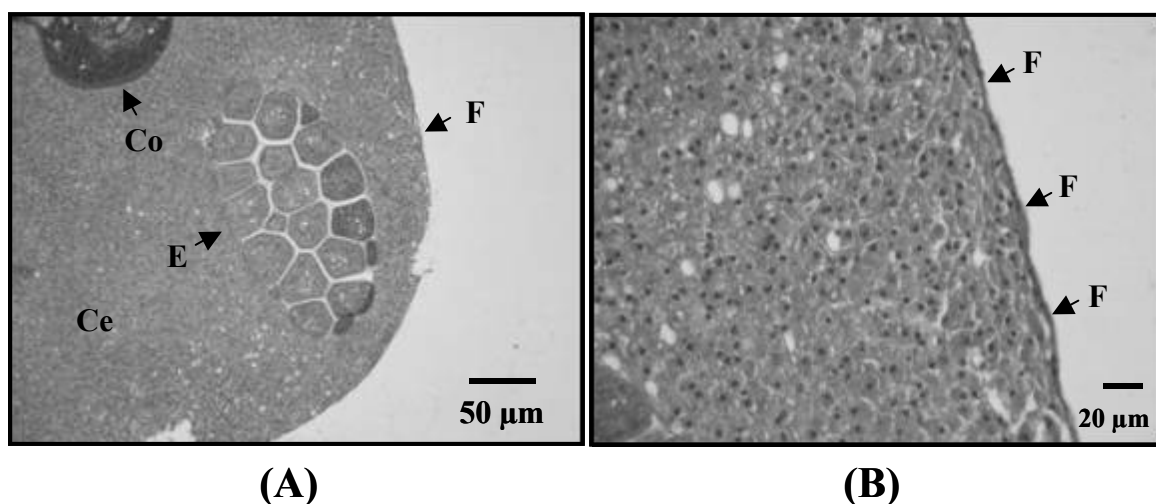


Figure 60. A section of an adult female *M. ostreae* observed in the gills of *C. gigas* involved with haemocyte-like cells (A). Magnification of the cells surrounding the copepod (B). Co, *M. ostreae*; E, copepod eggs; Ce, haemocyte-like cells; F, cell-like wall. H & E staining.

In *C. angulata* progeny, the prevalence of *M. ostreae* was 75.0 % (Figure 61A) whereas in AG and GA hybrids a slightly lower prevalence was observed (64.1 and 55.1 %, respectively), but not significantly different from *C. angulata* progeny (G-test; $p > 0.05$). A significantly lower prevalence (G-test. $p < 0.01$) was observed in *C. gigas* progeny (20.0 %) comparatively to AA, AG and GA groups. Significant differences in the infestation intensity were also observed among groups (Kruskal-Wallis test, $p < 0.01$). Pairwise comparisons revealed no significant differences in infestation intensity between AA, AG and GA groups (Nemenyi, $p > 0.05$). The number of *M. ostreae* per oyster in *C. angulata* progeny ranged from 1 to 13, and in both AG and GA hybrids the number of copepods ranged between 1 and 8. However, the infestation intensity was significantly lower (Nemenyi, $p < 0.05$) in *C. gigas* (between 1 and 5 copepods per oyster) in comparison with the other groups (Figure 61B).

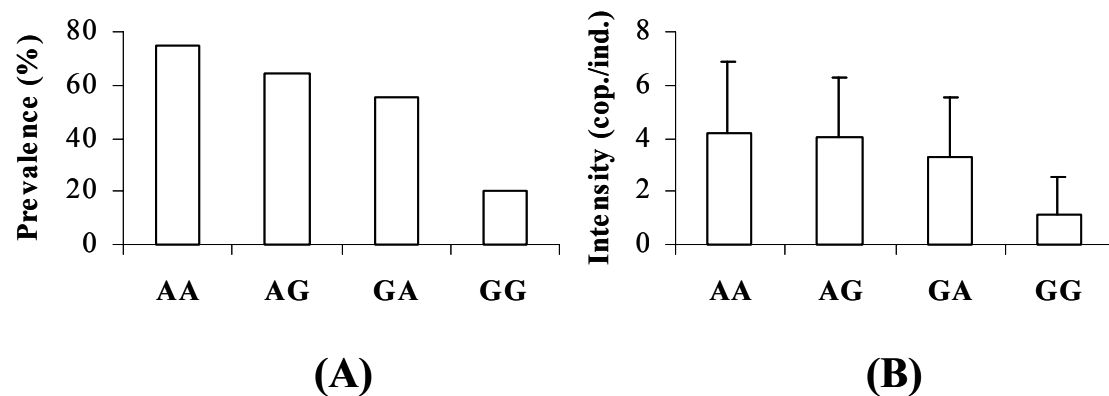


Figure 61. *M. ostreae* prevalence (A) and mean infestation intensity (B) observed in AA, AG, GA and GG groups.

Comps (1972) reported the presence of a copepod attached to the gills of *C. angulata* in Marennes-Oléron bay (Charente Maritime, France). According to His (1979) the copepod observed by Comps was *M. ostreae* and it was also observed in *C. gigas* in 1977 in Arcachon Bay (France). It is presumed that *M. ostreae* was introduced together with the parasitic copepod *Mytilicola orientalis* from Japan during the introduction of *C. gigas* into France (His, 1979). His (1979) reported a prevalence (between 4 and 40 %) and an infestation intensity (between 1 and 4 copepods per oyster) of *M. ostreae* in *C. gigas* close to the results obtained in the present work. The results of the present study are in agreement with Comps (1972) which reported gill lesions in the region where copepods were attached. Although the damage done by a single copepod may be unimportant, high infestation intensities may have some impact on oyster populations (Comps, 1972). In addition, gill lesions caused by *M. ostreae* even if small can facilitate the infection by other pathogenic organisms. Moreover, it has recently been suggested that ectoparasites can immunosuppress its invertebrate host (Yang and Cox-Foster, 2005).

His (1972) observed that *C. gigas* had a higher clearance rate than *C. angulata* as well as higher valve activity. These results suggest that the former taxon can filter a higher volume of water than the later taxon, increasing the chances of capturing *M. ostreae* nauplius. However, a lower prevalence and infestation intensity was observed in *C. gigas* comparatively to *C. angulata*, indicating that *C. angulata* is more susceptible to *M. ostreae*. The reciprocal hybrids were intermediate in terms of prevalence and infestation intensity although skewed toward *C. angulata* progeny. It is noteworthy that *M. ostreae* was never observed in natural beds of *C. angulata* in Sado and Mira estuaries (F. Ruano,

personal communication), which suggest that this parasitic copepod may have been introduced into Ria Formosa with the introduction of *C. gigas*.

Mytilicola-like parasites were observed in the intestine of *C. angulata* progeny as well as in the intestine of both reciprocal hybrids (AG and GA). Two oysters (a *C. angulata* specimen and an AG hybrid specimen) were simultaneously infected with *M. ostreae* and *Mytilicola*-like copepods. Lesions in the digestive epithelium of the infected oysters were observed near the site of *Mytilicola*-like copepods attachment.

The comparison of the defence mechanism of *C. angulata* and *C. gigas* can contribute to a better understanding of the higher resistance of *C. gigas* observed in the present study but also to irido-like viruses mentioned above.

7.3. CONCLUSIONS

OsHV-1 DNA was detected by PCR in *C. angulata* larval batches in which high mortality rates were observed. Although Arzul *et al.* (2001a) had already demonstrated the susceptibility of *C. angulata* larvae to OsHV-1 infections throughout transmission assays, this is the first record of a natural outbreak of OsHV-1 during the larval rearing of *C. angulata*. These results suggest that OsHV-1 may be the causative agent of recurrent high mortalities that have been observed during larval rearing of *C. angulata* (data not shown). In addition, the virus was also detected in AG and GA hybrid larvae associated with high mortality rates. Moreover, the analysis of larval samples by PCR using different DNA extraction methods, primers and PCR strategies allowed the following methodological findings:

- PCR inhibition was observed in larvae with one day after fertilization, which highlights the need to check for inhibition specially at early development stages;
- A higher percentage of PCR inhibition was observed in samples in which DNA was extracted from fixed material using combination of proteinase K/Tween 20 digestion followed by heating comparatively to a method where the last step was replaced by a phenol/chloroform extraction;
- Some primer pairs (e.g., C2/C6 and Gp3/Gp4) proved to be more suitable for epidemiological surveys than others (e.g., C13/C5, B3/B4 and C9/C10).

From an epidemiological point of view, the major findings of the present study were:

- OsHV-1 was detected in a sample collected from a batch of larvae that managed to settle, metamorphose and reach sexual maturity. However, it is not known if surviving oysters were infected;
- The high mortality rates as well as the detection of OsHV-1 did not occurred in synchronous way in the different tanks, suggesting that the larvae were infected at different moments (assuming horizontal transmission) or the conditions that triggered viral replication and onset of disease did not occurred at the same time in all tanks (assuming that the larvae were already infected).

The results of the detection of OsHV-1 DNA in samples from *C. angulata* and *C. gigas* broodstock were inconclusive since the storage conditions (*i.e.*, material fixed in ethanol and fixed in Davidson's fixative) may have reduced the quantity and quality of viral DNA. Hence, the results obtained in the present work cannot be use to support or undermine the vertical transmission hypothesis (Le Deuff *et al.*, 1996; Arzul *et al.*, 2002; Barbosa-Solomieu *et al.*, 2005).

The main findings from the zoosanitary analyses performed in samples of *C. angulata*, *C. gigas* and their reciprocal hybrids grown in Ria Formosa (Portugal) were:

- A higher parasite load (protozoans and metazoans) was observed in *C. angulata* and in the reciprocal hybrids in comparison with *C. gigas*;
- A significantly higher prevalence and infestation intensity of the copepod *M. ostreae* was observed in *C. angulata* and in the reciprocal hybrids comparatively to *C. gigas*;
- A new bacterial infection named “bacterial heart infection” that mainly affects the heart was observed in an AG hybrid specimen;
- Cytopathological changes associated with viral gametocytic hypertrophy were observed in an GA hybrid specimen.

FINAL CONSIDERATIONS

The Portuguese oyster *Crassostrea angulata* was a species of major economic importance in Europe up to the early 1970's, when mass mortalities almost led to its disappearance. In order to counteract the crisis and sustain the European oyster farming industry, the Pacific oyster *C. gigas* was introduced. Despite the successful introduction of *C. gigas* and its good aquacultural performance in terms of growth rate and disease resistance, this species might not be fully adapted to its environment as it notably suffers from summer mortality, a phenomenon notably related to reproductive allocation (Degremont *et al.*, 2007). Moreover, since the European oyster farming industry is mainly based on the production of *C. gigas*, diversification is desirable. Nowadays, there are only a few remaining populations of *C. angulata* located mainly in southern Europe (Spain and Portugal). Hence, these populations may constitute an important genetic resource for the European oyster farming industry. However, these *C. angulata* populations are endangered by the expansion of *C. gigas* aquaculture (Huvet *et al.*, 2000; 2004). Moreover, some *C. angulata* populations (e.g., from Sado River) experienced in the last decades a considerable reduction in population size due to pollution and putative diseases, but in the last years they managed to recover (Ruano, 1997). The phenotypic and genetic consequences of this reduction of population size remains unknown. In addition, it is also not known if these populations are still susceptible to the putative pathogenic agents responsible for the mass mortalities that occurred in the early 1970's. On the other hand, although some trials were conducted in France to evaluate the aquacultural potential of *C. angulata* x *C. gigas* hybrids (Bougrier *et al.*, 1986; Soletchnik *et al.*, 2002) there was no information available in Portugal. In this context, the general aim of this thesis was to evaluate the aquacultural potential of some of the remaining populations of *C. angulata* in comparison with *C. gigas* (being cultured in Charente Maritime, France) as well as with their reciprocal hybrids.

The development of a DNA mitochondrial (mtDNA) marker that differentiate *C. angulata* from *C. gigas* populations (Boudry *et al.*, 1998) allowed the confirmation that there were still "pure" *C. angulata* populations in Spain and Portugal. Even though mitochondrial genome is maternally inherited, populations in which only *C. angulata* haplotypes are observed can be considered as "pure" populations, since no *C. gigas* could have taken part in recruitment as female parents. Moreover, since cupped oysters are alternative hermaphrodites, the absence of *C. gigas* haplotypes indicate that no *C. gigas* females/males took part in recruitment. However, large sample sizes should be used to ensure that *C. gigas* haplotypes can be detected if present at low frequencies. In the present study, the analysis of a large sample of cupped oysters from Mira and Sado

Rivers (109 individuals per population) revealed that all the individuals scored had *C. angulata* haplotypes. **These findings indicate that these *C. angulata* populations remain “pure” and hence they can be used for future breeding and conservation programs for this species.** In addition, a higher genetic variability for a mitochondrial gene was observed in a *C. angulata* population from Sado River comparatively to a *C. gigas* population from Seudre River. However, the differences observed at the mtDNA level may not reflect a lower nuclear DNA variability in the latter taxon. Indeed, a very high genetic variability for nuclear DNA was recently observed for *C. gigas* oysters from the same region (P. Boudry, personal communication). Indeed, several studies presented evidence for non neutral evolution of animal mtDNA (Gerber *et al.*, 2001). These may explain the lower nucleotide sequence variation observed in the *C. gigas* populations and hence indicate that mitochondrial genes of this species may be under selective pressure. Nevertheless, the results of the present study suggest that **there is no evidence for reduced genetic variation in the *C. angulata* population from Sado River.** However, further studies should be performed to confirm the previous findings using nuclear markers.

During both nursery and grow-out (in Ria Formosa, Portugal) phases, a higher growth and survival was observed in *C. gigas* progeny in comparison to *C. angulata* progeny. These findings are in agreement with the results obtained by other authors (Bougrier *et al.*, 1986; Huvet, 2000; Soletchnik *et al.*, 2002). However, it is noteworthy that the rearing conditions during the nursery phase may have been more favourable to *C. gigas* than to *C. angulata*, since oysters were grown using protocols design for *C. gigas*. Moreover, the rearing conditions during the grow-out phase were very similar to the ones used in previous studies (Bougrier *et al.*, 1986; Soletchnik *et al.*, 2002), which may also have been more favourable to *C. gigas* production. The optimal rearing conditions (*e.g.*, salinity, temperature, type and amount of food, emersion time) for *C. angulata* should be determine in order to better evaluate its aquacultural potential and optimise the production of this species. Both reciprocal hybrids of *C. angulata* and *C. gigas* had a performance in terms of growth rate and survival similar or inferior to the best parental line and hence no hybrid vigour was observed. On the other hand, **a higher proportion of aneuploid cells and missing chromosomes were observed in *C. angulata* than in *C. gigas* progenies.** Both parameters were negatively correlated with growth rate, which suggest that karyological disorders may explain the faster growth rate observed in *C. gigas* comparatively to *C. angulata*. Alternatively, the higher aneuploidy observed in *C. angulata* progeny in comparison with *C. gigas* progeny may have been caused by a weaker physiological condition of the latter taxon caused by adverse environmental conditions. The assessment of aneuploidy levels under different culture conditions may shed more on

light on the causes/effects of these karyological disorders. On the other hand, both reciprocal hybrids showed intermediate aneuploidy levels which support an additive genetic basis for this phenomenon. The results of the present study also suggest that there are **genetically-based differences in terms of shell morphology and pigmentation between *C. angulata* and *C. gigas***. Their reciprocal hybrids showed intermediate values for most of the characters analyzed, which support an additive genetic basis for these characters. However, a similar work should be done using progenitors from several *C. gigas* and *C. angulata* populations to confirm that the differences observed are taxon specific and not only population specific. Nevertheless, the differences observed in shell morphology (e.g., shell height to depth relationship) in the progenies studied suggest that the genetically-based differences for these traits may be used in genetic improvement programs to produce oyster with a more favourable shell shape. Moreover, some characteristics (e.g., yellow coloration) can be used to provide product label identification. However, genetic correlation between those characteristics (e.g., shell coloration) and other commercial important traits (e.g., growth rate, survival) should be studied before considering a selective breeding program.

Natural recruitment of *C. angulata*, *C. gigas*, *Ostreola stentina* and *Ostrea edulis* was observed both at Cacela-Velha and Tavira (Ria Formosa). An overlap in recruitment pattern in these taxa was observed during some periods of the setting season. This can be seen as negative aspects for the collection of cupped oysters in Ria Formosa since *O. stentina* will compete for collectors space. However, if collectors are placed at high tidal levels for some days after setting, the putative number of *O. stentina* can be drastically reduced, since this species has a lower resistance to air exposure comparatively to *C. angulata* and *C. gigas* (data not shown). Moreover, the external shell morphology of *O. stentina* is very similar to cupped oysters leading to a miss classification and consequently an incorrect estimation of recruitment. The analysis of haplotype frequencies and cytonuclear disequilibrium at each settlement period in both sites suggest that hybrids settled during almost all setting season. Moreover, the results obtained also suggest that ***C. angulata* and *C. gigas* recruitment overlap during most of the setting season**. These results support previous findings that there are no major reproductive barriers between the two taxa (Huvet *et al.*, 2004). These results also indicate the two taxa cannot be selectively collected in Ria Formosa by setting collectors at different periods. Consequently, the results obtained in the present study suggest that the recruitment of cupped oysters in Ria Formosa during most part of the year is a mixture between *C. angulata*, *C. gigas* and their hybrids. However, different haplotypes and allelic frequencies were observed for almost all setting periods. A higher proportion of individuals scored for markers associated with *C. gigas* were observed at the beginning of the setting

season, but the opposite was observed by the end of the season. These findings suggest that **genetic variation in timing of gonadal development and/or spawning may exist between the two taxa**, which can lead to partial temporal reproductive isolation. Another hypothesis is that the response to the environmental cues that trigger settlement behaviour may be different between *C. angulata* and *C. gigas*. The differences observed in the haplotype and allelic frequencies can also be explained by different environmental requirements or potential habitat selection during larval and/or spat stages of the two taxa.

The development of DNA extraction method from small samples allowed a better detection by PCR of OsHV-1 DNA. A considerable reduction in the amount of larvae required for detection of OsHV-1 comparatively to the method developed by Renault *et al.* (2000) was achieved (3 versus 50 mg). Hence, this method can be useful for detection of OsHV-1 when only small amounts of larvae are available. The adequacy of this method to detect OsHV-1 DNA in single larva should be tested, which can open new perspectives in studies concerning the transmission of the virus.

The trials conducted to detect irido-like virus DNA by PCR in *C. angulata* and *C. gigas* were unsuccessful. However, the method developed in the present study to extract DNA from archival Bouin fixed samples may allow further retrospective analyses. Recently, DNA from an irido-like virus infecting a *Nautilus* sp. specimen was successfully amplified and its nucleotide sequence determined (Gregory *et al.*, 2006). The sequence data reported by the previous authors as well as the experimental approach used (e.g., primer pairs, PCR conditions) can be useful to study oysters infected by irido-like viruses. It is noteworthy that no cytopathological changes characteristic of irido-like virus infections were observed in the present study in oysters from the different groups cultured in Ria Formosa. Indeed, despite several trials to detect the irido-like viruses by several laboratories (T. Renault, personal communication) this type of viruses were observed by the last time approximately two decades (Bougrier *et al.*, 1986). Hence, **further study should be performed to develop more sensitive tools to detect irido-virus DNA.** On the other hand, the question about the putative resistance of *C. angulata* populations remains unsolved.

An outbreak of OsHV-1 was detected during the larval rearing of *C. angulata*, *C. gigas* and their reciprocal hybrids. During the present work several trials were conducted to rear *C. angulata*, but the majority were unsuccessful due to high mortality rates (data not shown). This may have been caused by OsHV-1 infections and hence the analyses to detect this virus should be performed in further larval rearing trials of *C. angulata*. The zoosanitary analyses performed in samples of *C. angulata*, *C. gigas* and their reciprocal hybrids grown in Ria Formosa also revealed the presence of other pathogens and symbionts. **A higher parasite load (protozoans and metazoans) was observed in C.**

***angulata* and in the reciprocal hybrids in comparison with *C. gigas*.** These results suggest that *C. angulata* is more susceptible to parasites than *C. gigas*. A higher prevalence and infestation intensity by the parasitic copepod *Myicola ostreae* was observed in *C. angulata* progeny comparatively with *C. gigas* progeny. Moreover, the non observation of these copepod in *C. angulata* in Mira and Sado Rivers (F. Ruano, personal communication) suggest that it has been introduced in Ria Formosa with *C. gigas*. A putative host reaction to *M. ostreae* infestation was observed in some *C. gigas* specimens in which copepods were observed engulfed by haemocyte and fibro-like cells. These putative cellular host reaction is very different from other previous cellular responses described in mollusc bivalves. Hence, it should be further characterized and its importance assessed by using *M. ostreae* and *C. gigas/C. angulata* as species model. Although infestation by *M. ostreae* may have little impact on its host, there presence in high number in oyster gills may have a negative impact on consumers since the copepods can be easily observed reducing product quality. On the other hand, **a new bacterial infection named “bacterial heart infection” (BHI) that mainly affects the heart was observed in a *C. angulata* x *C. gigas* hybrid specimen.** A similar infection was also observed in a *C. angulata* specimen collected in Sado River. The pathogen that causes BHI should be characterized and its pathogenicity assessed. Moreover, the prevalence of BHI should be assessed in Sado River as well as in other regions where *C. angulata* is present.

A better aquacultural performance in terms of growth rate, survival and parasites resistance was observe in *C. gigas* comparatively with *C. angulata*. With exception of *C. angulata* shell shape no other traits of interest were observed comparatively with *C. gigas*. Other traits should also be investigated in *C. angulata* in comparison with *C. gigas* such meat flavour and stress resistance.

The results obtained in this thesis highlighted the genetic-based differences between *C. angulata* and *C. gigas*, which supports the distinctness of the two taxa. Hence, these taxa cannot be considered synonymous. The remaining populations of *C. angulata* located on Southern Portugal are endangered by the expansion of *C. gigas* aquaculture, since the areas where they are located have good conditions for cupped oysters farming and *C. gigas* seed is easily available. On the contrary, *C. angulata* seed is not available neither from natural collection nor from commercial hatcheries. In this context, farmers are impelled to introduce and culture *C. gigas* which endangered remaining populations of *C. angulata*. Moreover, the results obtained in this thesis support previous evidence of natural hybridization between the two taxa. In this context, **measures should be implemented to prevent the introduction of *C. gigas* in areas**

where “pure” *C. angulata* populations still exist (where no commercial oyster production is currently performed on a large scale).

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Article 6 (published in The 1st International oyster symposium proceedings)

かき研究所 ニュース No.18

The 1st International Oyster Symposium Proceedings**The taxonomic status and origin of the Portuguese oyster*****Crassostrea angulata* (Lamarck, 1819)**Frederico M. Batista ^{1,2}, Alexandra Leitão ^{1,3}, Arnaud Huvet ⁴, Sylvie Lapègue ⁵, Serge Heurtebise ⁵ and Pierre Boudry ⁵¹ Instituto Nacional de Investigação Agrária e das Pescas (INIAP/IPIMAR), CRIPSul, Av. 5 de Outubro, 8700-305 Olhão, Portugal.² Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Universidade do Porto, Largo Prof. Abel Salazar, 2, 4099-003 Porto, Portugal.³ Departamento de Genética e Biotecnologia, Centro de Genética e Biotecnologia da Universidade de Trás-os-Montes e Alto Douro, CGB/UTAD, P-5000-911 Vila Real, Portugal.⁴ Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER), Unité Mixte de Recherche Physiologie et Ecophysiologie des Mollusques Marins, Centre de Brest, B.P. 70, 29280 Plouzané, France⁵ Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER), Laboratoire de Génétique et Pathologie (LGP), 17390 La Tremblade, France**Abstract**

The taxonomic status of the Portuguese oyster *Crassostrea angulata* (Lamarck, 1819) and the Pacific oyster *C. gigas* (Thunberg, 1793) has often been a matter of controversy. Based on larval shell morphology, experimental hybridization and electrophoretic studies of enzyme polymorphism several authors have considered these two species as being synonymous. During the recent years, several genetic studies based on mitochondrial DNA and microsatellites data have provided evidences that the two taxa are genetically distinct although closely related. Furthermore, karyotype analysis has also supported the close genetic similarity of these taxa in comparison with other cupped oyster species. However, a recent comparative analysis of restriction enzymes banding patterns highlighted differences between all chromosomes pairs of *C. angulata* and *C. gigas* with the exception of chromosomal pair 10. In addition, significant phenotypic differences between the two taxa were observed in terms of aquaculture production and eco-physiological characteristics. The two cupped oyster *C. angulata* and *C. gigas* were long assumed to be native to the northeastern Atlantic and Asia, respectively. Different hypotheses have been proposed to explain the apparent geographically distant distribution of these close related taxa. Phylogenetic analysis firmly places both Portuguese and Pacific oysters within an Asian *Crassostrea* clade supporting the hypothesis of the introduction of *C. angulata* from Asia to Europe. Pure populations of *C. angulata* were observed in Taiwan as well as presumed mixed populations of *C. angulata* and *C. gigas* in Northern China. Remarkably, using equivalent amount of research on both taxa, the level of genetic variability of *C. gigas*

(samples collected in different parts of the world) appears to be lower than that of *C. angulata* (samples collected in Portugal) based on cytochrome oxidase C subunit I haplotypes. This difference could be related to the dissemination of *C. gigas* mainly from Miyagi Prefecture to different regions in Japan and other places in the world where the Pacific oyster was introduced. These findings suggest that (1) *C. angulata* and *C. gigas* are very close related, but they cannot be considered synonymous and (2) the Portuguese oyster has an Asian origin.

Introduction

Cupped oysters belonging to the genus *Crassostrea* are among the most important commercial aquatic species in the world. These species have been harvested from the wild and cultivated since centuries. Among them we can find the Portuguese oyster *Crassostrea angulata* (Lamarck, 1819) and the Pacific oyster *C. gigas* (Thunberg, 1793). The two cupped oysters *C. angulata* and *C. gigas* were long assumed to be native of the northeastern Atlantic and Asia, respectively. The Portuguese oyster was a species of major economic importance in Europe from the late 19th century up to the early 1970's, sustaining the European oyster production for almost one century. Major mortalities between 1967 and 1973 almost wiped out the Portuguese oyster from Europe (Comps, 1988). The Pacific oyster was introduced in different regions and is presently farmed throughout the Americas, Africa, Australia, Europe, and Asia and is the marine organism with the highest annual aquacultural production in the world (FAO, 2005).

The taxonomic status of the Portuguese and the Pacific oysters has often been a matter of controversy. The main reason of their distinction into two different species was their separated geographical

distribution whereas some data (morphological, experimental hybridization or genetic) have suggested the two taxa as being synonymous. The objective of the present paper is to provide a review of the published studies about *C. angulata* and *C. gigas* that can help to clarify their relative taxonomic status and the origin of the Portuguese oyster. It is also discussed the role that *C. angulata* may have for the future development of the oyster production industry.

Morphological analysis

Classification of oysters based only on morphological analysis can be problematic, especially in the genus *Crassostrea*, due to the high variation and plasticity that is strongly influenced by environmental conditions (e.g., Galtsoff, 1964). Although some adult shell features can be used in the classification of *Crassostrea* species their utility is limited, especially in case of closely related taxa. Other morphological characteristics, like the morphology of late larval shells (Hu *et al.*, 1993) and anatomic features (Wang *et al.*, 2004), are usually considered more useful taxonomic characteristics. According to several authors the adult shells of *C. angulata* and *C. gigas* are indistinguishable (Menzel, 1974; Biocca and Matta, 1982) albeit no morphometric analysis were performed and the criteria's used to differentiate them were not specified. Furthermore, Ranson (1960) reported no differences between the morphology of larval shells of *C. angulata* and *C. gigas*, whereas he could separate other species using the same approach. Based on these observations Ranson stated that the Portuguese and Pacific oysters are the same species. Evseev *et al.* (1996) described the anatomy of *C. gigas* and found anatomical differences in the arrangements of the interlamellar septa when compared with the observations for the same features by Nelson (1960, in Evseev *et al.*, 1996) of *C. angulata*. A direct comparison of the anatomy of the two taxa using animals from different populations is still lacking in order to clarify these differences. Nevertheless, both taxa are morphologically very similar which suggests that they are very close related.

Growth and ecophysiological comparisons

Significant phenotypic differences between Portuguese and Pacific oysters have been reported in several studies in the last decades. Production yield is an important economic trait in shellfish farming that takes into account survival and growth. A higher production yield in the natural environment has been observed for *C. gigas* when compared with *C. angulata* (Parache, 1989; Soletchnik *et al.*, 2002). Héral *et al.* (1986) observed that the production was always higher for *C. gigas* than for *C. angulata* at different values of overall stocking biomass in the Marennes-Oléron bay, France. These differences were mainly attributed to the faster growth of *C. gigas*, but are also related in some cases with a higher mortality observed in *C. angulata* (His, 1972;

Bougrier *et al.*, 1986; Soletchnik *et al.*, 2002). Some ecophysiological parameters have been compared between the two taxa in order to better understand the differences observed in growth rate. His (1971) observed that the Pacific oyster had a higher clearance rate than the Portuguese oyster. This author also observed that below 10°C the difference in valve activity between these taxa increased and that only *C. gigas* was active at 2°C. It was also observed that juveniles of the Pacific oyster had higher oxygen consumption rates than juveniles of the Portuguese oyster (Goulletquer *et al.*, 1999). Haure *et al.* (2003) did not observe the same differences in the clearance and oxygen consumption rates, but only in terms of feeding time activity. These ecophysiological results suggest that *C. angulata* and *C. gigas* have different strategies for the allocation of the available energy, which could explain their dissimilar growth.

Parasitic-disease susceptibility

Major mortalities between 1967 and 1973 almost led to the disappearance of the Portuguese oyster from Europe (Renault, 1996). The "gill disease" has been identified as one of the cause of *C. angulata* high mortalities, that started in France in 1966 and the disease was characterized by the appearance of gill lesions (Arvy and Franc, 1968; Alderman and Gras, 1969; Marteil, 1969; Comps, 1970). This mortality episode was followed by a second period of mass mortalities, but this time without distinctive clinical signs (Comps, 1988). Different agents were implicated in the mortalities such as fungi, protozoans and viruses (Arvy and Franc, 1968; Besse, 1968; Gras, 1969; Comps and Duthoit, 1976). Irido-like viruses are regarded as one of the most probable causes of the mortalities of *C. angulata* and they were designated as gill necrosis virus (Comps and Duthoit, 1976) and haemocyte infection virus (Comps, 1988). However, these viruses were not isolated neither experimental transmission studies were performed to demonstrate their pathogenicity (Comps, 1988). It was also reported that the oyster stocks were very high in some regions prior to the mortalities, increasing the risk and impact of presumed pathogens (Héral *et al.*, 1986). Large-scale introduction of a replacement species in France, the Pacific oyster *C. gigas*, was decided in 1970 in order to overcome the crisis (Grizel and Héral, 1991). It was point out that smaller scale introductions that started in 1966, may have led to the introduction of the putative agent that caused the mortalities (Renault, 1996). The irido-like viruses associated with the mortalities were also observed in the Pacific oyster (Comps and Bonami, 1977; Comps and Duthoit, 1976), but no losses were detected in this species during a period when the Portuguese oyster suffered mass mortalities (Comps, 1988). Hence, these results suggest that *C. angulata* and *C. gigas* have different susceptibility to the putative disease responsible for the mortalities that almost wiped out the Portuguese oyster from Europe.

Recent unpublished studies revealed different

levels of prevalence and intensity of a parasitic copepod of *C. angulata* and *C. gigas* that is found mainly in the gills. These results also suggested that there is a host response to the copepod that may explain the differences observed in prevalence and intensity.

Genetic differentiation and variability

The high genetic similarity between *C. angulata* and *C. gigas* observed by several authors using allozymes markers supported the hypothesis that the Portuguese and Pacific oysters should be classified as the same species (Mathers *et al.*, 1974; Buroker *et al.*, 1979; Biocca and Matta, 1982; Mattiucci and Villani, 1983). However, studies on the mitochondrial cytochrome oxidase subunit I (COI) gene have shown clear genetic differences between the two taxa (Boudry *et al.*, 1998; O'Foighil *et al.*, 1998). An average of 2.3% differences in mitochondrial COI nucleotide sequence suggests that populations of *C. angulata* and *C. gigas* may have diverged several hundred thousand years ago (Hedgecock *et al.*, 2004). Huvet *et al.* (2000) revealed low but significant genetic differences between Portuguese and Pacific oysters populations using microsatellites markers (mean Wright's fixation index; $F_{st} = 0.022$). These authors reported that the genetic differentiation observed in pairs of populations of the two different taxa were twice as large as in pairs of populations of the same taxon. A more recent study based on a satellite DNA highlighted the genetic similarity between *C. angulata* and *C. gigas* (López-Flores *et al.*, 2004). All these genetic studies clearly indicate close phylogenetic ties between the Portuguese and Pacific oysters. Nevertheless, studies using mitochondrial and microsatellites markers have shown that there are low but clear genetic differences between them. It is noteworthy that similar mitochondrial differences were also observed between populations of the American oyster *C. virginica* from the Atlantic and Gulf coasts of America (estimated to differ by about 2.6% in nucleotide sequence), but they are not considered two different species (Reeb and Avise, 1990).

Using equivalent amount of research on both taxa, the level of mitochondrial genetic variation of *C. gigas* (samples collected in different parts of the world) appears to be lower than that of *C. angulata* (samples collected in Portugal) based on PCR-RFLP haplotypes of a COI fragment (Boudry *et al.*, 1998; Lapègue *et al.*, 2004) (figure 1). For the same mitochondrial gene, O'Foighil *et al.* (1998) observed no polymorphism in individuals of *C. gigas* with different origins whereas a high polymorphism was observed in *C. angulata* from Sado estuary, Portugal. The work of Buroker *et al.* (1979) has also showed a higher genetic variation (estimated by the mean heterozygosity) in *C. angulata* from Sado estuary (Portugal), when compared with other *Crassostrea* species. Nevertheless, Huvet *et al.* (2000) using microsatellites markers observed similar levels of

genetic variation in both taxa. The low mitochondrial variability of *C. gigas* when compared with *C. angulata* could be related with the dissemination of *C. gigas* basically from Miyagi Prefecture to different regions in Japan (O'Foighil *et al.*, 1998) and other places in the world where the Pacific oyster was introduced.

Cytotaxonomy

Oysters of the genera *Crassostrea* have a diploid chromosome number of $2n = 20$ which is a common feature in the *Ostreidae* family (Thiriot-Quiévreux, 2002). Comparative standard karyological analysis of *C. angulata* and *C. gigas* supported the close genetic similarity of these two taxa when compared with other cupped oyster species (Leitão *et al.*, 1999a). A study in which the G-banding technique was applied to chromosomes of *C. angulata* and *C. gigas* confirmed the high similarity between karyotypes of both taxa, but showed differences which agreed with their taxonomic separation (Leitão *et al.*, 1999b). A more recent study by Leitão *et al.* (2004) confirmed the previous findings, through the application, this time, of a restriction endonucleases banding technique that put in evidence differences between the banding pattern in all chromosomes pairs of *C. angulata* and *C. gigas* with the exception for chromosome pair 10. From a cytogenetics point of view the two taxa are indeed very similar, but can be distinguished.

Reproductive isolation

Pre- and post-zygotic mechanisms that can contribute to the biological isolation of *Crassostrea* species have been addressed in several studies. There are apparently no pre-zygotic barriers to interspecific hybridization between geographically or ecologically isolated species since interspecific fertilization often occurs with at least moderate success (Gaffney and Allen, 1993). On the other hand, asymmetric fertilization success was observed between sympatric species of the genus *Crassostrea* (Banks *et al.*, 1994). High fertilization rates in crosses of *C. angulata* with *C. gigas* have been reported by different authors (Imai and Sakai, 1961; Menzel, 1974; Huvet *et al.*, 2001). Molecular analysis of six-hour old embryos revealed no evidence of preferential fertilization between gametes from the same taxon when spermatric competition was allowed between taxa (Huvet *et al.*, 2001). Asynchronous spawning can also lead to reproductive isolation (pre-zygotic isolation). In the American oyster *C. virginica* (Gmelin, 1791) genetic variation in the timing of gonadal maturation and spawning was observed among distinct populations (Barber *et al.*, 1991). The results of Huvet (2000) and Soletchnik *et al.* (2002) based on the analysis of sexual maturation of *C. angulata* and *C. gigas* suggests that asynchronous spawning may occur. In addition, differences between these two taxa in the minimum temperature at which eggs or sperm are released were reported

by Lubet (1994) based on the work of Le Dantec (1968). It is noteworthy that there are evidences that eggs and sperm from one *Crassostrea* species can induce spawning in another one under laboratory conditions (Galtsoff and Smith, 1932), which can lead to synchronous spawning. Strong post-zygotic barriers to interspecific hybridization in the genus *Crassostrea* such as the viability and fertility of the hybrids are usually reported (Gaffney and Allen, 1993). Normal viability of the F1 hybrids between the Portuguese and Pacific oysters has been observed by several authors (Imai and Sakai, 1961; Menzel, 1974; Bougrier *et al.*, 1986; Huvet *et al.*, 2002). According to Menzel (1974) meiosis in the F1 hybrids of the Portuguese and Pacific oysters as well as mitosis in the F2's embryos appeared normal. However, Numachi (1966, in Gaffney and Allen, 1993) have previously reported that F1 hybrids of both taxa display normal viability and fertility, but the F2 progeny did not survive to settlement. These results were not corroborated by Huvet *et al.* (2002) that managed to produce F2 hybrids that showed normal fertilization rates, developmental yields, and settlement rates. A recent study provided evidence for the existence of hybridization between the Portuguese and Pacific oysters in the natural environment where the two taxa were put in contact recently (Huvet *et al.*, 2004).

The different studies about the mechanisms that can contribute to the biological isolation of *C. angulata* and *C. gigas* suggest that there are no major barriers that can prevent the genomes of these taxa from merging. More studies (*e.g.*, deficits of hybrid forms in the natural environment) are needed in order to confirm these findings.

Origin of the Portuguese oyster

The different geographical distribution, *C. angulata* being present in the northeastern Atlantic and *C. gigas* in Asia, before the voluntary introduction of *C. gigas* in Europe (Grizel and Héral, 1991) in the early 1970s, lead to the question of the origin of these two close related taxa. Three hypothesis have been proposed to explain their geographically distant distributions: (i) *C. angulata* (Iberian peninsula and Morocco), *C. gigas* (China, Japan, to Sakhalin Island) and *C. cattuckensis* (India) derived from a fossil ancestor *C. gryphoides* and toward the end of the Miocene and later, tectonic events produced land barriers, isolating three populations, resulting in the distribution of the three species (Stenzel, 1971); (ii) *C. angulata* was transported from Europe to Asia some centuries ago by European merchants ships (Menzel, 1974); (iii) *C. angulata* was introduced in Europe by undocumented anthropogenic transfer during the earliest days of circumglobal navigation from the Far East (Ranson, 1960).

Based on mitochondrial DNA sequence data, O'Foighil *et al.* (1998) estimated a divergence time

of 1 to 2 million years for the Portuguese and Pacific oysters, long after closure of the Tethyan Seaway estimated in approximately 7 million years ago. Although this study may overestimate oysters divergence times, it clearly undermines Stenzel's hypothesis, suggesting that the closure of the Tethyan Seaway occurred before the appearance of the last common ancestor of the Portuguese and Pacific oysters. According to Ranson (1948, in Edwards, 1976) there are no evidences of the presence of *C. angulata* on the Miocene, Pliocene and Quaternary beds of Portugal, which supports the hypothesis of a recent introduction of *C. angulata* in to Europe from another region. On the other hand, Lawrence (1995) argued that the fossil record does not promote the notion that the Portuguese and Pacific oysters may have been imported into the eastern Atlantic by humans. In fact, *C. angulata* have been reported to exist for at least 2200 years BP in Spain (Ruiz *et al.*, 2004). It is also possible that other *Crassostrea* species, native of the eastern Atlantic, have derived from *C. gryphoides*. Those species, that are no longer present in Europe, could have been incorrectly identified in the late Holocene as *C. angulata*. Another possibility is that in some palaeontological studies, other species of oysters were mistakenly classified as *C. angulata* because of the high morphologic plasticity of oysters. The close genetic relationship between *C. angulata*, *C. gigas*, and other *Crassostrea* species from Asia (figure 2) such as the Kumamoto oyster *C. sikamea* and the Suminoe oyster *C. ariakensis* suggests that the Portuguese oyster has an Asian origin (Buroker *et al.*, 1979; O'Foighil *et al.*, 1998; Boudry *et al.*, 2003). Moreover, studies using mitochondrial markers reported the presence of pure populations of *C. angulata* in Taiwan (Boudry *et al.*, 1998) as well as presumed mixed populations of *C. angulata* and *C. gigas* in Northern China (Yu *et al.*, 2003; Lapègue *et al.*, 2004). Consequently, Taiwan has been proposed as a possible origin of European *C. angulata* populations (Boudry *et al.*, 1998; Huvet *et al.*, 2000). These findings suggest that the Portuguese oyster has an Asian origin and is probably a case of recent undocumented anthropogenic introduction.

Final considerations

The current knowledge about the Portuguese and Pacific oysters show that they are very close related, but also that there are clear genetic and phenotypic differences between them. Consequently, these two taxa cannot be considered synonymous and more data about putative barriers to gene flow between the two taxa is needed in order to define their taxonomic status. Different lines of evidence suggest that the Portuguese oyster is of Asian origin and implicate Taiwan as the possible origin for the European *C. angulata* population (Boudry *et al.*, 1998). A better knowledge on the current

distribution of *C. angulata* in Asia can provide new insights about the origin of the Portuguese oyster, namely in regions where the most common historic commercial routes between Europe and Asia were established during the earliest days of circumglobal navigation.

Other cases of close related bivalves molluscs taxa that are genetic and phenotypically distinct, but interbreed producing sexually viable offspring, have been reported. One of the best studied cases are the mussels of the *Mytilus* complex composed by the close related but genetically distinct taxa *M. edulis*, *M. galloprovincialis* and *M. trossulus* (McDonald *et al.*, 1991). Two of these species, *M. edulis* and *M. galloprovincialis*, co-occur in western Europe and readily interbreed and produce hybrid zones with parental genotypes, high frequencies of F1 and F2 hybrids, and mussels of mixed genetic ancestry (Hilbish *et al.*, 2002; Bierne *et al.*, 2003). Another case of close related taxa that hybridize in the natural environment is the hard clams *Mercenaria mercenaria* and *M. campechiensis* that occur on the east coast of North America (Bert and Arnold, 1995). Despite the taxonomic difficulties posed by these taxa, hybridization between them and hybrid zones offer excellent opportunities to study speciation and processes, which contribute to reproductive isolation (Gardner, 1997). Evidence of natural hybridization between *C. angulata* and *C. gigas* throughout the study of wild cupped oyster population located in the south of Portugal where the two taxa are in contact due to recent anthropogenic transfer opened new perspectives to study the evolutionary history of the *Crassostrea* genus (Huvet *et al.*, 2004). The development of nuclear markers will be of crucial importance to study natural hybridization between these two cupped oysters.

The differences observed between the close related taxa *C. angulata* and *C. gigas* in growth, ecophysiologic characteristics, and parasitic-disease susceptibility also opens new perspectives to study the genetic basis of growth and disease resistance. On the other hand, *C. angulata* can also be seen as a valuable genetic resource for the development of selective breeding programs as well as in the context of production diversification and biodiversity preservation. The conservation of the remaining pure populations of *C. angulata* in Europe (Lapègue *et al.*, 2004) is of great importance since these genetic resources can be used for the development of the European oyster industry without the zoosanitary risks associated with the introduction of exotic oysters (Berthe and Boudry, 1999).

Acknowledgments

The authors thank Florence Rivet and Teresa Drago for helping in compiling some of the papers cited here.

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Article 7 (published in Marine Biology)

Mar Biol (2006) 150:103–110
DOI 10.1007/s00227-006-0333-1

RESEARCH ARTICLE

Phylogeographic study of the dwarf oyster, *Ostreola stentina*, from Morocco, Portugal and Tunisia: evidence of a geographic disjunction with the closely related taxa, *Ostrea aupaoria* and *Ostreola equestris*

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Received: 12 December 2005 / Accepted: 12 April 2006 / Published online: 12 May 2006
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Abstract Despite the economic importance of oysters due to the high aquaculture production of several species, the current knowledge of oyster phylogeny and systematics is still fragmentary. In Europe, *Ostrea edulis*, the European flat oyster, and *Ostreola stentina*, the Provence oyster or dwarf oyster, are both present along the European and African, Atlantic and Mediterranean, coasts. In order to document the relationship not only between *O. stentina* and *O. edulis*, but also with the other *Ostrea* and *Ostreola* species, we performed a sequence analysis of the 16S mitochondrial fragment (16S rDNA: the large subunit rRNA-coding gene) and the COI fragment (COI: cytochrome oxidase subunit I). Oysters were sampled from populations in Portugal (two populations), Tunisia (two populations) and Morocco (one population), identified

as *O. stentina* on the basis of shell morphological characters. Our data supported a high degree of differentiation between *O. stentina* and *O. edulis* and a close relationship between *O. stentina* and both *Ostrea aupaoria* (from New Zealand) and *Ostreola equestris* (from Mexico Gulf/Atlantic). The status of this geographic disjunction between these closely related species is discussed. Furthermore, although identified in a separate genus *Ostreola* by Harry (Veliger 28:121–158, 1985), our molecular data on *O. stentina*, together with those available for the other two putative congeneric species, *O. equestris* and *Ostreola conchaphila*, would favour incorporation of *Ostreola* in *Ostrea*. Finally, a PCR-RFLP approach allowed the rapid identification of *O. edulis* and *O. stentina*.

Introduction

Oysters are among the most familiar of all marine invertebrate taxa. However our knowledge of oyster phylogeny and systematics is fragmentary. This is principally due to the plastic growth patterns of these animals, which result in a wide range of overlapping, ecophenotypic variants (Ranson 1951; Quayle 1988; Yamaguchi 1994) that greatly reduce the value of analysis based on shell morphology. Besides that, many intentional or accidental anthropogenic transfers have emphasised this situation. Our current knowledge of oyster phylogeny and systematics is particularly limited for the subfamily *Ostreinae*, encompassing the flat oysters. In spite of the comprehensive reclassification of living oysters made by Harry (1985), numerous cases remained controversial until the use of DNA molecular methods that allowed the independent testing of

Communicated by S.A. Poulet, Roscoff

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pre-existing hypotheses. Concerning the *Ostreinae* in general, a major advance was realised by the phylogenetic analysis of Southern hemisphere flat oysters based on 16S sequences by Jozefowicz and O’Foighil (1998). Three ostreineid mitochondrial clades were evident, which were, however, incongruent with prevailing morphologically based interpretations of systematic relationships among *Ostreinae*. More recently, O’Foighil and Taylor (2000) showed that the brooding character originated once in the *Ostreinae*, and has been retained in all descendent lineages, providing novel insights into oyster evolution and systematics.

In several cases of misclassification or misidentification of oysters, DNA molecular data have provided valuable new insights. Hence, different oyster taxa initially described in separate geographical areas have since been grouped as a single species. Recently, Kenchington et al. (2002) suggested that *Ostrea edulis* and *Ostrea angasi* are conspecific. Conversely, DNA tools were used to correct misidentification of species and to confirm or revise their geographical range. For example, O’Foighil et al. (1999) confirmed the trans-oceanic range (New Zealand and Chile) of *Ostrea chilensis* using mitochondrial COI sequence data and proposed that dispersal by rafting was the most likely explanation for this distribution. Another example is the occurrence of the European flat oyster, *O. edulis*, in southwestern Western Australia, where *O. angasi* was supposed to be the only *Ostreinae* species present (Morton et al. 2003).

All over the world, numerous species (30–40 according to the classifications) of oysters of the genus *Ostrea* have been described. Their geographical range is particularly wide in warm and temperate waters of all oceans, with, however, a predominant tropical distribution (Jaziri 1990). In Europe, along the Atlantic and Mediterranean coasts, the European flat oyster, *O. edulis*, is the principal flat oyster species and an important economical marine resource. Hence, in 2003 almost 5,200 tons were produced in the world, mainly (99%) in Europe (Spain, France, Ireland, Netherlands, UK), representing about 28 million euros in value (FAO 2003). However, another species, *Ostreola stentina* (Payradeau, 1826), also known as the Provence oyster or dwarf oyster, is also not only present along the same coasts and on the southern Mediterranean coasts and southwestern coast of the Iberian Peninsula, but also along African Atlantic coasts as far as South Africa. Ranson (1967) also indicated its presence along the south coast of Argentina and the southeastern coasts of Australia, suggesting its ability to grow in waters with very different water temperatures. Several subspecies were also described in the Mediterranean

Sea as *O. stentina isseli* and *O. stentina pepratxi* by Bucquoy et al. in 1889 or *O. stentina syrica* by Pallary in 1933 but can be considered as synonymous names (Ranson 1967). Similarly, *O. parenzani*, *O. curvata*, *O. cristata* or *O. plicata*, previously described as different species, can be considered as one, *O. stentina* (Ranson 1967). Because of the particularly low maximal shell size of these oysters, between 40 and 50 mm at adult stage, *O. stentina* was not considered as a potential target for aquaculture and studies remain scarce. However, in the context of sympatry of these two species in Spain, *O. stentina* and *O. edulis* larvae were studied in order to determine the temporal occurrence of larvae from both species and, consequently, the settlement period (Pascual 1972). During a survey of oysters carried out in Portugal in Tejo, Sado and Mira estuaries, as well as Albufeira lagoon, and Algarve coast, aiming the identification of existing species and their eventual culture, Leal (1984) noted the abundant presence of *O. stentina* in Tejo and Sado estuaries. Then, allozymic markers allowed to clearly distinguish between the two sympatric species present in the Nador Lagoon in Morocco (Blanc et al. 1986), in the Bay of Cadiz in Spain, Mira estuary in Portugal (Amezcuca et al. 2001) and Mar Menor Lagoon in Spain (Gonzalez-Wangüemert et al. 2004). More recently, a molecular analysis based on a centromeric satellite DNA family clearly supported a high degree of differentiation between *O. edulis* and *O. stentina* (Lopez-Flores et al. 2004).

The principal aim of our study was to document the genetic relationship between *O. stentina* and other species of the genera *Ostrea* and *Ostreola*. The second one was to establish a rapid PCR-based method to easily differentiate *O. stentina* and *O. edulis*. Therefore, we performed sequence analysis of the 16S mitochondrial fragment (16SrDNA: the large subunit rRNA-coding gene) and the COI fragment (COI: cytochrome oxidase subunit I) on flat oyster individuals sampled in Portugal, Tunisia and Morocco, identified as *O. stentina*, on the basis of morphological characters. We compared the sequences obtained with the other *Ostrea* and *Ostreola* sequences published or submitted in databanks and then more especially with *O. edulis* in order to establish the differentiating PCR-RFLP method between the two species.

Materials and methods

Biological samples

A total of 214 dwarf oysters were sampled in 2003 from five locations, two in Tunisia (Gulf of Gabès), two in

Portugal (Sado and Mira estuaries) and one in Morocco (Dakhla Bay) (Fig. 1). In Tunisia, two samplings were performed, one in front of the Kneiss Islands (34°25'N, 10°10'E) abbreviated KN and one in Gannouche (34°01'N, 9°53'E) abbreviated GH, with, respectively, 74 and 8 dwarf oysters. In Tunisia, the oysters were found aggregated and low lying in the mudbank. In Portugal, 13 oysters were collected in Sado estuary (SA: 38°25'N, 8°39'W) and 101 oysters in Mira estuary (MI: 37°42'N, 8°44'W). In Morocco, 18 oysters were collected in the Dakhla Bay (MO: 23°42'N, 15°57'O).

The morphological identification was performed on the basis of Harry's (1985) criteria: (1) presence of chomata, (2) adductor muscle scar discoloured, (3) small size (less than 40 mm), (4) height greater than width, (5) no lamellae. Furthermore, and in parallel to the DNA sampling for molecular analyses, a biometric analysis (shell length) was performed on 1,399 oysters sampled every 15 days during 9 months in Tunisia, allowing the sampling of different classes of size. The same kind of measurement was performed on the 114 Portuguese oysters sampled for molecular analyses.

Furthermore, one sample of *Ostreola conchaphila* originating from Washington State, USA, was added to our COI sequencing analysis.

Amplification and sequencing

DNA extraction of ethanol-preserved gill fragments was performed by a phenol/chloroform method, as described by Moore (1993). We amplified the 16S mitochondrial fragment with primers described by Banks et al. (1993), according to the protocol detailed in Boudry et al. (1998). A partial COI fragment was also amplified using the primers and conditions detailed in Folmer et al. (1994). The PCR products

were purified with a High Pure PCR Product Purification Kit (Boehringer-Mannheim, Germany). The sequencing reaction, consisting of a first step of denaturation (2 min, 92°C) and 30 cycles (30 s, 95°C, denaturing; 30 s, 50°C, annealing; 1 min, 72°C, elongation), was performed with the Sequitherm EXCEL™ II DNA sequencing kit-LC (Epicentre Technologies). The fragments were separated on a Li-Cor® 4200 automated DNA sequencer. All the novel sequences were submitted to the Genbank nucleotide sequence database.

DNA sequence analysis

The 16S sequences obtained, together with some sequences already obtained by Jozefowicz and O'Foighil (1998) for *O. edulis*, *Ostreola conchaphila*, *Ostrea puelchana*, *Ostrea denselamellosa*, *O. chilensis*, *Ostrea aupouria*, *O. angasi*, *Ostrea algoensis*, *Ostreola equestris* (corresponding to *Teskeyostrea weberi* in their paper)—whose accession numbers are, respectively, AF052068, AF052071, AF052073, AF052067, AF052065, AF052064, AF052063, AF052062, AF052074—were aligned with CLUSTALW (Thompson et al. 1994). The same procedure was applied for COI sequences obtained, together with some sequences already obtained by Giribet and Wheeler (2002) for *O. edulis*, O'Foighil et al. (1999) for *O. chilensis*, *O. aupouria*, *O. angasi* and Kirkendale et al. (2004) for *O. equestris*—whose accession numbers are, respectively, AF120651, AF112289, AF112288, AF112287, AY376607. Pairwise sequence divergences between species were estimated with the DNADIST program in PHYLIP (Felsenstein 1989, <http://www.evolution.genetics.washington.edu/phylip/felsenstein.html>) according to Kimura's (1980) two-parameter model. Phylogenetic analyses were conducted using the program NEIGHBOR in PHYLIP. Bootstrap analysis with 100 replicates was performed with the SEQBOOT and CONSENSE programs in PHYLIP.

PCR-RFLP

According to the alignment of the *O. stentina* and *O. edulis* 16S sequences, two polymorphic sites were identified as candidate for restriction analysis because commercial digestion enzymes, *RsaI* and *Tru9I*, are available at these positions. Among them, for reading convenience, the restriction with *RsaI* was preferentially chosen as none (*O. stentina*) or only one (*O. edulis*) site was present in our sequences, instead of 6 and 9, respectively, for *Tru9I*. The digestion reaction was performed at 37°C during 90 min with 10 µl of PCR

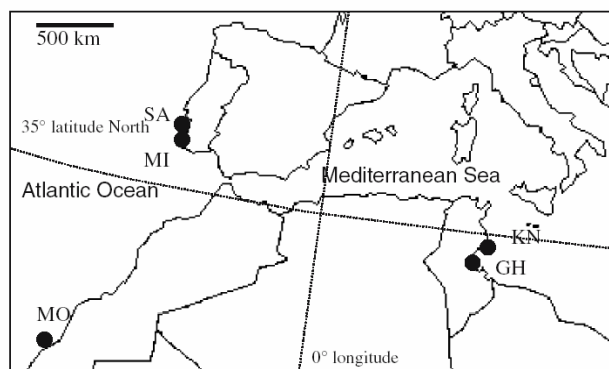


Fig. 1 Location of the samples: Gannouche (GH), Kneiss (KN) in Tunisia, Mira (MI) and Sado (SA) for Portugal, MO for Morocco

product and 10 µl of a mix encompassing 10 × buffer and 3 U *RsaI* enzyme. The resulting fragments were separated on 1% agarose gels and stained with ethidium bromide.

Results

Species identification

The biometric analysis on the Tunisian samples showed that the mean length of these oysters was 26 mm but never more than 47 mm. In Portugal, the oysters had a mean length of 29 mm (range 21–38 mm) in Sado estuary and 37 mm (range 23–52 mm) in Mira estuary. In Morocco, the oysters were not measured when sampled but described as “small”. According to the presence of the five criteria described by Harry (1985) and the biometric analyses, all these oysters were identified as *O. stentina*.

Sequence analysis

Three new different 16S sequences were detected encompassing, respectively, and without any exception samples from Tunisia, Portugal and Morocco. These sequences were, respectively, registered as Accessions DQ13178 for GH–KN, DQ13179 for MI–SA and DQ13180 for MO. The alignment of these new sequences with the other *Ostrea* species sequences allowed the reconstruction of a neighbour-joining tree presented in Fig. 2. The genetic distances (Table 1) confirmed that all the *O. stentina* samples we studied are genetically very close, but surprisingly also with the *O. aupaoria* and *O. equestris* samples sequenced by Jozefowicz and O’Foighil (1998). Hence the divergence between the three *O. stentina* sequences is always

below 2% with 1.8% between GH–NK and both MI–SA and MO. The Tunisian sequence is more differentiated from the Portuguese and Moroccan sequences than the latter two are with an underestimation of 0% divergence between them (as the estimator does not take into account the single indel difference). The divergence is also very low between the Tunisian, Portuguese or Moroccan *O. stentina* sequences and *O. aupaoria*, ranging from 1.3 to 1.6% according to the population of *O. stentina* considered. The same low divergence is observed between the *O. stentina* sequences and *O. equestris*, between 1.3 and 1.5%. Besides, the divergences between these *O. stentina* samples and all the other *Ostrea* species sequences are between 3 and 9% with a clear differentiation with *O. edulis* (between 7 and 8.2% divergence).

This close relationship between our five populations of *O. stentina* and *O. aupaoria* and *O. equestris* is confirmed by our analysis of the three *O. stentina* COI sequences (Accessions DQ13181Q for GH–NK, DQ13182 for MI–SA and DQ13183 for MO; Table 2, Fig. 3). The COI divergence is also very low between *O. aupaoria* and the Tunisian, Portuguese or Moroccan *O. stentina* sequences, ranging from 4.0 to 4.7%, and also with *O. equestris*, ranging from 3.3 to 4.2%. The divergences between these *O. stentina* samples and all the other *Ostrea* species sequences range from 14.9 to 24.1% with a clear differentiation with *O. edulis* (between 21.7 and 22.5% divergence). The new COI sequence for *O. conchaphila* was registered as Accession DQ464125.

Rapid detection of *O. stentina* specimens

Thanks to the PCR–RFLP analysis performed with the 16S–*RsaI* fragment–enzyme couple, *O. stentina* samples can be easily identified on agarose gels as they

Table 1 Pairwise sequence divergences, for the mt 16SrDNA fragment

	<i>Ostrea puelchana</i>	<i>Ostrea chilensis</i>	<i>Ostreola conchaphila</i>	<i>Ostrea denselamellosa</i>	<i>Ostrea edulis</i>	<i>Ostrea angasi</i>	<i>Ostrea algoensis</i>	GH–KN	MI–SA	MO	<i>Ostreola equestris</i>
<i>Ostrea chilensis</i>	0.0438										
<i>Ostreola conchaphila</i>	0.0273	0.0579									
<i>Ostrea denselamellosa</i>	0.0438	0.0676	0.0554								
<i>Ostrea edulis</i>	0.0683	0.0486	0.0754	0.0804							
<i>Ostrea angasi</i>	0.0732	0.0533	0.0803	0.0853	0.0045						
<i>Ostrea algoensis</i>	0.0663	0.0685	0.0710	0.0637	0.0684	0.0733					
GH–KN	0.0400	0.0619	0.0424	0.0676	0.0823	0.0881	0.0799				
MI–SA	0.0317	0.0562	0.0396	0.0642	0.0822	0.0880	0.0827	0.0185			
MO	0.0303	0.0520	0.0350	0.0615	0.0697	0.0747	0.0727	0.0185	0.000		
<i>Ostreola equestris</i>	0.0344	0.0603	0.0296	0.0602	0.0729	0.779	0.0659	0.0129	0.0156	0.0138	
<i>Ostrea aupaoria</i>	0.0371	0.0616	0.0363	0.0586	0.0819	0.0877	0.0796	0.0156	0.0129	0.0131	0.0077

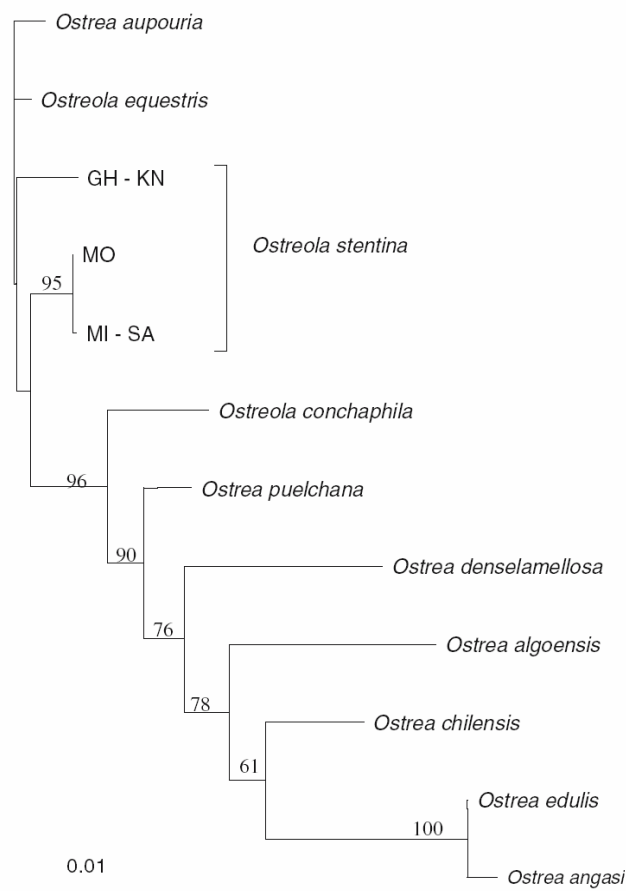


Fig. 2 Phylogenetic tree obtained from sequence divergence of a 489-base nucleotide mitochondrial 16S DNA fragment according to Kimura's (1980) model for three sequences of *O. stentina*, nine sequences by Jozefowicz and O'Foighil (1998) for *O. edulis*, *O. conchaphila*, *O. puelchana*, *O. denselamellosa*, *O. chilensis*, *O. eupouria*, *O. angasi*, *O. algoensis* and *O. equestris*. Numbers on the branches indicate bootstrap values superior to 50%

present one band (no restriction site), conversely to *O. edulis* samples that present two bands (one restriction site) (Fig. 4). All the 214 samples proved to be *O. stentina*.

Discussion

Ostrea edulis and *O. stentina* both inhabit southwestern coast of the Iberian Peninsula, Mediterranean Sea and African Atlantic coasts and they are sympatric in some areas (Leal 1984). In Nador Lagoon (Morocco), a cohort of 98 oysters supposed to be *O. edulis*, with 49 individuals characterised as fast-growing and 49 as slow-growing animals, was studied with allozymic markers (Blanc et al. 1986). The authors concluded that the fast-growing sample was *O. edulis*. Of the slow-growing oysters, only 19% were considered to be *O. edulis*, while 81% belonged to another species. Hence, the external morphology of *O. edulis* and *O. stentina* is very similar and this prevents their differentiation at the morphological level, especially when the oysters are small. *O. edulis* can reach 94 g and 95 mm in weight and size, respectively, after 13 months in culture, whereas *O. stentina* does not exceed 20 g in weight and 45 mm in size (Rosique et al. 1995). This is in agreement with our biometric analysis performed on the Tunisian flat oysters, which showed a mean length of 26 mm but never more than 47 mm, and on the Portuguese flat oysters, which showed a mean length of 29 mm in Sado estuary and 37 mm in Mira estuary. This is also in agreement with recent hatchery experiments (F. Batista, personal communication) that showed that *O. stentina* samples can reach at best 20 mm size in approximately 3 months. However, the potential range of overlapping between both species until the 40–50 mm would make another criteria useful for their distinction.

Allozymic markers were the first to clearly distinguish between the two sympatric species present in the Nador Lagoon in Morocco (Blanc et al. 1986), in the Bay of Cadiz in Spain and in Mira estuary in Portugal (Amezcuza et al. 2001) and in the Mar Menor Lagoon in Spain (Gonzalez-Wangüemert et al. 2004). Our molecular DNA analysis confirms this statement as the individuals of the five populations of small flat oysters

Table 2 Pairwise sequence divergences, for the mt COI DNA fragments

	<i>Ostrea chilensis</i>	<i>Ostrea edulis</i>	<i>Ostrea angasi</i>	<i>Ostreola conchaphila</i>	GH-KN	MI-SA	MO	<i>Ostreola equestris</i>
<i>Ostrea edulis</i>	0.1331							
<i>Ostrea angasi</i>	0.1370	0.0182						
<i>Ostreola conchaphila</i>	0.2229	0.1941	0.1916					
GH-KN	0.2300	0.2249	0.2404	0.1488				
MI-SA	0.2324	0.2233	0.2408	0.1599	0.0536			
MO	0.2150	0.2176	0.2330	0.1514	0.0439	0.0087		
<i>Ostreola equestris</i>	0.2112	0.2131	0.2182	0.1489	0.0337	0.0452	0.0419	
<i>Ostrea eupouria</i>	0.2137	0.2099	0.2185	0.1537	0.0439	0.0474	0.0400	0.0132

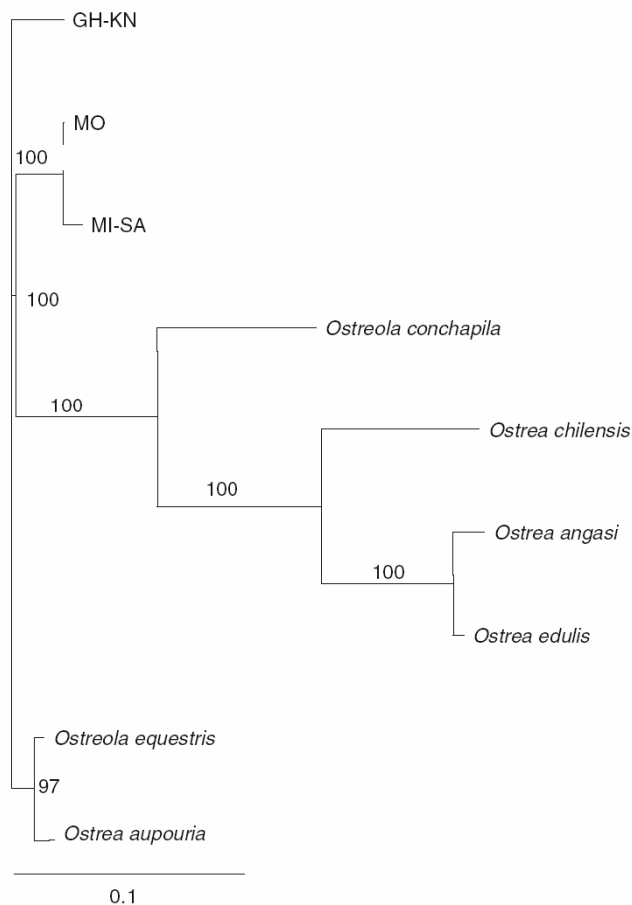


Fig. 3 Phylogenetic tree obtained from sequence divergence of a 660-base nucleotide mitochondrial COI fragment according to Kimura's (1980) model for three sequences of *O. stentina*, one sequence of *O. conchaphila* obtained in this study, one sequence by Giribet and Wheeler (2002) for *O. edulis*, three sequences by O'Foighil et al. (1999) for *O. chilensis*, *O. aupaoria*, *O. angasi* and one sequence by Kirkendale et al. (2004) for *O. equestris*. Numbers on the branches indicate bootstrap values superior to 50%

identified as *O. stentina* in Morocco, Portugal and Tunisia showed a very different sequence from *O. edulis* samples for both 16S (between 7 and 8.2% divergence) and COI (between 21.7 and 22.5% divergence) fragments, the latter being a faster evolving mt gene fragment than the former. As *O. edulis* may still represent a high commercial value in Europe (the value of farmed *O. edulis* production in 2002 was US\$24.3 million), its easy distinction with other sympatric species in Europe is of particular interest. Therefore, our easy PCR-RFLP technique allows a simple distinction between *O. edulis* and *O. stentina* (Fig. 4).

More surprising may be the very close relationship between *O. stentina* and both *O. aupaoria* and *O. equestris*: 1.3–1.5% divergence between *O. stentina* and each of *O. aupaoria* and *O. equestris* for 16S and 4–4.7 and

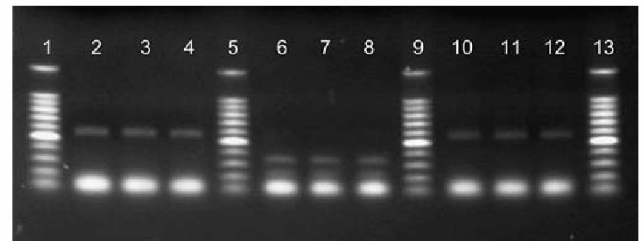


Fig. 4 Example of a rapid PCR-RFLP identification on agarose gel of *O. stentina* samples (two bands in columns 6, 7, 8) and *O. edulis* samples (one band in columns 2, 3, 4, 10, 11, 12) with the restriction by *RsaI* of 16S fragments. Columns 1, 5, 9 and 13 correspond to the 1 kb ladder

3.4–4.5% divergence between *O. stentina* and *O. aupaoria*, and *O. equestris*, respectively, for COI. This is particularly true between *O. stentina* and *O. aupaoria* as they do not belong to the same genus, although *O. stentina* and *O. equestris* do. Furthermore, *O. aupaoria* and *O. equestris* can be found, respectively, in New Zealand and on the coasts of Florida (USA), both on the Atlantic side and Mexico gulf side as well as the Florida Keys. *O. aupaoria* can be distinguished from the co-occurring and predominant *O. chilensis* by the anal appendage (Dinamini and Beu 1981). *O. equestris*, commonly known as the “crested” oyster, is described as having a shell with raised crenulated margins. Kirkendale et al. (2004) found a very close relationship between these two species with COI sequences analysis. Our study introduces a third species, *O. stentina*, in this geographic disjunction of three closely related species. This observation is but one of three such cases involving taxa in the brooding oyster 16S gene tree with *O. edulis*/*O. angasi* and *Cryptostrea permollis*/*O. puelchana* first discussed in Jozefowicz and O'Foighil (1998). Furthermore, on the basis of a repeat region in ITS-1, Kenchington et al. (2002) suggested that *O. edulis* and *O. angasi* are conspecific. In the case of *O. equestris*/*O. aupaoria* geographic disjunction, Kirkendale et al. (2004) ruled out historic transfers that have occurred or have been suspected on numerous occasions (Dinamini 1971; Edwards 1976; Buroker et al. 1979; Chew 1990; Carlton and Mann 1996; Boudry et al. 1998; O'Foighil et al. 1998; Lapègue et al. 2002) as possible origin of this disjunction observation. Indeed, their study of several populations in both species allowed them to conclude that New Zealand *O. aupaoria* and Gulf/Atlantic *O. equestris* are reciprocally monophyletic. This, together with their lack of shared COI haplotypes, is characteristic of populations that have not experienced recent gene flow (Avice 2000) and so no anthropogenic transoceanic introductions. In our case, a deeper comparison with the COI marker or even more polymorphic regions, between our *O. stentina*

populations and, respectively, *O. equestris* and *O. aupouria* ones is needed to allow the inference of the origin of such a geographic disjunction between our Mediterranean/African Atlantic species and the other two. However, a recent palaeontological study gives evidence for an old presence of *O. stentina* in the southwestern coast of the Iberian Peninsula (T. Drago, personal communication), as the author identified, according to Harry's (1985) criteria, *O. stentina*-like shells in Algarve (South Portugal) of age more than 6,000 years.

As observed by Kirkendale et al. (2004), *O. equestris*, *O. conchaphila*, and now *O. stentina*, the three constituent species of *Ostreola* genus, could not be considered as sister taxa in our 16S or COI trees. Hence, although *O. conchaphila* is closer to the *O. stentina*, *O. equestris* and *O. aupouria* group (3–4.2%) than to the other species (5.5–8%) for the 16S fragment, it is not as close as *O. stentina*, *O. equestris*, *O. aupouria* are among one another (0.8–1.6%). Again, although *O. conchaphila* is closer to the *O. stentina*, *O. equestris* and *O. aupouria* group (14.9–16%) than to the other species (19.2–22.3%) for the COI fragment, it is not as close as the *O. stentina*, *O. equestris*, *O. aupouria* are among each other (1.3–5.3%). Therefore, although identified in a separate genus *Ostreola* by Harry (1985), our molecular data agree with those of Kirkendale et al. (2004) and would favour incorporation of *Ostreola* in *Ostrea* as proposed by Coan et al. (2000). However, without considering the taxonomic debate, it clearly appears that *O. stentina*, *O. aupouria* and *O. equestris* have to be considered as a particular group in the Ostreinae and deserve particular attention, as it could, with its particular geographic disjunction in New Zealand, Mexico Gulf/Atlantic and Mediterranean/African Atlantic. This could help understanding a counter-subject of the phylogeny of oysters and more specially the Ostreinae ones.

Acknowledgements We would like to thank F. Ruano for helping us to obtain samples of *O. stentina* from Sado estuary and A. Bernoussi from INRH of Casablanca for providing us the oyster samples from Morocco. This work was made possible by a close collaboration between the University of Sfax and IFR-EMER and a FCT grant (SFRH/BD/8972/2002) and by a Marie Curie Training fellowship (PLUDAMOR, QLK5-CT-2000-60036) to F.M. Batista. This work was partly supported by the Région Poitou-Charentes (Convention No. 2001-RPC-A-212). The experimental procedure complied with the current national laws.

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Article 8 (accepted for publication in Aquaculture)

Evidence of response to unintentional selection for faster development and inbreeding depression in *Crassostrea gigas* larvae

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Keywords: larval stage, domestication, selection, inbreeding depression, *Crassostrea gigas*.

Abstract

Underlying consequences of domestication and artificial selection still remain largely unexplored in most aquacultured species. For species with a two phase life cycle, including the Pacific oyster *Crassostrea gigas*, most genetic studies have focused on the post-metamorphosis juvenile and adult stages, but relatively few considered the larval stage. To assess the consequence of hatchery practices on larval characters, especially growth, we performed a phenotypic study on larval progenies derived from crosses between Pacific oysters from natural beds and farmed Pacific oysters selected for desirable production traits such as rapid growth, for over seven generations. A set of three microsatellite loci was used to compare the genetic variability among the two parental broodstocks and to establish the relatedness between pairs of individuals within each broodstock. The mean relatedness of the hatchery broodstock was significantly different from expectations under the hypothesis of random association (i.e. no relatedness). On one hand, our results show a lower survival performance in the hatchery broodstock, which is associated with a multimodal distribution of growth rates. On the other hand, the hatchery broodstock had a higher proportion of success at metamorphosis. The results suggest that these larvae suffered from inbreeding depression, but that this was offset by better metamorphosis success. The combined effects are likely the result of unintentional selection for faster development in the hatchery through the practice of culling slow-growing larvae and a concomitant reduction in the effective population size leading to inbreeding depression.

Introduction

As pointed out by Darwin (1883), domestic animals were initially modified through unconscious selection, and population means were altered across generations by the selection of superior individuals for breeding. Therefore, domestication is commonly viewed as a continuing process by which humans, trying to achieve certain goals, modify traits they find desirable (Siegel 1993). According to Hale (1969), domestication may be globally defined "as that condition wherein the breeding, care and feeding of animals is more or less controlled by man".

Compared to the knowledge of terrestrial vertebrates, most aquatic species are very close to their wild progenitors, and thus either virtually undomesticated or at the very early stages of domestication (Mignon-Grasteau et al. 2005). For species of aquacultural interest, domestication currently consists largely of the development of reliable rearing methods that set the stage for control of the life cycle and subsequent genetic improvement through selective breeding (Vandeputte and Launey 2004). However, this emphasis on the technical aspects of husbandry and propagation does not preclude genetic change resulting from intentional or unintentional selective processes and adaptation to culture conditions.

In some species, however, these technologies have matured to the point where selective breeding programs have been implemented for the genetic improvement of fish and shellfish species (e.g. Gjerde 1986; Gjerdrem 1997; Knibb 2000; Davis and Hetzel 2000; Hulata 2001; Langdon et al. 2003). Through selection on traits such as growth and disease resistance, selective breeding programs logically aim at producing healthy seedstock with improved production performance (Keys et al. 2004). Selective programs can also aim at narrowing the variance of traits so that all individuals perform similarly (i.e. canalization). Unintentional side effects affecting other traits can arise by indirect selection, through genetic correlations between target and non-target traits (Pascual et al. 2004) or genetic drift in small breeding populations (Hedgecock and Sly 1990; Gaffney et al. 1992). In aquatic species, these indirect responses to selection are all the more important because genetic variance is typically high, usually due to a life cycle characterized by high fecundity, large population sizes and external fertilization with broadcast spawning. Furthermore, cultured populations are sometimes established using few breeders and have small genetically-effective population sizes promoting genetic drift (Allendorf et al. 1987). These small effective population sizes also lead to increased homozygosity and to chance mating between relatives even if mating is random and this can reduce fitness-connected traits, through inbreeding depression (Charlesworth and Charlesworth 1987; Falconer and Mackay 1996). The rapid accumulation of inbreeding

seems to result frequently in the degradation of hatchery-propagated breeding stocks (Bentsen and Olesen 2002).

In contrast to fin fish (Busack and Currens 1995; Roberge et al. 2006), little is known about intentional and unintentional consequences of domestication of bivalves from a genetic point of view. For these species, the life cycle includes a critical larval phase (Pechenik 1999). Studies of selection in bivalves have focused on juvenile and adult development stages, but relatively few give consideration to the larval stage (Lannan 1972, 1980; Hedgecock et al. 1995, 1996; Pace et al. 2006).

In many cultured bivalve species, mortality is high in the larval phase making it most likely to be affected by artificial rearing conditions, and domestication selection, since the later stages are usually grown under more natural conditions (i.e. tidal and coastal areas). Selection of fast growing larvae, by discarding smallest growing larvae (i.e. culling) is a common practice in shellfish hatcheries (Loosanoff and Davis 1963; Lipovsky 1984). This practice is of interest to producers because it reduces the time to metamorphosis and its variability, but it can also contribute to a loss of genetic diversity in cultured populations (Taris et al. 2006). Many studies tackled the heritability for larval growth in marine shellfish (Haley et al. 1975; Longwell 1976; Newkirk et al. 1977; Losee 1978; Newkirk 1980; Jones et al. 1996; Ernande et al. 2003; Dégremont 2003). Even if growth is generally considered as a trait with low to moderate heritability (Toro and Newkirk 1990), the majority of studies quoted above support the hypothesis that larval growth could respond to selection. However no studies have confirmed this point, especially when considering the combined influence of selection and inbreeding depression in populations with small effective sizes. Here we report an experiment on the Pacific oyster *Crassostrea gigas*, the most widely cultured shellfish species worldwide, for which the hatchery-based production is important and in continuous progress (Goulletquer 2005). Aiming at obtaining a deeper insight to selective process related to the domestication of this species, we studied progenies of parental oysters originating from natural beds and others resulting from seven generations of hatchery domestication and selection for growth at juvenile and adult stages. We studied the development of larvae resulting from these different crosses by measuring larval growth, survival and settlement success. Concurrently, a set of three microsatellite loci using a PCR-multiplex technique was used to compare genetic variability between the two parental populations and to establish the relatedness between pairs of individuals within each population. Hence, our experiment aimed at studying how several generations of propagation in commercial hatcheries influences the evolution of larval traits through selection and/or inbreeding.

Material & Methods

Parental oysters

Two groups of parental oysters were used in the study: the first one (n = 47) was sampled from a natural bed in Charente-Maritime (France), the second one (n = 37) was sampled from one of the broodstock populations of the commercial hatchery Grainocéan (Charente-Maritime, France). This broodstock results from seven generations of closed hatchery matings. This population was subjected to individual-level selection to improve growth performance at juvenile and adult stages, and also subjected to the typical hatchery practice of culling larval cultures.

Crosses

Crosses were performed within and between the two types of parental oysters. These crosses produced four progenies: two within-strain crosses (females W x males W and females H x males H) and two reciprocal hybrid crosses (females W x males H and females H x males W) where “H” stands for “Hatchery” and “W” for “Wild”.

For each parental broodstock, spermatozooids from all males were collected by stripping the gonad and pooled. Using the same procedure, oocytes were pooled for each broodstock. For the hatchery broodstock, gametes from 17 males and 20 females were collected. The wild broodstock was composed of 14 males and 33 females. Gamete concentrations were estimated using Thoma and Malassez slides coupled to the SAMBA™ IPS image processing software for both spermatozooids and oocytes. Fertilization was performed at a ratio of 100 spermatozoa per oocyte, 10^6 oocytes being used for each of the four matings. Three hours post-fertilization, embryos from each cross were transferred to three rearing tanks (5×10^6 embryos /tank; 3 replicated tanks/condition).

Gill fragments were individually sampled and preserved in ethanol for all parental oysters for further DNA analyses as described in Taris et al. (2005).

Larval rearing

Larvae were reared in 30-l tanks filled with 1 μ m filtered sea water (temperature 24 °C, salinity 28–30‰) and were fed a standard diet, consisting of a mixture of *Isochrysis* aff. *galbana* (T-iso) and *Chaetoceros gracilis* according to a three-phase rationing (Taris et al., 2006). Larval concentration was reduced to 10 larvae.ml⁻¹ one-day post-fertilization. We estimated the number of larvae in each tank by counting 5 water samples according to the procedure described in Utting and Spencer (1991). Two hundred larvae from each tank were also collected to measure their maximum shell length using the image processing system (SAMBA™ IPS 4.40, Samba Technologies). The larval size measurements were

performed every 2-3 days. When the first pediveliger larvae (i.e. ready-to-settle larvae) were observed, the largest larvae were retained by sieving on a 220- μm mesh (i.e. height greater than 280 μm) and transferred to 220- μm mesh-bottomed raceways with ground oyster shell. The remaining larvae were returned to the larval rearing tanks. This procedure was performed every two days. We estimated settlement success as the ratio of the number of successfully metamorphosed juveniles to the number of pediveliger larvae put into the settlement raceways for each progeny 10 days post-settlement. Three estimates of the number of juveniles (= total weight of a cohort / mean individual weight) were calculated for each cross and each settlement cohort.

DNA analyses

Genetic polymorphism was estimated for individuals from both the hatchery population and wild population using a set of three microsatellite loci (CG49 and CG108 from Magoulas et al. 1998; L10 from Huvet et al. 2000) in multiplex PCR conditions as described in Taris et al. (2005). Number of alleles, observed heterozygosity (H_o) and expected heterozygosity (H_e) (Nei 1987) per locus within population were determined by using GENETIX 4.05 (Belkhir et al. 2004). Deviation from Hardy-Weinberg expectations was estimated in each population within locus by using f , the Weir and Cockerham's (1984) estimator of F_{is} . Significance levels were tested using the permutation procedures available in GENETIX. Allelic richness was also determined to make direct comparisons of the mean number of alleles among populations irrespective of sample size (Fstat program; Goudet 1995). In addition, the frequency of null alleles was estimated per locus. The method used was based on the maximum likelihood approach developed by Kalinowski and Taper (2006), implemented in ML-RELATE (Kalinowski et al., 2006).

Pairwise relatedness coefficient

For both broodstocks, we estimated the relatedness between all potential pair of parents using Queller and Goodnight (rxyQG) (Queller and Goodnight 1989) and Lynch and Ritland (rxyLR) (Lynch and Ritland 1999) coefficients, which use population-level allele frequencies to determine the probability that two individuals share alleles that are identical by descent using the program IDENTIX (Belkhir et al. 2002). To evaluate the patterns of relatedness, a Monte Carlo resampling procedure implemented in IDENTIX was also used with 1000 permutations in order to compare the observed distribution of r with that expected under the null hypothesis of no relatedness. In parallel, a second program (ML-RELATE, Kalinowski et al., 2006), accommodating null alleles, was used. ML-RELATE relies on likelihood calculations (Wagner et al. 2006) to estimate relatedness (rxy ML).

Phenotypic data analysis

Larval size-frequency distributions were examined through modal analysis. Initially the distributions were plotted using size class intervals of 10 µm. This interval was chosen since it was larger than the error of measurements and minimized the number of adjacent empty classes. The size-frequency histograms were smoothed using a weighted moving average at the third order to rule out spurious peaks (Frontier and Pichod Viale 1991). We performed the modal analysis using a combination of Bhattacharya's method (Bhattacharya 1967) and NORMSEP (Hasselblad 1966) to decompose complex size-frequency distributions into a series of best-fit normal curves. Bhattacharya's method was used to first obtain an initial number of modes and their approximate means. The NORMSEP method (for SEParation of the NORMally) allowed refining the results using maximum likelihood approaches. We used Fisat (2002) for examination of this modal analysis. We tested for normality by means of the Kolmogorov-Smirnov goodness-of-fit test.

The coefficient of variation for larval length was analyzed for significant differences between crosses at each sampling date using a non-parametric procedure (Kruskal-Wallis test, PROC NPAR1WAY, SAS/STAT[®] Software, SAS Institute Inc. 1999). Survival was calculated as the ratio between the number of larvae at day 3 and day 20 and analyzed as Poisson data using a log link function (SAS macro GLIMMIX; Littell et al. 1996). This link function was used to model responses since the dependent variable is assumed to be nonlinearly (Poisson distribution) related to the predictors, such as Log link: $f(z) = \log(z)$; McCullagh and Nelder 1989). For this purpose, we used the following model:

$$Y_{ij} = \mu + \text{cross}_i + \text{rep}_j + \varepsilon_{ij}$$

where Y_{ij} is the dependant variable, μ is the overall mean, rep_j is the random replicate effect nested within crosses ($j = 1-3$), cross is the effect of the 4 experimental crosses ($i = 1-4$) and ε_{ij} is the residual error. Significance tests were based on F statistics for fixed effects (= cross effect), whereas tests for random effects (= replicate effect) were based on likelihood ratios between sub-models, which asymptotically follow a chi-squared distribution (Self and Liang 1987).

Results

Genetic analysis of parental oysters

Population-level diversity (Table 1). The allelic richness in the hatchery broodstock ranged from 9 to 13 per locus. In the wild broodstock, we observed an average of 31.3 alleles corresponding to a mean reduction of allelic diversity of about 68 % for the hatchery broodstock (from 49.9 to 76.2 % per locus). Regarding both observed and expected

heterozygosity estimates, we found systematically higher values for the population from natural environment (superior to 0.80 versus 0.66 for H_o , 0.96 in multilocus analysis versus 0.77 for H_e). Considering F_{is} estimates for each locus, significant positive values, indicative of heterozygote deficiencies, were observed in both populations, except for the hatchery broodstock at locus Cg108.

Relatedness coefficients.

Pairwise relatedness (r) values for both Queller and Goodnight ($r_{xy}QG$) and Lynch and Ritland ($r_{xy}LR$) estimators were calculated among individuals within each parental population. The permutation tests using relatedness values supported the presence of kin structure within hatchery broodstock. The mean pairwise coefficient (both $r_{xy}LR$ and $r_{xy}QG$) is significantly distinguishable from its expected distribution under hypothesis of random association (i.e. no relatedness) (Figure 1; $P = 0.004$ and $P = 0.008$ respectively). On the contrary for the wild parental population, no significant departure from the expected distribution was observed (Figure 1; $P = 0.573$ and $P = 0.134$). The (r) values obtained from likelihood calculations ($r_{xy}ML$), accommodating for null alleles, are also distinguishable between populations ($r = 0.13$ for hatchery broodstock; $r = 0.05$ for the wild broodstock).

Phenotypic analysis of larval oyster

Size distribution over time. The larval length-frequency distribution of each progeny varied over time from hatching to the ready-to-settlement stage. To avoid the inevitable bias related to differences in larval density (due to different fertilization and survival rates between crosses, see below), we only present in Figure 2 the replicates showing, for the four progenies, a similar larval density at day 3 (380 000, 394 400, 388 800, 395 600 for H x H, H x W, W x H and W x W respectively).

Tests for normality using the Kolmogorov-Smirnov goodness-of-fit test showed strong evidence of deviation ($P < 0.05$) for the H x H replicate and this from day 10 post-fertilization. This was supported by the use of the Fisat software that decomposes complex size-frequency distributions into a series of best-fit normal curves. Values of modal decomposition are shown in Table 2. The H x H progeny stood apart from the others, clearly showing a multimodal distribution as early as day 10 post-fertilization. This multimodality was more distinctly observed three days after fertilization (modal components represented respectively 28 % and 72 % of the total effective). At the same sampling dates, the three other crosses showed an almost exclusive unimodal curve. From day 10 to 20 post-fertilization, a polymodal structure was confirmed for the H x H progeny. The histograms reveal two markedly different peaks in the size distribution. A

first peak represents larval size ranging from 110 to 175µm (32 % of the total effective). From day 10 to 17 post-fertilization, the growth rate of this group was virtually zero. The second represents a cohort of faster growing larvae (68 % of the population at day 20) having a daily growth rate of 15µm (from day 10 to 17 post-fertilization). To a smaller degree, a bimodal distribution was also observed for the W x W progeny at days 15 to 17 post-fertilization. Finally, the hybrid modes showed intermediate values 17 days post-fertilization, ranged between the bigger modes from the H x H and W x W progenies.

Temporal changes in the coefficient of variation for larval length. In relation to the length-frequency distribution previously described, the temporal changes in the coefficient of variation of mean larval length varied among crosses (Figure 3). The H x H progeny were more variable than three others crosses as early as day 8 post-fertilization ($\chi^2 = 9.36$, $P = 0.02$). ANOVAs performed at further dates showed that the coefficient of variation of larval length differed significantly between progenies. The coefficient of variation of the H x H larvae reached a maximum of 22.63 % at day 17 post-fertilization, resulting from a progressive increase. The W x W progeny ended the larval period with a lower value (CV = 15.37 %) but intermediate coefficient of variation compared to the values of the two hybrids progenies (CV = 12.69 % (H x W); CV = 12.19 % (W x H)).

Survival.

Mean survival of larvae from day 3 to day 20 for each cross type is illustrated in Figure 4. No significant survival was observed between crosses up to day 17. H x H progeny showed significantly lower survival than the other progenies from day 17. Even though the W x W larvae had the best mean percentage survival of all crosses, it was not significantly different from the two hybrids.

Settlement timing and success.

For all progenies, the settlement lasted 12 days (from day 20 to 32 post-fertilization as reported on the figure 5 where the effectives were pooled by interval of two days, hence starting from day 22). For the H x H progeny, 50.04 ± 12.78 % of pediveliger larvae were present in the two first days of pediveliger collection and 78.52 ± 9.5 % after four days. After this, we observed few residual larvae. In contrast, the temporal distribution of the effective of pediveliger larvae were bell-shaped for the three others progenies (17.22 ± 12.42 % (H x W), 2.89 ± 3.58 % (W x H), 24.11 ± 16.83 % (W x W) for the first two days of collection). Furthermore, the results of global assessment for settlement success was respectively 90.7 % H x H, 72.3 % W x W, 78.1 % H x W and 68.7 % W x H.

Discussion

Despite the potential importance of processes acting at early development stages, relatively few studies have focused on the evolution of larval traits due to domestication-related selective pressures. Some studies dealt with the life history of *Crassostrea gigas* (Ernande et al. 2003) but did not tackle the question of the consequences of selection for growth in hatcheries, where genetic drift has also been reported (Hedgecock and Sly 1990; Hedgecock et al. 1992).

Our experiment, describing phenotypic patterns for larval traits in crosses with a known history of selection and data for the genetic relatedness of the parental oysters allows us to discuss some interesting trends and patterns.

Larval phenotypic trends

First, based on the larval size-frequency histograms, we observed two different patterns according to the origin of the progeny. These patterns are confirmed by the temporal changes of the coefficient of variation of larval size. The H x H progeny is different from the three others from day 8 post-fertilization. This clearly reflects important variability in the size distribution for this progeny that is not present in the other groups. At day 10 post-fertilization, a bimodal component can be observed whereas the three other progenies showed unimodal distributions. With time, this apparent distinction became more defined until it formed two distinct groups at day 15 and 17 post-fertilization that seem to correspond objectively to slow and fast growing larvae. Interestingly, the W x W progeny also presented a bimodal distribution at day 15 and 17 post-fertilization, while our two outbred progenies did not. This supports the hypothesis that inbreeding depression could be a driving evolutionary force in wild oyster populations (Hedgecock et al., in press). More microsatellite markers would be needed to detect significant relatedness in our W sample and validate this hypothesis.

The second trend can be observed through both values of survival and settlement success. The mean percentage of survival at days 17 and 20 (i.e. just before the first days of settlement) distinguished the H x H progeny from all others progenies. Only 41.6 % of the whole larvae population survived to this date. However, this relatively smaller proportion of larvae settled within a 3-day period with a high rate of settlement success of 90.7 %. It might be deduced that the group of slow growing larvae (size ranged from 110 to 175µm) that produced the second mode in the size distribution in this cross died before reaching metamorphosis. This is supported by the temporal distribution of pediveliger larvae, which should exhibit an increase at the end of the period if these smaller animals had survived because they would be expected to produce a second wave of pediveliger

larvae. Our data, however, indicate that only the portion of ‘fast growing’ larvae appear in the settling H x H population.

Significance of larval trends: inbreeding depression versus response to selection?

We favor the hypothesis that inbreeding depression explains this ‘slow’ growing (and finally dying) sub-population in only the H x H cross. Inbreeding depression has already been studied in bivalves, recording the performance in progenies of sib families or selfing hermaphrodites (with expected inbreeding coefficients $0.25 < F < 0.5$). It was observed at the larval stage in *Ostrea edulis* (Bierne et al. 1998), *Crassostrea virginica* (Longwell and Stiles 1973), *Pecten maximus* (Beaumont and Budd 1994), *Argopecten circularis* (Ibarra et al. 1995), and *Crassostrea gigas* (Hedgecock et al. 1995; Launey and Hedgecock 2001). Launey and Hedgecock (2001) have clearly demonstrated the high load of deleterious mutations carried by *C. gigas*. The phenotypic trends observed in our study may result in the expression of this genetic load, resulting from the breeding of related individuals.

The hatchery population exhibited mean values of r_{xy} almost six times higher than the wild broodstock regarding both Queller and Goodnight and Lynch and Ritland coefficients and almost three times higher based on likelihood estimator. Oysters within this population are genetically more related than expected in a randomly mating population. This seemed to be sufficient to lead to the expression of inbreeding depression at larval stage. Inbreeding depression notably may arise because the inbreeding increases the probability that an individual homozygous for segregating recessive alleles (Lynch and Walsh 1998). Deleterious recessive effects are thought to be major cause of inbreeding depression (Charlesworth and Charlesworth 1999), and especially for *C. gigas* (Launey and Hedgecock 2001). Furthermore, under additive gene action, trait mean and variance of the hybrid progeny should be intermediate between those of the “pure” lines (Lynch and Walsh 1998). In our experiment, both hybrid lines did not present a multimodal size distribution (Figure 2), showed similar survival rates and time to settlement pattern than the W x W progeny (Figures 4 and 5) and expressed intermediate growth values. This supports the hypothesis of the recessive nature of the load of deleterious mutations carried by *C. gigas*, affecting only the inbred fraction of our H x H progeny.

Evans et al. (2004) found, after two growing seasons, significant inbreeding depression of yield and individual growth rate observed in families with a weak value inbreeding coefficient $F = 0.0625$ ($P < 0.01$). This study does not include the larval stage, but underlines the fact that inbreeding depression may be observed with low values of F , knowing that inbreeding coefficient of an individual is equal to the relatedness coefficient of the two parents.

Conversely, in the H x H progeny, we also observed a group of fast growing larvae that exhibited the best percentage of settlement success. The mortality of ‘slow’ growing larvae might have lead to a reduced larval density in the rearing tanks from day 15, potentially explaining that the remaining larvae grew faster. However, this possible density effect did not equally affect all H x H larvae (see Table 2), supporting the hypothesis that our progenies might be genetically differed for their ability to grow and successfully settle. In order to further investigate this response to selection for fast growing larvae, simulation based on the breeder’s equation may be used. This equation describes the relationship between a response to selection for a given trait, the heritability of that trait, and the intensity of the selection applied, such as:

$$\Delta \mu = h^2 S$$

where $\Delta \mu$ is the gain in the mean phenotype across generations, S is the selective pressure defined as the within-generation difference between the mean phenotype after an episode of selection (but before reproduction) and the mean before selection, and h^2 is the heritability (Lynch and Walsh 1998). If we consider a value of selective pressure of $20\mu\text{m}$ (a plausible value, considering the effect of culling, Taris et al. 2006), coupled with a heritability equal to 0.16 (Dégremont 2003), we obtain a gain of $3.2\mu\text{m}$ per generation. Knowing that our hatchery broodstock resulted from seven generations of closed hatchery mating, and assuming a constant selective pressure, the gain in size after seven generations should be approximately $20\mu\text{m}$. This fits well with the observed difference of about $20\mu\text{m}$ between the sub-population of fast growing H x H larvae and the highest modal mean of the W x W larvae. This, together with the intermediate position of the two hybrid progenies, supports the hypothesis that fast growing larvae could efficiently be selected for and thus supports the observed phenotypic trend in the present experiment. Furthermore, this contrasting observation of inbreeding depression balanced with an effective response to selection is potentially avoided in hatcheries since the culling of slow growing larvae is commonly performed. Thus, by culling this part of the larval population, hatcheries might preserve a substantial diversity held by heterozygous individuals. Paradoxically, culling could preserve fitness in hatcheries, at least on the short-term by eliminating the most inbred larvae.

Alternatively, it is difficult to exclude a correlated response at larval stage to selection at adult stage. The relative performance of our progenies at later stages is currently being investigated to further document their difference in growth performances. However, genetic correlations between larval and post-larval traits are usually low or absent (Ernande et al. 2003) supporting the hypothesis of a decoupling between life-stages in order to break fitness trade-offs between adaptations to different tasks (Moran 1994). This hypothesis is therefore unlikely to explain our results. Further studies are required to

corroborate the hypothesis of effective selection for larval growth due to larval culling in hatcheries.

Phenotypic and genetic correlations between larval growth and settlement success.

In a quantitative genetic study of early life history traits in *C. gigas*, Ernande et al. (2003) observed negative genetic correlation between larval development rate and size at settlement, on the one hand, and metamorphosis success, on the other. They proposed that these correlations mean that fast developing genotypes settle and metamorphose early, but have a low survival probability during metamorphosis. They interpreted this as a possible cost of metamorphosing early. Our results do not support these results as the fast growing larvae of the H x H progeny showed the highest settlement success of our four tested progenies, showing that the negative genetic correlation observed by Ernande et al. (2003) may vary between genotypes or could be modified by selection. It should be noted that such a high settlement success (90.7 %) is rarely observed in the hatchery where the experiment was performed, where most experiments involve wild broodstock (Robert and Gérard 1999). More studies are needed to further document genetic correlations between pre- and post-metamorphic traits in *C. gigas*.

Conclusion

Larval development of our H x H progeny might reveal two concomitant opposite effects: first, expression of the genetic load in a significant portion of the population, and on the other side, response to selection of fast growing larvae, associated with a high metamorphosis success.

Further studies are required to validate this observation which appears to be essential in terms of conservation of genetic diversity for species under artificial selection. For this purpose, the genotyping of larvae (not only their parents) is needed to determine the heterozygosity among sub-populations and demonstrate the importance of genetic load and inbreeding depression at larval stage (Bierne et al., 1998; Launey and Hedgecock, 2001).

In hatcheries, larvae of *C. gigas* are size-selected by culling, affecting genetic diversity of the resulting population (Taris et al. 2006). Here we propose that differential selection, coupled with a presumed inbreeding effect, may co-occur in hatcheries. Culling can (1) lead to favor more heterozygous genotypes (expression of non-additive genetic variance), and (2) select for faster growing larvae as additive variance exist for that trait. Culling appears to make more complex the effect of culling when significant inbreeding is present in the population. The results reported here are one more step toward understanding the underlying consequences on artificial size-selection at larval stage and open new

perspectives on the strategies that may be suggested for genetic management of bivalves in hatcheries.

Acknowledgements

This work was partially funded by the BRG (Bureau des Ressources Génétiques), the Ministère de l'Ecologie et du Développement Durable (Convention n°14-C/2003) and the Conseil Général of Charente-Maritime. The authors wish to thank the Ifremer hatchery team in La Tremblade for technical assistance during the larval and post-larval rearing and, more specially, Pascal Phelipot. We also are grateful to Grainocéan for providing the hatchery broodstock and to Mark Camara for useful comments and reading of this manuscript.

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Table 1: Genetic variability of parental populations at three microsatellite loci (N = sample size; A = number of allele (Allelic Richness for the wild population was determined per locus based on minimal sample size of 37 diploid individuals); H_o = observed heterozygosity; Hnb = unbiased heterozygosity; *Null allele freq.* = frequency of null alleles using maximum likelihood estimate; F_{is} estimates according to Weir and Cockerham (1984). F_{is} values are followed by a significance test based on 1000 permutations; (ns) corresponds to non significant values of p, * of $p < 0.05$ and ** $p < 0.001$ after Bonferroni correction on locus).

Locus	Parameter	Populations	
		Hatchery	Wild
Cg108	N	37	46
	A	9	37 (34.49)
	H_o	0.59	0.89
	Hnb	0.61	0.97
	<i>Null allele freq.</i>	0	0.04
	F_{is}	0.03 ^{ns}	0.09 ^{**}
L10	N	37	47
	A	8	37 (33.64)
	H_o	0.65	0.89
	Hnb	0.8	0.97
	<i>Null allele freq.</i>	0.09	0.02
	F_{is}	0.19 [*]	0.08 [*]
Cg49	N	37	46
	A	13	28 (25.99)
	H_o	0.73	0.8
	Hnb	0.91	0.94
	<i>Null allele freq.</i>	0.09	0.05
	F_{is}	0.20 ^{**}	0.15 ^{**}
Multilocus	N	37	46
	A	10	34 (31.37)
	H_o	0.66	0.86
	Hnb	0.77	0.96
	F_{is}	0.15 ^{**}	0.10 ^{**}

Table 2: Modal decomposition of the size-frequency distributions of the larval progenies from the four crosses using a combination of Bhattacharya's method and NORMSEP.

Day	n	HxH				WxW				WxH				H x W											
		Mode	Mean	SD	Effective	n	Mode	Mean	SD	Effective	n	Mode	Mean	SD	Effective	n	Mode	Mean	SD	Effective					
			size		(%)			size		(%)			size		(%)			size		(%)					
3	356	1	94.8	7.2	100.0	344	1	91.3	5.0	100.0	394	1	94.8	7.1	100.0	360	1	94.9	7.2	100.0					
6	299	1	116.4	12.3	100.0	308	1	115.1	8.0	100.0	324	1	119.3	8.9	100.0	330	1	117.3	7.2	100.0					
8	284	1	133.3	13.4	38.9	308	1	137.1	11.9	100.0	318	1	140.6	11.2	100.0	297	1	143.8	11.0	100.0					
		2	152.4	8.5	61.2																				
10	261	1	127.3	14.1	40.0	298	1	146.5	12.7	100.0	317	1	152.9	11.4	100.0	276	1	151.9	12.8	100.0					
		2	166.5	11.1	60.0																				
13	233	1	138.2	18.1	28.4	253	1	169.6	15.2	100.0	282	1	174.6	11.9	100.0	257	1	177.7	16.3	100.0					
		2	187.6	15.5	71.6																				
15	214	1	128.5	12.0	15.9	253	1	133.4	7.5	2.7	260	1	199.1	17.1	100.0	217	1	210.6	16.9	100.0					
		2	164.7	20.9	15.9		2	190.5	16.1	97.3															
		3	217.2	16.7	68.2																				
17	182	1	126.1	12.0	11.5	244	1	180.6	12.4	14.9	260	1	229.2	24.3	100.0	217	1	240.7	24.1	100.0					
		2	173.5	29.2	20.7		2	223.7	16.9	85.1															
		3	247.1	18.0	67.8																				
(n		=				mean				number				of				larvae				per			
																		tank				/			
																						1000)			

Figure 1: Permutation testing of the significance of the relatedness measure estimated by Queller and Goodnight (rxyQG) (1989) and Lynch and Ritland (rxyLR) (1999) coefficient. Arrows indicate observed values.

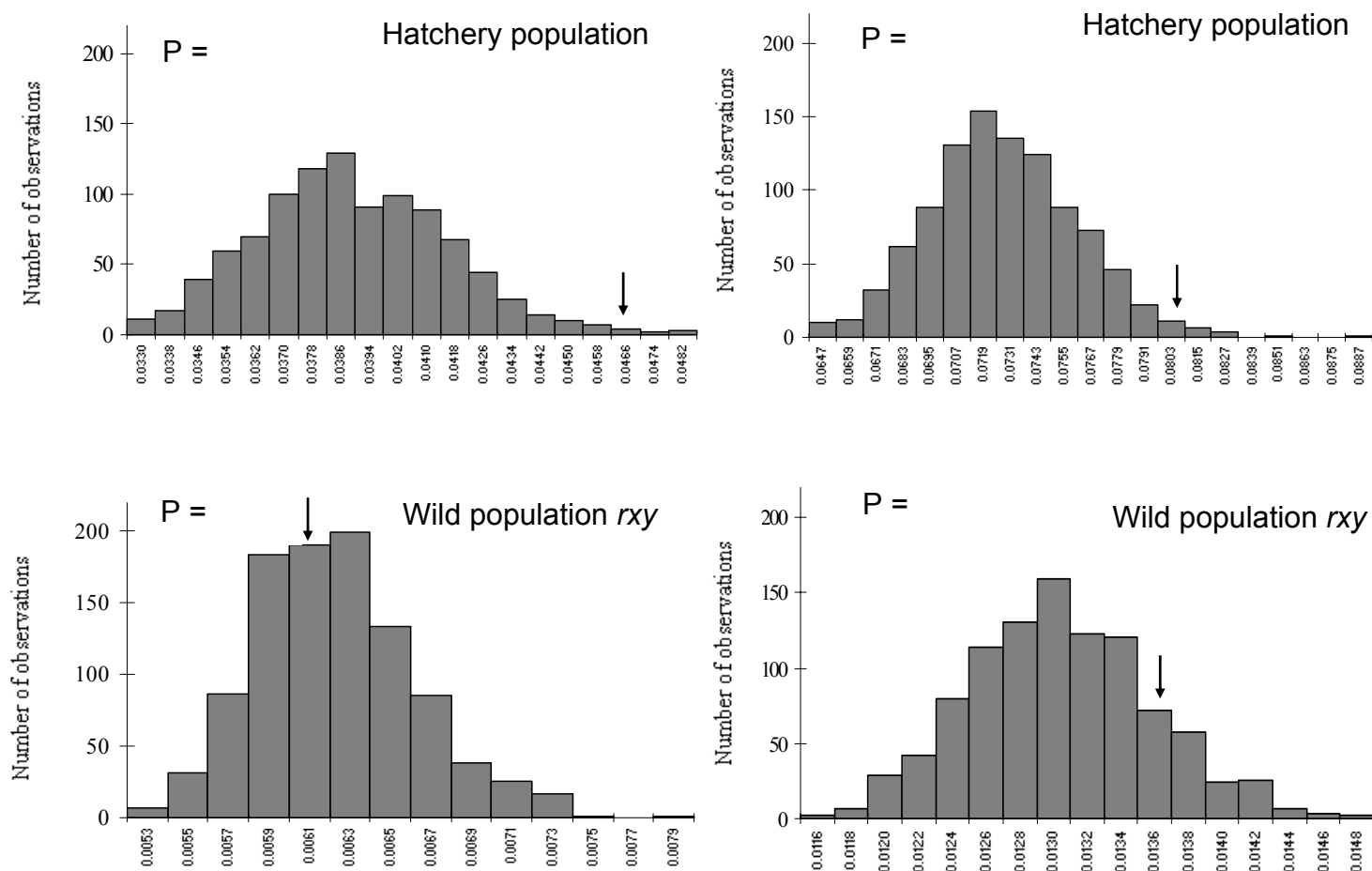


Figure 2: Larval size-frequency histograms for the four crosses (HxH ; WxW ; WxH ; HxW) through the rearing period (each arrow indicates the mean of modal component determined using FiSat, the x-axis represents the range of size, the y axis represents the percentage of larval number).

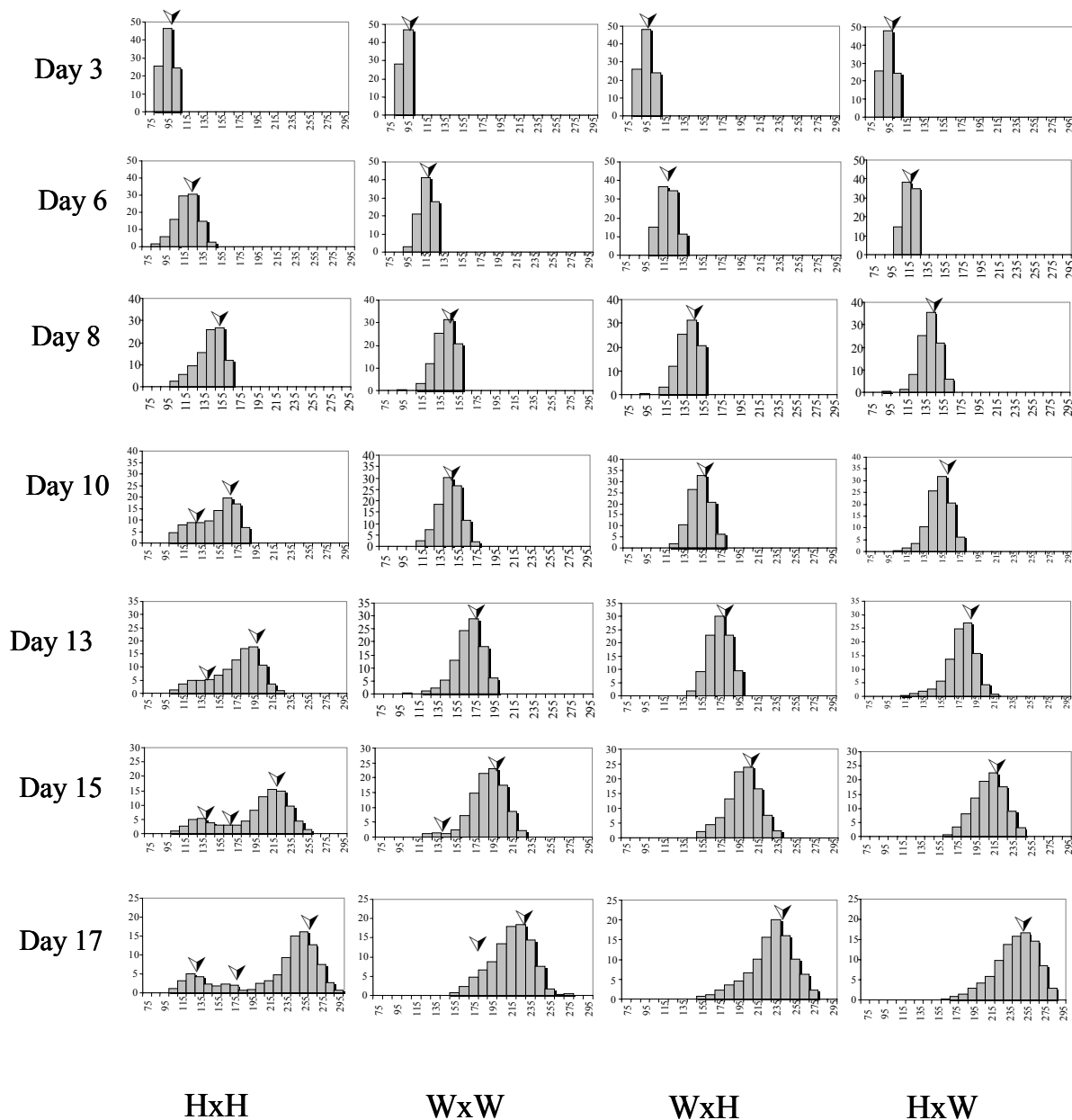


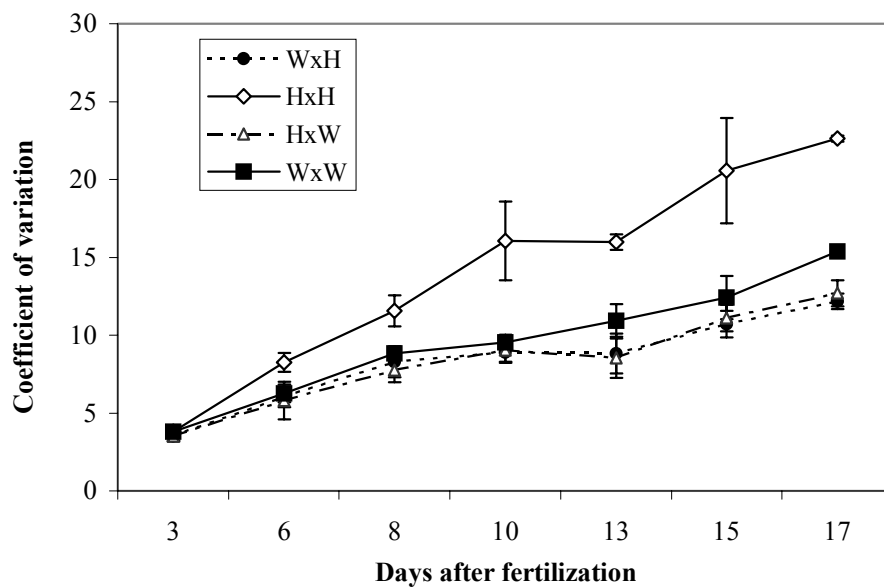
Figure 3: Temporal changes in the coefficients of variation for larval size.

Figure 4: Mean survival of larvae from day 3 to day 20 for each cross type. No significant survival was observed between crosses up to day 15. HxH progeny showed significantly lower survival than the other progenies from day 17.

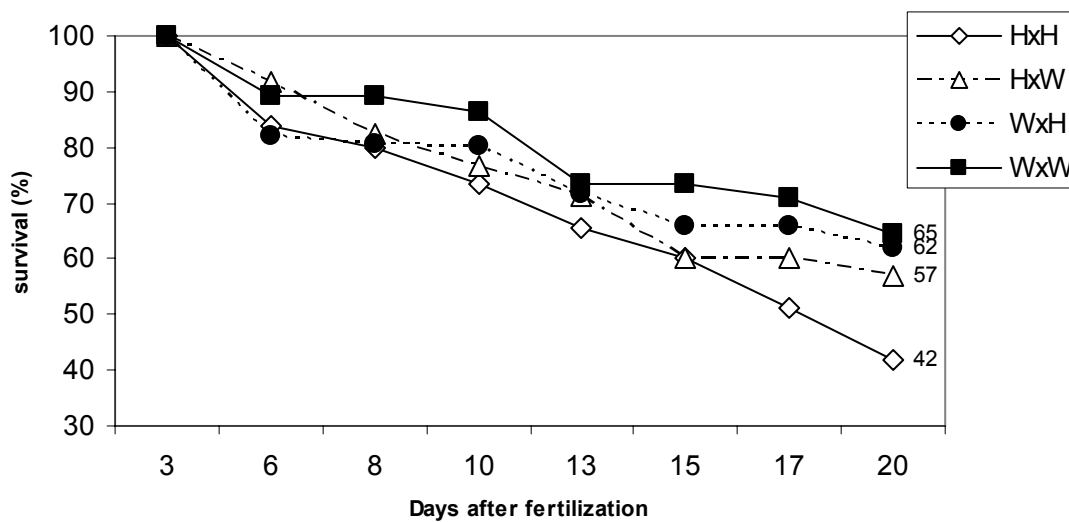
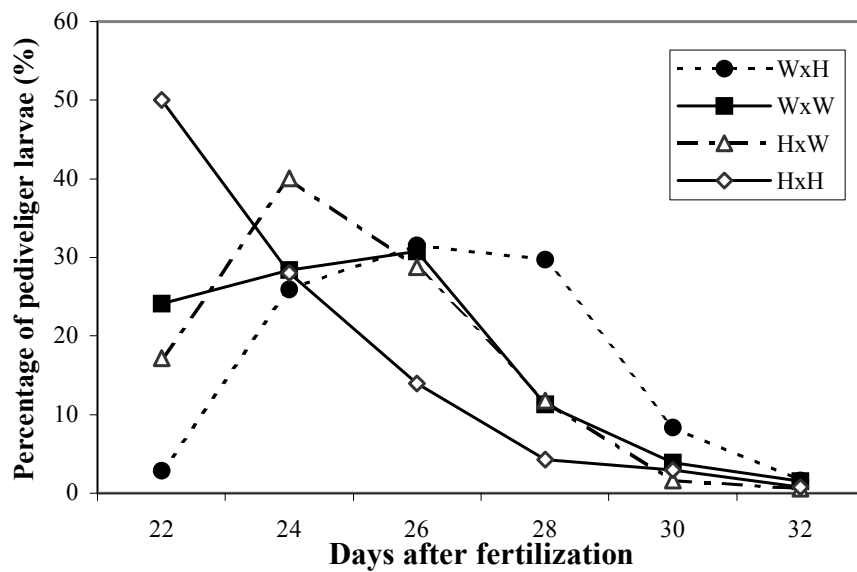


Figure 5: Temporal evolution of pediveliger larvae effectiveness of the four progenies.

PUBLICATIONS, TALKS AND POSTERS WITH RESULTS OF THE THESIS

Publications published and submitted

- Lapègue, S.; Batista, F.M.; Heurtebise, S.; Yu, Z.; Boudry, P. (2004). Evidence for the presence of the Portuguese oyster, *Crassostrea angulata*, in Northern China. *Journal of Shellfish Research* 23 (3): 759-763.
- Batista, F.M.; Taris, T.; Boudry, P.; Renault, T. (2005). Detection of ostreid herpesvirus-1 (OsHV-1) by PCR using a rapid and simple method of DNA extraction from oyster larvae. *Diseases of Aquatic Organisms* 64: 1-4.
- Batista, F. M.; Leitão, A.; Huvet, A.; Lapègue, S.; Heurtebise, S.; Boudry, P. (2006). The taxonomic status and origin of the Portuguese oyster *Crassostrea angulata* (Lamarck, 1819). *The 1st International Oyster Symposium Proceedings* 18: 3-10.
- Batista, F. M., Arzul I., Pepin, J.-F., Ruano, F., Friedman, C. S., Boudry, P., Renault, T., (2007). Detection of Ostreid herpesvirus 1 DNA by PCR in bivalve molluscs: A critical review. *Journal of Virological Methods* 139: 1-11.
- Batista, F. M.; Leitão, A.; Fonseca, V. G.; Ben-Hamadou, R.; Ruano, F.; Henriques, M. A.; Guedes-Pinto, H.; Boudry, P., Individual relationship between aneuploidy of gill cells and growth rate in the cupped oysters *Crassostrea angulata*, *C. gigas* and their reciprocal hybrids (submitted).
- Batista, F. M.; Ben-Hamadou, R.; Fonseca, V. G.; Taris, N.; Ruano, F.; Henriques, M. A.; Boudry, P., Comparative study of shell shape and muscle scar pigmentation in the closely related cupped oyster taxa *Crassostrea angulata*, *C. gigas* and their reciprocal hybrids (submitted).

Talks

- Batista F. M.; Leitão A.; C. C. Monteiro, P. Boudry (2005). Que futuro para a ostra Portuguesa? ExpoMar, 3-6 de Março de 2005, Olhão, Portugal.
- Batista, F. M.; Boudry, P.; Lapègue, S.; Heurtebise, S.; Monteiro, C. C. (2005). Is the Portuguese oyster *Crassostrea angulata* in Southern Europe endangered by the expansion of the Pacific oyster *C. gigas*? 8th International Conference on Shellfish Restoration, Brest, France, October 2nd - 5th 2005.

Posters

- Batista, F. M.; Leitão, A.; Huvet, A.; Lapègue, S.; Heurtebise, S.; Boudry, P. (2005). The taxonomic status and origin of the Portuguese oyster *Crassostrea angulata* (Lamarck, 1819). *The 1st International Oyster Symposium*, 13-14 July, Tokyo, Japan.

- Batista, F. M.; Fonseca, V.G.; Ben-Hamadou, R.; Taris, N.; Henriques, M. A.; Boudry, P. (2006). Hybridization and morphological differences between the close related oyster taxa *Crassostrea angulata* and *C. gigas*. International Symposium Genetics in Aquaculture IX, Montpellier, France, 25-30 June 2006.
- Leitão, A.; Chaves R.; Batista, F. M.; Santos, S.; Guedes-Pinto, H.; Boudry, P. (2006). Cytogenetic characterisation of *Crassostrea gigas* x *C. angulata* F1 hybrids: Restriction enzyme digestion chromosome banding and comparison of the aneuploidy levels of the two taxa and their hybrids. International Symposium Genetics in Aquaculture IX, Montpellier, France, 25-30 June 2006.