Universidade do Algarve

Biology and hatchery production of Chamelea gallina, Spisula solida and Venerupis corrugata, to support restocking and stock enhancement programs

Sandra Maria Duarte Joaquim

Tese de Doutoramento em Ciências do Mar, da Terra e do Ambiente

(Especialidade em Tecnologia de Aquacultura)

Trabalho efetuado sob a orientação de:

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

We should take care not to make the intellect our god; it has, of course, powerful muscles, but no personality.

Albert Einstein

To Íris, Duarte and Vitor.

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Resumo

A conservação dos recursos marinhos tem tido uma importância crescente ao longo do tempo. Contudo, nos últimos anos, temos assistido com grande preocupação ao declínio dos principais recursos em todo o mundo. Apesar das medidas de gestão introduzidas pela administração para ajustar as capturas, que visam a conservação dos mananciais, as populações de amêijoa-branca, *Spisula solida*, de pé-de-burrinho, *Chamelea gallina* e de amêijoa-macha *Venerupis corrugata* no Algarve mostram sinais evidentes de sobre-exploração. A acção sinergética da pressão exercida pela pesca com a taxa de crescimento elevada, o tempo de vida curto e a variabilidade no recrutamento destas espécies, levou a grandes flutuações inter-anuais na abundância dos mananciais, dificultando a sua recuperação natural. De forma a inverter esta tendência negativa e reconstruir as populações será necessária uma intervenção activa, e o estabelecimento de medidas de gestão dos recursos.

A presente tese visou contribuir para a resolução deste problema, aprofundando o conhecimento da biologia e produção em maternidade destas espécies, por forma a contribuir para a sua promoção para a moluscicultura, incentivando a diversificação desta actividade e por forma a apoiar futuros programas de repovoamento ou aumento dos mananciais com indivíduos produzidos em maternidade. Para atingir este objectivo, foram identificadas as melhores populações para recolha dos reprodutores e o período reprodutivo óptimo das espécies para a indução artificial da postura. Posteriormente, este trabalho focou-se na introdução de *S. solida* e *C. gallina* como novas espécies em aquacultura e no incremento da produtividade e redução de custos na produção de pós-larvas de *V. corrugata* em maternidade. Foi igualmente efectuada uma experiência piloto de aumento dos mananciais de *S. solida*.

Os reprodutores de *C. gallina* e *S. solida* foram recolhidos das populações selvagens alvo, por forma a promover a variabilidade genética dos indivíduos obtidos em maternidade e consequentemente, das populações selvagens alvo dos futuros programas de repovoamento ou aumento dos mananciais. Contudo, o mesmo não foi possível para *V. corrugata*, uma vez que a redução do tamanho da população da Ria Formosa não permitiu a obtenção de reprodutores. Assim, após a avaliação da variabilidade genética da população da Ria de Aveiro, verificou-se que esta era uma alternativa viável, uma

vez que, por um lado, apresentava uma elevada plasticidade genética e por outro lado, as duas populações eram geneticamente similares.

Os resultados obtidos no estudo do ciclo reprodutivo, bem como do consequente armazenamento e utilização das reservas energéticas mostraram que as três espécies apresentam estratégias reprodutivas diferentes. A amêijoa branca pode adoptar estratégias flexíveis em termos da gestão das reservas energéticas, e modificar a resposta reprodutiva quando ocorrem oscilações anormais na temperatura da água do mar. Esta flexibilidade reprodutiva tem implicações interessantes na produção, uma vez que permite a manipulação no acondicionamento dos reprodutores. *C. gallina* apresenta uma estratégia reprodutiva oportunista, enquanto *V. corrugata* é mais conservativa, contudo ambas as espécies têm um período natural de postura muito alargado e uma taxa de desenvolvimento gonadal elevada, o que permite a obtenção de larvas em maternidade, sem ser necessário um período de acondicionamento extenso com custos elevados de produção. Por outro lado, os resultados obtidos relativos à relação entre a condição dos reprodutores, a qualidade dos ovócitos e a viabilidade larvar de *V. corrugata* mostraram que é possível manipular os reprodutores por forma a obter múltiplas desovas anuais.

A produção em maternidade das espécies *C. gallina* e *S. solida* foi possível com a tecnologia (sistema estático - Batch) e alimento tradicionalmente utilizados em maternidades de bivalves. Apesar de não haver constrangimentos que impedissem a produção de ambas as espécies, os resultados obtidos mostraram que *C. gallina* pode ser mais atractiva para a produção aquícola, bem como para programas de repovoamento. Esta espécie apresentou um período larvar curto e o efeito das reservas vitelinas fizeram-se sentir até à metamorfose, o que diminui significativamente o aparecimento de problemas durante o período larvar, bem como os custos associados à produção. A possibilidade de produzir juvenis desta espécie de uma forma fácil e económica, bem como a ausência de mortalidade nesta fase, faz de *C. gallina* um candidato à produção aquícola como um produto complementar ou alternativo da moluscicultura, bem como a acções de repovoamento. A produção de larvas de *V. corrugata* foi testada em sistemas aquícolas de recirculação (RAS) concebidos à escala laboratorial e comparada com a produção nos sistemas tradicionais (Batch). Os resultados obtidos neste estudo mostraram que a produção larvar desta espécie em RAS pode ser efectuada em altas densidades sem detrimento da sobrevivência, aumentando ainda a taxa de crescimento e

diminuindo o tempo de cultura larvar. A produção em RAS possibilitará assim uma redução significativa dos custos, permitindo a produção em massa de pós-larvas, o que beneficia a espécie como um forte candidato à diversificação da actividade da moluscicultura na Ria Formosa e torna a recuperação das populações sobre-exploradas mais realista.

A baixa fecundidade de *S. solida* observada neste estudo pode ser vista como problemática para a produção em maternidade. Face a este constrangimento, e num esforço de contribuir para reconstruir os mananciais sobre-explorados de *S. solida*, foi delineada uma experiência piloto que visou determinar a exequibilidade de um plano de acções para o aumento dos mananciais, que consistiu no transplante de indivíduos para zonas com interdição temporária de pesca. Os resultados obtidos mostraram que o aumento de indivíduos num local, aumenta o valor residual reprodutivo de cada amêijoa relativa à sua condição pré-transplante. Por outro lado, o transplante de indivíduos com um comprimento abaixo do tamanho mínimo legal de captura pode ser mais vantajoso, uma vez que, estes têm a oportunidade de desovar mais de que uma vez antes de serem capturados, contribuindo assim para o incremento da produção de larvas e para o consequente povoamento das áreas adjacentes. O transplante de indivíduos para áreas protegidas de pesca surge assim como uma boa estratégia de aumento de mananciais, que em conjugação com medidas de gestão do recurso pode contribuir para uma exploração sustentável dos bancos naturais de *S. solida*, na costa algarvia.

Finalmente, os resultados obtidos nesta tese encorajam a diversificação da aquacultura como forma de promover esta actividade e de apoiar futuros programas de repovoamento e/ou aumento dos bancos naturais sobre-explorados, contribuindo para a gestão dos recursos.

Palavras-chave: *Spisula solida*, *Chamelea gallina*, *Venerupis corrugata*, aquacultura, repovoamento, incremento dos mananciais.

Abstract

Despite the management measures introduced by the fisheries administration to adjust catches in order to ensure the conservation of the stocks, beds of *Spisula solida, Chamelea gallina and Venerupis corrugata* in Algarve show evident signs of depletion. To reverse this negative trends an active intervention may be necessary in order to restore the populations of those three species. The present thesis aimed to increase the knowledge on the biological processes and hatchery production of these species, to support future restocking/stock enhancement programs for wild stock management as well as to promote them as candidates for the shellfish aquaculture industry, encouraging the diversification in this activity. To achieve this objective, first the best founder population for aquaculture purposes was defined, for each species, in order to contribute to the conservation of the genetic variability of the target wild populations to be restocked. Thereafter, the optimal reproductive time of the species for artificial spawning induction in aquaculture was defined, based on the characterization of the reproductive cycle of the three studied species as well as its nutrient storage and exploitation strategy. Particular emphasis was given to the evaluation of any eventual relationships between the broodstock condition, oocytes quality and the larval viability of *V. corrugata*. The introduction of the new species *C. gallina* and *S. solida* in aquaculture was based on the existing Batch technology. However, the production of *V. corrugata* larvae was evaluated in an optimized laboratory-scale recirculating aquaculture system and compared with the traditional larval rearing system. Furthermore, a pilot experiment was designed to determine the feasibility of a stock-enhancement strategy, which consisted of transplanting individuals of *S. solida* from natural clam beds to a closed fishing area. Finally, the main results of this thesis encourage the diversification of aquaculture as a way to promote this activity and to support future restocking and stock enhancement programs for the target species.

Keywords: *Spisula solida*, *Chamelea gallina*, *Venerupis corrugata*, aquaculture, restocking, stock enhancement.

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List of Abbreviations and Symbols

Δt	Time interval in days
AFDW	Ash free dry weight
ANOVA	Analysis of variance between groups method
Batch	Static water method for bivalve larvae production
Bp	Base pair
C. cal	*Chaetoceros calcitrans*
CFU	Colony forming units
Chlo	Chlorophyll
CI	Condition index
d	Days
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dDTTP	Dideoxythymidine Triphosphate
df	Degrees of freedom
DGRM	Directorate-General for Natural Resources, Safety and Maritime Services
dGTP	Deoxyguanosine triphosphate
D-larvae	Veliger larvae
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DO	Dissolved oxygen
EDTA	Ethylenediamine tetraacetic acid
ETOH	Ethanol
FAO	Food and agriculture organization
Fst	Fixation value
GI	Gonadal index
G_{ST}	Genetic statistic describing differentiation of populations
h	Nei's genetic diversity
H	Shannon's information index
Hs	Genetic diversity within populations
Ht	Total genetic diversity

KCl	Potassium chloride
K-W	Kruskal–Wallis
L	Litres
L_f	Mean shell length at the end of the experiment
L_i	Mean shell length at the beginning of the experiment
LS	Legal-sized
$MgCl_2$	Magnesium chloride
mtDNA	Mitochondrial DNA
M-W	Mann-Whitney
n0	Observed number of alleles
NaOH	Sodium hydroxide
ne	Effective number of alleles
NH_4^+	Ammonium
Nm	Effective migrants number by generation (genetic flow)
NO_2^-	Nitrites;
NO_3^-	Nitrates
OM	Organic matter
P	*P* value
P	Pearson correlation
PCR	Polymerase chain reaction
PUFA	Polyunsaturated fatty acids
R	Correlation coefficient
RAPD	Random Amplified Polymorphic DNA
RAS	Recirculating aquaculture system
RAS 10	Recirculating aquaculture system (10 larvae per mL)
RAS 200	Recirculating aquaculture system (200 larvae per mL)
RAS 40	Recirculating aquaculture system (40 larvae per mL)
RFLP	Restriction fragment length polymorphism
SGR_L	Length specific growth rate
SGR_W	Weight specific growth rate
Skt	*Skeletonema costatum*

SL	Shell length
SST	Seawater temperature
SSW	Sterile seawater
Taq	Thermostable DNA polymerase
TBE	Tris borate EDTA
TCA	Trichloroacetic acid
TCBS	Thiosulfate citrate bile salts
T-iso	*Isochrysis* aff. *galbana*
Tris–HCl	Tris- hydrochloric acid
TSA	Tryptic soy agar
U.V.	Ultra violet
US	Undersized

Chapter 1

General Introduction

Dredge

The conservation of marine resources has been taking progressively importance over time. Even so, we have witnessed with great concern a decline of the main resources worldwide. According to FAO (2012b), the declining global marine catch over the last few years together with the increased percentage of overexploited marine stocks and the decreased proportion of non-fully exploited species around the world convey the strong message that the state of world marine fisheries is worsening and has had a negative impact on fishery production. Most of the stocks of the top ten species, which account in total for about 30 percent of world marine capture fisheries production, are fully exploited and, therefore, have no potential for increases in production, while some stocks are overexploited (FAO, 2012b). Overexploitation not only causes negative ecological consequences, but it also reduces marine species production, which further leads to negative social and economic consequences (Pauly et al., 2002, FAO, 2012b). This reduced productivity of the world's coastal and marine capture fisheries is a widespread cause of concern for governments (Valdimarsson and James, 2001; Hilborn et al., 2003; Myers and Worm, 2003) since the demand of marine products in 2020 will require strict management plans to rebuild stock abundance and to restore their full and sustainable productivity (Worm, 2009). The Johannesburg Plan of Implementation that resulted from the World Summit on Sustainable Development demands that all overexploited stocks should be restored to the level that can produce maximum sustainable yield by 2015 (United Nations, 2004). However, although there are well-managed stocks and good progress is being made in reducing exploitation rates and restoring overexploited marine stocks and ecosystems through effective management actions in some areas, many fisheries will reach, in the near future, the limit of sustainability (Delgado et al., 2000) and according to FAO (2012b) the Johannesburg Plan target seems unlikely to be met if no additional measures are taken.

Marine bivalves are among the most important invertebrate resources in the world and their exploitation supports important fisheries and aquaculture activities (FAO, 2012a). However, many bivalve stocks worldwide have collapsed due to both anthropogenic and natural causes, such as increased commercial fishing efforts (Arnold, 2008), recreational and commercial watercraft activities (Lorenz et al., 2013), recruitment failure (Beukema et al., 2010), mass mortality (Chaney and Gracey, 2011) and habitat

degradation (Arnold, 2001). These impacts not only affect the fishery yields but also may compromise the productive potential of ecosystems.

Overfishing is harmful for bivalve populations in many ways; promotes the decrease in population densities, causes recruitment failures due to the unsuccessful fertilization and limit the population recovery (Marelli et al., 1999). Most bivalves' stocks that have suffered severe depletions were important fishery resources, such as: the fishery for scallop *Patinopecten yessoensis* in northern Japan declined from >20,000 y^{-1} in the early 1900s to relatively trivial catches from 1955–1973 (Bell et al., 2005); the stock collapse of hard clams *Mercenaria mercenaria* in Long Island that accounted for 45% of the national catch in the 70s (Malouf, 1989; Manzi, 1990); the oyster *Crassostrea virginica* in Chesapeake Bay, whose production in the 90s was a mere shadow of former levels, with landings at approximately 1% of peak historical levels (Rothschild et al., 1994; Hargis and Haven, 1999; Wesson et al., 1999).

Management efforts to restrain stock collapse have been implemented in many bivalve fisheries, but even when harvesting pressure is removed or when habitat loss is reversed, there is no assurance that the affected populations will rebound. When population density decreases below a threshold level, recruitment failure may occur and population recovery may be constrained by dispensatory effects, rendering the population effectively sterile (Marelli et al., 1999; Stoner and Ray-Cup, 2000). As a result, natural recovery of the population may be delayed and active intervention may be necessary to restore stocks to reproductive viability (Arnold et al., 1999 and Wilbur et al. 2000). The techniques to employ must be applied according to the populations recovery needs.

Bell et al. (2005) defined two different strategies to recover depleted populations: restocking and stock enhancement. Restocking involves the release of larvae and juveniles produced in hatchery to rebuild the reproductive biomass of the severe depleted wild populations to a level where the fishery can once again provide regular harvests. On the other hand, when the natural supply of juveniles fails to reach the carrying capacity of the habitat, recruitment may be inadequate to increase the productivity of an operational fishery. This situation can be redressed if a stock enhancement effort such as the transplanting of adults is implemented to augment the biomass of spawning (Bell et al., 2005; Lorenzen, 2005). The processes of restocking

and stock enhancement should be sequential for any given species. Thus, overfished populations could be restored by restocking and then managed for optimum productivity through stock enhancement (Bell et al., 2005).

Bivalve restocking/enhancement programs have been implemented worldwide. Approaches include habitat rehabilitation (Wallace et al., 1995; Luckenbach et al., 1999), stock management programs with seeding efforts (Arnold et al., 2005), direct early larvae and larvae ready to settle release (Preece et al., 1997; Arnold et al., 2002, Arnold, 2008) and the introduction of cultured juveniles or adults (Tettelbach and Wenczel et al., 1993; Peterson et al., 1996; Arnold et al., 2002, Bell et al., 2005, Uki, 2006). Habitat rehabilitation to restore *C. virginica* stocks has been tried with success in U.S. waters (Luckenbach et al., 1999) and other stock enhancement programs were performed in order to restore or supplement populations of the hard clam *M. mercenaria* (Arnold et al., 1999). Restocking of depleted scallop beds and stock enhancement programs has been successful in U.S. (Arnold et al., 1999; Frischer et al., 2000), New Zealand (Duncan and Dredge, 2001), Japanese (Kitada and Fujishima, 1997) and French (Fleury et al., 1997) waters.

FAO (2012b) argues that the integrate planning and management of fisheries and aquaculture is vital to their future development and sustainability. The development of bivalve restocking programs is largely supported by the technological progress in aquaculture focused on increasing productivity, reducing costs and large-scale production (Black and Pickering, 1998; Bell et al., 2008). Moreover, the restocking programs may imply the introduction of new species in aquaculture. According to López et al. (2008a), aquaculture diversification is a strategic challenge that must be oriented towards the culture of native species, given that dozens of these commercially interesting species are exploited exclusively by fisheries. On the other hand and from a production point of view, these new species introduced in aquaculture can also be candidates to alternative or complementary products for the shellfish aquaculture industry promoting the diversification in this activity. However, some ecological and physiological fundamentals are needed as criteria before selecting and introducing new species in aquaculture. As candidate for aquaculture, an organism must meet high productivity (i.e. to be short-living, to have high productivity, to have a high growth rate, to steadily develop at high population densities), high resistance to environmental

factors, high fecundity, high tolerance to diseases and other harmful factors, among others (Vertbitskii, 2008). Apply the research on biological basis of key aspects of bivalve life (e.g. conditioning and gamete fertilization success, larval nutrition and immuno-stimulation, metamorphosis and settlement success, maturation of seed) using different biological approaches and understanding the effect of biotic and abiotic factors in the organisms is essential to provide the success of the introduction of new species in aquaculture. Likewise, the knowledge on the reproductive activity of the species as well as in its nutrient storage and exploitation strategy provides a valuable insight into the biology of the species and is important for assessing the potential of these species for aquaculture and for the sustainable management of wild stocks (Sbrenna and Campioni, 1994; Massapina et al.,1999; Camacho et al., 2003; Gribben et al., 2004; Peharda et al., 2006). Furthermore, it is also of utmost importance to understand if there is any relationship between broodstock condition and larval viability. Indeed, according to Massapina et al. (1999), the physiological and biochemical variability in bivalve larvae can always be expected in hatchery and the broodstock condition and the consequent oocytes quality are important factors that sustain the larval viability (Boudry et al., 2002; Gosling 2003; Helm et al., 2004). Thus, the knowledge of the optimal reproductive time of the species can also guarantee high quality larvae and consequent juveniles quality production, fundamental for the success of the restocking programs.

On the other hand, restocking/stock enhancement programs based on larvae or juveniles produced in aquaculture require new technological approaches that allow mass production. However, the production of bivalve seed in hatcheries and nurseries is a relatively new industry for which most methods have been developed using empirical approaches by adapting methods across species and measuring the resulting effect in terms of growth and survival. Furthermore, the technology used in bivalve hatcheries has not greatly progressed and there are some limitations concerning technological aspects. For example, most larval production in commercial hatcheries is performed at low individual density with static water methods limiting its productivity (Helm et al., 2004). In order to stabilize and increase larval survival, the development of new technologies and its integration into commercial hatcheries must progress. To date, only a few bivalve species have benefited from technological innovations, such as flow-through rearing, re-circulating systems or automated monitoring, etc. (Magnesen et al., 2006; Rico Villa et al., 2008; 2009). The optimization of these new technologies will

allow hatcheries to use rearing methods that better fulfill the biological requirements of bivalves at early life stages than traditional methods. These innovative new methods will ultimately contribute to reliable production of better quality seed at lower cost for restocking programs.

Other issue that must be addressed is related to population genetics. Hatchery practices may have deleterious effects on the genetic makeup of the released stock, and consequently a deleterious effect on the wild population. (Ward 2006). Maintenance of broodstock in hatcheries may result in the loss of genetic variation in hatchery progeny compared with the natural population from which the broodstock were derived (Ryman & Laikre, 1991; Bartley et al., 1995). Gene flow, the interbreeding among wild and hatchery-outplanted clams may also occur, making organisms more homozygous (Machordom et al., 1999; Arnaud-Haond et al., 2004; Ward, 2006) resulting in negative effects through the exposure of deleterious recessive genes to selection or by eliminating increased fitness arising from over-dominance (Ward, 2006). Moreover, supportive breeding can reduce the effective population size (Ne) of the supplemented population especially if the number of broodstock used for artificial propagation is small and the stocked juveniles make-up a large proportion of all offspring (Ryman and Laikre, 1991; Waples & Do, 1994). Conscientious broodstock selection and husbandry could nonetheless minimize or eliminate these negative effects, enhancing the creation of positive outcomes from restocking. In order to develop sustainable aquaculture and reliable restocking programs, to ensure appropriate broodstock management programs and effective breeding programs, it is of utmost importance to study the range of genetic variation, genetic structure, diversification trend, and other factors affecting genetic structure of the different populations of the target species.

Exploitation of bivalves that occurs in soft beds constitutes one of the most important artisanal fisheries in Portugal (Gaspar, 1996). According to the Directorate-General for Natural Resources, Safety and Maritime Services (DGRM) (2012), the annual official landings of clams in 2011 was around 1 000 tons (t) for Portugal mainland. Along the Algarve coast, several species of clams with commercial interested are exploited namely *Spisula solida*, *Chamelea gallina*, *Donax* spp., *Venerupis corrugata* (=*V. pullastra*; =*V. senegalensis*), *Ruditapes decussatus* and *Cerastoderma edule*. Of these, the first three species only occur with high densities in shallow subtidal coastal areas whereas the

remaining species occurs in Ria Formosa coastal lagoon. In coastal areas the clams are caught using dredges operated from boats or with hand-dredges in the case of *D. trunculus.* In Ria Formosa several fishing techniques are used namely hand-picking, use of rudimentary tools (hand rakes, harvest knife and shovels), and hand-dredges. Clam fisheries are managed by a set of output controls, input controls and technical measures (Oliveira et al., 2013). Notwithstanding, despite the management measures introduced by the fisheries administration to adjust catches to the conservation status of the stocks, beds of the white clam *S. solida*, the striped venus *C. gallina* and the pullet carpet shell *V. corrugata* shows evident signs of depletion. This may be due to the synergistic action of fishing pressure coupled with the rapid growth rate and short lifespan of these species and high variability in recruitment, which leads to large inter-annual fluctuations in stock abundance (Gaspar, 1996; Kraan et al., 2008; Beukema et al., 2010). As a result, in some years, the abundance of these species decreases dramatically, threatening the biological and economic sustainability of the fishery. Moreover, the natural recovery of stocks may take several years and since the fishery is not stopped, bivalves' stocks became more and more depleted. The most worrying situation is related to the stocks of *V. corrugata*. This species was once abundant in the Ria Formosa lagoon (south coast of Portugal), however, in the early 1980s this population declined dramatically (Massapina and Arrobas, 1991) and nowadays the recovery has not take place yet. Indeed, despite the high commercial value of this species, the lack of interest presented by local fishermen of Ria Formosa in harvesting this species leads us to suppose that the population density has decreased below a threshold level, such that natural recovery does not appear to be possible. To reverse the negative trends abovementioned an active intervention may be necessary in order to rebuild the populations of those three species.

In this context, the present study aimed to increase knowledge on the biology and hatchery production of *C. gallina*, *S. solida* and *V. corrugata*, to support future restocking and stock enhancement programs for wild stock management as well as promoting the target species as candidates for the shellfish aquaculture industry, encouraging the diversification in this activity. To achieve this, the following specific objectives were pursued:

- To assess the best founder population for aquaculture purposes;

- To define the optimal reproductive time of the three target species for artificial spawning induction in aquaculture;
- To find relationships between broodstock condition and the oocytes quality that allow forecast the larval viability;
- To evaluate the feasibility of aquaculture production of *C. gallina* and *S. solida* and optimize the methods to produce *V. corrugata* in hatchery;
- To evaluate the success of a stock-enhancement strategy for *S. solida*.

This thesis is structured in seven major sections, 5 of them containing the compilation of articles published or submitted for publication in the scope of the present study.

Chapter 2 aimed to define the best founder population for aquaculture purposes in order to contribute to the conservation of the genetic variability of the target wild populations for restocking. With this purpose, the broodstock for aquaculture production of *C. gallina* and *S. solida* were obtained from the target natural populations. However, the same was not possible for *V. corrugata*, since the broodstock available from the natural population to be restocked, Ria Formosa population, revealed to be insufficient due to overfishing. In this context, this chapter aimed to evaluate the genetic diversity and structure of the pullet carpet shell populations from Ria Formosa and Ria de Aveiro and to assess if the latter could be a viable alternative as founder population for aquaculture purposes and for promoting successful restocking actions of the depleted population of Ria Formosa.

In **Chapter 3** aimed to characterize the reproductive cycle of the three studied species (*C. gallina, S. solida* and *V. corrugata*) as well as its nutrient storage and exploitation strategy. This information allowed the definition of the optimal reproductive time of the species for artificial spawning induction in aquaculture.

Chapter 4 aimed to understand if there is any relationship between the broodstock condition, oocytes quality and the larval viability. This knowledge has implications for the implementation of profitable aquaculture of the species, since it will allow the efficient management of the various steps of production. This study was only performed for *V. corrugata* since for the other species (*C. gallina* and *S. solida*), the successful spawning induction methodology prevented the obtention of sufficient unfertilized oocytes for the biochemical quality analysis.

Chapter 5 aimed to develop/improve the rearing techniques to produce quality larvae and juveniles of the three target species in artificial conditions. The introduction of the new species *C. gallina* and *S. solida* in aquaculture was based on the existing technology for traditional species. Since *V. corrugata* larvae production had been previously established by traditional aquaculture technologies, the feasibility of rearing *V. corrugata* larvae with an optimized laboratory-scale recirculating aquaculture system was evaluated and compared with the traditional larval rearing system.

In this study, the low fecundity of *S. solida* was found to be an obstacle for hatchery production which weakened the possibility of rearing larvae and/or juveniles artificially, for restocking programs. Consequently, it was therefore decided to go further in an effort to contribute to rebuild relatively high-density patches of *S. solida*. **Chapter 6** deals with the description of a pilot experiment designed to determine the feasibility of a stock-enhancement strategy that consisted of transplanting individuals from natural clam beds to a closed fishing area.

Finally, **Chapter 7** is the general conclusion of the study, summarizing the main results obtained in the previous sections and giving particular emphasis to their significance for supporting future restocking and enhancement programs for the target species. Mention is made of some further developments of this study that could contribute to improve wild stock management plans as well as to future progresses in *C. gallina*, *S. solida* and *V. corrugata* aquaculture production.

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Chapter 2

Genetic diversity of two Portuguese populations of the pullet carpet shell *Venerupis senegalensis*, based on RAPD markers: contribution to a sustainable restocking program

Bivalve

Joaquim, S., Pereira, J., Leitão, A., Matias, D., Chaves, R., Guedes-Pinto, H., Chícharo, L., Gaspar, M.G., 2010. Genetic diversity of two Portuguese populations of the pullet carpet shell *Venerupis senegalensis*, based on RAPD markers: contribution to a sustainable restocking program. Helgol. Mar. Res., 64, 289–295 (*Published*).

Abstract

The pullet carpet shell *Venerupis senegalensis* (=*V. pullastra*) is a commercially important species in Portugal, Spain, France, and Italy. In Portugal, this species was once abundant in the Ria Formosa (southern Portugal). However, in the early 1980s, its abundance declined dramatically due to overfishing. In order to reverse this negative trend, the genetic sustainable management of the wild stocks of *V. senegalensis* should be performed by promoting successful restocking actions and the development of an aquaculture commercial production program of this species. In order to find the best broodstock for aquaculture purposes and therefore minimize the deleterious effects of hatchery practices, we analyzed the genetic diversity of the natural population to be restocked (Ria Formosa) but also of another potential genetically close population (Ria de Aveiro) by RAPD. Similar and substantive percentage of polymorphic loci, effective number of alleles, Nei's gene diversity, and Shannon's diversity index was found within both populations. This high genetic variability within populations suggests that they might have a gene pool with sufficient genetic plasticity to support changes in the environmental conditions. Analyses of population genetic structure also revealed a small genetic differentiation between the two populations. The high genetic variability of the natural population to be restocked makes it the preferential broodstock for aquaculture purposes. However, the Ria de Aveiro population could also be a viable alternative, due to its genetic plasticity and the genetic similarity of both populations. The results of this study can be useful to the sustainable management of wild stocks as well as in promoting successful restocking actions based on aquaculture production.

2.1 Introduction

The pullet carpet shell *Venerupis senegalensis* (=*V. pullastra*) is an Atlantic–Mediterranean warm-temperate species that inhabits sandy to muddy bottoms, usually from the low tide mark to a depth of 40 m (Macedo et al., 1999). This species is commercially exploited in Portugal, Spain, France, and Italy. In Portugal, *V. senegalensis* occurs in estuaries and coastal lagoons. Presently, this species is more abundant on the western coast, namely in the Ria de Aveiro coastal lagoon (Maia et al., 2006). The pullet carpet shell was also once abundant in the Ria Formosa lagoon

(southern Portugal). However, in the early 1980s, the abundance of this species declined dramatically due to overfishing (Massapina and Arrobas, 1991).

Despite the high commercial value of this species, the lack of interest presented nowadays by local fishermen in harvesting this species leads us to suppose that the population density in Ria Formosa has decreased below a threshold level, such that natural recovery does not appear to be possible. An active intervention seems, therefore, necessary to restore stocks to reproductive viability. In order to do so and try to reverse this negative trend, a project to produce larvae and juveniles of *V. senegalensis* has been implemented aiming to test different technical approaches to restock this species in the Ria Formosa. This restock action can be performed in already existent bivalve-beds of Ria Formosa. In this way, *V. senegalensis* can be valorized as an aquaculture product and as a possible alternative to *Ruditapes decussatus*, the most important bivalve produced in this lagoon.

Hatchery practices may have deleterious effects on the genetic makeup of the released stock, and consequently a deleterious effect on the wild population, such as the increasing genetic homogeneity (Ward, 2006). The decline of productivity and viability of the naturally spawning of three species of wild Pacific salmonids when hybridized with hatchery produced species of lower fitness (Reisenbichler and Rubin, 1999) and the genetic homogenization of the pearl oyster stocks by the impacts of transplants (Arnaud-Haond et al., 2004) are two examples of that. Conscientious broodstock selection and husbandry could nonetheless minimize or eliminate this negative effect, enhancing the creation of positive outcomes from restocking. Since wild populations represent the primary source of genetic variability for aquaculture stocks (Alarcón et al., 2004), the broodstock should be obtained from the natural population to be restocked or, if polymorphism of this population is proven to be low and the population to be in genetic decline, from the genetically close population. In order to develop sustainable aquaculture and conscientious restocking programs, to ensure appropriate broodstock management programmes and effective breeding programmes, it is of utmost importance to study the range of genetic variation, genetic structure, diversification trend, and other factors affecting genetic structure of the different populations of this species.

To the author's best knowledge, no population genetic studies were performed until now in populations of *V. senegalensis*. Only a study with ELISA and PCR–RFLP data was performed for species identification (Fernández et al., 2002). The Random Amplified Polymorphic DNA (RAPD) method has been frequently applied to reveal population genetic variation, divergence, and biogeography (Schaal and Leverich, 2001). This low cost technique, using short oligonucleotides of arbitrary sequence for amplification of discrete regions of the genome, is simple, not requiring much in up-front resources, no need for prior DNA sequence knowledge, and represents the entire genome (nuclear and mtDNA; Williams et al., 1990; Star et al., 2003). In marine bivalves, RAPD technique has been extensively used in population genetic studies, in oysters (e.g., Liu and Dai, 1998), mussels (e.g., Star et al., 2003; Toro et al., 2004), and scallops (e.g., Heipel et al., 1998), and proved to be an appropriate tool at the molecular level for the identification of the divergence between populations or sibling species (Yu et al., 2004). In order to assess the most appropriate *V. senegalensis* founder population to develop an aquaculture production program of this species and for restocking purposes and since no information is currently available on Portuguese population genetic structure of *V. senegalensis*, the Ria Formosa population (to be restocked) and the Ria de Aveiro population were analyzed by RAPD techniques.

2.2 Materials and methods

Sample collection

Samples of *V. senegalensis* were hand-collected from two locations in Portugal: Ria Formosa (southern coast - 37º01′N; 07º49′W) and Ria de Aveiro (western coast - 40º42′N; 08º40′W; Fig. 2.1), both are shallow water mesotidal lagoons with semidiurnal tidal regimes that constitute the major hydrodynamic forcing (Nobre et al., 2005; Dias et al., 2000). These lagoons, that distance 500 km from each other, have several channels and a large intertidal area covered by sand, muddy sand-flats, and salt marshes (Falcão and Vale, 1990; Picado et al., 2009). Ria Formosa has an extension of 55 km and a maximum width of 6 km (Newton and Mudge, 2003). The lagoon is separated from the Atlantic Ocean by several barrier islands and two peninsulas. The tidal range varies from 1.35 m on neap tides to 3 m on spring tides, and the coefficient of renovation for the lagoon is 3.2 for a spring tide and 1.0 for a neap tide. The freshwater inputs are

almost negligible and salinity remains close to 36% (Falcão and Vale, 1990; Águas, 1986). The Ria de Aveiro is 45 km long and 10 km wide, being connected to the Atlantic Ocean by only a narrow channel (Picado et al., 2009), and the tidal amplitude is 0.6 m in neap tides and 3.2 m in spring tides (Dias et al., 2000). This lagoon has an important freshwater input coming from the Vouga and the Antuã rivers (Moreira et al., 1993; Dias et al., 2000). These two ecosystems are currently used for clam production and fish aquaculture ponds.

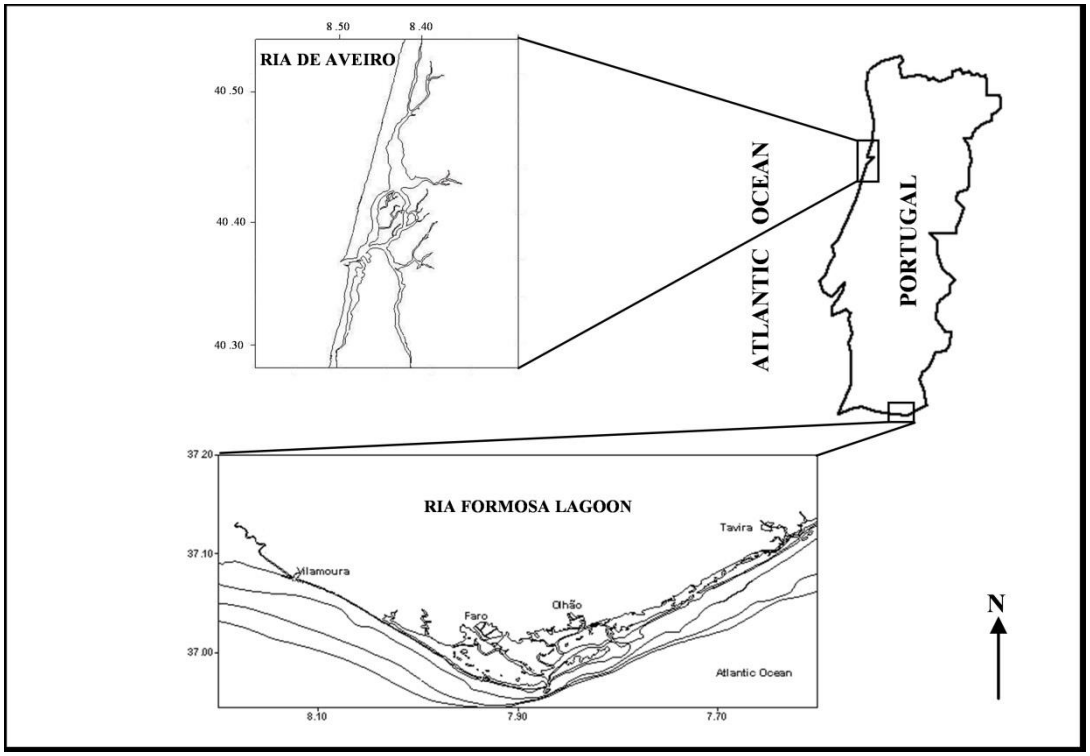

Figure 2.1 Ria Formosa and Ria de Aveiro locations.

Sample processing and DNA extraction

The adductor muscles of 20 individuals (35–37 mm) of each location were dissected from fresh specimens and immediately stored at 70% ethanol until DNA extraction. Genomic DNA was extracted from approximately 15 mg of the muscle tissue following Pereira (2008) by using a QuickGene DNA tissue kit and a Quickgene - 810 automatic nuclei acid isolation system (Fuji Photo Film Co., Ltd, Life Science Products Division, Tokyo, Japan).

RAPD amplification

Since the reproducibility has been pointed out as the major RAPD's limitation (Harris, 1999), a series of optimization experiments were conducted, in which 20 decanucleotide RAPD primers (Operon Technologies, Alameda, CA, USA) were screened in which 6 primers were selected on their ability to amplify reproducible and easily scorable bands. The amplifications were performed in a 25 µl of reaction volume containing 1 × 19 Taq polymerase buffer (Tris–HCl 200 mmol/L pH 8.3, KCl 500 mmol/L, 0.5% Triton-X 100), 2.0 mM $MgCl_2$, 100 µM of each dNTPs (dATP, dCTP, dGTP, dDTTP; Invitrogen, Life Sciences), 0.2 µM of primer, 0.5 U Taq polymerase (Fermentas, Canada), 50 ng of genomic DNA, and water. Initial denaturation was for 3 min at 94ºC, followed by 44 cycles of 1 min at 94ºC, 1 min at the optimal annealing temperature (36ºC), 2 min at 72ºC, and a final extension step of 10 min at 72ºC. The samples were finally cooled at 4ºC. Amplification products were analyzed by electrophoresis on 2% agarose TBE (Tris borate EDTA) gels stained with ethidium bromide and then visualized with ultraviolet light, and image were saved in UVIDOC (UVItec). A DNA ladder mix (Fermentas, Canada) was used as a molecular ruler. To avoid inaccuracy in scoring due to differences in gels, two lines of control molecular ladder were included in each set of samples for each gel.

Data analysis

Amplified fragments were scored as binary data, i.e., presence as one and absence as zero. The differences in band intensities were not taken into account to avoid errors introduced by competition among priming sites during the initial rounds of PCR (Bachmann, 1997). The matrix of RAPDs data was analyzed on the basis of the following genetic parameters: number of polymorphic loci, percentage of polymorphic loci, observed number of alleles (n0), effective number of alleles (ne), Nei's genetic diversity (h), Shannon's information index (H), G_{ST}, and the effective migrants number by generation (Nm; Wright, 1951), using POPGENE program version 1.32 (Yeh et al., 1997; available at http://www.ualberta.ca/~fyeh/). TFPGA program version 1.3 (Miller, 1997; available at http://www.marksgeneticsoftware.net/tfpga.htm) was also used to calculate the Nei's (1978) genetic distance and fixation F_{st} value, over loci, based on Lynch and Milligan's (1994) Taylor expansion estimate and assumed a Hardy –

Weinberg equilibrium. Significant levels were assessed by permutation of the alleles (multilocus genotypes) within populations.

2.3 Results

The 6 decanucleotide RAPD primers generated a total of 222 reproducible and clear amplification bands, with fragments ranging in molecular size from approximately 200–1,500 bp. The bands produced per primer ranged from 32 (OPE-02 primer) to 40 (OPE-08 and OPE-11; Table 2.1), and the average band loci per primer was 37.17. The percentage of polymorphic loci found was 86.55% in the Ria Formosa *V. senegalensis* population and 87.44% in the Ria de Aveiro one. The number of effective alleles per locus was 1.3545 and 1.3219 for Ria Formosa and Ria de Aveiro populations, respectively. The values of genetic diversity and the Shannon's information index for Ria Formosa (h=0.2276; H=0.3608) and for Ria de Aveiro (h=0.2105; H=0.3394) revealed a high level of gene diversity in both populations and did not vary considerably between them, (Table 2.2). The Nei's (1978) values (0.064) revealed a small genetic distance between the two populations.

Table 2.1 Primers selected for RAPD analyses.

Primer	Sequence (5´-3´)	%GC	Tm	No of score bands
OPE-02	GGTGCGGGAA	70	36	32
OPE-03	CCAGATGCAC	60	36	35
OPE-06	AAGACCCCTC	60	36	39
OPE-08	TCACCACGGT	60	36	40
OPE-11	GAGTCTCAGG	60	36	40
OPE-16	GGTGACTGTG	60	36	37

The value of the total genetic diversity (H_t) was 0.246 and the genetic diversity within populations (H_s) was 0.2190. The G_{st} index value was 0.1097, meaning that most of the genetic variation was found within populations (89.03%). Assuming Hardy–Weinberg

equilibrium, F_{st} value (0.1659) showed no evidence of genetic differentiation between populations. The genetic flow (N_m) between the two populations was 4.057.

Table 2.2 Genetic variability at the two *Venerupis senegalensis* populations detected by RAPD.

Population	Polymorphic *loci* (%)	Observed number of alleles (n_0)	Effective number of alleles (n_e)	Nei's gene diversity (h)	Shannon's information index (H)
Ria Formosa	86.55	1.8655	1.3545	0.2276	0.3608
Ria de Aveiro	87.44	1.8744	1.3219	0.2105	0.3394

2.4 Discussion

Assessment of genetic variation is an important step toward the implementation of species conservation strategies. Ward (2006) summarized that the high level of genetic variability in invertebrates strongly depends on its life form, geographic range, and larval dispersal mechanism. A variety of genetic markers has been used to determine aquatic populations structure. These include the use of allozyme, RAPD, restriction fragment length polymorphism (RFLP), and microsatellite markers (Yan et al., 2005). RAPD markers have been widely used for estimating genetic diversity and genetic structure in many species and have proved to be a powerful tool (Bussell, 1999; Ouborg et al., 1999; Chen et al., 2005). In our study, this methodology was efficient in obtaining information on the population genetics of *V. senegalensis*.

Analysis of RAPD data from the 40 individuals revealed similar and substantive percentage of polymorphic loci, effective number of alleles, and Nei's gene diversity within *V. senegalensis* populations. The values obtained in our study are slightly lower than the ones found for other bivalves, such as in mussel (*Mytilus galloprovincialis*) populations in Southern California (Li Ma et al., 2000) or in European populations of the razor clam *Ensis siliqua* (Fernández-Tajes et al., 2007), but are similar to the one's obtained in another veneroid species (*R. decussatus*) populations in Algarve coast (Southern Portugal; Pereira, 2008). Shannon's diversity index is well suited to the analysis of RAPD data as it is relatively insensitive to the bias produced by failures to detect heterozygous individuals (Dawson et al., 1995). The values obtained with this

index confirmed the high genetic variability in both populations. They were, however, lower than those observed in populations of *R. decussatus* by Pereira (2008), which might be explained by the larger populations size of that study. The high genetic variability found in both *V. senegalensis* populations suggests that they have a gene pool with sufficient genetic plasticity to support changes in the environmental conditions without endangering the species survival, even in the case of an overfished population like the Ria Formosa one's. Indeed, the ability of a species to respond adaptively to environmental changes and, therefore, to long-term survival depends on the levels of genetic variability within populations (Qian et al., 2001; Sofia et al., 2006). Fernández-Tajes et al. (2007) also reported a high genetic variability for an *Ensis siliqua* Portuguese population which has suffered strong declines due to overfishing.

The results of the population genetic structure of *V. senegalensis* analyses revealed a small genetic differentiation between the two populations. Similar levels of genetic differentiation, using the same methodology, have been recorded between populations of other bivalves (Star et al., 2003; Casu et al., 2005; Fernández-Tajes et al., 2007; Pereira, 2008). The main factors that might determine the population genetic structure of bivalve species include mating and reproductive system, gene flow (dispersal of larvae), genetic drift, environmental selection or adaptation, bottlenecks, and founder effects among others (e.g., Bierne et al., 1998; Holmes et al., 2004). The high values of genetic variability within populations and low levels of genetic variation among populations observed in this study can be a consequence of the reproductive system of *V. senegalensis*. As in most marine bivalves, it is characterized by high fecundity, large population sizes, external fertilization with broadcast spawning, and extensive larval dispersal (Bierne et al., 1998). At a first view, the results geared for the studied populations suggest a ''canonical'' model of population genetics known as panmixia, without inbreeding, proposed for other bivalve populations (e.g., Bierne et al., 1998; Fernández-Tajes et al., 2007). This model requires an intense gene flow (N_m) between populations without geographic barriers in order to allow them to evolve together (Armbruster, 1997, 1998; Ward, 2006). Traditionally, levels of $N_m > 1$ are thought to be sufficient to prevent population genetic differentiation (Slatkin, 1987). Thus, the number of migrants between the studied populations (Nm=4.057) may suggest that larval exchange could be responsible for the genetic similarities between them.

Fernández-Tajes et al. (2007) reported a gene flow of the same magnitude among Galician populations of *E. siliqua* and they suggested that these populations acted as a single interbreeding population, and Pereira (2008) also obtained an N_m=2.9 between two populations of *R. decussatus* from Ria Formosa and from a near lagoon (Ria de Alvor). However, some authors (Whitlock and McCauley, 1999) believe that these results should be interpreted carefully, since N_m is calculated based on the F_{st} value, and the genetic plasticity of both populations (high genetic variability) can contribute to their similarity. *V. senegalensis* is an actaeplanic species, i.e., with potential for dispersal (Ropes, 1979; Havenhand, 1995; Casu et al., 2005), and Ria Formosa and Ria de Aveiro, despite the fact that they are shallow lagoons systems, have a high tidal flow that allows a large water renovation of both lagoon systems and that may promote larval drift to the coastal waters, could be a possible vector responsible for larvae dispersal and consequent genetic similarity of the studied populations. Nevertheless, we believe that the short larval life spans of *V. senegalensis* that is of around 15 days, the 500 km that geographically distanced Ria Formosa from Ria de Aveiro and other oceanic physical constraints (e.g., variation in water temperature, salinity, predation, and currents) can restrict the dispersal capacity of larvae. These facts do not support the previous hypotheses of larval exchange between the two *V. senegalensis* populations.

Nonetheless, natural dispersal is not always the operating transport mechanism (Levin, 2006). The genetic structure of natural populations can be significantly affected by anthropogenic activities (Palumbi, 2001; Simmons et al., 2006; Pereira, 2008). Nowadays, there is no knowledge of market exchanges involving *V. senegalensis* between the two studied sites owing to the overfishing of Ria Formosa population, but an historical involvement of fishermen from Aveiro in Algarve fisheries (Oliveira FXd'A, 1906; Garrido and Carvalho, 2007) and the crescent importance of bivalve market in both sites can be a possible vector to contribute to the transplantation of *V. senegalensis* between those two lagoons and consequently to the genetic similarity between the two studied populations. This hypothesis can lead us to suggest that the high levels of N_m between the two populations can result from historically genotypic and allelic similarities between the individuals from these regions and do not reflect the present day value of N_m. This hypothesis was already conjectured by Star et al. (2003) for New Zeland *Perna canaliculus* populations.

Bell et al. (2005) have showed that the knowledge of spatial population structure is pivotal to efficient management of marine invertebrate fisheries. According to Gaffney (2006), three primary genetic concerns should be taken in consideration before implementing shellfish restoration projects: (1) identification and use of the correct genetic material for producing hatchery lines; (2) maintenance of genetic variability in hatchery stocks; and (3) maintaining N_e in the wild population. Given that *V. senegalensis* population of Ria Formosa still presents a high intrapopulation genetic variability, it should be the one preferentially selected as broodstock for aquaculture purposes and for subsequent restocking program. However, if the broodstock available from this population reveals to be insufficient due to severe overfishing, Ria de Aveiro population could be a viable alternative. Indeed, we have showed in this study that by one hand genetic plasticity is high and by other hand both population presented genetic similarity. Furthermore, when designing restocking actions based on aquaculture production, we should nevertheless always try to ensure the preservation of the genetic variability in the hatchery stocks and maintaining the effective size (N_e) of the wild population to be restored. In addition to the favorable genetic factors, the similarities between Ria Formosa and Ria de Aveiro in terms of habitat (both shallow water mesotidal lagoon with a large intertidal area covered and with similar semidiurnal tidal regimes, salinity, and sediment) can also positively influence the success of a restocking program. The results of this study provided useful information on the genetic structure of two Portuguese populations of the pullet carpet shell. This data can be useful to the sustainable management of wild stocks as well as in promoting successful restocking actions based on aquaculture production. The protection of genetic diversity in the Ria Formosa population should, however, always be a primary aim of any restocking program to be performed.

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Chapter 3

Characterization of the reproductive cycle of *Chamelea gallina*, *Spisula solida* and *Venerupis corrugata*

Spawning of *Venerupis corrugata*

Chapter 3.1

Biochemical and energy dynamics throughout the reproductive cycle of the striped venus *Chamelea gallina* (Linnaeus, 1758)

Joaquim, S. Matias, D., Matias, A.M., Moura, P., Roque, C., Chícharo, L, Gaspar, M.B. Biochemical and energy dynamics throughout the reproductive cycle of the striped venus *Chamelea gallina* (Linnaeus, 1758) (*Submitted to Invertebrate Reproduction and Development*).

Abstract

The striped venus *Chamelea gallina* (Linnaeus, 1758) is an important commercial bivalve species in Europe, especially in Iberian Peninsula and Mediterranean Sea. However, the large inter-annual fluctuations in stock abundance and periodic recruitment failure threat the biological and economic sustainability of this fishery. The detailed knowledge of the species reproductive cycle and its reproductive strategies is crucial to fishery management and to develop restocking/enhancement programs and to support advances in *C. gallina* aquaculture. The knowledge of *C. gallina* reproductive cycle including the characterization of the main energetic nutrients storage and utilization was improved in the present study. The influence of environmental factors (seawater temperature - SST and chlorophyll *a* – Chlo *a*) in condition index, histological, and biochemical composition (glycogen and total lipids) of *C. gallina* populations from Algarve coast (southern Portugal) were analised. The reproductive cycle of *C. gallina* was significantly influenced by SST and food availability and followed a seasonal cycle. This species comprised a ripe stage in spring followed by spawning that began in the middle of spring and extended during summer. After a short period of inactivity, clams progressed to the onset of gametogenesis in November coincided with a SST decrease and with the onset of phytoplankton bloom. Condition index (CI) did not reflect the reproductive cycle of *C. gallina*, Generally, CI followed the same trend of Chlo *a* and increased when clams were in gametogenesis and ripe stages following the phytoplankton bloom. However, thereafter, CI remained at high levels during the most part of the spawning period. Glycogen was positively correlated with gonadal index and Chlo *a*, which is typical from an opportunistic species. High total lipids values were recorded throughout the gonad ripeness and spawning, decreasing in the end of the spawning and in the rest period. Total lipids contributed most to the energy reserves and therefore these parameters. This kind of information could be most helpful for establishing effective management measures for *C.gallina* fishery and for assessing the potential of this species for aquaculture. The extended spawning period of the species will allow obtaining larval quality for a long period of the year, by artificial spawning induction of wild broodstock. Moreover, to shorten the gametogenic period of the species and thus make more profitable the production of *C.*

gallina in hatchery, the production plans should take into account the conditioning of broodstock in autumn, after the resting period.

3.1.1 Introduction

The striped venus *Chamelea gallina* (Linnaeus, 1758) is an infaunal bivalve species that occurs in the infralittoral zone. *C. gallina* is distributed throughout the Black Sea and Mediterranean (Poppe and Goto, 1993), the Adriatic Sea (Orban et al., 2006) and south of the Iberian Peninsula (Gaspar and Monteiro, 1998). Although this species inhabits a variety of sediment types it is preferentially distributed on the coastal well-sorted fine sand biocenosis (Perés and Picard, 1964). *C. gallina* is an important commercial bivalve species in Europe, especially in Iberian Peninsula and Mediterranean Sea. Along the Andalusia-Algarve coast, the exploitation of this species supports one of the most important fisheries (Gaspar and Monteiro, 1999; Gaspar et al., 1999; Chícharo et al., 2002a,b; Delgado et al., 2013). Officially, the annual landings exceed 3 000 tons (t) for this species in this region (Galisteo et al., 2012; DGRM, 2012), with a market price between 4 and 7 € /kg.

The synergistic action of fishing pressure coupled with the rapid growth rate and short lifespan of *C. gallina* leads to large inter-annual fluctuations in stock abundance and periodic recruitment failure (Gaspar, 1996). As a result, in some years the abundance of this species decreases dramatically, threatening the biological and economic sustainability of this fishery. The fishery management and the development of restocking programs or the promotion of stock enhancement supported by advances in *C. gallina* aquaculture could be an efficient strategy to rebuild stocks. However, to be able to do that, a detailed knowledge of the species reproductive cycle and its reproductive strategies is crucial.

The reproductive cycle of *C. gallina* have been previously studied in the Mediterranean Sea (Marano et al., 1980; Bodoy, 1983; Ramón, 1993; Erkan, 2009; Erkan and Sousa, 2002), in the Black Sea (Dalgic et al., 2009), in the Adriatic Sea (Poggiani et al., 1973; Salvatorelli,1967), in the Andalusia coast, particularly, in the Gulf of Cadiz (Rodríguez de la Rúa et al., 2003; Rodríguez de la Rúa, 2008; Delgado et al., 2013) and in the Algarve coast (Gaspar and Monteiro, 1998). However, no information on the

relationship between the reproductive cycle and the biochemical and energy dynamics was provided. Only Orban et al. (2006) studied the biochemical composition of *C. gallina*, but in a nutritional and commercial quality point of view.

Effectively, the relationship between the reproductive cycle and the energy storage and utilization cycles has already been reported by several authors for a wide variety of bivalves (e.g. Barber and Blake, 1981; Fernández-Castro and Vido-de-Mattio, 1987; Joaquim et al., 2011; Massapina et al., 1999; Ojea et al., 2004; Pérez-Camacho et al., 2003; Matias et al., 2013). Energy reserves are of considerable importance in reproduction and seasonal energy storage and utilization in bivalves are closely correlated to environmental conditions and the annual gametogenic cycles (e.g. Delgado et al., 2004; Holland, 1978; Ojea et al., 2004; Tlili et al., 2012). The timing and rate of energy storage in bivalves are mainly regulated by temperature and food availability (Joaquim et al., 2011). An accumulation of energy prior to gametogenesis, during the periods where food is abundant is the most ordinary model. This energy is then used for the gametogenic synthesis, when metabolic demand is high (Mathieu and Lubet, 1993) and later released during the spawning process (Albentosa et al., 2007). Carbohydrates constitute the most important and available energy reserve in bivalve adults, being glycogen the main component for supplying energy demands (Fernández-Castro and Vido-de-Mattio, 1987) for the reproductive cycle (e.g. Newell and Bayne, 1980; Pazos et al., 2005) whilst lipids play an important role in the gamete formation and are the main reserve of oocytes and bivalve larvae (Labarta et al., 1999; Matias et al., 2011). Many studies have shown that proteins did not contribute significantly to gametogenesis (Joaquim, et al., 2008; Joaquim et al., 2011; Matias et al., 2013). According to Beninger and Lucas (1984) and Albentosa, et al. (2007), proteins are mainly used in structural functions and represent an energy reserve, particularly in situations of low glycogen levels, or severe energy imbalance.

The present study improves the knowledge of *C. gallina* reproductive cycle including the characterization of the patterns of nutrient (glycogen and total lipids) storage and utilization. This information could be useful in the future for both the aquaculture production of this species and for implementing programs to enhance and restore natural stocks to reproductive viability.

3.1.2 Material and Methods

Sample collection

Sixty adult specimens of *C. gallina* (24 to 30 mm shell length) were collected monthly, during 2009, by local dredgers, from the Algarve coast (southern Portugal) off Ilha da Culatra (36º98′42″N and 7º83′36″W) at water depths ranging from 3 to 10 m (Fig. 3.1).

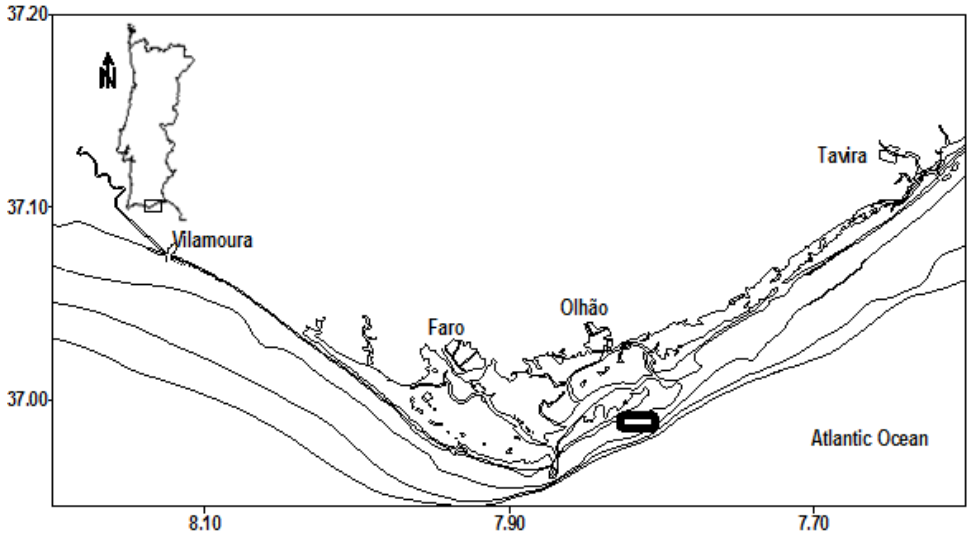

Figure 3.1 Location on the Algarve coast where *Chamelea gallina* were collected.

Sea surface temperature and Chlorophyll a

Monthly data on sea surface temperature (SST) were collected from the Portuguese Hydrographic Institute buoy nearest the sampling area and chlorophyll *a* (Chlo. *a*) data were derived from satellite remote sensing data, collected from the Giovanni online data system (MODIS-Aqua 4 km, monthly processed data, available at http://disc.sci.gsfc.nasa.gov/giovanni/overview/index.html, developed and maintained by the NASA Goddard Environmental Sciences Data and Information Services Center – GESDISC) (Acker and Leptoukh, 2007).

Laboratory analysis

In the laboratory, clams were placed in 0.45 μm-filtered seawater at 20 °C for 24 h to purge their stomachs, before condition index, histological, and biochemical analyses. Following the 24 h purging period, each clam was dissected and wet meat weight was determined.

Histology

Twenty individuals of each sex (when distinguishable) from each monthly sample were examined histologically to determine the gametogenic stages in both sexes. The visceral mass was separated from siphons and gills and fixed in San Felice solution for 48 h, then transferred to 70% ethyl alcohol (ETOH) for storage. Tissues from these samples were dehydrated with serial dilutions of alcohol and embedded in paraffin. Thick sections (6–8 μm) were cut on a microtome and stained with haematoxylin and eosin. The histologically prepared slides were examined using a microscope at 40× magnification and each specimen was assigned to a stage which represented the gonadal state. Clam reproductive maturity was categorized into six stages using a scale proposed by Gaspar and Monteiro (1998) (Fig. 3.2 and Fig. 3.3). When more than one developmental stage occurred simultaneously within a single individual, the assignment of a stage criteria decision was based upon the condition of the majority of the section.

A mean gonadal index (GI) was calculated using the method proposed by Seed (1976):

$$GI = \frac{\sum \text{ind. each stage x stage ranking}}{\text{total ind. each month}}$$

For each of the stages a numerical ranking was assigned as follows: inactive (0); early active gametogenesis (3); late active gametogenesis (4); ripe (5); partially spawned (2); spent (1). The GI ranged from 0 (all individuals in the sample are in rest stage) to 5 (all individuals are in ripe stage).

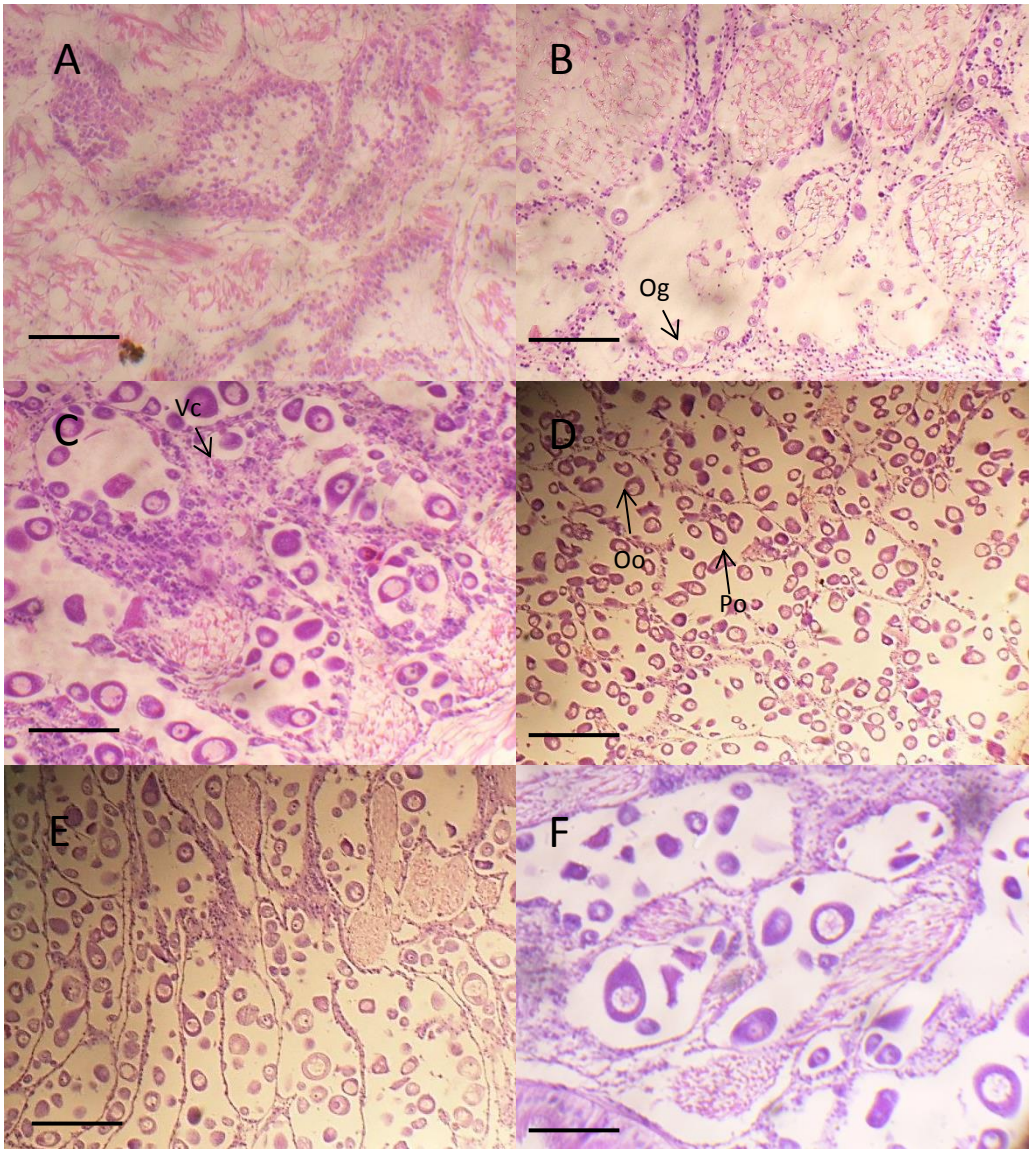

Figure 3.2 Photomicrographs showing stages in the development of *Chamelea gallina* female gonad. A. Inactive. B. Early active; Og – Ovogonia. C. Late active; Vc - Vesicular cell. D. Ripe; Oo – Oocytes; Po - Pedunculated oocyte. E. Partially spawned. F. Spent. Scale bar: 200 µm in A, B, C and F; 100 µm in D and E.

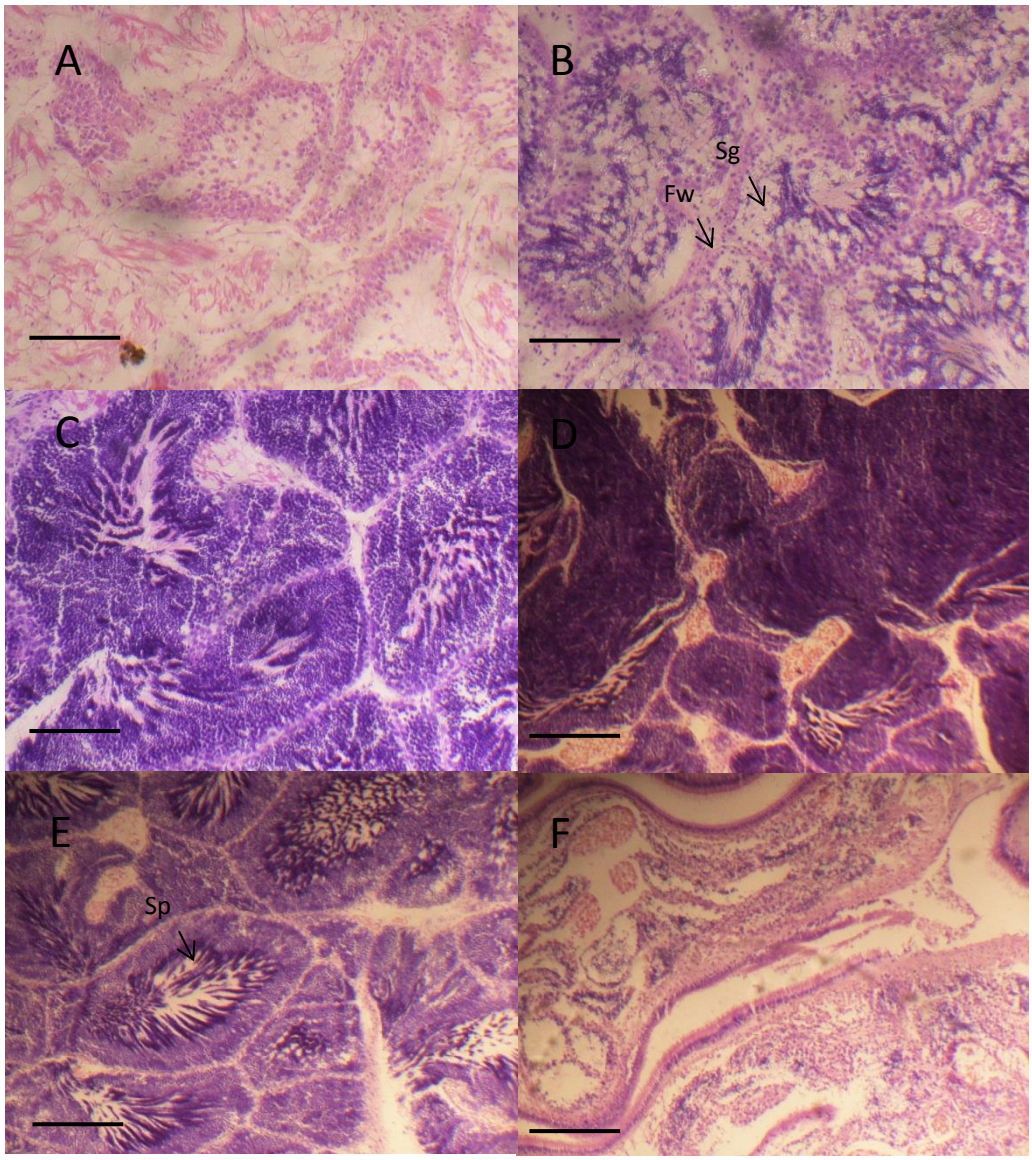

Figure 3.3 Photomicrographs showing stages in the development of *Chamela gallina* male gonad. A. Inactive. B. Early active; Sg – Spermatogonia; Fw - Follicule wall. C. Late active. D. Ripe. E. Partially spawned; Sp - Spermatozoa. F. Spent. Scale bar: 200 µm in A, B, C, and D, E; 100 µm in F.

Condition index

The dry meat and shell weights of 20 clams from each monthly sample were determined after oven drying at 80 °C for 24 h. Meat samples were then ashed at 450 °C in a muffle furnace, ash weight determined, and organic matter weight calculated as the ash free dry

meat weight (AFDW). The condition index (CI) was calculated according to Walne and Mann (1975):

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

Whenever the sex was possible to determine for all individuals analyzed in each sampling period by the observation of gonad smears under the microscope, the CI was calculated for females and males separately, otherwise its was determined for the entire sample without distinguish gender.

Biochemical composition

The meat of ten clams (5 females and 5 males, between March and July) from each monthly sample was frozen and stored at −20°C for biochemical analyses. For each specimen, glycogen content was determined from dried (80°C for 24 h) homogenate using the anthrone reagent (Viles and Silverman, 1949) and total lipids were extracted from fresh homogenized material in chloroform/methanol (Folch et al., 1957) and estimated spectrophotometrically after charring with concentrated sulphuric acid (Marsh and Weinstein, 1966). Duplicate determinations were performed in all cases and values are expressed as a percentage of AFDW. Caloric content of lipids and glycogen in tissues was calculated using the factors 33 KJ g^{-1} (Beninger and Lucas, 1984) and 17.2 KJ g^{-1} (Paine, 1971), respectively.

Statistics

Seasonal variations in histological parameters, condition index and biochemical composition were analyzed by one-way ANOVA or Kruskal–Wallis ANOVA on ranks whenever the assumptions of analysis of variance (ANOVA) failed. Percentage data were arcsine transformed to normalize variance (Sokal and Rohlf, 1981). Multiple pairwise comparisons were performed using the post-hoc parametric Tukey test or the non-parametric Dunn's test in order to detect significant differences between monthly consecutive samples. The *t*-test or the Mann-Whitney, whenever the assumption of *t*-test failed, was used to analyze the differences between sexes for all studied parameters. The Pearson or the Spearman (if the residuals are not normally distributed) correlation coefficient was used to determine the degree of association between parameters. Results

were considered significant at P<0.05. The statistical analyses were performed using the SIGMASTAT 3.11 statistical package.

3.1.3 Results

Sea surface temperature and Chlorophyll a

The evolution of the monthly SST and Chlo *a* during the experimental period in the sampling area is shown in Figure 3.4. The SST followed a seasonal cycle, ranging between 14.8±0.4ºC in February and 22.2±2ºC in August. Regarding Chlo *a*, the lowest values were registered in September and October (0.5 ±0.3 mg.m^{-3} and 0.5±0.2 mg.m^{-3}, respectively), coinciding with high SST values, whereas the highest value was observed in March (7.0±4.0 mg.m^{-3}), before the raising of SST. As expected, SST was negatively correlated with the Chlo *a* (Spearman's, r=−0.70, *P*<0.01) (Table 3.1).

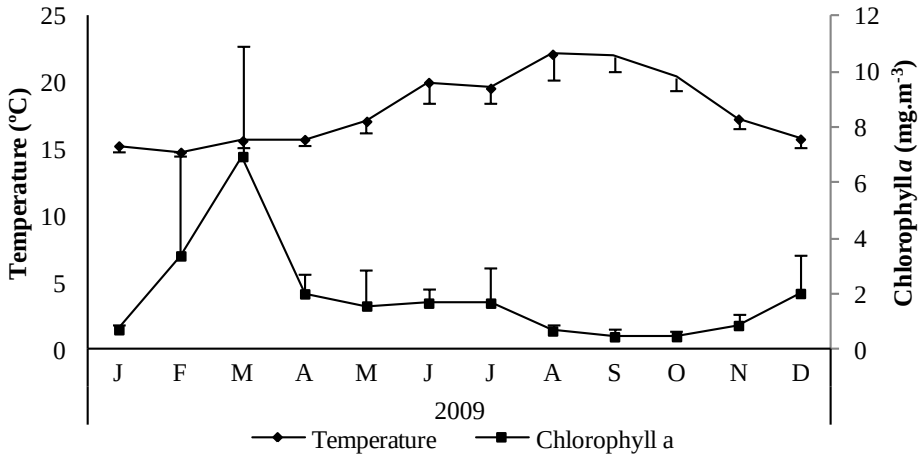

Figure 3.4 Monthly values (mean ± SD) of sea surface temperature (SST) and chlorophyll *a* in Algarve coast during 2009 (*statistically significant differences, *P*<0.05 found between consecutive samples).

Gametogenic cycle

The sexes were clearly separated and no hermaphrodites were found. Both sexes showed synchronism in gonadal development and no significant differences were found between GI of males and females (M-W, *P*>0.05). The reproductive cycle of *C. gallina* was characterized by a seasonal pattern (Fig. 3.5) and GI was negatively correlated with

SST (Pearson's, r=−0.76, P<0.01) (Table 3.1). The development of gametes took place in winter, coincided with the phytoplankton bloom.

Table 3.1 Results of Pearson and Spearman correlations between studied parameters (r, correlation coefficient, P, P value, n.c., no correlation was found).

	Chlorophyll *a*	Gonadal index	Condition index	Total lipids	Glycogen	Total energy
Temperature	$r = -0.70$ $P < 0.01$	$r = -0.76$ $P < 0.01$	n.c.	n.c.	$r = -0.62$ $P < 0.05$	n.c.
Chlorophyll *a*		$r = 0.80$ $P < 0.001$	$r = 0.68$ $P < 0.01$	n.c.	$r = 0.58$ $P < 0.05$	n.c.
Gonadal index			n.c.	n.c.	$r = 0.60$ $P < 0.05$	n.c.
Condition index				n.c.	n.c.	n.c.
Total lipids					n.c.	$r = 0.91$ $P < 0.001$
Glycogen						n.c.

A significant correlation was also found between GI and Chlo *a* (Spearman's, r=0.80, P<0.001). The population reached its peak of reproductive effort between March and May for both sexes, represented by the highest values of GI (4.5 and 4.6 in March for female and male, respectively, and 4.2 in May for both sexes), however, with a decline in April (GI=3.9, for both sexes) following the abrupt decrease of Chlo *a* (Fig. 3.6). In this month, spawning began for both sexes and intensified during summer as SST increased. After a short period of inactivity, between October and November (which coincided with the decrease of SST), the onset of phytoplankton bloom occurred and clams progressed to the gametogenesis.

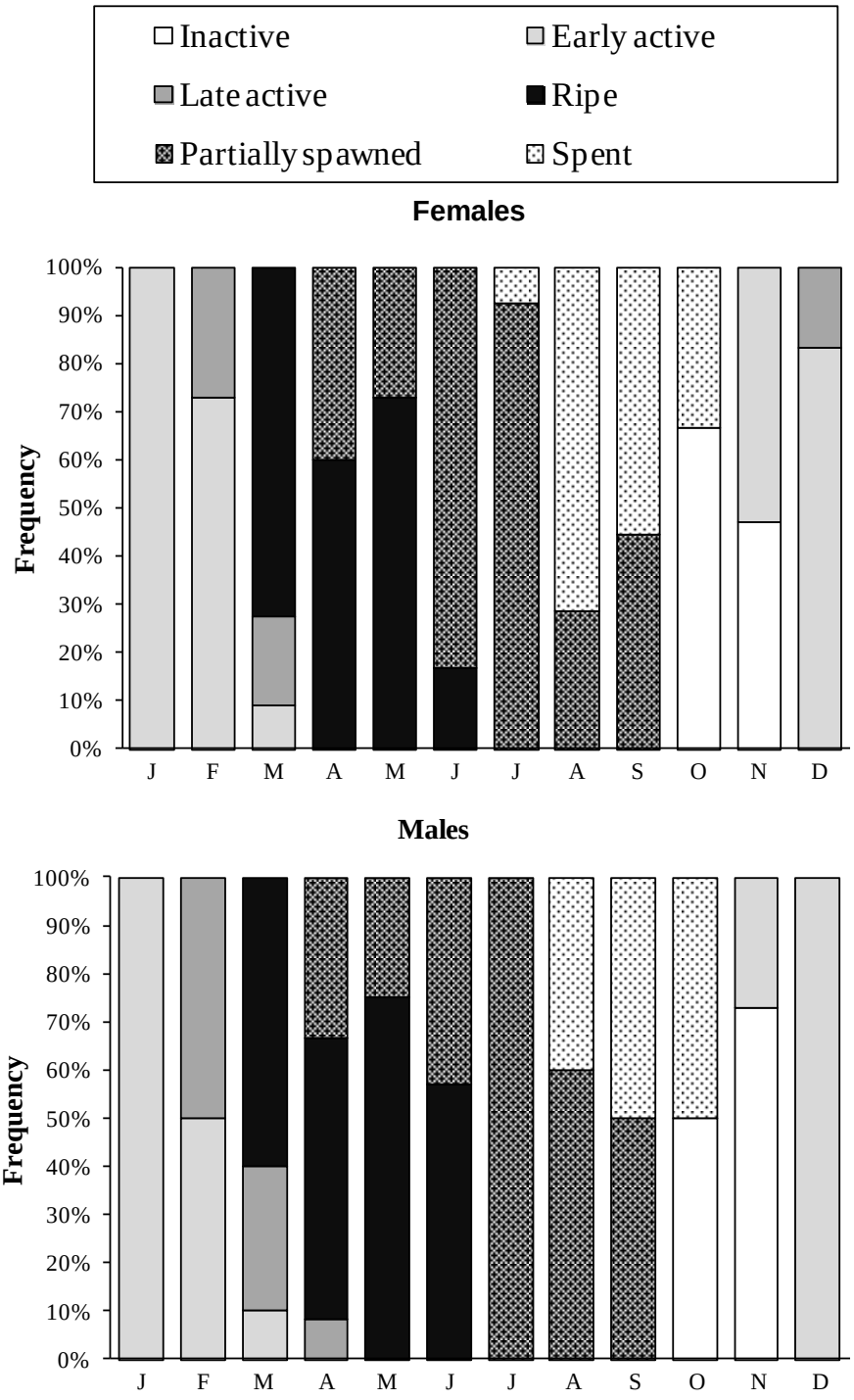

Figure 3.5 Monthly variations in gonadal development of *Chamelea gallina*, during 2009. Females (top) and Males (bottom).

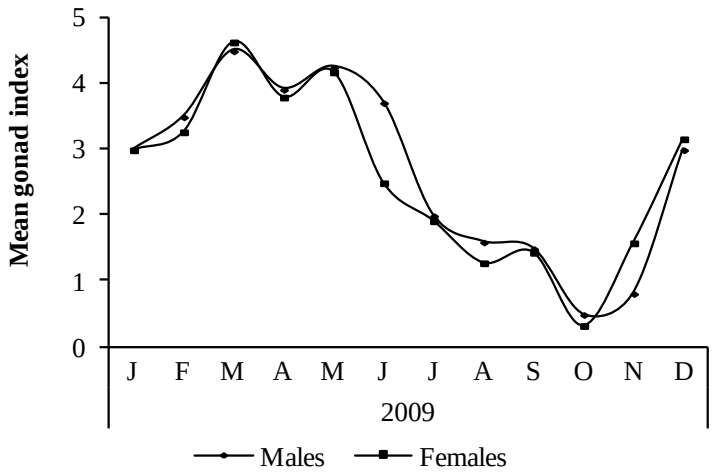

Figure 3.6 Monthly variations in gonad index (GI) of *Chamelea gallina* females and males (mean, n = 20), during 2009.

Condition index

Between January and March, CI increased with the ripeness of the gonad and the phytoplankton bloom and remained generally high afterwards until July, *i.e.* during the most part of the spawning period (Fig. 3.7). Generally, CI followed the same trend of Chlo *a* and a significant positive correlation (Spearman's, r=0.68, P<0.01) was found between these two parameters. Although the CI of females was lower than CI of males, no significant differences were found between sexes, except in July (t-test, t=2.19, P<0.04). Notwithstanding, the highest value of CI (5.00±0.81) was registered for males in July when all individuals were in spawning while for females the highest value of CI (4.57±0.81) was recorded one month earlier. After July, CI decreased reaching its lowest value (2.75±0.37) in August. At the end of spawning, this parameter started to increase again until the end of the year, except in November. Condition index exhibited statistically significant differences (K–W., H=109.2, $d.f.$=11, P<0.001) only in the consecutive months of July and August. No significant correlations were found between CI and both GI and SST (Table 3.1).

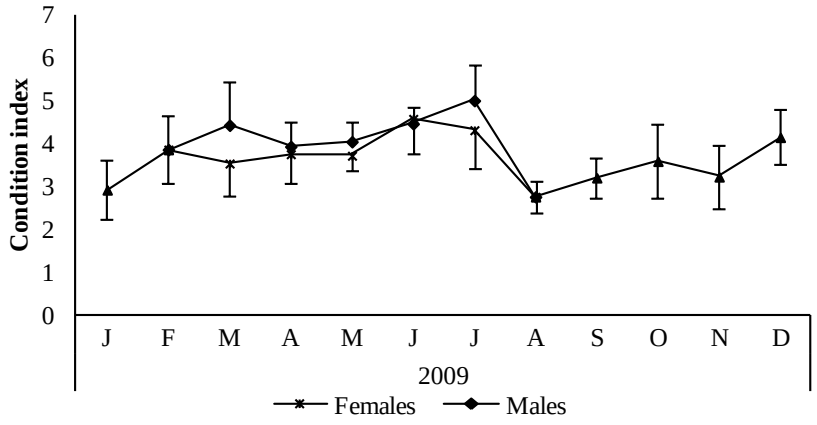

Figure 3.7 Condition index (mean ± SD) of *Chamelea gallina*, during 2009.

Biochemical composition

Glycogen increased between January and February with the increase of phytoplankton bloom, decreasing afterwards until April (Table 3.2). In May, glycogen increased again and significant differences were found between sexes (M-W., t=61.0, n (*small)*=9, n (*big*)=10, P<0.03). Thereafter, the glycogen reserves decreased during spawning, reaching the lowest value in August (20.0±15.1 μg mg^{-1} AFDW). In the next months and until the end of the year, this content showed an increase trend following temperature decrease and the phytoplankton bloom onset. Indeed, glycogen was positively correlated with Chlo *a* (Spearman's, r=0.58, P<0.05) and negatively correlated with the SST (Pearson's, r=-0.62, P<0.05) (Table 3.1). No statistical correlation was found between glycogen and CI; however this biochemical content correlated with GI (Pearson's, r=0.61, P<0.05). The highest value of glycogen for females was found in March (74.0±14.2 μg mg^{-1} AFDW) and for males in May (95.8±8.0 μg mg^{-1} AFDW). Total lipids content differed between sexes in April (t-test, t=-2.41, $d.f.$=15, P<0.03) and June (t-test, t=-3.20, $d.f$=16, P<0.01). Despite no statistical significant correlations were found between total lipids and the GI, this content peaked throughout the gonad ripeness and the spawning period of the species (in April and June) and decreased at the end of the spawning and during the resting period. Females and males reached the highest values of total lipids in April (98.5±12.7 μg mg^{-1} AFDW and 83.4 ±13.0 μg mg^{-1} AFDW, respectively). The lowest (24.0±5.0 μg mg^{-1} AFDW) total lipid values were found in December (Table 3.2). No significant

correlations were found between total lipids and glycogen content, neither with CI nor with the studied environmental parameters (SST and Chlo *a*). Total lipids contributed most to the energy content which explain the positive correlation found between these parameters (Pearson's, r=0.91, P<0.001) (Table 3.1). No correlation was observed between energy and the other studied parameters. Males reached the highest (4.2 kJ mg^{-1} AFDW) energy value in June and females in April and June (3.6 kJ mg^{-1} AFDW). The lowest (2.0 kJ mg^{-1} AFDW) energy values were recorded in October/December (Table 2.2). The energy of females differed from males in June (t-test, *t*=-2.49, *d.f*=16, *P*<0.03). Statistically significant differences were observed between consecutive sampling periods for all the biochemical constituents studied (glycogen: ANOVA, *d.f.*=11, *F*=20.63, *P*<0.001; total lipids: ANOVA, *d.f.*=11, *F*=35.21 *P*<0.001; energy: K-W., *H*=101.5, *d.f.*=11, *P*<0.001) (Table 3.2).

Table 3.2 Mean values (±sd) of glycogen, total lipids (µg mg^{-1} AFDW) and energy (kJ g^{-1} AFDW) of *Chamelea gallina* during the experimental period (a, b statistically significant differences, *P*<0.05 found between consecutive samples and between sexes, respectively).

Month	Sex	Glycogen (µg mg^{-1} AFDW)		Total lipids (µg mg^{-1} AFDW)		Energy (kJ mg^{-1} AFDW)	
Jan		43.6 ± 19.0	a	46.1 ± 12.2	a	2.4	a
Feb		78.7 ± 12.7		72.6 ± 15.4		3.9	
Mar	♀	74.0 ± 14.2		60.1 ± 4.7		3.4	
	♂	74.8 ± 14.9	a	44.2 ± 13.0	a	2.8	
Abr	♀	39.6 ± 9.1		83.4 ± 13.0		3.6	
	♂	34.9 ± 9.2	a	98.5 ± 12.7	a	4.1	
May	♀	62.8 ± 31.7	b	56.9 ± 12.4	b	3.1	
	♂	95.8 ± 8.0	a	50.5 ± 9.2	a	3.4	
Jun	♀	51.2 ± 18.1		77.5 ± 11.4		3.6	b
	♂	51.5 ± 12.1		95.2 ± 11.6		4.2	a
Jul	♀	41.9 ± 20.6		55.5 ± 20.5		2.7	
	♂	47.3 ± 12.0	a	45.2 ± 7.3	a	2.4	
Aug		20.0 ± 15.1	a	55.2 ± 12.6	a	2.3	
Sep		46.1 ± 16.1		36.4 ± 3.7		2.1	
Oct		25.5 ± 7.9	a	42.9 ± 6.9	a	2.0	
Nov		72.1 ± 0.3		43.4 ± 12.0		2.8	
Dec		66.9 ± 14.3		24.0 ± 5.0		2.0	

3.1.4 Discussion

The reproductive activity of bivalves, which includes a sequence of events from gametogenesis to spawning, is controlled by the interaction between endogenous and environmental factors (Enríquez-Díaz et al., 2009; Normand et al., 2008). The major role has traditionally been assigned to the temperature and food availability (e.g. Eversole, 1989; Grant and Creese, 1995; Gribben et al., 2004). The SST and Chlo *a* patterns and values observed in this study were typical from temperate climates and were similar to the reported by Delgado et al. (2013) for a near area (Gulf of Cadiz). The seasonal SST pattern was characterized by relatively low sea water temperatures during the winter that increased during spring and early summer, stabilized in the end of the summer, and decreased in autumn. The negative correlation found between SST and the Chlo *a* had already been reported by Falcão et al. (2007) for the studied area. According to these authors, when SST is lower, an upwelling event occurs, giving rise to the phytoplankton bloom registered from late winter until early spring.

As has been reported for other bivalve species (e.g. Gabbott, 1973; Xie and Burnell 1994; Albentosa et al., 2007, Ojea et al., 2004), the reproductive cycle of *C. gallina* was significantly influenced by SST and food availability. The GI followed a seasonal cycle and a negative correlation was found between this parameter and the SST. This species comprised a ripe stage in spring followed by spawning that began in the middle of spring and extended during summer. A similar reproductive cycle with an extended spawning period was already described by Gaspar and Monteiro (1998) and Delgado et al. (2013) for populations of *C. gallina* from the same area of study and from the near Gulf of Cadiz, respectively. The population reached its peak of maturation (ripe stage) in March and the decline of GI in the next month was a consequence of the spawning onset. Apart from the start of the SST increasing during April, spawning onset may also be triggered by the sharp decline of chlorophyll *a* in this month, since a significant correlation was found between GI and Chlo *a*. However, the expected sequential development of gonadal stage was interrupted in May with the gametogenesis re-initiation and consequent gonad recovers. Delgado et al. (2013) also detect interruptions in gametogenesis, however, these authors reported that this event was not unique and intra-gonadal and inter-individual asynchrony in the spawning period was observed. In our study, although the extended reproductive period of the species, no signals of

successive asynchrony in spawning were detected. The recovery of the gonad was an isolated episode after which spawning intensified for both sexes until the end of summer as SST increased and with low food availability. Nevertheless, Gaspar and Monteiro (1998) reported that, twenty years ago, spawning of this population began in May and the gonad index of this population, once it fell in April never rose again to a second peak in the same year. These small differences in the reproductive cycle of the species can be inherent to environmental factors associated with the different geographical location on the first case and with environment changes over times in the other. The extended spawning period and the maintenance of a synchronized gonadal development observed between males and females during the spawning period ensures the reproductive success of the species since gametes will be expelled into the water column simultaneously for a long period, augmenting the probability of fertilization. This synchronism also had previously been reported by Gaspar and Monteiro (1998) for the species. After a short period of inactivity between October and November, clams progressed to the onset of gametogenesis that occurred in November coincided with a SST decrease and with the onset of phytoplankton bloom. The development of gametes with the proliferation of gonias intensified in winter following the phytoplankton bloom, reaching the ripe stage in March. The gametogenesis results were generally consistent with the previous findings by Gaspar and Monteiro (1998) and Delgado et al. (2013).

Condition index is generally considered to reflect the reproductive activity of bivalves (Fernández-Castro and Vido-de-Mattio, 1987; Massapina et al., 1999; Ojea et al., 2004). This relationship has been observed in several bivalve species from the Portuguese coast (e.g. Gaspar and Monteiro, 1998; Joaquim et al., 2011; Moura et al., 2008). In this study, CI did not reflect the reproductive cycle of *C. gallina*, since no significant relationship was found between these parameters, nor was observed a correlation between CI and SST. Generally, CI followed the same trend of Chlo *a* and a significant correlation was found between these two parameters. CI increased when clams were in gametogenesis and ripe stages following the phytoplankton bloom but also reflected the abrupt Chlo *a* decrease in April and the gametogenesis re-initiation in the next month. However, thereafter, CI did not followed the reproductive cycle since it remained at high levels during the most part of the spawning period. Nevertheless, this phenomenon

was not observed by other authors for *C. gallina* (Gaspar and Monteiro, 1998;, Moschino and Marin, 2006; Orban et al., 2006). According to these authors, CI only increased with the progress of gametogenesis declining during the spawning period. Although the CI of females was lower than CI of males, this difference was not statistically significant, except in July. In the next month, CI decreased until the lowest value and started to increase again even in the end of the spawning period of the species. It is also generally accepted that CI is highly influenced by the energy storage and exploitation strategy of bivalve species (Delgado and Pérez-Camacho, 2005; Joaquim et al., 2008, 2011). Orban et al. (2006) found a relationship between CI and the biochemical constituents of this species in the Adriatic Sea. However, again, in our study this fact cannot be confirmed for *C. gallina*, since no statistically correlation was found between CI and the biochemical contents studied (total lipids and glycogen).

The relative amounts of glycogen (20.0 to 95.8 μg mg^{-1} AFDW) measured in *C. gallina* were lower, in term of the proportions, to those previously described by Orban et al. (2006) for this species, however the amount of total lipids (23.95 to 98.5 μg mg^{-1} AFDW) was similar. Several studies on bivalves have shown that sexual maturity is related to energy supply from previously stored reserves or the ingestion of available food and consequently is closely linked with the biochemical composition (Pérez - Camacho et al., 2003; Sastry, 1979). The reproductive cycle translates a seasonal pattern of biochemical composition that can vary among populations and species (Albentosa et al., 2007). Glycogen is one of the main energy reserves in adult bivalves. In this study, this energy reserve was positively correlated with GI, which is typical from an opportunistic species, in which the gamete production occurs coupled with the phytoplanktonic blooms and following the SST decrease. Indeed, glycogen was positively correlated with Chlo *a* and negatively correlated with SST. Delgado et al. (2013) also referred *C. gallina* as an opportunist species in terms of energy storage and utilization cycles. The abrupt decrease of Chlo *a* in April, has probably forced the species to a remarkable consumption of the glycogen content reflecting its use in the formation of gametes. However, in May, it was observed a recover of the glycogen content, accompanied by the gametogenesis re-initiation and consequent gonad recover. Thereafter, this content decreased gradually with the spawning evolution and the rising of SST, reaching the lowest value at the end of spawning. Although the extended

reproductive effort, *C. gallina* retained some glycogen reserves as an energy source for the maintenance of their physiological state during the resting period. This is an important strategy for survival of the species, since some bivalves, such as the *Ruditapes decussatus* almost depletes its energy reserves in the reproductive period, which leads to their debilitation and consequent dead (Matias et al., 2013). Several authors (e.g. Beninger and Lucas, 1984; Mouneyrac et al., 2008; Ojea et al., 2004) have reported that lipid seasonal variations are inversely related to glycogen, due to the conversion of glycogen into lipids, biosynthesized during the formation of gametes (Gabbott, 1975). In conservative species, this process is detected by a negative correlation between total lipids and glycogen content, since the reserve accumulation is lagged in time with gametogenesis. In *C. gallina*, an opportunist species, the glycogen accumulation occurred simultaneously with the *novo* synthesis of lipids during the gametogenesis process which justifies the lack of significant correlation between this two biochemical contents. Although the highest values of total lipids were recorded throughout the gonad ripeness and spawning, decreasing in the end of the spawning and in the rest period, no significant correlations were found between total lipids and GI, neither with CI nor with the environmental parameters (SST and Chlo *a*). Contrary, Orban et al. (2006) found coincident fluctuations for the biochemical contents and the CI of *C. gallina*. The erratic variation of total lipids shown in the reproductive period may be related to production and simultaneous release of gametes. Total lipids contributed most to the energy reserves and therefore these parameters showed the same trend along the year. No correlations were found between energy and the other studied parameters.

Besides contribution to increase the knowledge on the reproductive biology of *Chamelea gallina*, this work provides the first data on the consequent energy reserves storage and depletion in this species. This kind of information could be most helpful for establishing effective management measures for this fishery and for assessing the potential of this species for aquaculture. The extended spawning period of the species will allow obtaining larval quality for a long period of the year, by artificial spawning induction of wild broodstock. Moreover, to shorten the gametogenic period of the species and thus make more profitable the production of *C. gallina* in hatchery, the

production plans should take into account the conditioning of broodstock in autumn, after the resting period.

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Chapter 3.2

The reproductive cycle of white clam *Spisula solida* (L.) (Mollusca: Bivalvia): Implications for aquaculture and wild stock management

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

Abstract

The dynamics of the white clam's (*Spisula solida*) reproductive cycle along with its nutrient storage and exploitation strategy in the Algarve coast (Portugal) was studied throughout the year 2003. The timing of gametogenic development and spawning of *S. solida* were analysed through histological preparation using qualitative and quantitative criteria. Condition index and biochemical composition were determined in order to provide information on energy storage and utilization. Seawater temperature is a primary environmental factor determining reproductive development and spawning of *S. solida*; reproductive activity occurred during low temperatures. The spawning period began in late winter as a consequent response to the increase in seawater temperature and extended through spring. During this period, the condition index and the gonadal index decreased. In June, most of the population was spent and big resting oocytes appear dispersed in the gonad. In summer, the specimens were found to be in the resting phase and condition index increased to its maximum value as a consequence of reserves storage. Gametogenic activity was initiated coincident with decreasing temperature in September, but a sudden increase of this environmental parameter in October disturbed the gametogenic process and a second spawning occurred. In this period, the synchronism between males and females of the population was lost. The striking consumption of glycogen reserves developed during the previous August and consequent biosynthesis of lipids during gamete formation occurred. In the following two months, reproductive synchronism was restored, and storage of reserves and gametogenesis took place concurrently. In December the entire population was in the ripe stage of gonadal development. Moreover than a consequence of gametogenesis during autumn/ winter, lipid behavior reflected the energy accumulation process and its conversion to somatic development in spring/summer. The reproductive strategy adopted by *S. solida* makes possible broodstock manipulation in terms of conditioning in aquaculture. The information obtained in this study is important for assessing sustainable management of wild stocks as well as for estimating its potential for aquaculture production.

3.2.1 Introduction

The white clam *Spisula solida* (Linnaeus, 1758) has been reported from off the south of Iceland, and the Norwegian Sea, to the Atlantic coast of the Iberian Peninsula, Morocco and Madeira (Tebble, 1966). This species is of commercial importance in Ireland (Fahy et al., 2003), Spain (Peña et al., 2005) and Portugal (Gaspar and Monteiro, 1999) where it is the target of a dredge artisanal fishery, and *S. solida* may have commercial fishery potential in other countries including France and Morocco (Gaspar and Monteiro, 1999). However, in the past decade the synergistic action of the intensive harvest of *S. solida*, coupled with the rapid growth rate and short lifespan of the species has resulted in large inter-annual fluctuations in stock abundance and periodic recruitment failure (Joaquim et al., 2008). In order to try to reverse this negative trend, it is of utmost importance to improve the management of the fishery and to develop restocking programs supported by aquaculture advances to rebuild the reproductive viability of depauperate populations.

Knowledge of the reproductive cycle of *S. solida* is fundamental for developing management strategies (e.g. protect spawning stock and/or larval settlement) (Shaw, 1965; Manzi et al., 1985; Sbrenna and Campioni, 1994) and is crucial for establishing successful hatchery based production (Gribben et al., 2004; Peharda et al., 2006). The reproductive cycle of *S. solida* was investigated by Gaspar and Monteiro (1999) for a population off Vilamoura (southern Portugal). In their study, the seasonal gonadal development was followed using qualitative histological methods. However, the subjectivity inherent to these methods has led us to think that the use of a quantitative histological method (oocyte diameter) would be important to confirm patterns observed from qualitative analyses. Barber and Blake (1981) suggest that qualitative staging is necessary to describe the reproductive events pertaining to gamete development but that detailed quantitative information eliminates the subjectivity and semantic problems associated with qualitative description and thereby enhance our ability to extract ecologically meaningful information.

Several authors have reported the relationship of the reproductive cycle with energy storage and utilization cycles and with local environmental conditions in a wide variety of bivalves (e.g. Costa Muniz et al., 1986; Fernandez Castro and Vido de Mattio, 1987;

Massapina et al., 1999; Camacho et al., 2003). In general, reserves accumulate prior to gametogenesis in the form of glycogen, lipid and protein substrates, and subsequently are utilized in the production of gametes when metabolic demand is high (Mathieu and Lubet, 1993). However, that relationship has not been studied in *S. solida*.

The main objectives of this study were to determine from reproductive staging of histological preparations (using qualitative and quantitative criteria), from condition index analyses, and from biochemical composition assays, the reproductive cycle and the pattern of energy storage and utilization in a population of *S. solida* from the Algarve (southern Portugal).

3.2.2 Materials and methods

Sample collection

Forty-five adult specimens (30 to 33mm shell length) were collected monthly during 2003 by local dredgers from a site off Fuzeta (Algarve, southern Portugal; Fig. 3.8) at water depths ranging from 4 to 8 m. Mean monthly surface seawater temperature (SST) data were provided by Tunipex. SST was measured daily with an YSI multiparameter probe.

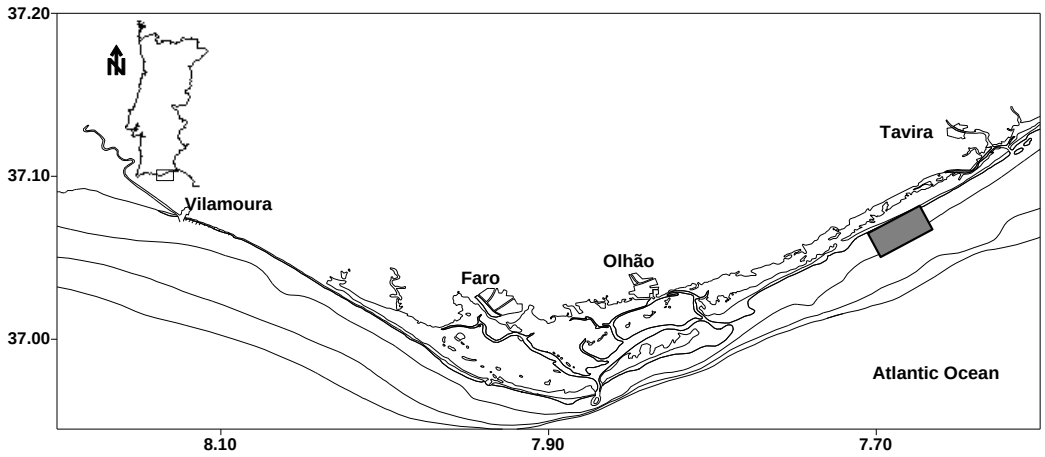

Figure 3.8 Location on the Algarve coast where *Spisula solida* were collected.

Laboratory analysis

In the laboratory, clams were placed in 0.45 µm-filtered seawater at 20 °C for 24 h to purge their stomachs before histological, condition index and biochemical analyses.

Following the 24 h purging period, each clam was dissected and wet meat weight was determined.

Histology

Ten individuals of each sex from each monthly sample were examined histologically to determine the gametogenic stages of both sexes. The visceral mass was separated from siphons and gills and fixed in San Felice solution for 24 h, then transferred to 70% ethyl alcohol (ETOH) for storage. For microscopic examination, tissues from these samples were dehydrated with serial dilutions of alcohol and embedded in paraffin. Thick sections (6–8 μm) were cut on a microtome and stained with haematoxylin and eosin. The histologically prepared slides were examined using a microscope at 40× magnification and each specimen was assigned to a stage which represented the gonad condition. Clam reproductive maturity was categorized into six stages using the scale proposed by Gaspar and Monteiro (1998).When more than one developmental stage occurred simultaneously within a single individual, the staging criteria decision was based upon the condition of the majority of the section. For each one of those stages a numerical ranking was assigned as follows (Gosling, 2003): Inactive (0); Early active (3); Late active (4); Ripe (5); Partially spawned (2); Spent (1). A mean gonad index (GI) was then calculated using the method proposed by Seed (1976):

$$GI = \frac{\sum \text{ind. each stage x stage ranking}}{\text{total ind. each month}}$$

The GI ranged from 0 (all individuals in the sample are resting) to 5 (all individuals are ripe). The images of each slide were recorded with a Nikon DSFi 1 camera and subsequently analysed using the freely available image analysis software Image J 1.38s. In order to obtain quantitative data the diameter of all oocyte with visible nuclei from five randomly selected female images were was measured.

Condition index

Dry meat and shell weights of 20 clams from each sample were determined after oven drying at 80°C for 24 h. Meat samples were then ashed at 450°C in a muffle furnace, ash weight determined, and organic matter weight calculated as the ash free dry meat

weight (AFDW). The condition index (CI) was calculated according to Walne and Mann (1975):

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

Biochemical composition

The meat of five clams of each monthly sample was frozen and stored at −20°C for biochemical analyses. For each specimen, protein was determined using the modified Lowry method (Shakir et al., 1994), glycogen content was determined from dried (80°C for 24 h) homogenate using the anthrone reagent (Viles and Silverman, 1949) and total lipids were extracted from fresh homogenised material in chloroform/methanol (Folch et al., 1957) and estimated spectrophometrically after charring with concentrated sulphuric acid (Marsh and Weinstein, 1966). Duplicate determinations were performed in all cases and values are expressed as a percentage of AFDW. Caloric content of protein, lipid and carbohydrates in tissues was calculated using the factors 17.9 kJ g^{-1} (Beukema and De Bruin, 1979), 33 kJ g^{-1} (Beninger and Lucas, 1984) and 17.2 kJ g^{-1} (Paine, 1971), respectively

Statistics

Seasonal variations in condition index, biochemical composition and histological parameters were analysed by one-way ANOVA or Kruskal–Wallis ANOVA on ranks whenever the assumptions of analysis of variance (ANOVA) failed. Percentage data were arcsine transformed to normalize variance (Sokal and Rohlf, 1981). Multiple pair comparisons among means were performed using the post-hoc Tukey test. The Pearson correlation coefficient was used to determine the degree of association between parameters. The statistical analyses were performed using the SIGMASTAT 3.11 statistical package.

3.2.3 Results

Temperature

The evolution of mean monthly SST during the experimental period is shown in Figure 3.9. SST ranged between 14.5 ºC and 22.4 ºC with a minimum in February and a maximum in August. Unusual SST variations were observed in July and October.

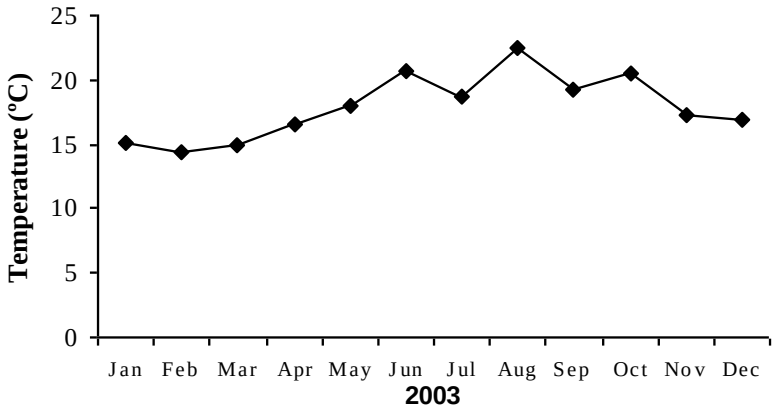

Figure 3.9 Monthly average values of sea surface temperature (SST) in Algarve coast during 2003.

Gametogenic cycle

The sexes were clearly separated and no hermaphrodites were found. Generally, both sexes showed a synchronism in gonadal development until August (except in March), however differences were found between males and females in the following three months. For this reason we decided to analyse the reproductive cycle of both sexes separately (Fig. 3.10). In January 70% of males and 50% of females were in the late active stage while the remaining specimens were ripe.

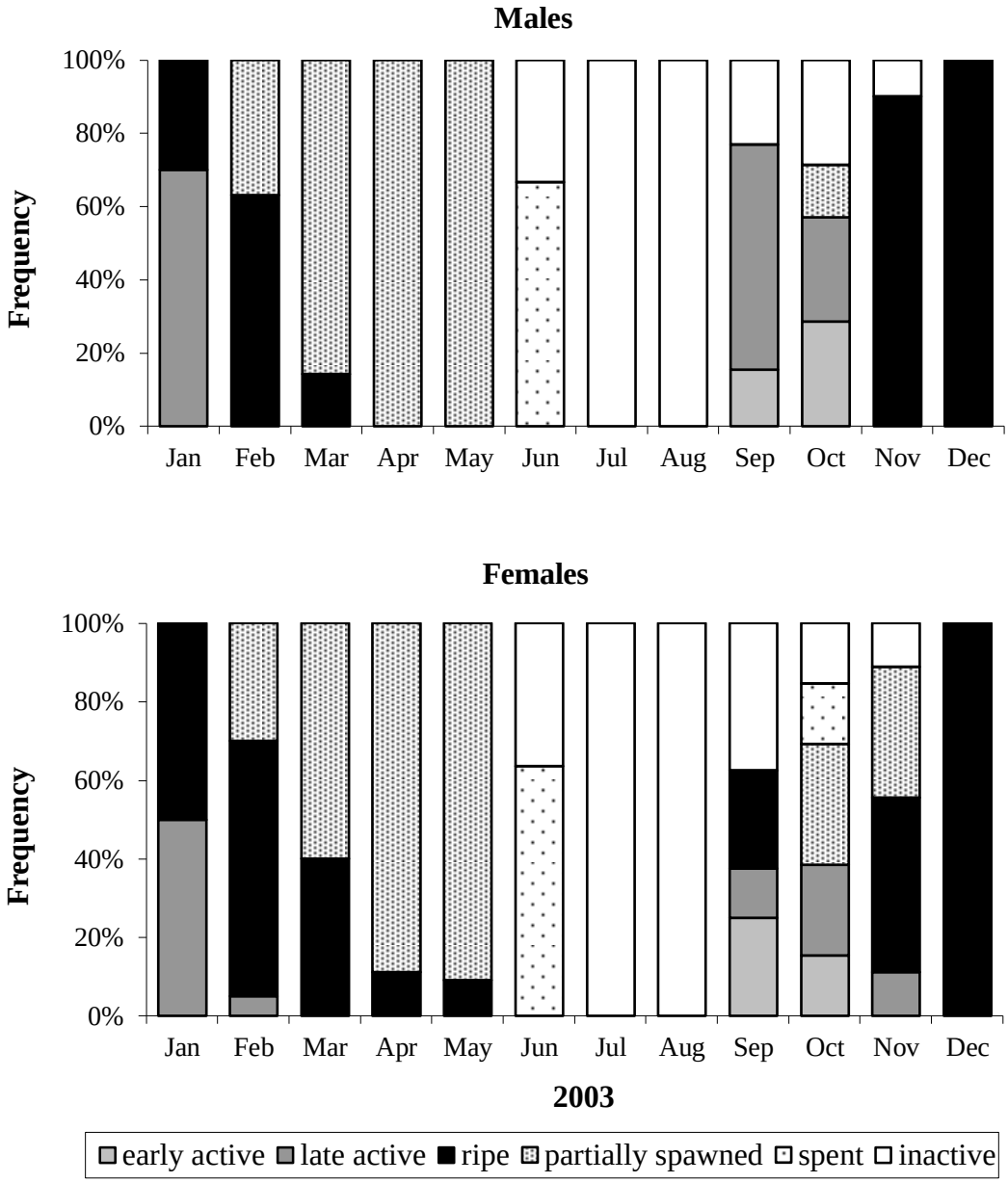

Figure 3.10 Monthly variations in gonadal development of *S. solida* during 2003. Males (top) and Females (bottom).

The onset of spawning occurred coincident with increased SST in February. Spawning continued until the end of May with a peak in April and May when almost all the individuals had spawned. In June the majority of the population was in the spent stage (approximately 65%) whereas the remaining individuals were inactive. The population was sexually inactive in July and August and a new gametogenic cycle began in September. Between September and October gametes in both sexes developed quickly, and various stages of gonadal development coexisted in this period. This heterogeneity

of stages coincided with a sharp decrease of SST in September followed by an increase in October. During this month a second spawning event occurred with 14% of males and 30% of females in the spawning stage. Spawning continued in November. However, during this month only females were found in the spawning stage whereas the majority of males were ripe. In December, all the specimens observed were in the ripe stage. Gonad index (GI) (Fig. 3.11) followed the same pattern as gonad development; no significant differences were found between males and females GI (K–W, H=0.0749, $d.f.$=2, P<0.05). The monthly GI was negatively correlated with SST (Pearson's r=−0.63, P<0.02) (Table 3.3).

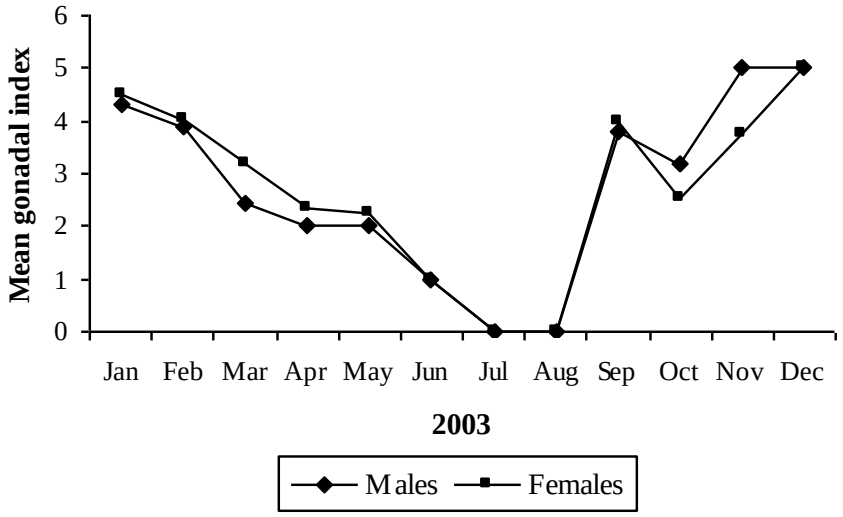

Figure 3.11 Mean gonad index of *S. solida* during 2003.

We measured 4960 oocyte diameter of *S. solida*. The mean oocyte diameter (Fig. 3.12), decreased from January to April, except in March, and increased afterwards reaching a maximum value of 40.4±6.8 μm in June. By July/August all residual oocytes had been resorbed.

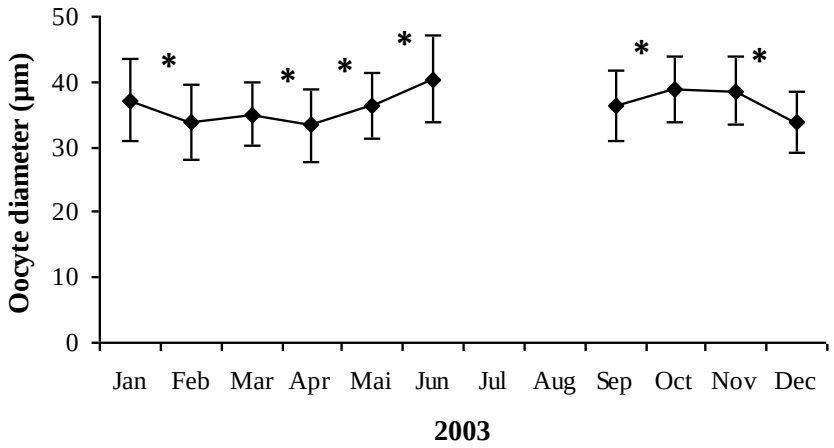

Figure 3.12 Monthly variations in oocyte diameter. Values are means±S.D. (*, statistically significant differences, $P<0.05$ found between consecutive months).

After the resting period, the oocyte diameter increased until October with values decreasing after November. Statistically significant differences were found among samplings (K–W, $H=566.3$, $d.f.=9$, $P<0.001$) and among females in the same sampling period (ANOVA, $P<0.05$), except in June (ANOVA, $P<0.05$). Oocyte diameters ranged from 10 to 49 µm (Fig. 3.13). Quantitative measurements of oocyte development support the patterns observed in the qualitative stagings (Fig. 3.10).

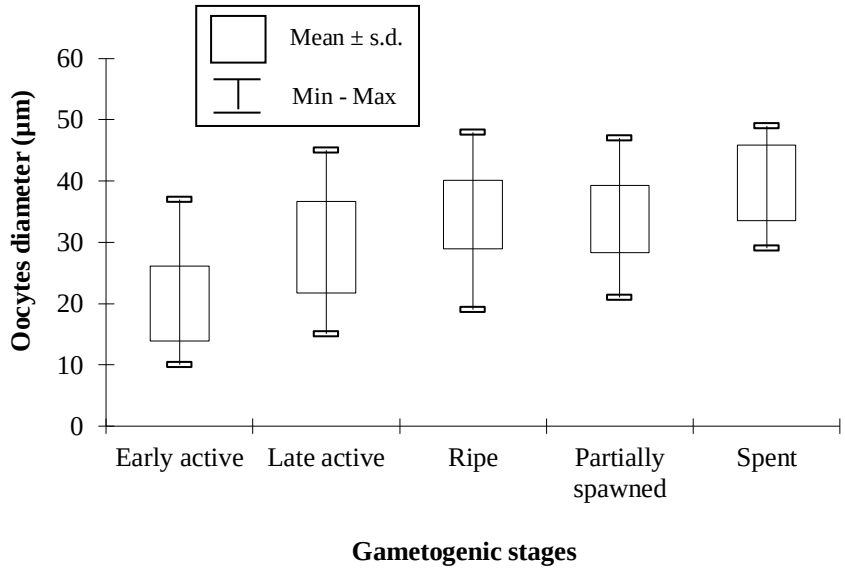

Figure 3.13 Reproductive development of *S. solida*. Average oocyte diameters relating to each stage of gonadal development.

The early active stage was characterized by a mean oocyte diameter of 20.0±6.1 µm. The mean oocyte diameter increased during gonadal maturation, attaining 29.2± 7.5 µm

and 34.5±5.6 μm in late active and ripe gonads, respectively. In the partially spawned stage the mean oocyte diameter decreased slightly to 33.8±5.5 μm. However, in the spent stage the mean oocyte diameter increased again (39.7±6.2 μm) due to the presence of degenerating oocytes. There were statistically significant differences among all stages of gonadal development considered (K–W, H=629.8, $d.f.$=4, P<0.001).

Table 3.3 Results of Pearson correlation between studied parameters (r, correlation coefficient, P, P value, n.c., no correlation was found).

	Gonadal index	Oocyte diameter	Condition index	Proteins	Total Lipids	Glycogen	Total energy
Temperature	r= -0.63 p= 0.02	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
Gonadal index		n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
Oocyte diameter			n.c.	n.c.	n.c.	n.c.	n.c.
Condition index				n.c.	n.c.	n.c.	n.c.
Proteins					n.c.	r= -0.57 p= 0.05	n.c.
Total Lipids						n.c.	r= 0.69 p= 0.01
Glycogen							n.c.

Condition index

Between January and February, CI increased with the ripeness of the gonad and decreased afterwards until late May during spawning (Fig. 3.14). During the resting period, CI started to increase. However this trend reversed in July probably due to the decrease of temperature observed during that month. The highest value of CI (9.51±0.94) was registered in August when all individuals of both sexes were in the resting stage. In September, with the onset of gametogenesis, CI decreased. This negative trend lasted until November and was related to the abnormal SST variation observed during autumn 2003. At the end of the year CI again started to rise. Condition index exhibited statistically significant differences (K–W., H=155.2, $d.f.$=11, P<0.001) among months. No statistical correlation was found between CI and GI, nor was a correlation observed between CI and SST (Table 3.3).

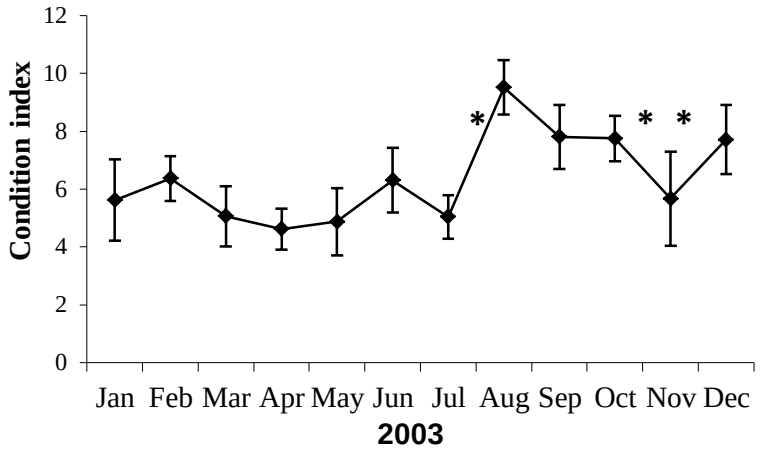

Figure 3.14 Mean condition index (±S.D.) in *S. solida* during the experimental period (*,statistically significant differences, P<0.05 found between consecutive months).

Biochemical composition

Proteins were the predominant clam constituent followed by total lipids and glycogen (Table 3.4). The highest value of protein content was recorded in July (384.4±59.8 µg mg^{-1} AFDW) and the lowest value was registered in September (227.2±38.6 µg mg^{-1} AFDW). Protein and glycogen contents were inversely correlated (Pearson's r=−0.57, P=0.05) (Table 3.3).

Table 3.4 Monthly mean values (±S.D.) of ash-free dry weight (mg), proteins, glycogen, total lipids (µg/mg AFDW) and total energy (kJ g^{-1} AFDW) of *S. solida* during the experimental period (*statistically significant differences, P<0.05 found between consecutive months).

Month	AFDW (mg)	Protein (µg mg^{-1} AFDW)	Glycogen (µg mg^{-1} AFDW)	Total lipids (µg mg^{-1} AFDW)	Total energy (kJ mg^{-1} AFDW)
Jan	178,05	289,5 ± 91,4	72,8 ± 30,7	72,1 ± 25,7	8,81
Feb	305,155	266,2 ± 59,2	77,1 ± 12,2	105,6 ± 29,48	9,58
Mar	267,165	364,3 ± 136,0	44,0 ± 21,6	106,2 ± 41,2	10,78
Apr	212,65263	333,5 ± 74,1	23,9 ± 9,5	136,1 ± 52,1	10,87
May	280,315	241,4 ± 70,2 *	55,5 ± 33,0	119,6 ± 31,8	9,22
Jun	217,67368	361,1 ± 86,0	22,2 ± 6,2	81,6 ± 23,1	9,54
Jul	289,1	384,4 ± 59,8	7,1 ± 2,6 *	121,5 ± 37,7	11,01
Aug	520,395	269,2 ± 84,6	97,4 ± 17,9 *	86,5 ± 20,9	9,35
Sep	372,165	227,2 ± 38,6	24,5 ± 7,5	82,4 ± 31,6	7,21 *
Oct	361,715	287,1 ± 54,9	21,9 ± 10,5	138,9 ± 45,9 *	10,10
Nov	280,245	327,8 ± 51,1	16,4 ± 11,0	73,4 ± 25,0	8,57
Dec	323,64	325,6 ± 84,4	43,2 ± 12,0	94,9 ± 27,1	9,70

Glycogen decreased from February to July and presented a small peak in May. The lowest value of glycogen (7.1±2.6 µg mg^{-1} AFDW) was observed in July and the highest in August (97.4±17.9 µg mg^{-1} AFDW). Between August and November the glycogen content decreased sharply. Generally, the total lipid and glycogen patterns were similar, but with two months mismatched. The lowest (72±25.7 µg mg^{-1} AFDW) and the highest (138.9±45.9 µg mg^{-1} AFDW) total lipid values were reached in January and October, respectively. The pattern of total energy content was quite similar to that observed for total lipids (Pearson's r=0.69, P=0.01) (Table 3.3). Statistically significant differences among samples were observed for all the biochemical constituents (proteins: ANOVA, P<0.001; glycogen: K–W., H=96.9, $d.f.$=11, P<0.001; total lipids: K–W., H=39.2, $d.f.$=11, P<0.001). No correlations were observed between biochemical contents and the other parameters studied (CI, GI and SST) (Table 3.3).

3.2.4 Discussion

An important topic in marine ecology is the inter-annual variation in recruitment of many species, including economically important shellfish (Honkoop, et al., 1998). These variations can be inherent to the physiological viability of larvae that depend on environmental factors and on broodstock condition (Massapina, et al., 1999). Timing of the reproductive cycle of bivalves from gametogenesis to spawning is controlled by an interaction of environmental and endogenous factors (Sastry, 1979). Temperature as traditionally been assigned a major role to determining reproductive development and spawning (e.g. Eversole, 1989; Grant and Creese, 1995; Gribben et al., 2004). In this study, a clear negative relationship between SST and reproduction in *S. solida* was described. The reproductive cycle of *S. solida* follows an annual cycle, comprising a ripe stage in winter followed by spawning that begins in late winter and extends through spring and is triggered by the rise of SST. The resting phase occurs during the summer. Till that moment, both sexes showed a synchronism in gonadal development and spawning concordant with Gaspar and Monteiro (1999) for other Portuguese *S. solida* populations. However, in the following months the gonad developed and matured extremely fast, leading to a second spawning episode. This phenomenon was not reported in Gaspar and Monteiro (1999) study but was reported for a population of *Callista chione* from the southwestern coast of Portugal (Moura et al., 2008) and may

have been caused by the unexpected oscillations in SST registered between midsummer and early winter. It is possible that the increase of SST that occurred in October, just after the initiation of gametogenesis, started the spawning process. This fact supports the idea stated by Gaspar and Monteiro (1999) that *S. solida* does not spawn at a definite temperature but rather responds to an increase in seawater temperature. Despite the differences in gametogenic development between sexes in this specific period, the synchronism between sexes was reestablished in December. The maintenance of synchronized gonadal development is fundamental to the reproductive success of gonochoric species, since sperm and eggs will be expelled in the water column simultaneously during the spawning period, augmenting the probability of fertilization (O'Connor and Heasman, 1995).

Results of the gonadal index analyses used in the present study reflect the relative variations of the gonad based upon qualitative staging and confirmed the negative relationship found between gonadal development stages of the *S. solida* population (males and females) and SST. A similar correlation was found by Mladineo et al. (2007) for the horse-bearded mussel *Modiolus barbatus*.

Quantitative analysis of oocyte diameter was used by several authors as a good descriptor of annual trend of the reproductive cycle of bivalves (e.g. Lango-Reynoso et al., 2000; Gribben, 2005; Peharda et al., 2006). Sastry (1979), Lango-Reynoso et al. (2000) and Moura et al. (2008) have associated oocyte size with the stages of the gametogenic cycle for *Argopecten irradians*, *Crassostrea gigas* and *C. chione*, respectively. In our study, a similar relationship was defined for *S. solida* where the oocytes diameter increased as reproductive development progressed; small oocytes occurred in the early stages whereas large oocytes corresponded to the spent stage. These oocytes are reabsorbed within the gonad by lysis process usually described as oocyte atresia (Lango-Reynoso et al., 2000). Although statistically significant differences in the oocyte diameter among stages were observed, no significant correlation between these two parameters was obtained. Therefore, the relationship between oocyte diameters and reproductive stages in *S. solida* is unclear, as it was pointed out by Heffernan and Walker (1989), Gribben et al. (2004) and Moura et al. (2008) for other bivalve species.

Condition index is generally considered to reflect reproductive activity (Fernandez Castro and Vido deMattio, 1987; Massapina et al., 1999; Ojea et al., 2004). The relationship between these two parameters was observed in several species from the Portuguese coast including *Ensis siliqua* (Gaspar andMonteiro,1998), *Donax trunculus* (Gaspar et al.,1999), *Venus striatula*/*Chamelea gallina* (Gaspar and Monteiro, 1998), *C. chione* (Moura et al., 2008) and *S. solida* (Gaspar and Monteiro, 1999). In our study, the CI tracked gonadal development, increasing during gametogenesis and decreasing during spawning. The CI increased immediately after spawning indicating a rapid recovery and an accumulation of reserves that may be used in the next gametogenic cycle. Even though the CI pattern generally followed the reproductive stage, it did not statistically correlate with GI. This result suggests that, in *S. solida*, CI is highly influenced by the pattern of energy storage.

Many studies on marine invertebrates have shown that the reproductive cycle and environmental conditions are reflected by variations in biochemical composition (Costa Muniz et al., 1986; Fernandez Castro and Vido de Mattio, 1987; Massapina et al., 1999; Camacho et al., 2003). The present study demonstrated for the first time the seasonal change in biochemical composition of the white clam *S. solida*. The relative amount of protein (227 to 384 μg mg^{-1} AFDW), glycogen (7 to 97 μg mg^{-1} AFDW) and total lipids (72 to 139 μg mg^{-1} AFDW) measured in *S. solida* were similar to those described in the literature for other bivalves (Robert et al.,1993; Marin et al., 2003; Ojea et al., 2004). The protein pattern observed suggests the utilization of somatic protein as an energy reserve in situations of nutritional stress and energy imbalance or during gonad maturation, as described by other authors (Gabbot and Bayne, 1973; Bayne and Newell, 1983; Beninger and Lucas, 1984). Moreover, the negative correlation found in the present study between proteins and glycogen demonstrated that *S. solida*, like other bivalves (Barber and Blake, 1981; Camacho et al., 2003) use proteins as a source of energy for maintenance when carbohydrate reserves have already been depleted. However, glycogen is the main energy reserve in adult bivalves and can be simultaneously an energy source for growth and also stored in specific cells as energetic reserve during the vitellogenic process (Deslous-Paoli and Heral, 1988; Marin et al., 2003). Although no statistical correlation was found between CI and glycogen, these parameters peak simultaneously in August. In several conservative species such as *A.*

irradians concentratus (Barber and Blake, 1981), *Argopecten purpuratus* (Martinez, 1991) and *Ruditapes decussatus* (Ojea et al., 2004), gametogenesis depends largely on the amount of glycogen stored. However, in other species, the storage reserves and the gametogenetic cycle exhibited an opportunistic activity (e.g. *Ostrea edulis*, Bayne, 1976). In our study, the glycogen pattern suggest that *S. solida* can adopt both strategies, since in September to October the rapid gonadal development and spawning process provoked a striking consumption of the glycogen stored during the resting period; in the next two months, the storage reserves and the gametogenesis took place simultaneously. Mladineo et al. (2007) found similar results for *M. barbatus*. It has long been evident that glycogen and lipids are inversely correlated and that lipid loss accompanies spawning (Beninger and Lucas, 1984; Beninger and Stephan, 1985; Robert et al., 1993). This relationship is usually attributed to the conversion of glycogen to lipids biosynthesized during the formation of gametes (Gabbot, 1975). In the present study, glycogen loss was in general synchronous with total lipid accumulation, although with some mismatching. However, total lipids peaked both in gametogenesis/spawning and in resting periods. These results and the correlation found between total lipids and total energy, suggest that more than a consequence from the gametogenesis in autumn/winter, the total lipids behavior can reflect the energy accumulation process and its consumption during somatic development in spring/summer when food is abundant.

According to Urrutia et al. (1999) and Ojea et al. (2004) when food is plentiful, surplus energy is used by animals for growth of somatic tissues and also for gonad development. During the gametogenic and spawning period the energy balance was stable and after a small decrease in May/June, the accumulation of total lipids reinforced the total energy reserves. A depletion of energy occurred in September when the sudden temperature oscillation coincided with a rapid increase in gonad development. The description of the *S. solida* reproductive cycle obtained in this study provides a valuable insight into the biology of this species and is important for the sustainable management of wild stocks as well as for future aquaculture progress with this species. For example, the reproductive response adopted by *S. solida* when abnormal temperature oscillations occur can have interesting implications for the implementation of profitable aquaculture, since broodstock may be susceptible to manipulation via conditioning.

Detailed knowledge of the gametogenic and spawning periods and consequent energy storage, as described in this study, can also provide the optimal reproductive time for artificial spawning induction in aquaculture. From a management point of view, this information is important to obtain a good quality of larvae and consequently to promote successful restocking actions based on aquaculture production. The knowledge provided in this study also is relevant to regulate the dredge fishery, since global climatic changes foreseen for the future can give rise to significant alterations in the reproductive cycle pattern of this species with implications for effective management.

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Chapter 3.3

Reproductive activity and biochemical composition of the pullet carpet shell *Venerupis senegalensis* (Gmelin, 1791) (Mollusca: Bivalvia) from Ria de Aveiro (northwestern coast of Portugal)

Joaquim, S., Matias, D., Matias, A.M., Moura, P., Arnold, W.S., Chícharo, L., Gaspar, M.B., 2011. Reproductive activity and biochemical composition of the pullet carpet shell *Venerupis senegalensis* (Gmelin, 1791) (Mollusca: Bivalvia) from Ria de Aveiro (northwestern coast of Portugal). Sci. Mar. 75, 217-226 (*Published*).

Abstract

The present study characterizes the reproductive cycle of *Venerupis senegalensis* (=*V. pullastra*) from Ria de Aveiro (Portugal) as well as its nutrient storage and exploitation strategy. The reproductive cycle followed a seasonal cycle that correlated negatively with sea surface temperature, and comprised a ripe stage in winter followed by a spawning period that began in late winter and ended in the early summer. This extended spawning may be an advantageous strategy for the species because it ensures a continuous supply of settlers. Gametogenesis began in late summer/early autumn and intensified with the decrease in temperature during autumn. The condition index increased even during the spawning period, which indicates that there is rapid recovery and that reserves are accumulated during late summer and used later in the gametogenic process. Proteins did not contribute significantly to gametogenesis and the glycogen pattern is typical of conservative species, since gametogenesis depends largely on the amount of glycogen stored. The lipid storage and utilization cycle showed that gametogenesis took place in autumn/winter and that energy reserves were accumulated in summer.

3.3.1 Introduction

Venerupis senegalensis (=*V. pullastra*) is commercially exploited in Portugal, Spain, France and Italy (FAO, 2006-2010). Presently in mainland Portugal, *V. senegalensis* is most abundant in the Ria de Aveiro lagoon on the west coast, although Maia et al. (2006) reported that between 2001 and 2006 this population declined due to overfishing and recruitment failure. This species was also once abundant in the Ria Formosa lagoon; however, in the early 1980s these populations declined dramatically for the same reasons (Massapina and Arrobas, 1991). This not only threatened the sustainability of the fishery, but also compromised the productive potential of both of these important lagoonal ecosystems (Joaquim et al., 2008a). An active intervention may be necessary not only to restore stocks to reproductive viability in both Ria de Aveiro and Ria Formosa, but also to provide alternative or complementary products for the shellfish aquaculture industry. Intensive hatchery production of juveniles will therefore be necessary. However, inherent physiological variability among bivalve larvae can always be expected in production situations (Massapina et al., 1999), and

understanding the source of this variability is crucial for reducing it. Broodstock condition is an important factor that contributes to this variability (Magnesen and Christophersen, 2008). The relationship between the reproductive cycle and energy storage and utilization cycles has already been reported by several authors for a wide variety of bivalves (e.g. Barber and Blake, 1981; Fernandez Castro and Vido de Mattio, 1987; Massapina et al., 1999; Pérez-Camacho et al., 2003). In general, reserves accumulate prior to gametogenesis in the form of glycogen, lipid and protein substrates, and are used in the production of gametes when metabolic demand is high (Mathieu and Lubet, 1993). Previous works have studied the reproduction of *V. senegalensis* (Villalba et al., 1993; Maia et al., 2006; Cerviño-Otero et al., 2007a) and its biochemical composition (Alvarez-Seoane, 1960; Gonzalez, 1975; Albentosa et al., 2007). Nevertheless, the reproductive cycle is influenced by local environmental conditions such as food availability and temperature (e.g. Gabbott, 1976; Pérez-Camacho et al., 2003). Therefore, understanding the natural reproductive cycle of *V. senegalensis* is essential for establishing successful hatchery-based production. Maia et al. (2006) reported the reproductive cycle of a Ria de Aveiro population of *V. senegalensis*; however, no information on the relationship between the reproductive cycle and biochemical composition was provided.

In the present study we characterize the reproductive cycle of *V. senegalensis* from the Ria de Aveiro, including patterns of nutrient storage and utilization. This information could be useful in the future for both the aquaculture production of this species and for implementing programs to enhance and restore natural stocks to reproductive viability.

3.3.2 Materials and Methods

Collection of samples

Adult specimens of *Venerupis senegalensis* were collected fortnightly during 2006 and 2007 by local dredgers from a site in Ria de Aveiro (40°42′N; 08°40′W, western Portugal; Fig. 3.15) at water depths ranging from 4 to 8 m. Monthly sea surface temperature (SST) data were measured with a YSI multiparameter probe (Model 556 MPS).

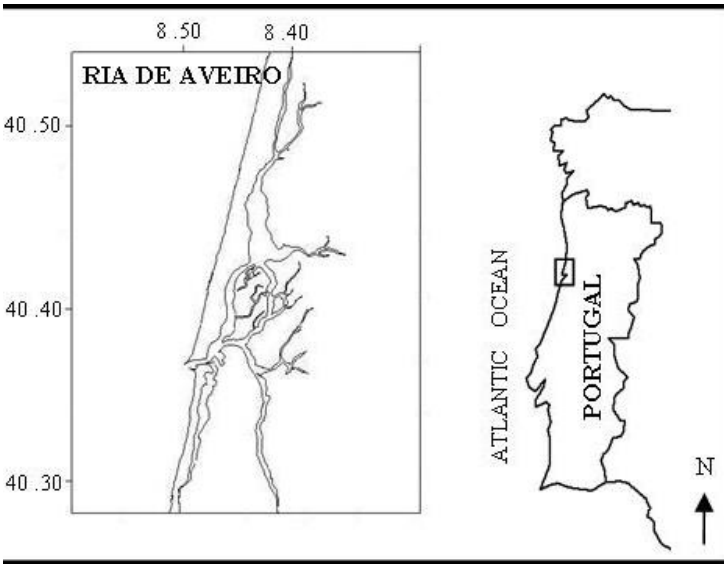

Figure 3.15 Ria de Aveiro location.

Laboratory analysis

The laboratory analyses were only performed for standard animals with a shell length ranging from 36 to 38 mm to avoid the influence of growth, and thus minimize bias from increasing somatic weight and focus on the accumulation or loss of organic matter associated with reproduction (Beninger and Lucas, 1984). In the laboratory, clams were placed in 0.45 μm-filtered seawater at 20°C for 24 h to purge their stomachs before histological, condition index and biochemical analyses. Following the 24 h purging period, each clam was dissected and the wet soft tissue weight was determined on a top-loading digital balance (precision of 0.01 g) after 15 minutes on a sloped plane covered with absorbent paper.

Histology

Ten males and ten females from each fortnightly sample were examined histologically to ascertain gonadal development. The visceral mass was separated from siphons and gills and fixed in San Felice solution for 24 h, then transferred to 70% ethyl alcohol (ETOH) for storage. For microscopic examination, tissues from these samples were dehydrated with serial dilutions of alcohol and embedded in paraffin. Thick sections (6-8 μm) were cut on a microtome and stained with haematoxylin and eosin. The histologically prepared slides were examined using a microscope at 40× magnification and each specimen was assigned to a stage which represented the gonad condition.

Clam reproductive maturity was categorized into six stages using the gametogenic scale proposed by Gaspar and Monteiro (1998):

Inactive. Sexes are indistinguishable microscopically due to the total absence of follicles and gametes.

Early active. The connective tissue is abundant and the size of follicles is small. In females the oocytes are attached to the basal membrane by the stalk. In males spermatocytes proliferate toward the lumina.

Late active - Interfollicular connective tissue scarce. Follicles become larger. In females most oocytes are free in the lumina while some are still attached to the basal membrane. Appearance of sperm in the lumina, which form weak columns with tails orientated toward the center.

Ripe - The connective tissue has been replaced by follicles that are full of ripe gametes. In females the oocyte tale is oval or polygonal shape and in males the lumina are packed with spermatozoa.

Partially spawned - The gametes are discharged. Depending on the degree of spawning the follicles are more or less empty. The follicle walls are broken. There are many empty spaces between and within the follicles.

Spent - Abundant interfollicular connective tissue. Occasional residual sperm or oocytes present.

When more than one developmental stage occurred simultaneously within a single individual, the staging criteria was based on the condition of the majority of the section. For each of the stages a numerical ranking was assigned as follows: inactive (0); early active (3); late active (4); ripe (5); partially spawned (2); spent (1). A mean gonad index (GI) was then calculated using the method proposed by Seed (1976):

$$GI = \frac{\sum \text{ind. each stage x stage ranking}}{\text{total ind. each month}}$$

The GI ranged from 0 (all individuals in the sample are resting) to 5 (all individuals are ripe).

Condition index

Dry soft tissues and shell weights of 20 clams from each sample were determined after oven drying at 80°C for 24 h. Soft tissue samples were then ashed at 450°C in a muffle furnace, the ash weight was determined and the organic matter weight calculated as the ash-free dry soft-tissue weight (AFDW). The condition index (CI) was calculated according to Walne and Mann (1975):

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

Biochemical composition

The soft tissue of five clams of each fortnightly sample was frozen and stored at −20°C for biochemical analyses. For each specimen, protein was determined using the modified Lowry method (Shakir et al., 1994), glycogen content was determined from dried (80°C for 24 h) homogenate using the anthrone reagent (Viles and Silverman, 1949) and total lipids were extracted from fresh homogenized material in chloroform/methanol (Folch et al., 1957) and estimated spectrophometrically after charring with concentrated sulphuric acid (Marsh and Weinstein, 1966). Duplicate determinations were performed in all cases and values were expressed as a percentage of AFDW. The caloric content of protein, lipid and carbohydrates in tissues was calculated using the factors 17.9 kJ g^{-1} (Beukema and De Bruin, 1979), 33 kJ g^{-1} (Beninger and Lucas, 1984) and 17.2 kJ g^{-1} (Paine, 1971) respectively.

Statistics

Significant differences in the condition index, biochemical composition and histological parameters during the study period were tested using one-way ANOVA. Whenever the assumptions of analysis of variance were not met, the Kruskal–Wallis ANOVA on ranks test was performed. Prior to any analyses, percentage data were arcsine transformed to normalize variance (Sokal and Rohlf, 1981). Multiple pairwise comparisons were performed using the post-hoc parametric Tukey test or the non-parametric Dunn's test in order to detect significant differences between fortnightly consecutive samples. The Spearman correlation coefficient was used to determine the

degree of association between parameters. The statistical analyses were undertaken using the SIGMASTAT 3.11 statistical package.

3.3.3 Results

Temperature

The evolution of the monthly SST during the experimental period in Ria de Aveiro is presented in Figure 3.16. A seasonal cycle in SST was observed, and ranged between 11.7 in February 06 and 20.7ºC in May 07.

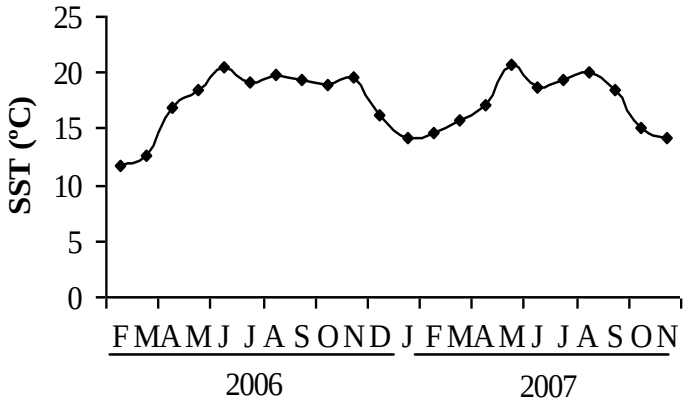

Figure 3.16 Monthly values of sea surface temperature (SST) in Ria de Aveiro from February 2006 to November 2007.

Gametogenic cycle

The sexes were clearly separated and no hermaphrodites were found. Both sexes generally showed synchronism in gonadal development. The reproductive cycle of *V. senegalensis* was characterized by a seasonal pattern (Fig. 3.17).

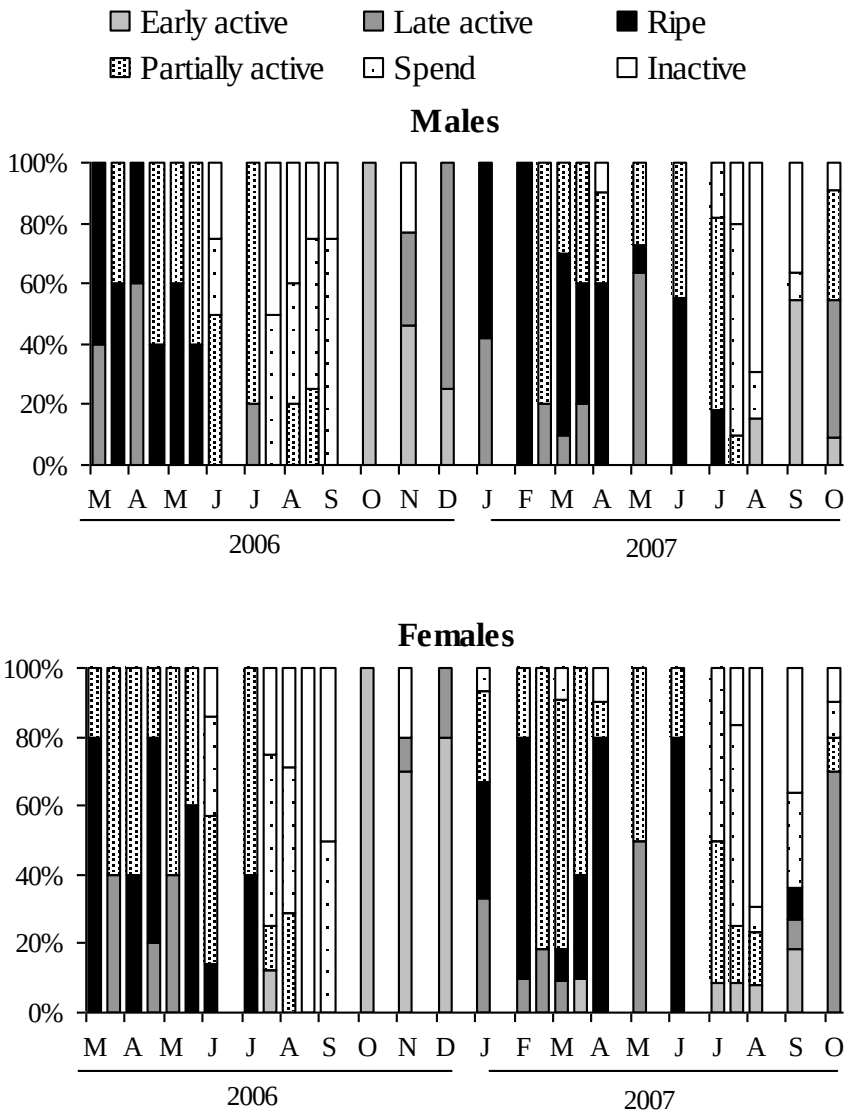

Figure 3.17 Monthly variations in gonadal development of *V. senegalensis* during 2006 and 2007. Males (top) and Females (bottom).

The GI was negatively correlated with SST (Spearman's, $r=-0.65$, $P<0.001$) (Table 3.5). The onset of the gametogenic cycle occurred in October 06 and August 07; however, there was a short inactive period (the maximum sexual inactivity, 70%, was observed in August 07). The development of gametes intensified during the autumn, which coincided with the decrease in SST. Spawning began in winter, intensified during spring as SST increased, and continued until August. However, in summer most of the population had already spawned and was inactive. Nevertheless, in spite of the seasonal

pattern, *V. senegalensis* did not show continuous gonad development, in which stages would occur sequentially, especially during the spawning period. For example, in late March 06, about 60% of males were in the "ripe" stage whereas the remaining males were "partially spawned".

Table 3.5 Results of Spearman correlation between studied parameters (r, correlation coefficient, *P*, P value, n.c., no correlation was found).

	Gonadal index	Condition index	Proteins	Total lipids	Glycogen	Total energy
Temperature	*r*= - 0.65 *P*< 0.001	*r*= 0.55 *P*= 0.01	*r*= - 0.65 *P*< 0.001	n.c.	n.c.	*r*= - 0.65 *P*< 0.001
Gonadal index		*r*= - 0.65 *P*< 0.001	n.c.	n.c.	*r*= - 0.65 *P*< 0.001	n.c.
Condition index			*r*= - 0.74 *P*< 0.001	n.c.	*r*= 0.71 *P*< 0.001	n.c.
Proteins				n.c.	n.c.	*r*= 0.57 *P*< 0.01
Total lipids					*r*= 0.54 *P*= 0.01	*r*= 0.53 *P*= 0.01
Glycogen						n.c.

In the following sample, 60% of the population restarted gametogenesis with the development of new gametes, whereas 40% of the population remained in the "ripe" stage. This phenomenon occurred in both study years and for both males and females, and can be observed in the series of alternating increases and decreases in the GI (Fig. 3.18) during the spawning period. Indeed, in microscopic examinations of the gonadal tissues it was common to observe different stages of gonadal development in the same individual (Fig. 3.19), which indicates simultaneous spawning and recovery of the gonad. The gonad index followed the same pattern as gonad development; no significant differences in GI were found between sexes (ANOVA, P=0.877) or between years (ANOVA, P=0.073).

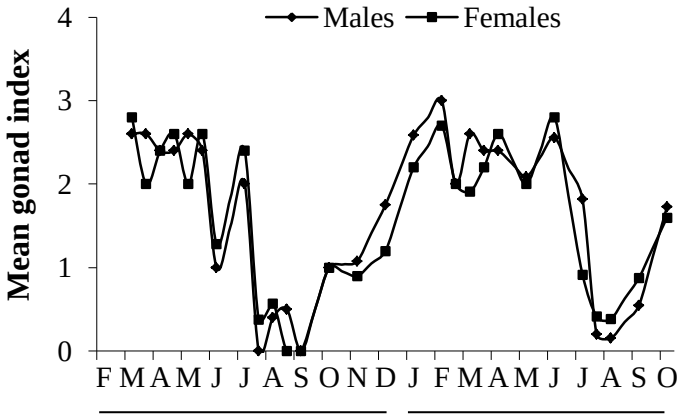

Figure 3.18 Gonad index (GI) of *V. senegalensis* during the experimental period.

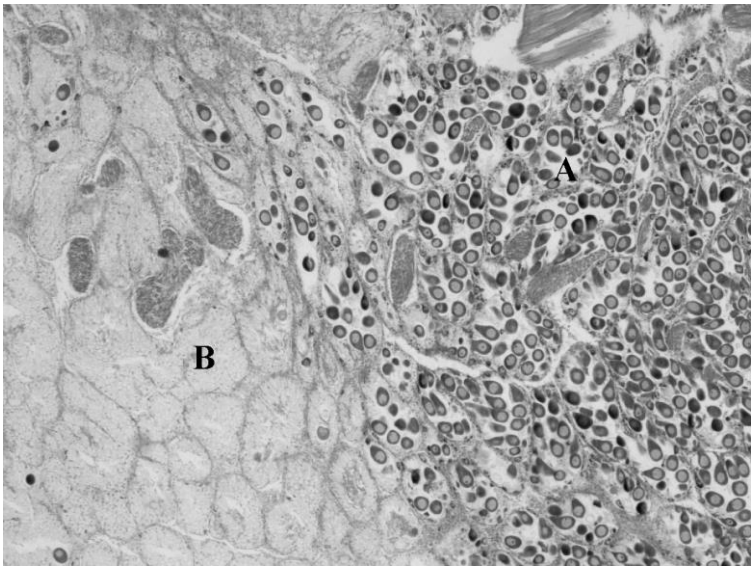

Figure 3.19 Detail of different stages of gonadal development coexisting in the same *V. senegalensis* individual. A, early/late active; B, spent.

Condition index

The condition index showed statistically significant differences among samples (K-W., H=689.0, *d.f.*=33, P<0.001) but not between years (K-W., H=2.7, *d.f.*=1, P=0.102). SST was positively correlated with CI (Spearman's r=0.55, P=0.01). Between early February and late March, CI decreased with the beginning of spawning. After early May 06 and in May 07, this index generally trended upward until late September 06 and early October 07 respectively, which corresponded to the maximum SST. Exceptions were

found between late May and early July 06 and in late August 07, when the CI decreased slightly (Fig. 3.20). After late September 06, the CI generally decreased until late January 07 (with the exceptions of early November 06) following the temperature trend and the increase in the mean gonad index. The highest CI value (18.09±3.37) was registered in late September 06. In that month, the majority of clams (both males and females) were in the "spent" or "inactive" stage. The CI was negatively correlated with the GI (Spearman's, r=−0.65, P<0.001) (Table 3.5).

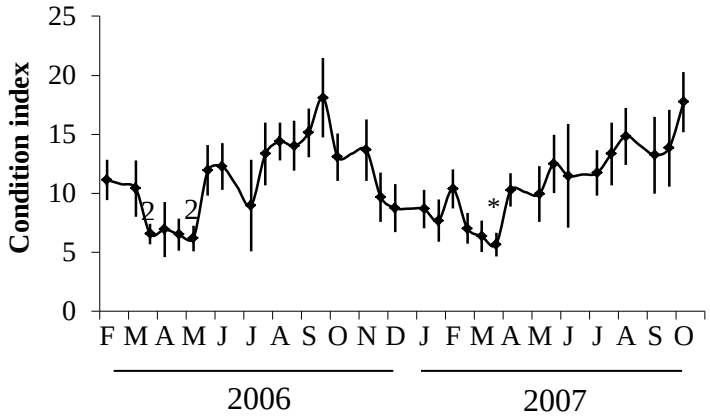

Figure 3.20 Mean condition index (± S.D.) in *V. senegalensis* during the experimental period (* statistically significant differences, *P*<0.05 found between consecutive months).

Biochemical composition

Proteins were the predominant dry tissue constituent of the clams followed by total lipids and glycogen (Table 3.6). The highest protein content value was recorded in early March 07 (377.8±85.9 μg mg^{-1} AFDW) and the lowest in late May 06 (142.2±48.8 μg mg^{-1} AFDW). Protein content was inversely correlated with SST (Spearman's r=−0.65, P<0.001) and with CI (Spearman's r=−0.74, P<0.001) (Table 3.5). Glycogen was generally low and stable between February and May of both years (Table 3.6), and increased until early July in 2007 but between late July and late September in 2006. The lowest glycogen value (10.9±5.2 μg mg^{-1} AFDW) was observed in late March 07 and the highest in late September 06 (135.3±27.6 μg mg^{-1} AFDW). After that, the glycogen content decreased until the end of the year. Glycogen was positively correlated (Spearman's r=0.71, P<0.001) with CI and total lipids (Spearman's, r=0.54, P=0.01) and negatively correlated (Spearman's, r=-0.65, P<0.001) with the GI (Table 3.5).

Table 3.6 Mean values (±sd) of shell length (mm), proteins, glycogen, total lipids (μg mg^{-1} AFDW) and total energy (kJ g^{-1} AFDW) of *V. senegalensis* during the experimental period (* statistically significant differences, $P<0.05$ found between consecutive months).

Year	Month	Lenght (mm)	Protein (μg mg^{-1} AFDW)	Glycogen (μg mg^{-1} AFDW)	Total Lipids (μg mg^{-1} AFDW)	Total energy (kJ mg^{-1} AFDW)
2006	Feb	36.5 ±	259.6 ± 27.6	20.8 ± 4.4	67.3 ± 15.7	7.2
		--	--	--	--	--
	Mar	35.5 ±	296.6 ± 47.2	16.4 ± 3.0	91.2 ± 27.0	8.6
		37.0 ±	324.7 ± 45.2	17.4 ± 2.6	103.3 ± 25.5	9.5
	Apr	36.5 ±	235.5 ± 88.2	15.7 ± 3.5	125.4 ± 17.9	8.6
		37.8 ±	350.8 ± 15.0	18.2 ± 4.4	105.1 ± 34.1	10.1
	May	37.3 ±	358.2 ± 97.2	21.9 ± 9.5	107.2 ± 28.4	10.3
		37.6 ±	142.2 ± 48.8 *	12.2 ± 3.0	86.3 ± 25.0	5.6
	Jun	36.9 ±	175.7 ± 33.3	53.3 ± 29.2	117.2 ± 14.3	7.9
		--	--	--	--	--
	Jul	36.2 ±	252.2 ± 53.9	37.3 ± 21.6	107.1 ± 27.3	8.7
		38.0 ±	250.7 ± 49.0	11.7 ± 6.1	104.1 ± 35	8.1
	Aug	36.4 ±	216.2 ± 37.2	18.4 ± 16.0	110.6 ± 21.3	7.8
		36.8 ±	228.2 ± 38.3	32.5 ± 21.3	91.5 ± 37.5	7.7
	Sep	37.5 ±	240.2 ± 30.3	107.9 ± 23.6	119.9 ± 22.4	10.1
		37.9 ±	246.9 ± 16.3	135.3 ± 27.6	136.1 ± 30.2	11.2
	Oct	37.4 ±	237.0 ± 74.5	78.7 ± 44.2	100.0 ± 13.0	8.9
		--	--	--	--	--
	Nov	36.9 ±	251.6 ± 21.3	83.7 ± 14.5	113.7 ± 12.2	9.7
		37.1 ±	274.2 ± 25.6	66.6 ± 10.4	146.4 ± 18.1	10.9
	Dec	36.7 ±	354.5 ± 30.0	82.0 ± 39.7	156.4 ± 17.3	12.9
		--	--	--	-- *	--
2007	Jan	36.9 ±	300.0 ± 31.5	42.5 ± 22.8	85.7 ± 24.4	8.9
		36.5 ±	377.3 ± 112.6	17.5 ± 2.8	77.2 ± 3.7	9.6
	Feb	37.2 ±	225.5 ± 36.0	15.5 ± 2.9	66.3 ± 25.9	6.5
		37.3 ±	351.7 ± 44.7	38.2 ± 30.1	115.1 ± 25.5	10.8
	Mar	36.4 ±	377.8 ± 85.9	19.8 ± 5.0	75.7 ± 25.6	9.6
		37.9 ±	273.2 ± 38.7	10.9 ± 5.2	92.4 ± 30.4	8.1
	Apr	37.4 ±	243.1± 61.0	37.1 ± 4.6	115.6 ± 25.0	8.8
		--	--	--	--	--
	May	38.2 ±	248.6 ± 67.1	15.7 ± 5.8	78.1 ± 20.0	7.3
		36.4 ±	199.1 ± 64.8	25.8 ± 7.6	90.7 ± 18.6	7.0
	Jun	38.2 ±	166.3 ± 32.1	33.7 ± 16.6	93.3 ± 26.3	6.6
		--	--	--	--	--
	Jul	38.7 ±	178.8 ± 40.3	98.3 ± 48.4	89.7 ± 32.4	7.9
		38.3 ±	280.1 ± 12.2	75.5 ± 23.5	99.3 ± 18.5	9.6
	Aug	37.3 ±	202.9 ± 55.9	76.7 ± 37	109.2 ± 32	8.6
		--	--	--	--	--
	Sep	37.7 ±	248.0 ± 12.6	28.2 ± 16.8	86.4 ± 22.2	7.8
		38.2 ±	248.6 ± 34.1	76.0 ± 37.5	130.5 ± 44.1	10.1
	Oct	38.4 ±	215.8 ± 41.8	47.6 ± 10.2	136.0 ± 35.6	9.2

The lowest (66.3±25.9 μg mg^{-1} AFDW) and the highest (156.4±17.3 μg mg^{-1} AFDW) total lipid values were reached in early February 07 and early December 06 respectively (Table 3.6). Proteins and total lipids contributed most to the total energy content (proteins: Spearman's, r=0.57, P<0.01; total lipids: Spearman's, r=0.53, P=0.01) (Table 3.5). Statistically significant differences among sampling periods were observed for all the biochemical constituents (proteins: K-W., H=160.2, *d.f.*=33, P<0.001; glycogen: K-W., H=223.7, *d.f.*=33, P<0.001; total lipids: K-W., H=133.6, *d.f.*=33, P<0.001; total energy: K-W., H=147.2, *d.f.*=33, P<0.001). No significant differences were observed

between years for proteins (K-W., H=1.0 *d.f.*=1, P=0.306) and glycogen (K-W, H=0.04, *d.f.*=1, P=0.843); however, total lipids (ANOVA P<0.001) and total energy (K-W, H=4.9, *d.f.*=1, P<0.05) showed significant differences between 2006 and 2007.

3.3.4 Discussion

The reproductive cycle of bivalves is controlled by the interaction between environmental and endogenous factors (Normand et al., 2008). In this study, gametogenesis of *V. senegalensis* was significantly influenced by water temperature, as has been previously reported for several other bivalve species (e.g. Gabbott, 1976; Xie and Burnell 1994; Albentosa et al., 2007). The seasonal SST pattern in Ria de Aveiro was characterized by relatively low temperatures during the winter that increased during early spring, stabilized in summer, and decreased in late autumn. The reproductive cycle of this species followed a seasonal cycle that correlated negatively with SST. It comprised a ripe stage in winter followed by an extended spawning period that began in late winter, was triggered by the rise of SST in spring and continued throughout the summer. A similar prolonged reproductive cycle was described by Villalba et al. (1993) for a population of *V. senegalensis* from Galicia (Spain) and for the Ria de Aveiro population by Maia et al. (2006). Moreover, Cerviño-Otero et al. (2007a) observed ripe and spawning stages throughout the year for a population from the O Grove (SW of Galicia). Extended spawning seems to be an advantageous reproductive strategy for the species because it ensures a continuous supply of settling larvae. Indeed, histological analyses showed gonias, maturing gametocytes and variable proportions of fully matured gametes simultaneously in the same individual. There was high intra-individual variation in gonadal maturation both in females and males. This phenomenon was reflected in the sequential development of gonadal stages, especially during the spawning period, when most of the population was "partially spawned" on a given sample date but had reinitiated gametogenesis on the following sample date (e.g. between late March and early April 06). Delgado and Pérez-Camacho (2007) also observed this great capacity for gonadal regeneration and the long reproductive period in *Ruditapes philippinarum*. Nevertheless, despite the intra-individual asynchrony, the synchronized gonadal development observed between males and females during this period is fundamental to the reproductive success of the species because sperm and

oocytes are expelled into the water column simultaneously during the spawning period, which increases the probability of fertilization (O'Connor and Heasman, 1995). This synchronism was reported by Maia et al. (2006) in Ria de Aveiro but not by Villalba et al. (1993) in Galicia. The resting phases occurred during the summer, although total inactivity of the population was rare. The proliferation of gonias (early active stage) began during late summer/early autumn and gametogenesis intensified as the SST decreased in autumn. These results were generally consistent with the previous findings by Maia et al. (2006), although these authors reported the onset of gametogenesis in December. The results of the gonad index analyses of the present study reflect the relative variations in the gonads based on qualitative staging. A similar correlation was found for the white clam *Spisula solida* (Joaquim et al., 2008b).

The condition index was positively correlated with the SST and inversely correlated with reproductive activity. The CI showed a seasonal cycle: it decreased during gametogenesis but increased again even during the spawning period. This pattern indicates that there is rapid recovery followed by an accumulation of reserves in summer during the resting phase. These reserves are then used in the next gametogenic cycle. The CI reached its maximum value in September 06 and October 07 at the beginning of gametogenesis. The relationship between CI and the reproductive cycle has been observed in several other bivalve species from the Portuguese coast (Gaspar and Monteiro, 1998; Moura et al., 2008). However, in the present work, the negative relationship between the CI and GI may not be a direct consequence of the reproductive cycle of the species, but rather a consequence of the energy storage and exploitation strategy. Joaquim et al. (2008b) found that the CI for *Spisula solida* is highly influenced by the energy storage pattern. Similarly, Delgado and Pérez-Camacho (2005) reported that the evolution of the dry soft-tissue weight in *R. decussatus* indicates the energy status of the organism and the volume of gonad produced. Several studies on bivalves have shown that gametogenesis is associated with an annual cycle of the accumulation and use of energy reserves, which is influenced by environmental parameters such as food availability and temperature (Fernandez Castro and Vido de Mattio, 1987; Massapina et al., 1999; Pérez-Camacho et al., 2003). Generally, energy is accumulated when food is abundant, and this energy is then used to synthesize gametes, which are liberated during the spawning process. This cycle translates into a seasonal pattern of

biochemical composition that can vary among populations and species (Albentosa et al., 2007). The relative amounts of protein (142 to 378 μg mg^{-1} AFDW), glycogen (11 to 146 μg mg^{-1} AFDW) and total lipids (66 to 156 μg mg^{-1} AFDW) measured in *V. senegalensis* were similar to those previously described in the literature for this species (Alvarez-Seoane, 1960; Cerviño-Otero et al., 2007b).

Many authors have suggested that somatic protein is used as an energy reserve during gametogenesis in bivalves (Gabbott and Bayne, 1973; Liu et al., 2008), including in *V. senegalensis* (Albentosa et al., 2007; Cerviño-Otero et al., 2007b). In this study, although protein was negatively correlated with SST and CI, the relationship between protein and GI was not significant. Similar results were observed for the Pacific oyster *Crassostrea gigas* (Li et al., 2009). Moreover, the lack of a significant correlation between proteins and glycogen suggests that proteins are not generally used as an energy source for maintenance when carbohydrate reserves have been depleted; this is contrary to what González (1975) found for this species previously as well as the findings for other bivalves (Barber and Blake, 1981; Pérez-Camacho et al., 2003; Joaquim et al., 2008b). Nevertheless, the protein concentration followed the sudden decrease in the GI in May 06 and February 07, which suggests that in a stressful situation of energy imbalance provoked by the extended breeding period of the species, which includes simultaneous spawning effort and development of new gonias and maturing gametocytes, this species can use proteins as an energy reserve. Mao et al. (2006) reported excessive use of protein during and after the spawning period in *C. gigas*. According to Albentosa et al. (2007), in response to a starvation situation, females of *V. senegalensis* obtained most of their energy to maintain vital functions from the catabolism of proteins and in males this energy was obtained from the catabolism of proteins and lipids in equal proportions.

Glycogen is the main energy reserve in adult bivalves. It can be an energy source for growth and at the same time stored in specific cells as an energy reserve during the vitellogenic process (Marin et al., 2003). *V. senegalensis* accumulates glycogen in summer, before the intensification of the gametogenesis process that will take place in autumn/early winter, and uses it during spawning in late winter, spring and early summer, when this energy reserve will be totally depleted. This glycogen pattern was also observed for this species by Pérez-Camacho (1980) and for other typical

conservative species such as *A. irradians concentratus* (Barber and Blake, 1981), *Argopecten purpuratus* (Martinez, 1991) and *Ruditapes decussatus* (Ojea et al., 2004), since gametogenesis depends largely on the amount of glycogen stored. Indeed, this biochemical compound was correlated positively with CI and negatively with GI.

Several authors (e.g. Beninger and Lucas, 1984; Ojea et al., 2004; Mouneyrac et al., 2008) have reported a negative relationship between glycogen and lipids, probably due to the conversion of glycogen to lipids biosynthesized during the formation of gametes (Gabbott, 1975). In the present study, although there was a significant increase in lipids during autumn when an intensification of the gametogenesis process with glycogen losses occurs, there was generally positive synchronism between these two parameters. Therefore, contrary to the pattern usually reported for the majority of bivalves, in this study a positive relationship was observed between total lipids and glycogen. These results suggest that, more than a consequence of gametogenesis in autumn/winter, total lipid behaviour reflects the energy accumulation process. *V. senegalensis* accumulates both total lipids and carbohydrates during the early stages of gametogenesis. Lipids are depleted following spawning due to the gametes being expelled. The rapid decline in lipid content observed in January 07 during the gametogenesic process, may be explained by nutritional stress and energy imbalances during this period of low SST. The scarcity of food may also explain these results. In fact, Albentosa et al. (2007) reported that in *V. senegalensis* lipids contribute more than carbohydrates to the energy demands for sustaining vital functions during starvation periods. Although we have no data on the food availability in Ria de Aveiro for this year, some studies reported minimum values of chlorophyll a in January (Lopes and Silva, 2006; Lopes et al., 2007). This behaviour of total lipids may explain why there is no correlation between this parameter and the GI. Fernandez Castro and Vido de Mattio (1987) observed similar lipid and glycogen response cycles in *Ostrea puelchana*.

This study shows that in *V. senegalensis*, proteins and total lipids contribute significantly to the total energy supply, since they were all positively correlated. During the spawning period the energy balance was stabilized mainly by proteins, whereas during the gametogenesis process, the total lipids reinforced the total energy reserves. In summer, when food is abundant, a surplus of energy is available to be used for gonad

and somatic development, as has previously been suggested by Ojea et al. (2004) for *R. decussatus*.

In conclusion, the results of this study show that *V. senegalensis* is a conservative species, since gametogenesis takes place in autumn/early winter at the expense of reserves accumulated previously in late summer during a short period of time. Moreover, this species has a prolonged spawning period that starts in winter and ends in the middle of summer.

Besides the genetic plasticity and genetic similarity of the two populations reported by Joaquim et al.(2010), and which make the Ria de Aveiro *V. senegalensis* population a genetically viable broodstock for intensive hatchery production of juveniles, the extended spawning period of this *V. senegalensis* population has interesting implications for the implementation of profitable aquaculture. This extended natural spawning period implies that manipulation of broodstock in terms of conditioning, which should be initiated in late summer, would be relatively easy. Moreover, this species' great capacity for gonadal regeneration, coupled with its high gonadal development rate would provide larvae over much of the year without extensive and expensive broodstock conditioning. Information on the gametogenic and spawning periods and consequent energy storage can also provide the optimal reproductive time for artificial spawning induction in aquaculture. The results of this study can be used to adjust fishing effort by modifying management regulations, and also in aquaculture production to restore the Ria de Aveiro population and rebuild the depleted Ria Formosa population.

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Chapter 4

Is there any relationship between broodstock condition, oocytes quality and larval viability in pullet carpet shell *Venerupis corrugata* (Gmelin, 1791)?

Joaquim, S., Matias, D., Matias, A.M., Gonçalves, R., Vera, C., Chícharo, L., Gaspar, M.B. Is there any relationship between broodstock condition, oocytes quality and larval viability in pullet carpet shell *Venerupis corrugata* (Gmelin, 1791)? (*Accepted in Marine Biology Research*)

First divisions of an egg

Abstract

The pullet carpet shell is commercially exploited in Europe. Over-fishing and recruitment failure is causing the decline of populations and stock sustainability. The knowledge of *Venerupis corrugata* reproduction is essential to establish an intensive hatchery production of juveniles for restoring of natural beds. This work aimed to find a relationship between broodstock, oocytes and veliger larval viability. Adult specimens were induced to spawn by thermal stimulation. From each female, oocytes were taken for biochemical analysis (proteins, total lipids and carbohydrates) and the remaining oocytes were fertilised. The normal and abnormal veliger rates were calculated after embryos incubation. Spawning in hatchery with "wild" broodstock was possible for a long period, however, the subsequent larval hatching rate varied according to the oocytes quality. Two distinct periods of spawning were recorded: in February/March, with a higher number of oocytes released and in June/July with a weaker response to the spawning stimulation, however with more success in larval hatching rate. The organic matter of oocytes released can be used as a bench mark for estimating the larval hatching success.

4.1 Introduction

The pullet carpet shell *Venerupis corrugata* (= *V. pullastra* and *V. senegalensis*) is commercially exploited in Portugal, Spain, France and Italy (FAO 2012). In Portugal, this species occurs in coastal lagoons (mainly in Ria de Aveiro and Ria Formosa) and in estuaries (Tejo river) where is targeted by harvesters on foot or onboard fishing vessels, using bullrakes and/or mechanical dredges. However, in the last five years the populations of this species declined dramatically due to both over-fishing and recruitment failure (Joaquim et al. 2010, 2011), especially in Ria de Aveiro and Ria Formosa lagoons. Hatchery production of juveniles of pullet carpet shell can overcome this situation since it allows the enhancement/restoring of natural stocks, also valuing this species for farming.

In a previous study, it was found that the *V. corrugata* population from Ria de Aveiro was the most appropriate founder population to develop an aquaculture production program for restocking proposes due to its genetic plasticity (Joaquim et al. 2010).

However, even taking into consideration the genetic characteristics of broodstock, inherent physiological variability among bivalve larvae can always be expected in hatchery. Understanding the source of this variability is crucial for hatchery production. To this end, Joaquim et al. (2011) characterized the reproductive cycle of *V. corrugata* from Ria de Aveiro to assess the broodstock condition in order to establish a successful hatchery based production program. These authors showed that the reproductive cycle of this population followed a seasonal cycle negatively correlated with seawater temperature, comprising a ripe stage in winter followed by a long spawning period that began in late winter and ended in the early summer. This spawning strategy may be advantageous for the species, since it can ensures a continuous supply of settlers. However, is important to know if the oocyte quality changes over this period, since the endogenous reserves of eggs are important for providing energy during the embryogenesis before exogenous sources became available (e.g. Mann 1988; Dorange et al. 1989; Devauchelle & Mingant 1991; Sedano et al. 1995; Utting & Millicam, 1997; Cannuel & Beninger 2005; Fukazawa et al. 2005).

The reproductive success and profitability of hatcheries remain inconsistent, partly due to this variability of oocyte quality in bivalves (Boudry et al., 2002). Massapina et al. (1999) and Fukazawa et al. (2007) have showed relevant changes in oocyte quality during the spawning season in the oyster *Crassostrea gigas* and in the abalone *Haliotis discus hannai*, respectively. These authors have also reported that the oocyte quality and larval viability seems to depend on intrinsic broodstock characteristics. More specifically, Whyte et al. (1990) and Bransden et al. (2007) have referred that oocytes quality varies with the level of lipids stored during vitellogenesis. Proteins and lipids are the main constituents of oocytes from different species of marine bivalves (Sedano et al. 1995; Massapina et al. 1999; Nevejan et al. 2003). Most of the published data on oocyte quality have suggested that fatty acid lipids fraction are the main content of the gametes since it is generally accepted that vitelline reserves appear as lipids (*e.g*. Gallager et al. 1986*;* Whyte et al. 1990; Devauchelle & Mingant 1991; Caers et al. 1999b; Nevejan et al. 2003, Hendriks et al., 2003). According to Hendriks et al. (2003) *Macoma balthica* kept on a broodstock diet supplemented with PUFAs spawned a larger number of eggs per female and larger sized eggs compared to adults kept on a diet without PUFA supplementation. The PUFA content of eggs is dependent on a two-phase process

controlled at first by the environmental conditions during the fattening of broodstock and early gametogenesis (phospholipid PUFAs) and then by the conditions during the later stages of oocyte development (neutral lipid PUFAs) (Helm et al., 1991; Utting and Millican, 1997). Other studies demonstrate that maternal history modulates the expression and function of proteins in oocytes in a way that will impact offspring metabolism and survival (Chan et al., 2009; Heerwagen et al., 2010). Likewise early larval development has been correlated with the lipid content of oocytes from several species of bivalves (*e.g.* Helm et al., 1973; Bayne et al. 1975; Gallager & Mann 1986; Lee & Heffernan 1991; Massapina et al. 1999; Cannuel & Beninger 2005).

There are some studies about the reproductive cycle of *V. corrugata* (Villalba et al. 2011; da Costa et al. 2012) and/or its nutrient storage and exploitation strategy (Pérez-Camacho et al. 2003; Albentosa et al. 2007; Cerviño-Otero 2011; Joaquim et al. 2011; da Costa et al. 2012), however studies in oocyte quality are scare in this species and only Cerviño-Otero (2011) related the condition of the broodstock to the oocytes quality and the viability of D-larvae, but without information about the biochemical composition of oocytes.

This work aims to investigate if there is any relationship between the broodstock condition, oocytes quality and larval viability in pullet carpet shell *Venerupis corrugata* based on analysis of both biochemical composition and energy content of oocytes during the spawning season.

4.2 Material and Methods

Broodstock collection

Adult specimens of *Venerupis corrugata*, with a shell length >35 mm, were collected fortnightly during 2006 and 2007 by local hand dredgers operating from boats from a site in Ria de Aveiro (40º42′N; 08º40′W, northwestern coast of Portugal; Fig. 4.1) at water depths ranging from 4 to 8 m.

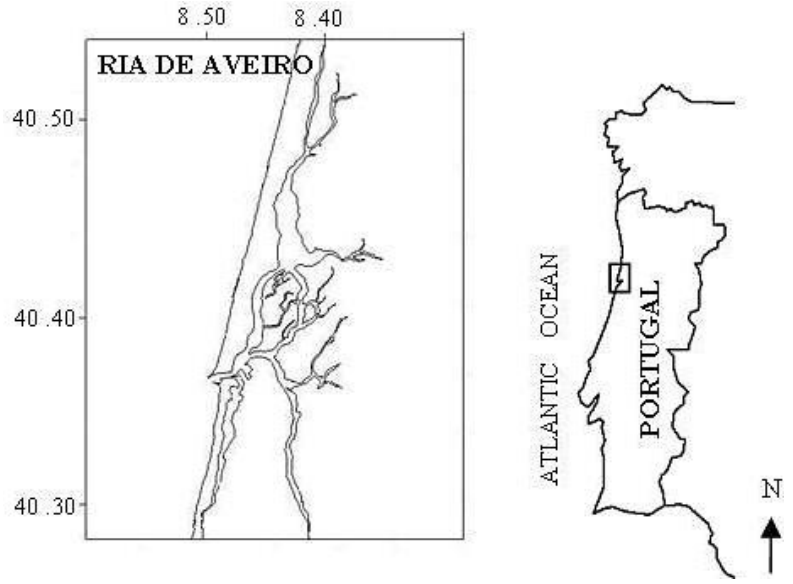

Figure 4.1 Ria de Aveiro localisation.

Spawning and D-larvae viability

In laboratory, clams were stabilized at 15ºC, during 24h, before being induced to spawn by thermal stimulation, through a rapid increase of temperature from 15 to 19±1°C, over 4 hours. To avoid uncontrolled fertilisation, females once identified were stored in individual containers for spawning. From each female, three samples of about 50 000 oocytes were taken, rinsed with iso-osmotic ammonium formate (3% $w.v^{-1}$) to remove salt, frozen and stored in liquid nitrogen for future freeze-drying and biochemical analysis.

The remaining oocytes were fertilised by addition of a mixture of sperm from various males, to provide a ratio of about ten spermatozoa per oocyte, on the microscopic field of view. One hour later, the fertilisation rate was determined. Only females that released a sufficient number of oocytes to biochemical analyses and incubation were used.

The eggs from each selected female were incubated in triplicate 5 L tanks, with 1 µm filtered and U.V. irradiated seawater, maintained at 20±1°C, at a density of 100 eggs per milliliter. After 24 h, the normal (D-larvae) and abnormal (misshapen or exhibiting defective swimming) veliger rates relative to the initial number of eggs (hatching rate) were calculated.

Biochemical composition of oocytes

The lyophilized oocyte samples were homogenized in 500 µl distilled and deionized water (dd) using a sonicator and then fractionated according to a micro-analytical extraction scheme developed by Holland & Gabbott (1971) and Holland & Hannant (1973). A separate sample (200 µl) of the initial homogenate was taken for analysis of proteins and total carbohydrates and another separate sample (200 µl) was taken to determine total and neutral lipids. Proteins were precipitated by cold 5% trichloroacetic acid (TCA) and washed in warm 1.0 N NaOH. Protein concentration was assayed by a modification of Lowry method (Bensadoun & Weinstein; 1976; Hess et al., 1978) at 750 nm using serum albumin as a standard.

Hydrolysed and unhydrolysed samples of TCA supernatant were used for the determination of total carbohydrates by a modification of the method of Folin & Malmros (Hess, et al. 1978). This component was quantified with a ferricyanate reduction reaction at 420 nm using glucose as a standard.

Total lipid content was extracted by the method of Bligh & Dyer (1959), taken up in 500 µl of chloroform and determined according Marsh & Weinstein (1966) using tripalmitin as a standard and the absorbance determined at 375 nm.

Duplicate determinations were performed in all cases and values were expressed as a µg (100 µg dry weight)$^{-1}$. The organic matter (OM) was calculated as the sum of proteins, total lipid and carbohydrates. The energy conversion factors used for these parameters were 35.24, 17.16 and 18.00 KJ.g^{-1}, respectively (Beukema & De Bruin, 1979).

Statistical analyses

Significant differences in biochemical composition of oocytes and in normal and abnormal hatching rates during the study period were tested using one-way ANOVA. Whenever the assumptions of analysis of variance were not met, the Kruskal–Wallis ANOVA on ranks test was performed. Prior to any analyses, percentage data were arcsine transformed to normalize variance (Sokal & Rohlf, 1981). Multiple pairwise comparisons were performed using the post-hoc parametric Tukey test or the non-parametric Dunn's test in order to detect significant differences among, replicates, females and fortnightly consecutive samples. The Pearson or Spearman (if the residuals

are not normally distributed) correlation coefficient was used to determine the degree of association between parameters. In order to define quality criteria, data of broodstock quality reported in Joaquim et al. (2011) were correlated with oocytes and larval parameters studied. Results were considered significant at $P<0.05$. The statistical analyses were undertaken using the SIGMASTAT 3.11 statistical package.

4.3 Results

Spawning data

A positive response to the spawning stimulation was obtained over a long period of the year (Table 4.1). Despite this positive response extended in time, two distinct periods were observed: a first intense response between February and March and a second less significant spawning event between late May and July of both years. The higheest number of females that spawned was obtained in early March 2007 (almost 50%), however, males did not respond with the same extent to spawning stimulation (only 10%). The highest number of oocytes released was obtained in early February 2007 ($63.8x10^6$), coinciding with the highest mean of oocytes released per female ($2.55\pm1.40x10^6$).

The total number of oocytes released per sampling was positively correlated with the percentage of females that responded to the spawning stimulation (Pearson $r=0.68$, $P<0.001$). Despites the mean number of oocytes released per female peaks when more females responded to the spawning stimulation, no significant correlation was found between these two parameters. Furthermore, a large variability in the number of oocytes released by each female intra-sampling was found. The positive correlation (Spearman's, $r=0.83$; $P<0.001$) (Table 4.2) found between males and females showed that they were synchronized in the spawning response.

Table 4.1 Spawning data and hatching rates during the experimental period. *[1] Eggs were not incubated due to embryos degradation, *[2] statistically significant differences, P<0.05 found between consecutive samples.

Year	Month	Spawning			Total number of oocytes realeased	Number of oocytes released per female	Fertilization rate	Normal veliger hatching rate	Abnormal veliger hatching rate
		Total	Females	Males					
		(total stimulated)	(% of total stimulated)	(% of total stimulated)	(10^6)	(10^6)	(%)	(%)	(%)
2006	Feb								
		112	26.8	24.1	12.817	0.427 ± 0.356	74.0 ± 15.6	12.3 ± 12.4	3.4 ± 6.0
	Mar	100	32	31	26.923	0.841 ± 0.842	88.0 ± 10.2	12.4 ± 7.4	3.1 ± 2.3
		208	0.0	7.7	0.0	0.0	--	--	--
	Apr	105	7.6	8.6	1.783	0.223 ± 0.178	95.0 ± 0.6	6.2 ± 4.3	2.7 ± 1.2
		137	9.5	8.8	7.120	0.548 ± 0.609	86.7 ± 7.2	4.4 ± 2.0	3.5 ± 4.3
	May	148				No spawning			
		139	18.7	15.1	19.905	2.212 ± 1.088	96.8 ± 2.1	5.7 ± 6.4 *[2]	5.3 ± 7.0
	Jun	163	16.6	14.7	25.120	1.142 ± 0.632	96.7 ± 2.2	53.1 ± 42.3	7.4 ± 5.0
		--	--	--	--	--	--	--	--
	Jul	306	11.1	7.8	24.005	0.889 ± 0.639	92.4 ± 6.8	33.5 ± 24.2	7.3 ± 5.5
		160				No spawning			
	Aug	160	2.5	1.3	1.713	0.857 ± 0.900	--	--	--
		250	0.0	0.4	--	--	--	--	--
	Set	200	3.5	4.5	1.005	0.168 ± 0.212	--	--	--
		167	1.2	7.2	0.333	0.166 ± 0.044	--	--	--
	Oct	--	--	--	--	--	--	--	--
		258	3.1	3.9	5.864	0.733 ± 0.696	--	--	--
	Nov	286				No spawning			
		219	6.8	6.0	7.401	0.493 ± 0.460	--	--	--
	Dec					No spawning			
		--	--	--	--	--	--	--	--
2007	Jan	264	4.2	0.0	17.247	0.157 ± 0.115	--	--	--
		295	18.7	17.9	19.626	0.785 ± 0.754	81.8 ± 14.9	53.6 ± 37.8	11.5 ± 8.3 *[2]
	Feb	202	39.1	45.0	63.762	2.550 ± 1.394	90.0 ± 5.2	37.9 ± 21.1	31.8 ± 12.6
		207	25.1	24.6	10.078	0.403 ± 0.237	79.0 ± 14.3	32.7 ± 27.1 *[2]	24.8 ± 19.2
	Mar	175	49.7	10.8	18.089	0.724 ± 0.494	89.4 ± 6.8	7.5 ± 8.4	38.2 ± 24.3
		222	29.7	22.5	20.292	0.812 ± 0.499	91.7 ± 8.3	9.1 ± 8.8	22.3 ± 14.4
	Apr	214				No spawning			
		--	--	--		--	--	--	--
	May	200	12.5	1.5	4.652	0.202 ± 0.214 *[1]	--	--	--
		340				No spawning			
	Jun	--	--	--		--	--	--	--
		231	4.3	7.8	12.300	1.367 ± 0.884	95.9 ± 2.3	57.1 ± 26.4	8.7 ± 6.4
	Jul	168	17.8	23.8	9.185	0.383 ± 0.387	88.5 ± 13.1 *[1]		
		127	3.9	4.7	0.838	0.168 ± 0.149	--	--	--
	Aug	--	--	--		--	--	--	--
						No spawning			
	Set	214				No spawning			
		210				No spawning			
	Oct	179				No spawning			

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Table 4.2 Results of Pearson (P) or Spearman (S) correlation between condition of broodstock, oocytes biochemistry and hatching rates (*r*, correlation coefficient, *P*, P value, n.c., no correlation was found).

		Broodstock											Oocytes					Larvae	
		Gonadal index			Spawned			Total		Total	Total number	Fertilization			Total	Organic	Total	Veliger rate	
		Males	Females	Total	Females	Males	Glycogen	Lipids	Proteins	energy	oocytes released	rate	Proteins	Carbohydrates	Lipids	matter	energy	Normal	Abnormal
Broodstock	Condition Index	--	--	--	P; r=-0.44 P<0.05	n.c.	--	--	--	--	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
	Males gonadal index		--	--	n.c.	n.c.	--	--	--	--	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
	Females gonadal index			--	S; r=0.49 P<0.05	n.c.	--	--	--	--	S; r=0.62 P<0.05	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
	Total gonadal index				n.c.	n.c.	--	--	--	--	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
	Spawned females					S; r=0.83 P<0.001	S; r=-0.46 P<0.05	n.c.	n.c.	n.c.	P; r=0.68 P<0.001	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
	Spawned males						n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
	Glycogen							n.c.	n.c.	n.c.	S; r=-0.51 P<0.05	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
	Total Lipids								n.c.	n.c.	P; r=-0.47 P<0.05	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
	Proteins									n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
	Total energy										P; r=-0.45 P<0.05	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
	Total number oocytes released											n.c.	S; r=0.58 P<0.05	n.c.	n.c.	P; r=0.60 P<0.05	n.c.	n.c.	n.c.
	Fertilization rate												n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
Oocytes	Proteins													n.c.	n.c.	S; r=0.73 P<0.05	P; r=0.70 P<0.05	n.c.	P; r=0.68 P<0.05
	Carbohydrates														P; r=0.91 P<0.001	S; r=0.57 P< 0.05	P; r=0.75 P<0.05	n.c.	n.c.
	Total lipids															S; r=0.72 P<0.05	P; r=0.77 P<0.001	n.c.	n.c.
	Organic matter																S; r=0.88 P<0.001	S; r=0.70 P<0.05	S; r=0.62 P<0.05
	Total energy																	S; r=0.62 P<0.05	n.c.
Larvae	Normal veliger rate																		n.c.

Biochemical composition of oocytes

Protein was the predominant oocyte constituent (21–38 µg(100µg dry weight)$^{-1}$) followed by total lipids (7–17 µg(100µg dry weight)$^{-1}$) and carbohydrates (1-4 µg(100µg dry weight)$^{-1}$) (Table 4.3). The protein content remained relatively high throughout the entire sampling period, and significant differences between consecutive samplings were only found between late February and early March 2006 (K.-W., H=97.814, df=13, P<0.001).The highest protein content value was recorded in early July 2006 (38.04±6.84 µg(100 µg dry weight)$^{-1}$) and the lowest in the first sampling (late February 2006 – 20.77±5.78 µg(100 µg dry weight)$^{-1}$). Significant differences were found among females in the same sampling time (P<0.05). The protein content of oocytes was positively correlated with the total number of oocytes released in each sampling (Spearman's, r=0.58, P<0.05).

The carbohydrates content increased until early April and then generally stabilized until early July 2006. In 2007, after an increase recorded in early February, the total carbohydrates tended to decrease until late March, and then increased again in June and July. The lowest value was recorded in late February 2006 (2.37±0.94 µg(100 µg dry weight)$^{-1}$) and the highest in early February 2007 (4.52±1.10 µg(100 µg dry weight)$^{-1}$). Significant differences were found between consecutive samplings (K.-W., H=124.522, df=13, P<0.001; late January and early February and late February 2007) and the carbohydrates content of oocytes are different among females of the same sampling (P<0.05).

In general, the total lipid content increased during the first year of sampling. In the following year, there was a trend towards a decrease in the first four months of the year except in early February and total lipids content of oocytes increased in June and July. The lowest value was recorded in late February 2006 (7.15±3.58 µg(100 µg dry weight)$^{-1}$) and the highest in early July (17.06±8.05 µg(100 µg dry weight)$^{-1}$) of 2007.

Table 4.3 Mean values (± S.D.) of proteins, carbohydrates, total lipids (µg(100 µg dry weight)$^{-1}$), organic matter (OM) (µg), and total energy (KJ µg^{-1}) of *V. corrugata* oocytes during the experimental period (*statistical significant differences, P<0.05 found between consecutive months).

Year	Month	Proteins µg(100 µg dry weight)$^{-1}$		Carbohydrates µg(100 µg dry weight)$^{-1}$		Total Lipids µg(100 µg dry weight)$^{-1}$	Organic matter (OM) µg(100 µg dry weight)$^{-1}$		Total energy (KJ µg^{-1})
2006	Feb	--		--		--	--		--
		20.77 ± 5.78	*	2.37 ± 0.94		7.15 ± 3.58	28.94 ± 6.53	*	5.74 ± 2.07
	Mar	28.68 ± 8.67		2.47 ± 0.73		8.27 ± 5.03	40.27 ± 12.09		7.70 ± 3.49
		Insufficient number of oocytes released							
	Apr	27.16 ± 8.61		2.91 ± 1.32		8.35 ± 3.10	39.90 ± 10.64		8.56 ± 2.50
		28.85 ± 7.22		3.06 ± 1.25		9.07 ± 4.87	39.93 ± 9.91		8.72 ± 2.50
	May	No spawning							
		28.22 ± 4.65		3.78 ± 1.65		10.91 ± 6.56	40.87 ± 5.57		8.88 ± 2.22
	Jun	32.22 ± 6.03		3.29 ± 0.98		10.96 ± 4.97	43.69 ± 6.68		9.00 ± 2.03
		--		--		--	--		--
	Jul	38.04 ± 6.84		3.53 ± 1.76		12.32 ± 5.30	48.59 ± 5.97		10.20 ± 1.05
		No spawning							
	Aug	Insufficient number of oocytes released							
		No spawning							
	Set	Insufficient number of oocytes released							
		Insufficient number of oocytes released							
	Oct	--		--		--	--		--
		Insufficient number of oocytes released							
	Nov	No spawning							
		Insufficient number of oocytes released							
	Dec	No spawning							
		--		--		--	--		--
2007	Jan	Insufficient number of oocytes released							
		33.22 ± 5.84		2.85 ± 0.67	*	9.66 ± 3.07	45.75 ± 6.76		9.70 ± 1.76
	Feb	33.61 ± 6.90		4.52 ± 1.10	*	13.57 ± 4.48	52.18 ± 12.13		10.84 ± 3.22
		31.59 ± 6.86		2.58 ± 0.82		9.21 ± 4.84	41.14 ± 8.01		9.15 ± 1.89
	Mar	31.94 ± 8.91		2.92 ± 1.08		7.83 ± 3.17	40.50 ± 6.76		8.29 ± 2.36
		34.69 ± 9.75		2.60 ± 0.97		7.93 ± 3.91	43.86 ± 10.92		8.70 ± 1.43
	Apr	No spawning							
		--		--		--	--		--
	May	Insufficient number of oocytes released							
		No spawning							
	Jun	--		--		--	--		--
		30.68 ± 5.67		3.23 ± 1.25		10.78 ± 4.43	44.46 ± 6.55		9.60 ± 2.27
	Jul	27.99 ± 8.54		4.43 ± 0.82		17.06 ± 8.05	43.93 ± 10.53		10.40 ± 2.24
		Insufficient number of oocytes released							
	Aug	--		--		--	--		--
		No spawning							
	Set	No spawning							
		No spawning							
	Oct	No spawning							

Significant differences were found among samplings (ANOVA, F=5.309, df=13, P<0.001) and females in the same sampling time (P<0.05), however, no significant differences were found between consecutive samplings. Generally, total lipids followed the same trend as the total carbohydrates, especially in 2007, which explains the positive correlation found between these two contents of oocytes (Pearson r=0.91, P<0.001).

Generally, the OM of oocytes remained high over the two years of sampling, except in February 2006, when the lower value was found (28.94±6.53 µg(100 µg dry weight)$^{-1}$). The highest (52.18±12.13 µg(100 µg dry weight)$^{-1}$) value was found in February 2007 (Table 4.3). Significant differences among samplings were found (ANOVA, F=4.573, df=15, P<0.001), however without consecutiveness, except between late February and early Marsh 2006. The OM reflects the proteins, total carbohydrates and total lipids contents variation along the sampling period, since these contents were correlated positively (Spearman's, r=0.73, P<0.05; r=0.57, P<0.05; r=0.72, P<0.05, respectively). A positive correlation was also found between the OM of oocytes and the number of oocytes released per female (Pearson, r=0.60, P<0.05).

The lowest value of energy of oocytes released was found in late February 2006 (5.74±2.07 KJ µg^{-1}) and the highest in early February 2007 (10.84±3.22 KJ µg^{-1}). The energy content of oocytes followed the same trend as the OM (Spearman's, r=0.88, P<0.001) and all the biochemical contents contribute positively to this parameter (proteins: Pearson, r=0.70, P<0.05; carbohydrates, Pearson, r=0.75, P<0.05; total lipids: Pearson, r=0.77, P<0.001).

Fertilisation and hatching rates

The fertilisation rate did not vary substantially along the sampling period (88.44±11.13%) (Table 4.1). Rather, the hatching rate of normal veliger varied considerably throughout the sampling period. Two peaks per year were recorded: one in the first two months of the year followed by a decline until May and another one between June and July. The lower value was recorded in Late April 2006 (4.4±2.0%) and the highest one in late June 2007 (57.1±26.4%). Significant differences were found in the hatching rate of normal veliger, inclusive between the consecutive samplings late May and early June 2006, late February and early March 2007, and late March and late June 2007 (K.-W., H=143.869, df=12, P<0.001) and between females within samplings. The hatching rate of normal veliger larvae was related to both OM (Spearman's, r=0.70, P<0.05) and total energy of oocytes (Spearman's, r=0.62, P<0.05) (Table 4.2). Abnormal veligers were present along all the larval culture, however with different intensities. In 2007 there were a higher percentage of abnormal veliger larvae. The lower value was recorded in early April 2006 (2.7±1.2%) and the highest one in late

March 2007 (38.2±24.3%). As for the normal veliger rate, significant differences were found between females within samplings and among samplings (K.-W., H=170.751, df=12, P<0.001), however between consecutive samplings only the late January and early February 2007 were different. The occurrence of abnormal larvae was positively correlated with the OM (Spearman's, r=0.62, P<0.05) and proteins content (Spearman's, r=0.68, P<0.05) of oocytes.

Relationships among broodstock, oocytes and D-larvae

Significant positive correlations were found between the gonadal index of females and their response to the spawning stimulation (Spearman's, r=0.49, P<0.05) (Table 4.2). However, condition index and the glycogen of broodstock were negatively correlated with the percentage of females that responded to the spawning stimulation (Pearson, r=-0.44, P<0.05; Spearman's, r=-0.46, P<0.05, respectively). The total number of oocytes released obtained in each spawning depended on the gonadal index of breeding females (Spearman's, r=0.62, P<0.05). However, glycogen, total lipids and total energy of broodstock were inversely correlated with the total number of oocytes released per sampling (Spearman's, r=-0.51, P<0.05; Pearson, r=-0.47, P<0.05; Pearson, r=-0.45, P<0.05). No significant relationships were found between the studied parameters that defined the quality of broodstock and the oocytes quality or the larval viability.

4.4 Discussion

The variability in spawning and larviculture success in hatcheries can be influenced by the reproductive process of bivalves. Moreover, it is widely accepted that embryogenesis of bivalves is fueled with the endogenous reserves provide to eggs during oogenesis (Lu et al. 1999; da Costa et al. 2011; Sánchez-Lazo & Martínez-Pita 2012). The broodstock of *V. corrugata* responded positively to the spawning stimulation in an extended period of the year and the synchronized response confirmed the synchronized gonadal development between males and females find in Joaquim et al.(2011). Cerviño-Otero (2011) found that three "wild" populations of *V. corrugata* from Galicia also had no sexual rest period and reported that, spawning in hatchery was possible throughout the year. In our study, in the first year of sampling (2006) spawning in hatchery was also possible in almost all year, however after July 2007, clams did not

respond positively to the spawning stimulation. Despite this extended response in time, two distinct spawning periods were identified: a first one more intense between February and May and a second one less significant in quantity, between late May and July. Furthermore, the response of females to the spawning stimulation was positively correlated with the gonadal index find in Joaquim et al. (2011). The prolonged spawning period of the species and the variability in the number of oocytes released by females in each sampling found in this study was a consequence of the great capacity for the gonadal regeneration of this species, especially during the spawning period. Indeed, the total number of oocytes released per sampling was positively correlated with the gonadal index of adults reported by Joaquim et al. (2011).These authors also reported that, in the same individual was frequently found gonias, maturing gametocytes and variable proportions of fully matured gametes. Cerviño-Otero (2011) also found a large spawning period and heterogeneity of gonadal maturation in the same sampling in all different Galician populations of *V. corrugata* studied. This can be an advantageous strategy for the species, since it ensures a continuous supply of settlers.

Nevertheless, the highest number of females that responded to the spawning stimulation was obtained, generally in a period of decreasing of the condition index and glycogen content, the main energy reserve in adults. Therefore, the glycogen and the total lipids of adults were also inversely correlated with the total number of oocytes released in each sampling. As already reported in Joaquim et al. (2011), the condition index of *V. corrugata* may be not a direct consequence of the reproductive cycle, but rather a consequence of the strategy of storage and exploitation of glycogen of this species. Cerviño-Otero (2011) also found that the biochemical composition of broodstock gives no information about the quantitative response to spawning stimulation.

Endogenous oocytes reserves are vital to promote survival and normal development throughout embryogenesis to first-feeding larvae and any deficiency could have serious implications (Sargent, 1995; Ojea et al. 2008; da Costa et al. 2011; Sánchez-Lazo & Martínez-Pita 2012). The biochemical composition of oocytes can be an indicator of the reproduction potential of broodstock and consequently contribute to the definition of quality criteria applied in hatcheries (Utting & Millican, 1997)). Although the oocytes of *V. corrugata* were obtained for an extended period of the year, their

biochemical composition varied in each sampling. Corporeau et al. (2012) reported that female Pacific oysters do not all produce oocytes of equal quality. The relative amount of protein (21 to 38 µg (100µg dry weight)$^{-1}$), carbohydrate (1 to 3 µg (100µg dry weight)$^{-1}$) and total lipids (7 to 17 µg (100µg dry weight)$^{-1}$) determined in *V. corrugata* oocytes were similar to those previously described in the literature for other bivalve species (Sedano et al. 1995; Kennedy & Newell 1996; Utting & Millican, 1997; Massapina et al. 1999; Nevejan et al. 2003; Cannuel & Beninger, 2005). The mean quantity of oocytes proteins remained consistently high throughout the spawning period. This fact and the positive correlation found between oocytes protein content and the total number of oocytes released in each sampling led us to think that this content was important at a structural level. The importance of proteins as a means to determine the bivalve oocytes quality was also suggested for the oyster *O. edulis* (Whyte et al., 1991) Corporeau et al. (2012) have identified several proteins that were differentially accumulated according to oocyte quality of *C. gigas*. According to these authors), oocyte proteins were at a similar rate and provide almost the same amount of energy as lipids in this species. Moreover, da Costa et al. (2011) reported that in the razor clam *Solen marginatus,* proteins are depleted as energy source throughout embryogenesis. However, in our study, no significant relationship was found between the proteins of oocytes and the larval viability, as was already been reported to *C. gigas* by Massapina et al. (1999). Rather, the occurrence of abnormal larvae was positively correlated with both oocytes proteins and OM contents.

The carbohydrates and total lipids contents of oocytes generally followed the same trend and the highest values of these contents were observed when the normal veliger hatching rate was higher. Despite this similarity, the initial quantity of endogenous carbohydrates and total lipids supplied to oocytes by the parents during vitellogenesis seems that do not have a primordial effect in *V. corrugata* D-larvae viability, since no significant relationships were found between these two oocytes contents neither with larval veliger rate nor with the parents' condition. Holland & Spencer, (1973), Massapina et al. (1999) and Matias et al. (2011) have already reported that there was no evidence of carbohydrate being used as an energy source in embryo development stages in *Ostrea edulis*, *C. gigas* and *Venerupis decussata*, respectively. Rather, the importance of the lipids constituents of oocytes in the first larval stages has been suggested by

several authors for other bivalve species (e.g. Le Pennec et al. (1990) and Devauchelle & Mingant (1991) for *Pecten maximus*, Napolitano et al. (1992) for *Placopecten magellanicus*, Massapina et al. (1999) and Cannuel & Beninger, 2005 for *C. gigas*). Although no correlations were found between any of the studied specific constituents of oocyte and the larval hatching rate, the OM, as a whole, and their energy proved to be of great importance to allow normal larval development. Indeed, in *V. corrugata*, proteins, carbohydrates and the total lipids contents of oocytes contributed significantly to both OM and total energy of oocytes, since they were all positively correlated. Massapina et al. (1999) have already found the same relationship between the organic matter and the energy content of oocytes and larval viability in *C. gigas*. Moreover, Sedano et al. (1995) and Acosta-Salmón (2004) reported that proteins and total lipids contributed significantly to the total energy content of oocytes of mussel *Mytilus galloprovincialis* and pearl oysters *Pinctada margaritifera* and *P. fucata*, respectively.

The hatching rate of normal veliger varied substantially throughout the spawning period and was lower than the rates reported by Cerviño-Otero (2011) for the same species. One of the reasons for these lower results can be related to the culture conditions, namely the temperature used for the incubation of eggs. Indeed, Ceviño-Otero (2011) incubated the *V. corrugata* eggs at a lower temperature (18ºC) than in the present study (20ºC). Two peaks in the hatching rate per year were recorded: one in the first two months of the year followed by a decline until May and another one, more important, between late May and July. Although there were not found any relationships linking the broodstock conditions, the oocytes quality and the D-larvae viability, if only this second peak is considered, the hatching rate coincided with the maximum level of oocytes quality and also with an increase of glycogen reserves and condition index in adults. During the first part of the spawning period (February to May) of *V. corrugata*, despites the high gonadal maturation of broodstock, it was not possible to trace quality criteria that define the D-larvae viability based on the broodstock condition. This reinforce the idea defended in Joaquim et al., (2011) that the condition index did not reflect the reproductive cycle, but rather instead the continuous storage and exploitation of glycogen. Thus, biochemical composition of broodstock gives no information about oocytes quality and consequent D-larvae viability. However, between late May and July, the highest normal larval rate was related to a highest oocytes quality generated by

a better condition of the broodstock. Indeed, the great capacity for gonadal regeneration and consequently continuous supply of gametes of the species (Joaquim et al. 2011), together to the high food availability (Lopes et al. 2007) and coupled to the stored reserves, allowed the species to produce oocytes with better quality.

In summary, spawning in hatchery with "wild" broodstock of the pullet carpet shell *V. corrugata* was possible in a long period of the year, however, the subsequent hatching larval success varied according to oocytes quality. The producers may have the chance to invest on "wild" broodstock, in two distinct periods of the year: in February/May, with more chance to obtain a higher number of oocytes, and in late May/July with a weaker response to the spawning stimulation, however with more successful larval hatching. This opportunity has interesting implications for the implementation of profitable aquaculture of the species, since allow manage its production with low costs. Indeed, as we forecasted in Joaquim et al. (2011), the long natural spawning period of *V. corrugata* implies that manipulation of broodstock in terms of conditioning will be relatively easy and neither extensive nor expensive.

The results of this study showed that OM of oocytes released can be used as a bench mark for estimating the larval hatching success. Such indices can be applied in hatcheries and aquaculture ongrowing systems and can also contribute to the knowledge of the pullet carpet shell reproduction potential.

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Chapter 5

Hatchery production of *Chamelea gallina*, *Spisula solida* and *Venerupis corrugata*

Spisula solida larvae

Chapter 5.1

New species in aquaculture: Are the striped venus *Chamelea gallina* (Linnaeus, 1758) and the white clam *Spisula solida* (Linnaeus 1758) potential candidates for diversification in shellfish hatchery?

Joaquim, S., Matias, D., Matias, A.M., Gonçalves, R., Chícharo, L., Gaspar, M.B. New species in aquaculture: Are the striped venus *Chamelea gallina* (Linnaeus, 1758) and the white clam *Spisula solida* (Linnaeus 1758) potential candidates for diversification in shellfish hatchery? (*Submitted to Aquaculture*)

Abstract

The white clam *Spisula solida* (Linnaeus, 1758) and the striped venus *Chamelea gallina* (Linnaues, 1758) support important fisheries in Europe. However, in last years, these fisheries were affected by inter-annual fluctuations in stock abundance and periodic recruitment failures. Aquaculture could contribute to address these problems through the production of larvae or juveniles to promote restocking programs. In the present study the potential of these species to the hatchery production was evaluated. Three different methods were tested to induce *C. gallina* and *S. solida* to spawning. *C. gallina* only spawned in open circuit and only fertilized oocytes were obtained. The more effective spawning induction method for *S. solida* was stripping, allowing to test the effect of temperature (15, 17, 19, 23, 25ºC) in eggs incubation. An inverse relationship between the hatching success and the temperature of embryos incubation was observed and the higher hatching rates of D-larvae were found at 15ºC and 17ºC. The larval rearing of the two species was possible with the technology traditionally used in hatchery and best results of survival and growth rates (*C. gallina* - K=5.47±0.67 µm day^{-1} and K=7.66±0.66 µm day^{-1} at 20ºC and 23ºC; *S. solida* - K=5.70±0.12 µm day^{-1} at 17ºC) were obtained fed larvae with *Isochrysis* aff. *galbana* (*T-iso*). First *C. gallina* and *S. solida* pediveliger larvae were found at day 8 and day 26, respectively. The high percentage of metamorphosed larvae found (66% in larvae fed with *T-iso* at 20ºC) even in the unfed treatment (42% at 20ºC) for *C. gallina* and for *S. solida* (70% at 17ºC) is an advantage for aquaculture production and restocking programs. After metamorphose, the *S. solida* postlarvae survival was quite low, however there was no mortality in *C. gallina* juveniles reared without substrate regardless the diet provided. Juveniles fed with the binary diet (*Chaetoceros calcitrans* (*C. cal*) + *T.iso*) and the monodiet *T-iso* showed relatively higher growth performances in shell length and weight (SGR_L(*T-iso*+*C.cal*)=1.38±0.20; SGR_w(*T-iso*+*C.cal*)= 4.35±0.43 and SGR_L(*T-iso*)=1.39±0.21; SGR_w(*T-iso*)=4.53±0.55) than juveniles fed with *C.cal* (SGR_L=1.26±0.08; SGR_w=3.93±02.80). There were no constrains that prevented the two species production, but the results of this study showed that *C. gallina* can be more attractive for aquaculture than *S. solida.*

5.1.1 Introduction

The white clam *Spisula solida* (Linnaeus, 1758) and the striped venus *Chamelea gallina* (Linnaues, 1758) are infaunal bivalve species that occurs in the infralittoral zone. *S. solida* has been reported from off the south of Iceland, and Norwegian Sea, to the Atlantic coast of the Iberian Peninsula, Morocco and Madeira Island (Tebble, 1966), whereas *C. gallina* is distributed throughout the Black Sea and Mediterranean (Poppe and Goto, 1993) and along the Algarve coast (southern Portugal). Although these species inhabits a variety of sediment types it is preferentially distributed on the coastal well-sorted fine sand biocenosis (Gaspar and Monteiro, 1999; Perés and Picard, 1964).They are important commercial bivalve species in Europe, especially in Iberian Peninsula and Mediterranean Sea. Along the Portuguese coast, the exploitation of *C. gallina* and *S. solida*, associated with the wedge shell *Donax trunculus* supports one of the most important fisheries (Chícharo et al., 2002a,b; Gaspar and Monteiro, 1999; Gaspar et al., 1999).

However, in the last years the synergistic action of the intensive harvest coupled with the rapid growth rate and short lifespan of these species has resulted in large inter-annual fluctuations in stock abundance and periodic recruitment failure (Joaquim et al., 2007). As a result, catches have dramatically decreased, threatening the biological and economic sustainability of this fishery. For this reason, over the last years great attention has been given in order to reverse this negative trend. For example, the knowledge acquired concerning the reproductive cycle of *S. solida* (Joaquim et al. 2008) was crucial to define management strategies including the development of stock-enhancement programs (Joaquim et al. 2007).

In this context aquaculture could, also, contribute to address these problems through the production of larvae or juvenile to promote restocking programs and therefore to enhance the stocks (Bell et al., 2005). According to López et al. (2008a), aquaculture diversification is a strategic challenge that must be oriented towards the culture of native species, given that dozens of these commercially interesting species are exploited exclusively by fisheries. On the other hand, from a production point of view, *C. gallina* and *S. solida* can be candidates to alternative or complementary products for the shellfish aquaculture industry and thus, promote the diversification in this activity.

However, as far as the authors' best knowledge, these species has never been produced in hatchery.

In general, the introduction of new species in aquaculture is based on the existing technology for traditional species (e.g. da Costa and Martínez-Patiño 2009; Ruiz-Azcona et al. 1986). The major objective in hatchery production is to improve the larval and newly settled seed growth and survival. Several environmental factors are important for bivalve growth and survival. Among them, phytoplankton supply and temperature have been considered as key factors affecting the bivalve physiological processes (Matias et al. 2009; Pérez-Camacho et al. 2003; Rico-Vila et al., 2009).

Hence, in the present study and in order to evaluate the potential of the species *C. gallina* and *S. solida* to the hatchery production, we present the results of the first investigations on the culture of these species from spawning to larvae and seed.

5.1.2 Material and methods

Algal culture

The microalgae *Isochrysis* aff. *galbana* (*T-iso*), *Chaetoceros calcitrans* (*C. cal*) and *Skeletonema costatum* (*Skt*) were cultured in 10-l flasks contained U.V.-sterilized, filtered (0.45 µm) and aerated seawater (salinity=33±1), enriched with f/2 medium (Guillard, 1975), in a temperature-controlled room at 20±2ºC under continuous illumination (9900 lux). Microalgae were harvested when the culture reached the end of the exponential growth phase. Cell density was determined by standard algal cell counts (Büker chamber) prior to feeding.

Broodstock collection and maintenance

A hundred and nine individuals of *C. gallina* and 694 individuals of *S. solida* measuring 26.8±2.84 mm and 31.5±2.26 mm in shell length and 8.8±3.4 g and 9.21±1.14 g in weight, respectively, were collected by dredge from a natural bed in Algarve coast (southern Portugal) off Ilha da Culatra (36º98′42″N and 7º83′36″W) between 3 and 10 m depth. Individuals were immediately transported to the IPMA Experimental Hatchery at Tavira. The broodstock was collected during the natural spawning period of the species, which for *C. gallina* begins in late spring and extends throughout all summer

(Gaspar and Monteiro, 1998) and for *S. solida* begins in late winter and extend through spring (Joaquim et al. 2008).

Spawning induction

Three different methods were tested to induce *C. gallina* and *S. solida* to spawning: *gonad stripping* - gametes were obtained by stripping the gonad of the mature clams; *thermal stimulation* - clams were exposed to 1 h alternate cycles of 15°C and 23°C for *C. gallina* and 14°C and 25°C for *S. solida*. Additional stimulation was provided by adding gametes obtained by gonad stripping and microalgae. A total of 5 cycles were performed; *open circuit* - individuals were maintained at 22±1ºC at a density of 2 Kg/m^2 for both species, in 50-L rectangular fiberglass tanks. The seawater flow used was 10 L per hour of sand-filtered seawater with aeration. Broodstock were fed daily with a constant mixed diet of *T-iso, C. calcitrans* and *S. costatum* in equal proportions at a ration of 6% mean dry meat weight of adults per day. Fertilised eggs were collected at the end of the circuit with a 30 µm sieve.

Eggs incubation

Oocytes obtained by gonadal stripping were incubated at 5 different temperatures (15, 17, 19, 23 and 25ºC) after fertilization in order to determine the effect of temperature in eggs incubations. Embryos were incubated in triplicate 200 mL beakers with filtered and U.V.-sterilized seawater at a density of 100 eggs mL^{-1}. The number of the different larval stages (morula, trochophore, normal (D-larvae) and abnormal (misshapen or exhibiting defective swimming) veliger larvae) was determined in 24, 31, 48 and 55 hours on three 1 mL aliquots for establish the embryonic chronogram of the species.

Eggs for the larval rearing trials were incubated in 80 L larval culture tanks with gently aerated and treated seawater (filtered at 0.45 µm and U.V.-sterilized) at 22±1 °C for *C. gallina* and 18±1ºC for *S. solida*, during 24h. These temperatures of incubation were chosen taking into account the requirements of *C. gallina* at the natural spawning period and the results of the incubation experiment in the case of *S. solida*. Embryos density in each container at this period was of 25 eggs mL^{-1}.

Larval rearing experiments

D-larvae were placed in 20 L larval culture tanks, in aerated (0.5 L min^{-1}), 0.45 µm filtered and UV-sterilized seawater, at the salinity of hatchery facilities seawater (33±1). The larval density was decreased from 7 until 3 larvae ml^{-1} along the experimental period and water was changed three times a week. The combined effect of temperature and diet on *C. gallina* larval rearing was studied by growing the larvae in two different temperatures, (20 and 23ºC) and three fed regimes (*T-iso*, *C. cal* and unfed). For *S. solida*, the effect of such diets was determined at 17±1ºC. Each experimental treatment consisted of triplicate tanks. To supply a permanent availability of remaining phytoplankton concentration around the larvae throughout the different feeding periods, larvae were fed daily with equal biomass proportions of *T-iso* and *C. cal* in a ration cell number of 50 and 68 cells μL^{-1} until day five, 75 and 100 cells μL^{-1} from day five to day 10 and 100 and 135 cells μL^{-1} until day fifteen, respectively.

For all larval rearing experiments, in each water change, larvae were collected from each tank and they were counted to estimate survival as a percentage (number of observed live larvae/initial number of larvae × 100) based on three sub-samples. The antero-posterior shell length was measured for 30 randomly sampled larvae from each replicate using images recorded with a microscope connected Nikon DSFi 1 camera, that were subsequently analysed using a freely available image analysis software Image J 1.38s. The presence of a foot was scored to determine larval development status. Larvae that showed a clearly visible foot bulging out of shell (pediveligers) were considered suitable to proceed to the post-larval culture.

Juvenile experiment

Postlarvae resulting from the larval trials were cultured without substrate in sieves in 80 L larval rearing tanks each of which had an inverted air supply. Seawater was 0.45 µm filtered and U.V.-sterilized and maintained at room temperature (22±1°C) and at the salinity of the hatchery facility seawater (33±1). When juveniles measured 3.7±1.9 mm of shell length and weighed 16.2±0.7 mg, a second set of experiments was carried out to test the influence of a binary diet (*T-iso* + *C. cal*) in equal proportion in terms of algae weight and two monospecific diets (*T-iso* and *C. cal*) on growth and survival. Juveniles were fed with 2% of mean live weight in dry weight of algae divided into four daily

doses. Water was changed three times a week. Each experimental treatment consisted of triplicate tanks with 30 juveniles per cm^2. Survival was measured as the percentage (number of observed live juveniles/initial number of juveniles × 100) weekly during the experimental period. Shell length and width was determined by measuring the maximum antero-posterior and the left-right axes, respectively, of 25 randomly sampled juveniles from each replicate using a Mitutoyo digital caliper (±0.02 mm). Fresh weight was determined individually on a top-loading digital balance (precision of 0.01 g).

Statistical analysis

Linear regressions were fitted to shell length over larval and juveniles growth trajectories to determine shell length growth equation for each treatment. Growth rate of larvae and juveniles were determined using respectively the formulas:

$$k = \frac{L_f - L_i}{\Delta t} \quad \text{and} \quad SGR = 100 \times \frac{\ln L_f - \ln L_i}{\Delta t},$$

where K is larvae growth rate; SGR is the specific growth rate; L_f and L_i are the mean shell length at respectively the beginning and the end of the experiment; Δt is the time interval in days between sampling i and f.

Survival and growth were analyzed by analysis of variance (One-way and Two-way ANOVA) or the *T*-test. Whenever the assumptions of ANOVA or *T*-test were not met, the Kruskal–Wallis ANOVA on ranks or the Mann-Whitney test was performed, respectively. Prior to any analyses, percentage data were arcsine transformed to normalize variance (Sokal and Rohlf, 1981). Multiple pair comparisons among means were performed using the post-hoc parametric Tukey test or the non-parametric Dunn's test in order to detect significant differences. The Spearman correlation coefficient was used to determine the degree of association between the hatching success and the temperature of embryos incubation. Results were considered significant at $P<0.05$. The statistical analyses were undertaken using the SIGMASTAT 3.5 statistical package.

5.1.3 Results

Spawning induction

C. gallina

Neither gonad stripping nor thermal stimulation was effective as spawning methods (Table 5.1). The oocytes obtained from the stripped females were unavailable, since this invasive method causes the disruption of the chorionic envelope of *C. gallina* oocytes. This species only spawned in the open circuit treatment. From the 109 clams that were induced, the first eggs were colleted after 24h after conditioning and spawning lasted 48h. All of the 204.445×10^6 eggs obtained were already fertilized when collected. The hatching rate after 24h was 56%. The resulting D-larvae were used in larval experiments.

S. solida

The more effective spawning induction method for *S. solida* was stripping. From the 223 stripped females, a total of 38.384×10^6 oocytes were obtained, corresponding to a mean number of 172.128 oocytes per female (Table 5.1). The fertilization rate was high (75%) and the normal veliger hatching rate was of 37%. The D-larvae obtained with this method were used in larval experiments. The thermal stimulation was also successful, however with less effectiveness. Furthermore, a large variability in the number of oocytes released by each female ($111.1 \pm 61.9 \times 10^3$) was found. From the 98 clams stimulated, only 6 females and 3 males were spawned. A total of 0.693×10^6 oocytes were obtained. The fertilization rate was lower than the obtained with the stripped method (52%) and the normal veliger hatching rate (D-larvae) was of 23%. *S. solida* clams did not spawn in open circuit.

Table 5.1 Details of *Chamelea gallina* and *Spisula solida* spawning induction.

Species	Type of induction	Total	Spawning		Total number of oocytes	Number of oocytes per female	Fertilization rate	Normal veliger hatching
		Total total stimulated	Females % of total stimulated	Males % of total stimulated	(10^6)	(10^3)	(%)	(%)
C. gallina	Gonad stripping	50	47,3	52,7	*[2]	--	--	--
	Thermal stimulation	109	0	0	--	--	--	--
	Open circuit	109	--	--	0,204 *[1]	--	100	73
S.solida	Gonad stripping	510	43,7	56,3	38,384	172,128	75	37
	Thermal stimulation	98	6,1	3,2	0,69	111,1 ± 61,9	52	23
	Open circuit	235	0	0	--	--	--	--

*[1] - Fertilized eggs.
*[2] - Unviable oocytes.

Effect of temperature in eggs incubation

This experiment was only performed with *S. solida*, since for *C. gallina* no unfertilized oocytes were obtained. Morula stage was present 24h after fertilization in all temperature of incubation and 55 hours later this stage was still present at 15 and 17ºC. Trochophore larvae were present in temperatures equal or higher than 17ºC at 24 hours after fertilization and were still present 55h after fertilization in all treatments, except at 25ºC (Table 5.2).

Table 5.2 Embryonic and larval chronogram of *Spisula solida* obtained at different temperatures. Percentage (mean ± SD) of morula, trochofore, normal and abnormal veliger stages.

Temperature (ºC)	Larval Stage	Hours			
		24	31	48	55
		(%)			
15	Morula	78.2 ±16.8	31.8 ± 8.5	8.9 ±2.0	2.0 ± 2.0
	Trochophore		28.9 ± 7.3	12.2 ±3.4	6.7 ± 1.3
	Normal veliger			58.9 ± 3.9	52.0 ± 2.7
	Abnormal veliger			2.2 ± 1.0	6.7 ± 2.8
17	Morula	30.7 ± 7.7	8.7 ± 2.9	4.2 ± 1.7	2.0 ± 1.7
	Trochophore	42.4 ± 4.7	14.2 ± 1.0	5.5 ± 3.9	3.6 ± 3.0
	Normal veliger		50.4 ± 4.3	57.6 ± 6.2	28.2 ± 12.1
	Abnormal veliger			3.1± 2.7	6.0 ± 2.7
19	Morula	31.3 ± 8.4	10.2 ± 2.7	2.4 ± 1.9	
	Trochophore	31.6 ± 1.34	10.2 ± 4.8	4.7 ± 4.0	0.2 ± 0.4
	Normal veliger	8.7 ± 3.0	36.4 ± 2.7	34.7 ± 15.5	10.0 ±3.5
	Abnormal veliger		6.9 ± 1.0	5.1 ± 2.7	3.1 ± 1.0
23	Morula	20.0 ± 0.9	9.3 ±2.8	1.6 ± 2.1	
	Trochophore	26.7 ± 8.5	13.0 ± 4.2	2.2 ± 2.3	0.4 ± 0.8
	Normal veliger	6.3 ± 3.3	3.0 ± 1.4	6.4 ± 2.1	
	Abnormal veliger	3.3 ± 1.9	14.0 ± 10.4	8.9 ± 2.5	3.1 ± 5.4
25	Morula	30.7 ± 8.7	18.7 ± 4.0	0.2 ± 0.4	
	Trochophore	11.3 ± 6.1	7.8 ± 3.4	1.1 ± 1.9	
	Normal veliger		0.4 ± 0.8	0.4 ± 0.4	
	Abnormal veliger				0.7 ± 0.7

The higher percentage of morula stage was found 24h hours after fertilization at 15ºC (78.2±16.8%) and the higher prevalence of trochophore stage (42.4±4.7%) was found also 24h after fertilization but with a higher temperature of incubation (17ºC). Normal veliger D-larvae appeared nearly 24h after fertilization at 19ºC and 23ºC and only over 48h after fertilization at 15ºC. However, the precocious appearing of normal veliger D larvae at 19 and 23ºC did not imply a higher hatching rate (Fig. 5.1). On the contrary, the higher hatching rates of D-larvae (58.9% and 57.5%) were found at the lower temperatures (15º and 17ºC, respectively) showing an inverse relationship between the hatching success and the temperature of embryos incubation (Spearman's, r=-0.94, P<0.001). At 25ºC the number of normal veliger rate was irrelevant. The abnormal larvae were also present in all treatments, but in low percentage (maximum of

14.0±10.4%, 31h after fertilization at 23ºC) and their presence was not related to the incubation temperature (Spearman's, P>0.05).

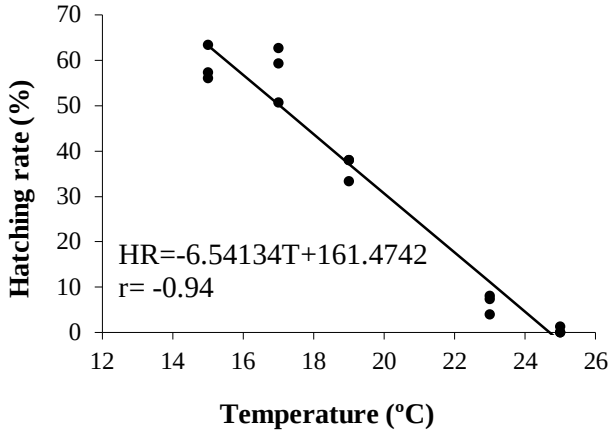

Figure 5.1 Relationship between incubation temperature (17, 19, 23 and 25ºC) and the hatching rate of *Spisula solida.*

Larval rearing

C. gallina

Survivorship of *C. gallina* larvae started to decrease substantially until below 45%, between the 3rd and the 8th day after fertilization, for all treatments (Fig. 5.2). After these days, mortality decreased significantly in all treatments until the end of the experiment. At the 13th day mean survival best results were observed in larvae fed with *T-iso* diet for the two tested temperatures (21.1±4.8% at 20ºC and 24.2 ± 2.2% at 23ºC). The lowest results were found in the unfed larvae (8.7±5.8% and 7.5±4.2%, at 20 and 23ºC, respectively) and larvae fed with *C. cal* at 20ºC (7.5±5.2%). There was no statistically significant differences among diets (P>0.05), between temperatures (P>0.05) and within diets and temperatures (P>0.05), except in day 6 and in day 13 after fertilization, if each day was considered separately. In the 6th day after fertilization, the survival of unfed larvae at 20ºC was statistically different from larvae fed with *C.cal* at 23ºC, larvae fed with *T-iso* at 20ºC and unfed larvae at 23ºC. Also in this day, larvae fed with *C.cal* showed significant differences between temperatures of rearing (ANOVA: F=8.183; *d.f.* = 5, P=0.001). In the last day of culture, survival of larvae fed with *T-iso* at 23ºC was statistically different from that fed with *C.cal* at 20ºC and the unfed larvae at 23ºC (ANOVA; F=5.007, *d.f.* = 5, P<0.05).

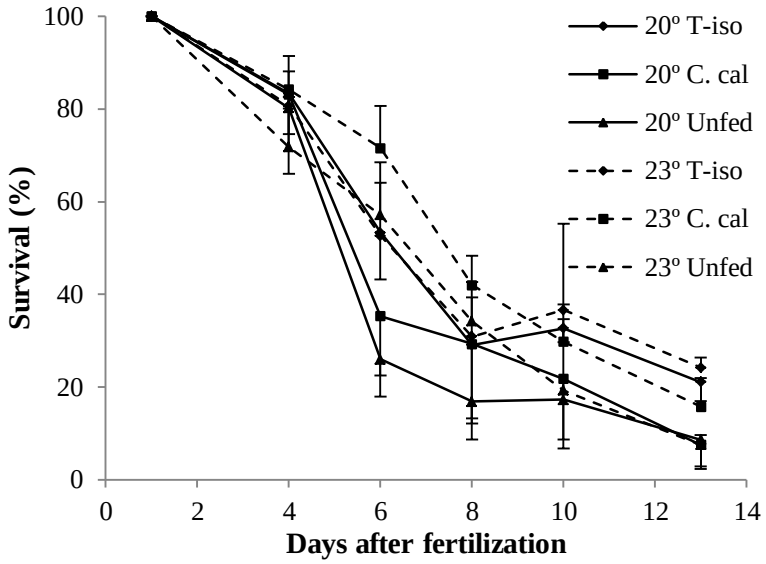

Figure 5.2 Survival of *Chamelea gallina* larvae reared at different temperatures (20ºC and 23ºC) and at different food regimes (*T-iso*, *C. cal* and unfed).

Larvae from all treatments showed an increase in shell length during the spawning period, including the unfed ones. Larvae fed with *T-iso* showed relatively higher growth performance in shell length (183.39±8.06 µm; K=5.47±0.67 µm day^{-1} and 212.58±7.95 µm; K=7.66±0.66 µm day^{-1} at 20ºC and 23ºC, respectively) than larvae fed *C.cal* and unfed treatments (134.28±1.24 µm, K=1.19±0.10 µm day^{-1}; 133.84±1.02 µm, K=1.10±0.08 µm day^{-1}; and 129.40±0.91 µm, K=0.73±0.08 µm day^{-1}; 134.37±3.61 µm, K=1.14±0.30 µm day^{-1}; for *C.cal* diet and unfed at 20ºC and 23ºC, respectively). The shell length growth and respective linear growth equations of *C. gallina* larvae, for all nutritional regimes are present in Figure 5.3. The slope of the equations also clearly showed the supremacy of *T-iso* diet for the larval growth, regardless of the rearing temperature (*T-iso* - 5.15 µm and 7.96 µm; *C. cal* - 1.40 µm and 1.28 µm; Unfed - 0.78 µm and 1.42 µm; respectively at 20ºC and 23ºC).

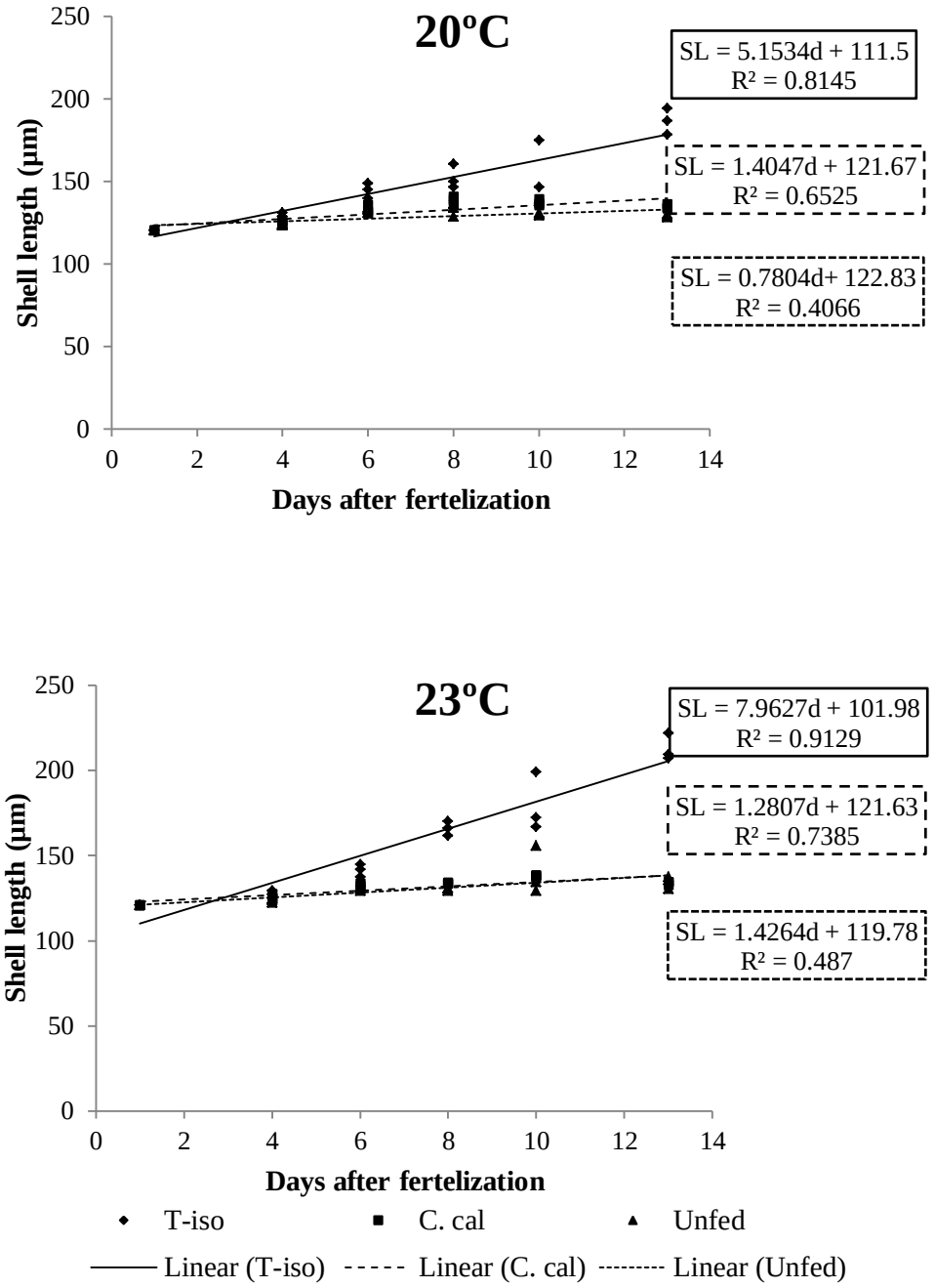

Figure 5.3 Growth in shell length (µm) and respective linear growth equations of *Chamelea gallina* larvae reared at different temperatures (20ºC and 23ºC) and at different food regimes (*T-iso*, *C. cal* and unfed).

Significant differences in shell growth length were found among diets (K-W; H=564.4, $d.f.$ = 2, P <0.001), between temperatures (M-W; =1701088, $n(small)$=1319, $n(big)$=1322, P<0.05) and among treatments (K-W; H=572.15, $d.f$ =5, P<0.001), except within temperatures of unfed and fed with *C. cal* larvae and between unfed and larvae fed with *C. cal* at 23ºC. The differentiation between treatments occurred at the 6th day. The results of the analyses of the interaction between diets and temperatures showed that only in the *T-iso* diet was felt the effect of temperature (M-W; T=17658, $n(small)$=442, $n(big)$=443, P<0.001). The temperature did not influence significantly the growth of larvae fed with *C.cal* (M-W; T=186468, $n(small)$=427, $n(big)$=431, $P>0.05$) and unfed larvae (M-W; T=204773, $n(small)$=448, $n(big)$=450, $P>0.05$). The presence of foot was detected between the day 8 and 10 after fertilization. At the end of the experiment (day 13), the highest percentage of foot, although with differences among replicates was observed in the larvae fed with *T-iso* at 23ºC (43.4±27.6%), while the lowest was observed in the unfed larvae (2.0±3.4 %) at 20ºC (Table 5.3).

Table 5.3 Percentage of metamorphic rate (mean ± SD) of *Chamelea gallina* larvae reared under different food regimes.

Temperature (ºC)	Food regime	Days after fertilization	Metamorphic rate (%)
20	T-iso	8	
		10	22.8 ± 8.4
		13	43.4 ± 27.6
	C.cal	8	1.4 ± 2.5
		10	32.2 ± 6.7
		13	32.6 ± 16.5
	Unfed	8	11.5 ± 1.4
		10	14.6 ± 10.4
		13	15.5 ± 21.4
23	T-iso	8	3.2 ± 0.3
		10	20.4 ± 1.2
		13	32.0 ± 5.0
	C.cal	8	
		10	0.6 ± 1.0
		13	8.3 ± 10.4
	Unfed	8	
		10	8.1 ± 14.1
		13	2.0 ± 3.4

S.solida

Survivorship of *S. solida* larvae suddenly decreased to about half in the first 5 days after fertilization for all diet treatment (53.5±10.6% - *T-iso;* 43.4±3.8% - *C. cal*; 45.6±7.6% - unfed) (Fig. 5.4). After this day, mortality decreased significantly, including in unfed treatment. The larvae fed with *T-iso* showed a slightly but statistically significant (Two Way Anova, F=24.84; df=8, P<0.001) higher survival than those fed with the other diets, until day 26. These differences were independent on what day of culture is present since there was not found a statistically significant interaction between days of culture and feeding regimes ($P>0.05$). The survivals of unfed and larvae fed with *C. cal* were statistically similar in this period (P>0.05). The foot only appeared (70%) in larvae fed with *T-iso*, at the 26th after fertilization and survival of fed larvae were 5.01±1.21% and 2.90±2.85%, for *T-iso* and *C.cal* diet regimes, respectively. On the 26th day, all larvae from unfed treatment were dead.

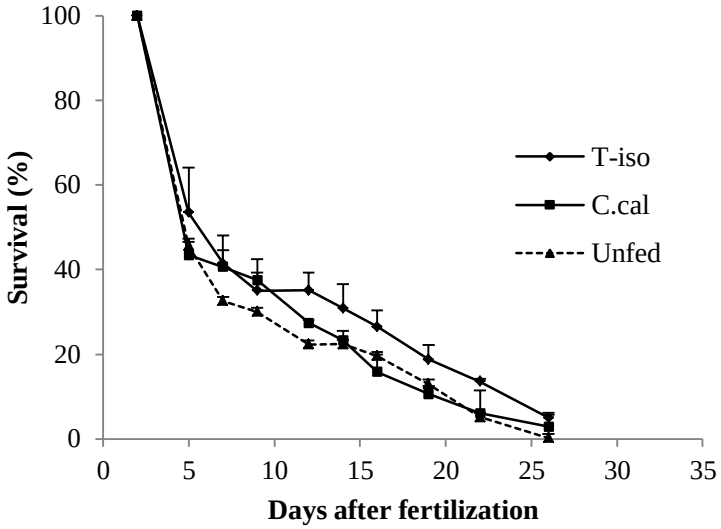

Figure 5.4 Survival of *Spisula solida* larvae reared at different food regimes (*T-iso, C. cal* and unfed).

The growth in shell length and respective linear growth equations of *S. solida* larvae, for all nutritional regimes are present in Figure 5.5. Larvae of all treatments showed an increase in shell length during the experimental period, including the unfed ones. However, the *T-iso* diet was much more effective than the others. Larvae fed with *T-iso* showed an increase in shell length and a growth rate (K=5.70±0.12 µm day^{-1})

substantially higher than larvae from the other treatment (K=1.72±0.33 µm day^{-1}; 1.64±0.24 µm day^{-1} for larvae fed with *C. cal* and unfed larvae, respectively). The slopes of the growth equation clearly showed this fact (6.04 µm - *T.iso*; 1.13 µm - *C. cal*; 1.34 µm - unfed). Larvae fed with *T-iso* and larvae fed with *C.cal* grow from an initial shell length of 75.16±6.67 µm to 213.03±29.81 µm and 107.46±10.75, respectively in 26 days of culture. The unfed larvae measured 108.72±8.15 µm, 22 days after fertilization. There were statistically significant differences in larval shell length among all treatments (K.W., H=917.39, $d.f.$=2, P<0.001) during all the experimental period, except between larvae fed with *C.cal* and unfed larvae that only differed on day 12 (K.W., H=117.96, $d.f.$=1, P<0.001) and day 14 (K.W., H=249.6, $d.f.$=1, P<0.001) after fertilization.

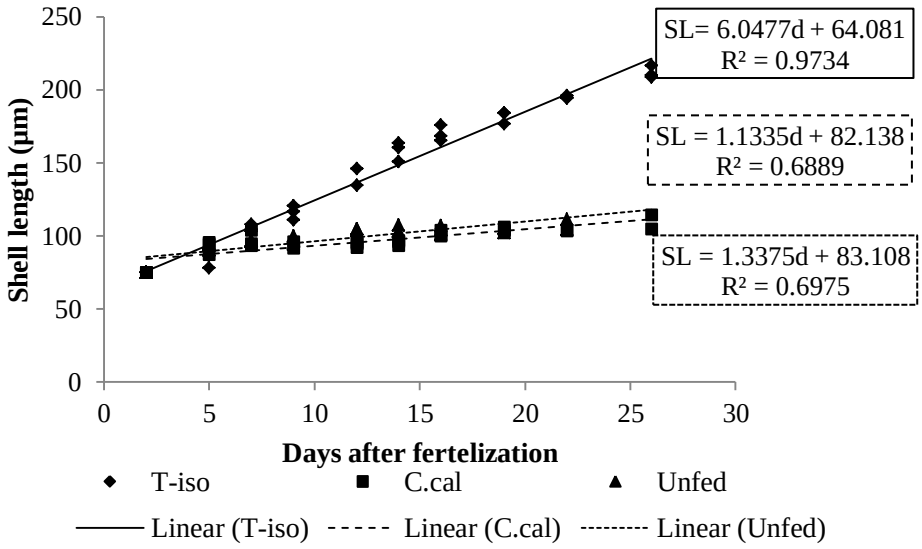

Figure 5.5 Growth in shell length (µm) and respective linear growth equations of *Spisula solida* larvae reared at different food regimes (*T-iso*, *C. cal* and unfed).

Juvenile production

The *S. solida* postlarvae survival after metamorphosed was quite low and just a few specimens were obtained, hindering the juvenile production experiment. All treatments were effective in terms of survival for seed of *C. gallina* production. There was no mortality, during this experiment 60 days-old (after fertilization) juveniles measured initially 3.82±0.17 mm in shell length and 15.49±1.55 mg in weight, grew about 5.4 mm and 241.0 mg respectively, in 65 days (Fig. 5.6). At the end of the experiment, juveniles

fed with the binary diet and *T-iso* showed relatively higher growth performances in shell length and weight (9.40±0.52 mm; SGR_L=1.38±0.20; 271.65±57.99 mg; SGR_w=4.35±0.43 and 9.40±1.46 mm; SGR_L=1.39±0.21; 289.95±104.00 mg; SGR_w=4.53±0.55, respectively) than juveniles fed with *C.cal* (8.74±0.54 mm; SGR_L=1.26±0.08; 207.91±23.87 mg; SGR_w=3.93±02.80). Although, generally there were no statistically significant differences among juveniles growth (shell length and weight) with different diets ($P \geq 0.05$), if only the last 30 days of the experiment were considerate, the growth in shell length and weight of *C. gallina* juveniles fed with *C.cal* were statistically different (shell length: K-W; H=47.070, *d.f.*=2, $P<0.001$; K-W; weight: H=34.836, *d.f.*=2, $P<0.001$) and lower than the growth of juveniles fed with the other tested diets.

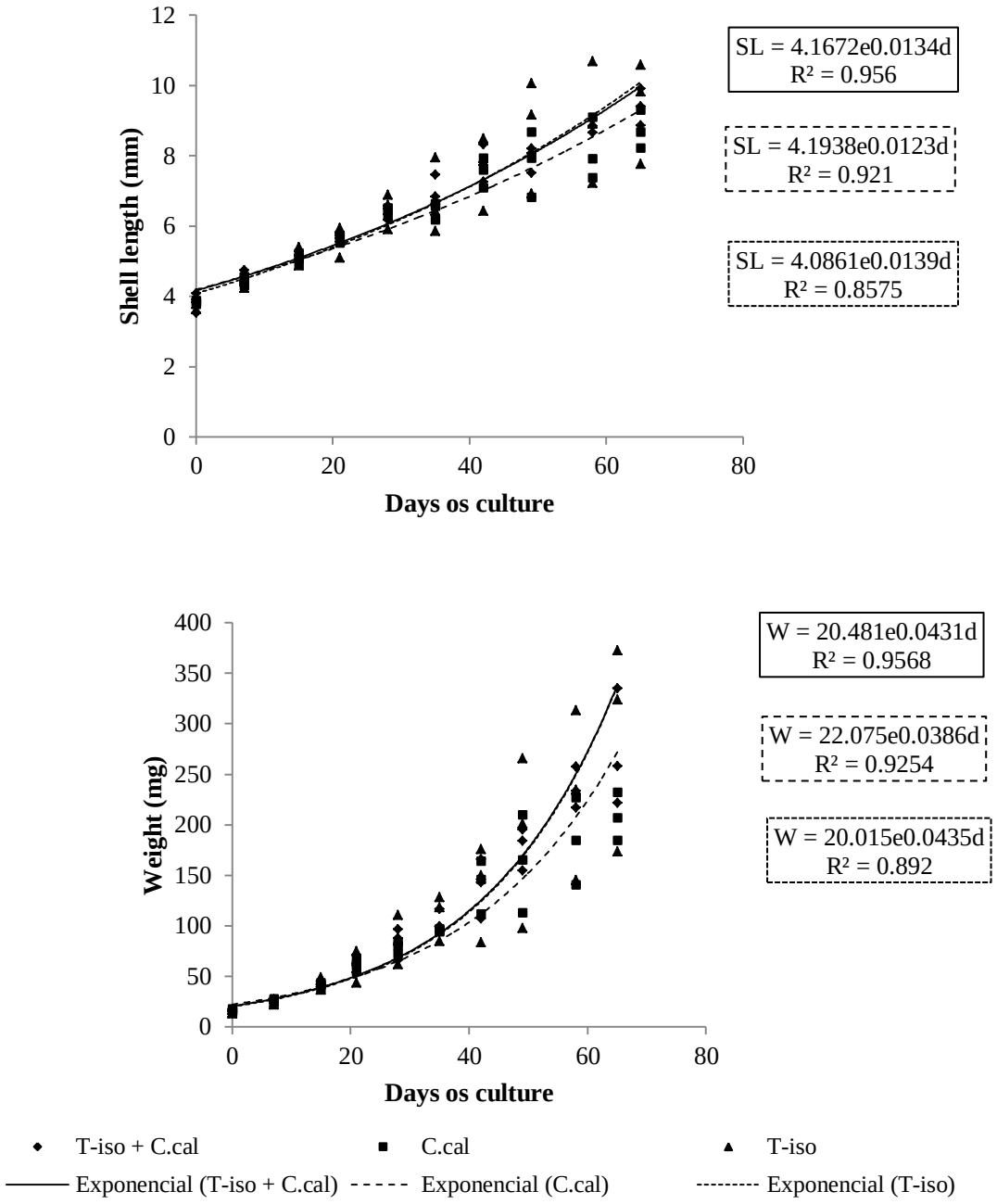

Figure 5.6 Growth in shell length (mm) and weight (mg) and respective linear growth equations of *Chamelea gallina* juveniles reared at different food regimes (*T-iso*, *C. cal* and unfed).

5.1.4 Discussion

In bivalves, different artificial stimuli are used to induce spawning. However, some lack of efficient stimuli still hinders the culture of several bivalve species (Mouëza et at., 1999). The techniques applied in this study (thermal stimulation, gonad stripping and open circuit) are often used in bivalve hatcheries. Usually, the stimulation with thermal cycles is the most effective method for spawning induction in bivalves, resulting in high hatching rates (O'Connor and Heasman, 1995). This methodology was only applied successfully in *S. solida,* however only a small amount (0.69 million) of fertilizable oocytes was obtained, whose hatching rate was 23%. The second method, gonad stripping, is used in species whose oocytes can be fertilized directly after dissecting the gonad, as it is the case of some oysters. Once again, this methodology was ineffective for *C. gallina* probably because, as in most bivalve species, oocytes are blocked in the ovaries at prophase of the first division of meiosis, and development reinitiates during spawning (Hamida, et al., 2004). On the contrary, gonad stripping was the most effective method to obtain gametes of *S. solida*. From the 223 stripped females, around 38 million of oocytes were obtained. The fertilization rate of oocytes was high (75%) and they generated 37% of normal D veliger. On the other hand, the addition of some water flow through an open system, to simulate the natural habitat hydrodynamics was crucial for the spawning success of *C. gallina*. This methodology was the only successful artificial stimuli tested with this species and allowed to obtained 0.24 million of fertilized eggs with the higher hatching rate observed (73%), however was not effective for *S. solida*.

Hatching rate assume an important role in bivalve culture control, since can reflect eggs quality (in terms of nutritional reserves) and consequently the success of embryonic and larval development. The succeeded method to obtained *C. gallina* eggs did not allow the full control of embryonic development and consequently the hatching rate, since only fertilized eggs and embryos in an advanced development phase were collected. In the case of *S. solida* it was possible to improve the hatching rate by testing the embryonic development incubated at different temperatures. The temperature range used in the present study took into account the seawater temperature observed during the natural spawning period of the species (15°C) and a temperature which could possibly shorten the eggs incubation in artificial conditions (25ºC). A relationship between temperature

and the chronology of *S. solida* larval development was found. Although a precocious appearing of D larvae occurred at 19ºC and 23ºC, the hatching rate was low. The greater hatching rate (≈60%) was found at the lower temperatures (15ºC and 17ºC) showing an inverse relationship between the hatching success and the temperature of incubation. At 25ºC the eggs incubation was not viable. Thus, to obtain a higher *S. solida* D larvae quality, the incubation of eggs should be performed between 15ºC and 17ºC.

The effect of microalgal diet on larval performance is difficult to generalize and seems to be species-specific (Brown et al. 1997; Pernet and Tremblay, 2004). In this study experiments were developed in order to evaluate the feasibility of *C. gallina* and *S. solida* larvae and juveniles in hatchery by assessing the nutritional adequacy of two different common microalgae used in bivalve hatcheries, *I. aff galbana* and *C. calcitrans* as monospecies diet. The shortest larval periods from hatching to metamorphosis obtained in this experiment were 8 days for *C. gallina* and 26 days for *S. solida*. The survival of *C. gallina* larvae was high in the first 5 days after fertilization, even in the unfed treatment. These results suggest that in this species, egg reserves can contribute to the maintenance of larvae beyond the period of embryonic development. Matias et al. (2011) reported similar results for *R. decussatus* larvae. Endogenous egg reserves are known to be important for survival and growth throughout embryogenesis until exogenous food sources become available (Cannuel and Beninger 2005; Fukazawa et al. 2005; Ojea et al., 2008). Survivorship of *C. gallina* larvae started to decrease substantially between the 3rd and the 8th day after fertilization, probably due to the adaptation to a different feeding regime in which yolk reserves and planktonic particles are used simultaneously by the young larvae (mixotrophic phase) (Labarta et al., 1999; Matias et al., 2011). After this adaptation period, mortality decreased in all treatments and at the end of the experiment mean survival best results were observed in larvae fed with *T-iso* diet for the two tested temperatures (20ºC – 20% and 23ºC – 24%). Conversely to *C. gallina*, the sudden decrease of larvae survival in *S. solida* it was observed within the first 5 days after fertilization, in unfed larvae as well as in larvae fed with the monospecies diets *T-iso* and *C.cal.* This suggests that the endogenous egg reserves do not contribute significantly to support the larval metabolism of this species. After this day, mortality decreased considerably, including in unfed larvae and although larvae fed with *T-iso* had a slightly better survival at the end of larval culture, its

percentage was low (5%). Indeed, the unfed larvae of *S. solida* survived 26 days and *C. gallina* survived until the end of the experiment (13 days). As has been reported for other species, such as *Crassostrea gigas* (Moran and Manahan, 2004), *Meretrix meretrix* (Tang et al., 2005) and *R. decussatus* (Matias et al., 2011), *S. solida* and *C. gallina* larvae can survive for long periods of time without phytoplankton, by using alternative sources of energy such as the eggs reserves or the dissolved organic material in seawater or bacteria as suggested by several authors (Gallager et al., 1994; Gomme, 2001; Manahan, 1990).

The growth rates of *C. gallina* larvae fed with *T-iso* at the two tested temperatures of rearing were substantially higher than those fed with *C. cal* and unfed larvae, and even higher than species traditionally produced in hatchery, such as *R. decussatus* (Ojea et al., 2008). This supremacy of the growth rate of larvae fed with the monodiet *T-iso* was also observed for *S. solida.* Indeed, this diet yielded high metamorphosis rates for the two bivalve species (*C.gallina:* about 66% at 20ºC and 55% at 23ºC; *S. solida:* about 70%). The results suggested that the microalgae *T-iso* have an adequate nutritional quality for larval rearing of *C. gallina* and *S. solida*. According to Matias et al. (2011) larvae of *R. decussatus* fed with *T-iso* accumulated significantly more organic matter reserves in their tissues, which have allowed them to overcome more successfully the critical phase of metamorphosis. Our results showed unequivocally that feeding *C. gallina* whichever was the rearing temperature and *S. solida* larvae with *C. cal* gave similar growth results as if no food was given to larvae. However, a significant percentage of competent larvae was obtained feeding *C. gallina* with *C.cal* at 20ºC (about 66%) and a small percentage (about 9%) of larvae were able to successfully begin metamorphosis at 23ºC. In contrast, both *C.cal* monodiet and the starvation regime did not allow the production of competent *S. solida* larvae after 26 days of rearing. This microalgae species is often reported as unable to feed early bivalve larvae due to its peculiar morphology with silica rods that could avoid ingestion during the first days of larvae life (Ferreiro et al., 1990; Matias, 2012). Metamorphosed larvae were observed in unfed *C. gallina* larvae at both temperatures of rearing (about 41% at 20ºC and about 10% at 23ºC), indicating that this species uses eggs reserves until metamorphosis. According to Albentosa et al. (1996) and Mayzaud (1976) one of the main effects of starvation in invertebrates is a decrease in metabolism down to

maintenance levels. Under such conditions, energy is provided by body reserves and/or the catabolism of tissues.

In summary, the larval rearing of the two studied species was possible in hatchery, with satisfactory results, especially in *C. gallina*, and are comparable with the larval rearing of traditional cultured species such as *Ruditapes decussatus* (Matias et al., 2011; Ruiz-Azcona, 1986) and *Pecten maximus* (Nicolas and Robert, 2001). No major zootechnique adjustments were necessary to obtain these results. The technologies applied are traditionally used and the culture of microalgae species used as food is already optimized in bivalve hatcheries. However, after metamorphose, the *S. solida* postlarvae survival was quite low and just a few specimens were obtained, thereby limiting seed production. The reason of this drastic mortality was due to the appearance of vorticella and accumulated microalgae and detritus at the edge of the shell that blocked the opening of the valves limiting the feeding and the movement of the larvae. Further research is essential to improve this production phase. The increase of the hydrodynamics inside the rearing tank or the use of proper substrate material may help to keep clean the valves and reduce mortality at postlarvae stage.

The rearing of *C. gallina* juveniles, without substrate was one hundred percent successful regardless the diet provided. The need to be buried hinders or even prevents the nursery rearing of some species of bivalve (da Costa and Martínez-Patiño, 2009). There was no mortality during the experiments and juveniles measured initially 3.82±0.17 mm in shell length and 15.49±1.55 mg in weight grew about 5.4 mm and 241 mg, in 65 days. *T-iso* has been reported as one of the best monospecies diet for larvae of many bivalve species (Helm and Laing, 1987; Southgate et al., 1998; Liu et al., 2009) but not suitable to postlarvae feed (Lora-Vilchis and Doktor, 2001; Okauchi, 1990; Rivero-Rodríguez et al., 2007). On the other hand, the monospecies diets of *Chaetoceros spp*. has been well documented as high food value for bivalve juveniles (Enright et al., 1986; Laing and Millican, 1986; Rivero-Rodríguez et al., 2007; Taylor et al., 1997). Nevertheless, in our study, as in the larval rearing, also for the juvenile phase, the microalgae *T-iso* demonstrated their nutritional suitability for *C. gallina* and growth of juveniles fed with *C. cal* was slightly lower than growth of larvae fed with *T-iso*. The mix diets are also often recommended for better results, however, in this study, growth of *C. gallina* juveniles fed with the binary diet (*T-iso* + *C. cal*) was very similar

to the one obtained with the monodiet *T-iso*. Other authors have also report that spat experienced maximum growth when fed with a single-algal diet comparatively with algal mixtures (Laing and Millican, 1986; Liu et al., 2009; Rivero-Rodríguez et al. 2007).

In conclusion, the two studied species *C. gallina* and *S. solida* have potential to be produced artificially with the techniques traditionally used for other bivalves and fed with microalgae species whose production is common in bivalve hatcheries. There were no constrains that prevented their production; however the results showed that *C. gallina* can be more attractive for aquaculture than *S. solida*. The possibility to obtain gametes of *S. solida* both by thermal shock, as by stripping is an advantage for the beginning of the process since both techniques allow the incubation control; however the low fecundity of the species can be an obstacle. The long larval period can improve the appearance of problems during this phase, increasing larval mortality and thus, make the rearing process less successful. Nevertheless, the high percentage of metamorphosed larvae achieved can be a great advantage if it is considered the restock of natural populations with larvae ready to settle. This restock technique was already efficaciously used with other species (Arnold et al., 2002; Preece et al., 1997). In this study, the production of *S. solida* juveniles was not viable and therefore further studies are needed to improve the zootechniques to enable this rearing phase. On the other hand, although the succeeded method to obtained *C. gallina* embryos did not allow the full control of spawning, the short larval period of the species is undoubtedly a great advantage in aquaculture. This fact reduces significantly the appearance of problems during the rearing, increasing larval success, making the process cheaper. Other advantage suggested by the results obtained in this study is the great contribution of yolk reserves of *C. gallina* during the larval period. The low mortality in the first days of larvae life and the high percentage of metamorphosed larvae found even in the unfed treatment are advantages for aquaculture production and for restocking programs. Indeed, it allows releasing early larvae or larvae ready to settle in periods where food availability is poor, because larvae may rely on other sources of energy for the maintenance of their metabolism. The high larval growth rate obtained with ordinary produced microalgae (*T-iso*) in bivalve hatcheries also benefits the production of this species. Finally, the possibility to produce juveniles in hatchery in an inexpensive and easy way makes *C.*

gallina also an interesting candidate to alternative or complementary products for the shellfish aquaculture industry. These juveniles may also be used in restocking programs by seeding natural beds.

Although more studies are necessary to improve production methodologies and thus maximize the success of larval and post-larval culture, the results achieved in this study can effectively contribute to the implementation of these species aquaculture, especially of *C. gallina*. This production can have a very favourable impact in terms of populations' preservation in areas where they support important fisheries, such as the Iberian Peninsula and the Mediterranean Sea.

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Chapter 5.2

The effect of density in larval rearing of the pullet carpet shell *Venerupis corrugata* (Gmelin, 1791) in a recirculating aquaculture system

Joaquim, S., Matias, D., Matias, A.M., Soares, F., Cabral, M., Chícharo, L., Gaspar, M.B. The effect of density in larval rearing of the pullet carpet shell *Venerupis corrugata* (Gmelin, 1791) in a recirculating aquaculture system. (*Submitted to Aquaculture Research*)

Abstract

The pullet carpet shell *Venerupis corrugata* is commercially exploited in several European countries. This species is an economic valuable fisheries target species, however, nowadays stocks are under strong fisheries pressure.. Lately, the production of juveniles at hatcheries is becamming an important contribution to the sustainability of the natural stocks. This production is based on the existing traditional technology (Batch system) for bivalve species. This metadology has limitations related to some technological aspects, as requires much labour and becomes very expensive. The main goal of this study was determine the feasibility of rearing *V. corrugata* larvae with a new methodology. Thus we compared the recirculating aquaculture system (RAS) with the traditional larval rearing system (Batch). Larvae were reared at three different initial densities (10, 40 and 200 larvae per mL) in the RAS and compared with the rearing in the Batch system at the traditional density of larval culture (10 larvae per mL). Physical, chemical and microbiologic parameters were monitored and larvae were sampled for survival, growth and metamorphosis rate determination. The mean survival rate of *V. corrugata* larvae at the end of the larval culture in RAS was 28.44 ± 16.15 %, higher than in the Batch system (17.35 ± 3.18 %); however significant differences were found among replicates of the same treatment in the RAS system. Furthermore, the RAS system achieved higher larval length and growth rates (RAS 200 = 224.53 ± 26.27 µm, K=11.34 µm day^{-1}; RAS 40 = 278.89 ± 29.00 µm, K=16.31 µm day^{-1}; RAS 10 = 278.95 ± 33.30 µm, K=16.31 µm day^{-1}) than the Batch system (189.47 ± 24.56 µm; K=8.18 µm day^{-1}). The larval growth was not affected by the initial density until 40 larvae per mL, however, 200 larvae per mL decreased the larval growth in length nearly 54 µm. The larval rearing time until the settling stage was shortened in 2 days (14 days), compared to the Batch (16 days) system and as for growth, also the metamorphic rate best results were found in RAS (especially in RAS 40 and RAS 10). The physical, chemical and microbiologic parameters suggested that the capacity of the RAS biofilter was not a limiting factor to the larval rearing and the tested densities were not excessive to disturb its stability. The *V. corrugata* larval rearing in RAS system constitute a significant reduction in the operating costs and can contribute positively to the the implementation of restocking programs based on hatchery produced settle larvae and also benefit the production of seed for aquaculture in commercial parks.

5.2.1 Introduction

The pullet carpet shell *Venerupis corrugata* (=*V. pullastra* and *V. senegalensis*) is an Atlantic–Mediterranean warm-temperate species that inhabits sandy to muddy bottoms, usually from the low tide mark to a depth of 40 m (Macedo et al. 1999). This species is commercially exploited in Portugal, Spain, France, and Italy (Joaquim et al. 2010). In Portugal, *V. corrugata* populations declined during the last decades due to overfishing and recruitment failure, even in areas where this species was once abundant, such as in Ria de Aveiro and Ria Formosa lagoons. Indeed, Ria Formosa population density has decreased below a threshold level, such that natural recovery does not appear to be possible (Joaquim et al., 2011). Despite the high market demand and high commercial value of this species, this decline has led to a lack of interest presented nowadays by local fishermen in harvesting it.

Thus, it appeared crucial that an active intervention may be necessary to restore stocks to reproductive viability in both Ria de Aveiro and, *a fortiori*, Ria Formosa. In order to reverse the decline situation of pullet carpet shell populations, a project which the main objective is to allow the enhancement/restoring of natural stocks based on hatchery production of larvae and/or juveniles, has been implemented.

The restock of natural populations with early larvae or larvae ready to settle in protected and overfished areas is a restock technique already efficaciously used with other bivalve species (Preece et al., 1997; Arnold et al., 2002). Some previous studies have been performed in *V. corrugata* larval production (e.g.Pérez Camacho et al., 1977; Fernández Reiriz et al., 2001; Nóvoa et al., 2002; Martínez Patiño et al., 2001; 2007). Cerviño-Otero (2011) reported the optimization of *V. corrugata* production in hatchery based on the existing traditional technology (Batch system) for bivalve species (e.g. Ruiz-Azcona et al. 1986; da Costa & Martínez-Patiño, 2009). However, this traditional culture has limitations related to technological aspects as the larval production has to be performed at low individual density (initial density = 5 - 10 larvae per mL) in a large volume of stagnant seawater that is completely replaced at least 3 times a week. This methodology requires much labour and becomes very expensive. In addition, the manipulation required leads to mechanical stress by sieving and the exposure of larvae to water quality changes in each renewal, increasing the contamination risk (Merino et al., 2009).

This static water method was developed in the 1960s by Loosanoff and Davis (1963) for *Crassostrea virginica* and Walne (1974) for *Ostrea edulis* and has not progressed much since then. Implementation of new methodologies of rearing could directly contribute to a more stable and reliable production of better quality larvae, reducing costs and therefore, making the restocking program economically feasible. The rearing of *V. corrugata* larvae in a recirculating aquaculture system (RAS) could signify technological progress in the hatchery process by reducing the labour (handling of larvae and tank cleaning) and energy costs, improving larval growth and survival. This system consists of a flow-through system linked into a recirculation system where water is treated and re-used. The use of RAS provides a higher quality control of culture, since the water in the system is not subject to fluctuations inherent to the natural water supply (Magnesen and Jacobsen, 2012).

RAS has been used in juvenile bivalve production (*Villosa iris,* Gatenby et al., 1996 and O'Beirn et al., 1998; *Argopecten irradians*, Widman, 1998; *Mercenaria mercenaria*, Pfeiffer and Rusch, 2000; *Hyriopsis myersiana,* Kovitvadhi et al., 2006; 2008; *Argopecten purpuratus,* Soria et al., 2007) with promising results in growth and survival, but at present, only a limited number of studies with this new rearing methodology have been performed on bivalve larvae of marine species (e.g. Merino et al., 2009; Magnesen and Jacobsen, 2012; Robert et al., 2013).

The main goal of this study was to determine the feasibility of rearing *V. corrugata* larvae whit different rearing larval densities in a recirculating aquaculture system (RAS), compared with the traditional larval rearing methodology (Batch).

5.2.2 Material and Methods

Rearing system design and components

The development of *V. corrugata* larvae was compared in two systems: a laboratory-scale closed recirculating system – RAS and the traditional larval rearing system – Batch.The RAS system included the culture and the sump unities (Fig. 5.7). The sump consisted in a 115-L glass reservoir (Length×Width×Height = 50×55×42 cm) that comprises four sections: the water inlet section (50×11×42 cm); the decanted cabinet (50×8×32 cm); the biofilter section (50×25×26 cm) and the resting cabinet (50×11×42

cm). Water from the culture unity was collected in the inlet section of the sump and flowed upwards into the decanting cabinet. Thereafter, water flowed from the top to the biofilter section which was composed of three layers: a bio balls layer, a filter wool (synthetic fibre saltwater proof) and a sponge layer. The filtered water passed to the resting cabinet whence was pumped to the culture unity at 1000 ml per minute after going through a U.V. tube.

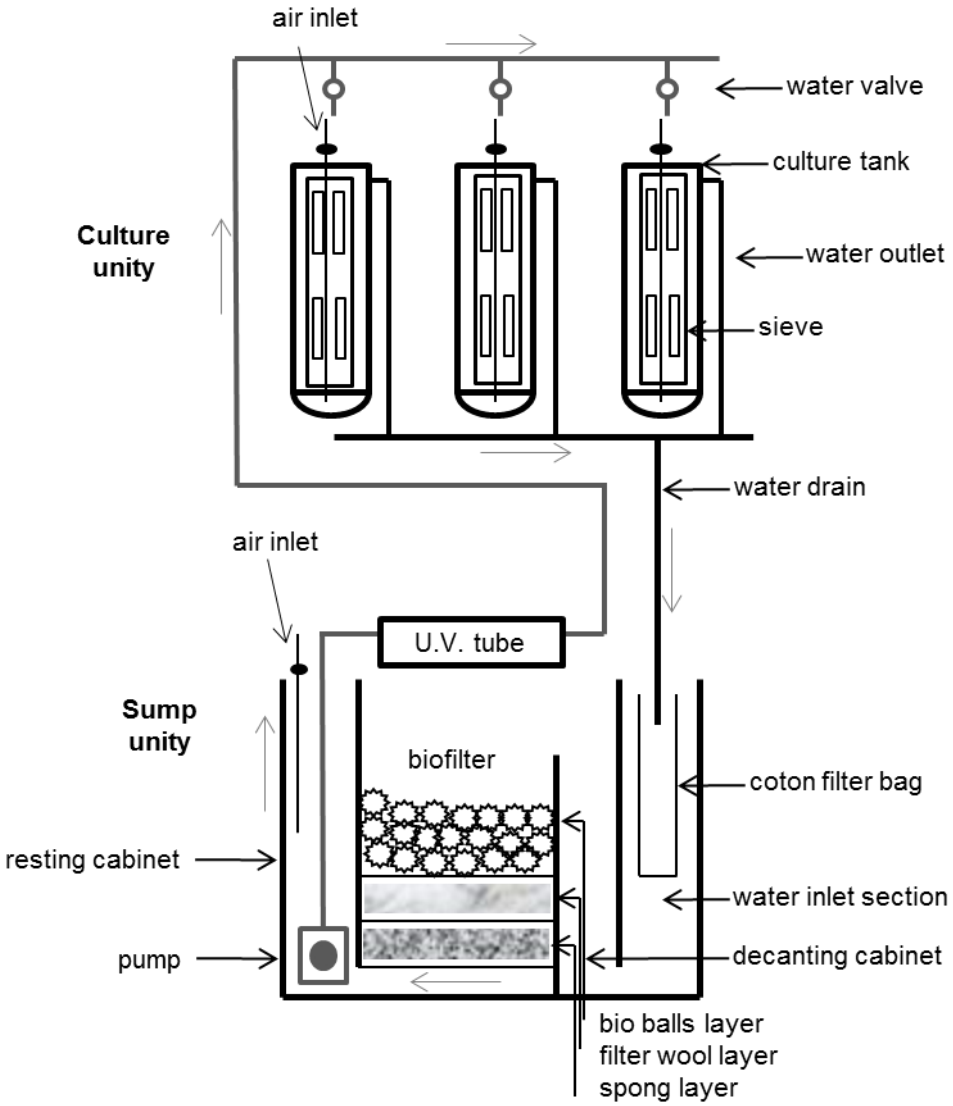

Figure 5.7 Schematic diagram of the recirculating systems used for *V. corrugata* larvae culture.

The culture unity comprised three acrylic cylindrical-concave tanks of 9L (height=42.5 cm and diameter=16.5 cm) for larval rearing. Each tank was aerated (0.5 L min^{-1}) and had a sieve along the cylindrical part of the tank. Although this sieve limits the volume available for larvae in 5 L, it offers a total surface area of 322 cm^2 of nylon mesh screen

that allows the flowing of the water, thus reducing the suction pressure across its surface and preventing larvae from being stuck against the sieve or discharged with the water flow. The water pumped from the sump unity entered inside each of the three rearing tanks at 300 mL per minute and was returned by gravity after filtered by a cotton filter bag of 1 µm to the water inlet section. This flow allowed a complete water exchange in each tank every half hour. To mature the biological filter, 1 µm filtered and U.V.-sterilized seawater at 33±1 of salinity was added to the RAS system eight weeks before the beginning of the experiment. The colonization process and the maintenance of the biological balance of the filter were guaranteed by the addition of nitrifying and heterotrophic bacteria and microalgae to the system. Three RAS systems were used to perform the experiment. The Batch system was composed by three independent cylindrical-conical tanks of 10L of capacity with continuous aeration (0.5 L min^{-1}).

Broodstock collection, spawning induction and eggs incubation

Adult specimens of *V. corrugata*, (shell length >35 mm), were collected in Ria de Aveiro (40º42′N; 08º40′W, northwestern coast of Portugal) at water depths ranging from 4 to 8 m. The broodstock was collected during the natural spawning period of the species, which began in winter, intensified during spring and continued until August.

Clams were induced to spawn by thermal stimulation, through a rapid increase of seawater temperature from 15 to 23±1°C, in alternate cycles of one hour. To avoid uncontrolled fertilization, females once identified were stored in individual containers for spawning. Oocytes were fertilized by addition of a mixture of sperm from various males. The eggs were incubated in 220 L tanks, with 1 µm filtered and U.V.-irradiated seawater, maintained at 20±1°C, at a density of 50 eggs per milliliter. After 48 h, the D-larvae were collected.

Larval rearing

To compare the efficiency of RAS with Batch system, three days old larvae after fertilization were reared at the traditional density of larval culture (10 larvae per mL) in the Batch and the RAS (RAS 10) systems. In addition, higher densities (40 (RAS 40) and 200 (RAS 200) larvae per mL) in RAS were tested. During the experimental period, the daily maintenance of RAS consisted in changing only 10% of the seawater to offset

the increase in salinity caused by evaporation and wash the sieves inside the rearing tank and the filter bag, to avoid clogging and overflowing. Sieves inside each tanks of the RAS system were changed whenever larval width would allow a larger mesh size. The Batch system had the usual maintenance in bivalve hatcheries: tanks were drained, cleaned and seawater was changed three times a week.

Larvae were fed with microalgae *Isochrysis* aff. *galbana* (*T-iso*) and *Chaetoceros calcitrans* (*C. cal*) over the experimental period. Microalgae were cultured according to standard batch methodology (Guillard, 1975) and harvested when the culture reached the end of the exponential growth phase. Food was provided using a peristaltic pump multichannel to the RAS and manually, once per day, in the Batch system. To supply a permanent availability of phytoplankton concentration around the larvae throughout the different feeding periods, larvae were fed daily with *T-iso* and *C.cal* in a ration cell number of 75 cells μL^{-1} of *T-iso* until day five after fertilization, 75 cells μL^{-1} of *T-iso* plus 25 cells μL^{-1} of *C. cal* between day five and day 10 after fertilization and 50 cells μL^{-1} of *T-iso* plus 50 cells μL^{-1} of *C. cal* until the end of the experiment.

Larval samples were collected on three occasions: on the first day of culture (3 days after fertilization), after 6 days (day 9 after fertilization) and at the end of the experimental period (day 14 after fertilization), for all larval rearing experiments (RAS and Batch). Larvae from each tank were counted to estimate survival as a percentage (number of observed live larvae/initial number of larvae x 100). The antero-posterior shell length was measured for 50 randomly sampled larvae from each replicate using images recorded with a microscope connected Nikon DSFi 1 camera, that were subsequently analysed using a freely available image analysis software Image J 1.38s. The presence of a foot was scored to determine larval development status. Larvae that showed a clearly visible foot bulging out of shell (pediveligers) were considered to be competent to continue to the post-larvae production.

Seawater physical and chemical analyses

Temperature, pH and dissolved oxygen (DO) were daily monitored during the experiment using GHL profilux electrodes and the datalogger function of the controller. Seawater in the larval rearing systems was sampled for chemical (nitrites, NO_2^-;

nitrates, NO_3^- and ammonium NH_4^+) analyses at the same time of larval sampling. These nutrients were analysed on a Spectroquant Nova 60A photometer, with a variation coefficient of ±1.0%.

Microbiological analyses

Sampling for microbiological (total bacteria and *Vibrionaceas*) parameters, monitored in the inflow and the outflow seawater of the rearing tanks, were carried out simultaneous with sampling for chemical analyses. *Vibrionaceas* were accounted at days 7th and 12th of culture. After seawater samples homogenization, ten-fold serial dilutions in sterile seawater (SSW) were done with a total volume adjustment of 10 ml. Plates were prepared in triplicate with Tryptic Soy Agar (TSA, OXOID®) supplemented with 1.5% NaCl and thiosulfate citrate bile salts sucrose agar (TCBS, OXOID®), and colonized by spreading 0.1 ml of each dilution. These plates were incubated at 23±1 ^{o}C and the total number of bacteria was counted after 2 and 7 days of incubation. The results were expressed as logarithm of colony forming units (CFU) per ml of seawater.

Statistical analysis

Linear regressions were fitted to shell length over larval and juveniles growth trajectories to determine shell length growth equation for each treatment. Growth rate of *V. corrugata* larvae was calculated using the formula:

$$k = \frac{L_f - L_i}{\Delta t}$$

Where K is larvae growth rate; L_f and L_i are the mean shell length at respectively the beginning and the end of the experiment; Δt is the time interval in days between sampling i and f.

The variation in the studied parameters was analyzed by analysis of variance (ANOVA). Whenever the assumptions of analysis of variance were not met, the Kruskal–Wallis ANOVA on ranks test was performed. Prior to any analyses, percentage data were arcsine transformed to normalize variance (Sokal & Rohlf, 1981). Multiple pair comparisons among means were performed using the post-hoc parametric Tukey test or the non-parametric Dunn's test in order to detect significant differences. Results

were considered significant at $P<0.05$. The Pearson or Spearman (if the residuals are not normally distributed) correlation coefficient was used to determine the degree of association between parameters. The statistical analyses were undertaken using the SIGMASTAT 3.5 statistical package.

5.2.3 Results

Larval development

The larval rearing time was different in the two studied systems. The larval culture lasted 14 days in RAS and 16 days in Batch system. The mean survival of *V. corrugata* larvae at day 14 ranged from 17.50±6.36 % in RAS 10 to 35.88±13.79 % in RAS 40 (Table 5.4). However, significant differences were found among replicates of the same treatment in the RAS system, as showed by the survival standard deviation values. These differences were substantially higher in RAS 200 and in RAS 40, and were mainly due to problems of system malfunction, namely the clogging of sieves and consequent overflowing. Nevertheless, the survival of larvae in Batch system at the end of the larval culture (17.35±3.18%) was not higher than the obtained in the RAS. Indeed, in RAS with 200 larvae per mL of initial density a survival of 50.65% was reached in one of the replicates.

Table 5.4 Survival and metamorphic rates of larvae reared in RAS with different larval stock densities (200, 40 and 10 larvae per mL) and Batch (10 larvae per mL) systems, along the experimental period.

Rearing system	Density (larvae per mL)	Days after fertilization	Survival (%)	Metamorphic rate (%)
RAS	200	9	46.65 ± 26.66	0
		14	31.95 ± 26.44	77.19 ± 4.24
	40	9	59.83 ± 5.83	0
		14	35.87 ± 13.79	96.80 ± 0.28
	10	9	40.00 ± 8.45	0
		14	17.5 ± 6.36	90.53 ± 10.18
Batch	10	9	71.00 ± 15.56	0
		14	27.45 ± 5.73	0
		16	17.35 ± 3.18	66.6 ± 3.25

The growth in shell length and respective linear growth equations of *V. corrugata* larvae rearing in the RAS and the Batch systems are presented in Figure 5.8. Significant differences were found between the two larval rearing systems (Anova, F=137.94, $d.f.$=3, P<0.001; Tukey: P<0.05). After 11 days of culture, larvae reared in RAS system showed substantially higher growth in length and growth rates (RAS 200=224.53±26.27 µm, K=11.34 µm day^{-1}; RAS 40=278.89±29.00 µm, K=16.31 µm day^{-1}; RAS 10=278.95±33.30 µm, K=16.31 µm day^{-1}) than larvae reared in Batch system (189.47±24.56 µm; K=8.18 µm day^{-1}).

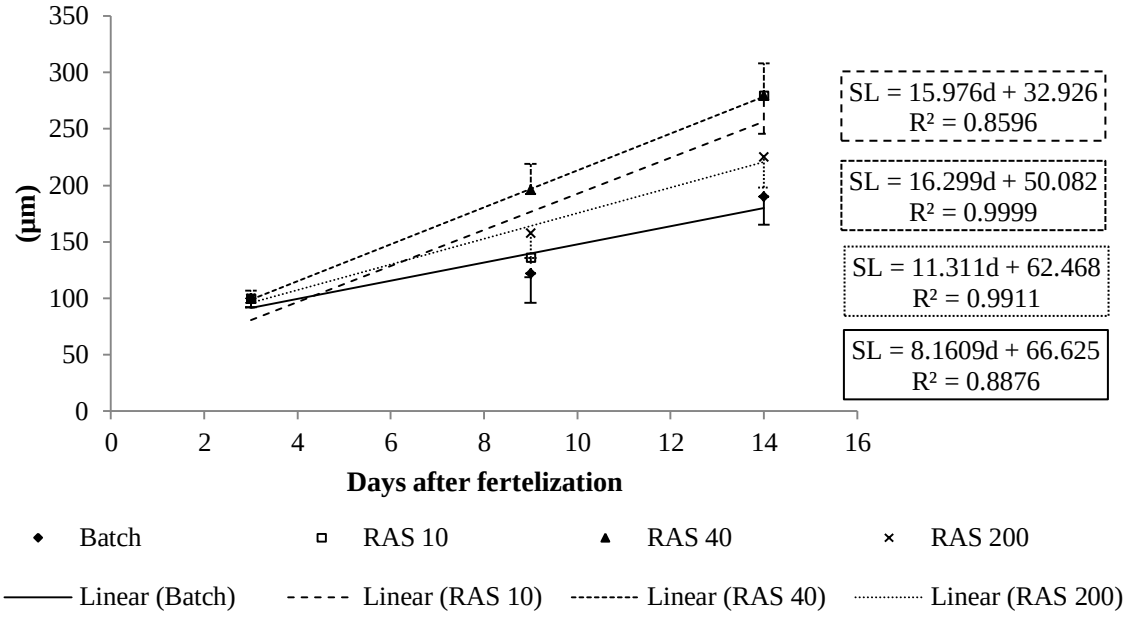

Figure 5.8 Length larval growth (µm) and respective linear growth equations of larvae reared in RAS with different larval stock densities (200, 40 and 10 larvae per mL) and Batch (10 larvae per mL) systems, along the experimental period.

Generally, the larval growth in RAS depended on larval stocking densities (K.W., H=109.67, $d.f$=3, P<0.001). Indeed, densities of 200 larvae per mL decreased the larval growth in length about 54 µm relatively to the other densities; however densities of 40 larvae per mL did not negatively affect the growth of larvae since it was similar to that of larval growth in RAS 10 (Anova, P>0.05). The slopes of the growth equation also clearly showed this fact (11.31 µm – RAS 200; 16.30 µm – RAS 40; 15.98 µm – RAS 10; Batch – 8.16 µm). At the third larval sampling day (day 14 after fertilization) the foot was detected in a large percentage in all treatments of RAS, contrasting with the Batch system where pediveliger (66.6±3.25) were only found at the 16th day after

fertilization (Table 5.4). Similarly to growth, the metamorphic rate best results were also found in RAS 40 (96,80±0,28%) and RAS 10 (90,53±10,18%).

Physical characterization of seawater

There was a statistically significant difference between seawater temperature in RAS and Batch systems (K.W., H=22.16; df=3, P<0.001; Dunn's, P<0.05). The seawater temperature remained between 19.1ºC and 21.7ºC in Batch system and was in mean 1.88±0.27ºC lower than in RAS (Table 5.5). In RAS, seawater temperature ranged between 20.9ºC in the first day and 23.6±0.1ºC at the end of the culture and there were no significant differences in temperature among densities treatments (K.W., P>0.05). As for seawater temperature, DO of Batch was also statistically different from the RAS system (K.W, H=27.03 df=3, P<0.001; Dunn's, P<0.05) and there were no differences in DO among densities in RAS (K.W, P>0.05). The DO in Batch system increased in the first three days of culture reaching values of 100% and remained unchanged during almost all the experimental period (Table 5.5). In the RAS system, DO remained stable between 72 and 78% during all larval rearing period, except in the 5th day when a rise occurred in all treatments (RAS 200=84.6%; RAS 40=88.2%; RAS 10=95%). The pH remained stable between 8.22 and 8.27 in Batch system (Table 5.5). In RAS, values of pH increased from 7.79±0 to 8.16±0.06 until day 4 in all treatments and remained stable for the rest of the experimental period in RAS 40 and RAS 10 treatment. In RAS 200 there was a decrease in pH from 8.11 to 7.79 between the day 3 and day 7 of the culture. This reduction in pH was corrected by the use of extra air diffuser stones in the sump unity. Also for the pH values there was a significant difference between Batch and RAS (K.W, H=28.76 df=3, P<0.001; Dunn's, P<0.05) and regardless of the decrease of pH in RAS 200 recorded between the day 3 and day 7, no significant differences were found among treatments in this system (K.W, P>0.05). Considering the physical parameters of seawater of all treatments, positive relationships were found between temperature and pH in RAS 40 (Pearson, r=0.62, P<0.05) and RAS 200 (Spearman's, r=0.77, P<0.05).

Table 5.5 Physical (temperature, dissolved oxygen and pH) parameters in RAS with different initial larval stock densities (200, 40 and 10 larvae per mL) and Batch (10 larvae per mL) systems, along the experimental period.

		Temperature (ºC)				Dissolved Oxygen (%)				pH			
		RAS			Batch	RAS			Batch	RAS			Batch
Initial larval rearing (larvae mL^{-1})		200	40	10	10	200	40	10	10	200	40	10	10
Days of culture	1	20.9	20.9	20.9	20.1	73.2	73.2	73.2	93.8	7.79	7.79	7.79	8.26
	2	22.0	21.9	22.2	19.9	74.5	73.5	73.9	95.3	8.08	8.10	8.09	8.27
	3	22.1	22.3	22.0	19.5	74.4	74.9	74.3	99.3	8.09	8.08	8.08	8.22
	4	21.6	21.8	21.8	19.6	74.6	71.8	72.3	103.9	8.11	8.16	8.22	8.27
	5	21.0	21.9	21.9	19.8	84.6	88.2	95.0	101.2	7.95	8.13	8.20	8.26
	6	20.5	21.5	21.4	19.1	71.7	72.7	72.6	100.7	7.95	8.20	8.23	8.27
	7	20.7	21.6	21.8	19.7	71.8	--	72.4	104.5	7.79	8.18	8.21	8.25
	8	21.6	21.7	21.8	19.7	73.8	76.1	74.2	101.0	8.18	8.15	8.20	8.27
	9	22.3	22.4	22.3	20.5	73.7	77.1	72.3	93.4	8.22	8.23	8.21	8.27
	10	22.6	22.6	22.7	20.9	74.5	78.3	71.4	92.3	8.18	8.21	8.21	8.25
	11	23.1	23.0	23.2	21.2	72.5	74.0	71.0	102.8	8.18	8.22	8.22	8.25
	12	23.6	23.5	23.7	21.7	71.4	71.4	71.8	102.9	8.16	8.21	8.18	8.26

Chemical characterization of seawater

The ammonia (NH_4^+) concentration remained below 0.1 mg L^{-1} in all treatments throughout the experimental period, except at the end of the culture in the higher larval stoking density (0.19 mg L^{-1}) (Table 5.6). In this treatment, the NH_4^+ concentrations followed the same trend as temperature and pH. The nitrites (NO_2^-) concentration increased throughout the experiment, except in the Batch system, however, never exceeded 0.059 mg L^{-1} (in RAS 40, at the end of the experiment). The trend of NO_2^- concentration was inverse of DO trend in all density treatments reared in RAS. The content of nitrates (NO_3^-) was similar in RAS 40 and RAS 10 and inverse to NH_4^+ trends. The higher concentration (0.88 mg L^{-1}) was recorded in the Batch system at day 7 of culture.

Table 5.6 Chemical (NO_3^- , NO_2^- , NH_4^+) and microbiological (total bacteria and *Vibrionaceas*) parameters in RAS with different initial larval stock densities (200, 40 and 10 larvae per mL) and Batch (10 larvae per mL) systems, along the experimental period.

			Chemical parameters			Microbiological parameters			
						Total bacteria		*Vibrionaceas*	
		Initial larval rearing (larvae mL^{-1})	NH_4^+	NO_2^-	NO_3^-	Inflow	Outflow	Inflow	Outflow
			(mg L^{-1})			(log CFU mL^{-1})			
Days of culture	1	RAS 200	0.1	0.01	0.36	3.6	3.6		
		RAS 40	0.1	0.01	0.35	3.6	3.6		
		RAS 10	0.1	0.01	0.36	3.6	3.6		
		Batch	0.1	0.01	0.35	3.6			
	7	RAS 200	0.1	0.03	0.54	5.0	6.4	2.5	3.2
		RAS 40	0.06	0.03	0.54	4.9	5.7	1.9	1.9
		RAS 10	0.01	0.02	0.54	4.6	6.2	0.0	1.4
		Batch	0.1	0.03	0.88	6.5		2.8	
	12	RAS 200	0.19	0.04	0.63	3.4	4.9	0.3	2.6
		RAS 40	0.08	0.06	0.45	2.3	5.4	0.0	2.6
		RAS 10	0.08	0.03	0.43	1.5	4.1	0.0	1.9
		Batch	0.07	0.04	0.39	7.4		2.4	

Microbiological survey of seawater

The total number of bacteria was higher in the outflow than in the inflow of the RAS rearing tanks (Table 5.6). The highest number of total bacteria was found in Batch system at the 12th day of culture (7.4 log CFU mL^{-1}) and the lowest, actually even lower than the initial number (3.56 log CFU mL^{-1}), in the RAS 10 inflow (1.5 log CFU mL^{-1}), at the same day. After 7 days of culture, the total of bacteria grown exponentially in all treatments either in inflow or outflow of rearing tanks of the RAS and in the Batch system, reaching values ranging from 4.6 to 6.5 log CFU mL^{-1}. However, at the end of the culture, the bacterial concentration decreased when compared to these values in RAS10 (1.5 log CFU mL^{-1}), RAS40 (2.3 log CFU mL^{-1}), and RAS 200 (3.4 log CFU mL^{-1}) inflow and in RAS10 (4.1 log CFU mL^{-1}), and RAS 200 (4.9 log CFU mL^{-1}) outflow, the only exception being RAS 40 (5.4 log CFU mL^{-1}) outflow. During the experimental period, the total of bacteria increased in Batch system. No relationships were found between initial larval stoking densities and the total bacteria in RAS system. No *Vibrionaceas* were found in the inflow or the outflow of RAS 10 rearing tanks, but in the other rearing densities of the RAS system, they were presented at the 7th day of culture (outflow - RAS 40 – 1.9 log CFU mL^{-1}; RAS 200 1.5 3.2 log CFU mL^{-1}), even

in the inflow after the sump treatment (RAS 40 – 1.9 log CFU mL^{-1}; RAS 200 – 2.5 log CFU mL^{-1}). However, at the end of the culture, the values of *Vibrionaceas* in the inflow were close to zero. The values of *Vibrionaceas* remained constant in the Batch system throughout the experimental period (2.8 and 2.4 log CFU mL^{-1}, at the 7^{th} and the 12^{th} days of culture, respectively).

5.2.4 Discussion

The production of larvae in artificial conditions is required to promote restocking programs and therefore to enhance the natural stock of the pullet carpetshell *V. corrugata* in the Ria formosa area. So far, the traditional Batch tecnique has been used to produce bivalve larvae. However, this technique also involves higher costs and may be discouraging to establish a restocking program. We evaluated and compared the use of RAS technology for the production of the pullet carpetshell larvae.

The results showed that the *V. corrugata* larvae could be reared in a recirculating aquaculture system with better performances than in Batch system. The mean survival rate of larvae at the end of the larval culture in RAS was 28.44 ± 16.15 %, higher than in the Batch system (17.35 ± 3.18 %). Indeed, in RAS 200 a survival of 50.65 % has been reached in one of the replicates. Previously, Merino et al. (2009), when studied the RAS larval culture system for *Argopecten purpuratus*, found, however, lower survival in RAS compared to a closed system. Cerviño-Otero (2011) found higher mean percentage of survival rates (56%) for *V. corrugata* larval rearing in Batch the system. However, this author found substantial differences between assays (87 to 19%) depending on the season and broodstock origin. In our study, the differences found among replicates of the same treatment in RAS, did not allow to find a statistical significant relationship between the survival and the tested stock densities of culture. These differences were substantially higher in larvae rearing at an initial density of 200 and 40 larvae per mL and were mainly due to the system malfunction, namely the clogging of sieves and consequent overflowing. In the first days of larval culture it was necessary to use rearing tank sieves with a 40 µm mesh screen that were clogged by clusters of waste and shells from dead larvae. This problem was solved by minimizing the time interval between sieves washing. Merino et al. (2009) and Magnesen and

Jacobsen (2012) also found large differences in survival between the larval tanks of a RAS system for scallop culture.

Larval survival is affected by several factors, being the seawater quality one of the main ones (Magnesen and Jacobsen, 2012). Every year massive larval losses with serious economic consequences are reported by commercial hatcheries due to seasonal variations in water quality that is pumped into the hatchery facilities. One of the advantages of the RAS system is the water stability regulated by water treatment processes. In an aquaculture system, the DO level in the water is one of the most important parameters of culture equilibrium (Timmons et al., 2002; Pillay and Kutty, 2005). In this study, the DO and the pH in Batch were always higher than in the RAS system. The accumulation of carbon dioxide, as a result of larvae and bacteria respiration process, may have contributed to the reduction of these parameters in the RAS system, specially the pH of RAS with an initial density of 200 larvae per mL. According to Timmons and Ebeling (2007) a decrease in pH can be restrictive for production when the larval biomass increases and the water exchange are feeble. Barros et al. (2013) described high sensitivity of *C. gigas* veliger larvae to values of 7.76 of pH, reflected by a decrease in survival and growth rates, as well as an increased frequency of prodissoconch abnormalities and protruding mantle. Moreover, the authors also found that mortality increased further when larvae are exposed to lower values of pH (7.37). On the other hand, Magnesen and Wahl (1993) defended that the reduction of pH to 7.8 is within the limits of tolerance for marine organisms. In our study, it was not possible to relate the reduction in DO and pH to larval survival, since the survival was not significantly different in the two tested systems. Although the pH decreased in RAS 200, between day 3 and day 7 of culture no significant differences were found between the different larval stocking densities tested in the RAS system. This decrease was solved by the use of extra stone diffusers. Merino et al. (2009) also used extra air diffusion in order to maintain DO level above 80%.

From the larvae metabolism results the discharge of various organic and inorganic compounds to the water of culture, such as ammonium, phosphorus, dissolved organic carbon and organic matter (Piedrahita, 2003; Sugiura et al., 2006). The high levels of nutrients cause quality deterioration of the receiving water bodies and may increase the occurrence of pathogenic microorganisms (Thompson et al., 2002). Ammonium is one

of the end products of protein metabolism and of bacterial activity (Walsh and Wright, 1995), and could be toxic for larvae, affecting survival, growth, and physiological parameters such as oxygen consumption (Crab et al., 2007). According to Colt and Armstrong (1981) and Lawson (1995), concentrations higher than 1 mg NH_4^+ per liter are toxic to most fishes, crustaceans and mollusks. In our study, the ammonia concentration remained below 0.1 mg L^{-1} in all treatments throughout the experimental period, except at the end of the culture in the higher larval stoking density reared in RAS (0.19 mg L^{-1}). Although the NO_2^- concentration increased during the experiment, except in the Batch system, it never exceeded 0.059 mg L^{-1} (in RAS 40, at the end of the experiment). The variation of NO_2^- concentration was inverse to the DO trend in all density treatments in the RAS system, reflecting the ammonia oxidation by the aerobic autotrophic bacteria. Nitrite is also considered toxic for mollusks but in concentrations above 5 mg L^{-1} (Basuyaux and Mathieu, 1999). The concentration of NO_3^- was similar in RAS 40 and RAS 10 and inverse to NH_4^+ trends and never exceeded 1 mg L^{-1}. These results suggest that the capacity of the RAS biofilter was not a limiting factor to the larval rearing and the tested densities were not excessive to disturb its stability. Although the exponential growth of total bacteria in all treatments of RAS at day 7 of culture reached higher numbers than that found by Magnensen and Jacobsen (2012) in a RAS system for *P. maximus* larval production, the low values determined in the last day of culture showed that the equilibrium of the biofilter was recovered. Moreover, the total number of bacteria was higher in outflow than in inflow of RAS rearing tanks and no significant relationships were found with larval stocking densities. Nitrification in the bacterial film of the biofilter is affected by a variety of parameters such as DO concentrations, organic matter, temperature, pH, salinity and turbulence level (Satoh et al., 2000; Chen et al., 2006). In the last days of culture, temperature was inverse to the DO trend; however, the variation of these parameters in RAS the system was not sufficient to disturb the sensitive nitrifying bacteria of biofilter. The highest concentration of nitrates (0.88 mg L^{-1}) and the highest number of total bacteria was recorded in the Batch system. Robert et al. (2013) reported maximum values of 2.1 mg L^{-1} of NO_3^- in a RAS system for *C. gigas* larvae reared at 50 larvae per mL. Nitrate is considered of low toxicity for most species (Epifano and Srna, 1975; Muir et al., 1991)

but bacterial blooms, specially a number of *Vibrio* species have been reported to cause problems in static larval cultures (Torkildsen et al., 2005, Romalde and Barja, 2010). No *Vibrionaceas* were found in RAS 10 rearing tanks, but in the other rearing densities of the RAS system, they were present at the 7th day of culture, even in the inflow after the sump treatment. However, at the end of the culture, the values of *Vibrionaceas* in the inflow were close to zero. Again, these results suggested that a peak of bacteria development was reached at day 7, but the equilibrium of sump unities was recovered after that. The higher number of bacteria and *Vibrionaceas* found in the Batch system could be a primary cause of mortality; however there was no evidence of relationship between the pathogenic microorganisms and mortality, since *Vibrionaceas* was not found in RAS10 and larval survival was not significantly different. Magnesen and Jacobsen (2012) also reported no evidence of this relationship for the *P. maximus* larval rearing in a RAS system.

Temperature is known as one of the more important factors to promote larval growth, but, on the other hand contributes to decrease survival by maximizing bacterial growth (Devakie and Ali, 2000; Robert and Nicholas, 2011). In this study, the seawater temperature in Batch remained between 19.1ºC and 21.7ºC and was 1.88 ± 0.27ºC lower than in the RAS system. Nevertheless, although this significant difference in systems temperature, no relationship with larval survival rate, was found. These results suggested/suggest that the harmful effect of higher temperature was minimized by the specific characteristics of the RAS system, namely the biofilter that promoted a constant water treatment. Furthermore, RAS larval rearing promoted a faster larval growth than the Batch system. After 11 days of culture, larvae reared in the RAS system showed substantially higher growth in length and growth rates than larvae reared in Batch. The larval growth rate found in Batch was similar to the ones reported by Cerviño-Otero (2011) for *V. corrugata* rearing in the same system, but the larval growth rates of larvae rearing in the RAS system were substantially higher. Moreover, the larval rearing time until the settle stage was lower in the RAS (14 days after fertilization) than in the Batch system (16 days after fertilization) and was also lower than the larval rearing time (19 to 20 days) reported by Cerviño-Otero (2011). At the 14th day of culture, over than 77% of larvae reared in the RAS system had foot. Merino et al. (2009) also found a reduction of about 6 days in RAS larval rearing for *A. purpuratus*. This fact represents a great

advantage to the larval production of the species, since the shortening of larval rearing time decreases the rate of larval mortality. Higher growth rates and decreasing of larval rearing time observed in RAS could be attributed to several factors that distinguish the two systems. The reduction in daily larval manipulation resulting in a significant lower amount of stress, more food availability, higher temperature and the stability of water quality in the RAS system are the more frequently mentioned factors (Magnesesn and Jacobsen, 2012; Merino et al., 2009; Soria et al., 2007; Robert et al., 2013). However, in our study, growth of *V. corrugata* larvae in RAS depended on larval stocking densities. The larval growth was not affected by the initial density until 40 larvae per mL, however, 200 larvae per mL decreased the larval growth in length nearly 54 µm. As for growth, also the metamorphic rate best results were found in RAS 40 and RAS 10 when compared to RAS 200. Several authors (Malouf and Breese 1977, Yan et al., 2006 and Sarkis et al. 2006) have found that an increase in bivalve larval stocking density resulted in an inhibitory effect on growth and competence in their rearing systems. In opposition, Rico-Villa et al. (2008) reported for *C. gigas* that larval stocking densities may undoubtedly be increased to 300 larvae mL^{-1} in a flow-through rearing system with similar growth performances compared to 5 larvae mL^{-1}. Further studies will be necessary to know the optimal larval stoking density of *V. corrugata* larval rearing, taking into account the RAS system capacity.

In summary, despite the great variability in survival among rearing tanks of the same system, we can conclude that the larval rearing of *V. corrugata* could be performed at higher larval stoking densities in a RAS system than in a Batch system, without decrease of survival. Furthermore, the RAS system achieved higher larval growth rates and lower larval rearing time until the settling stage than the Batch system. All these constitute a significant reduction in the operating costs to produce the pullet carpet shell in hatchery. The larval rearing in RAS system can contribute positively to the promotion of the implementation of restocking programs based on hatchery produced settle larvae, namely for injection into seawater column. The advantages of RAS system also benefit the seed production for aquaculture in commercial parks. Notwithstanding, challenges still exist; further work is required to maximize the pullet carpet shell larval production in RAS before the transition of technology to a commercial scale, such as achieve control the stabilization of water quality in a physical, chemical and

microbiological point of view, aiming to increase the survival of larvae and the definition of the optimal larval stoking density in the RAS system.

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Chapter 6

Rebuilding viable spawner patches of the overfished *Spisula solida* (Mollusca: Bivalvia): a preliminary contribution to fishery sustainability

Joaquim, S., Gaspar, M.B., Matias, D., Ben-Hamadou, R., Arnold, W.S., Rebuilding viable spawner patches of the overfished *Spisula solida* (Mollusca: Bivalvia): a preliminary contribution to fishery sustainability. ICES J. Mar. Sci., 65, 1-5 (*Published*).

Spisula solida experiment

Abstract

Populations of commercially important bivalves along the coast of Portugal are depleted as a consequence of natural and anthropogenic causes. A pilot experiment was designed to determine the feasibility of transplanting individuals from natural clam beds to a closed fishing area in an effort to rebuild relatively high-density patches of *Spisula solida*. For this purpose, clams were equally partitioned into two groups (undersize and legal clams) and transplanted at a density of 40 clams m^2 into two areas 50 m^2. Transplanted and control clams were sampled to estimate survival, condition index, biochemical composition, and reproductive condition. Generally, the physiological condition of clams was not affected by the method of transplanting. One year after transplanting, survival was 45%. The increase in local abundance of mature clams should facilitate successful fertilization and increase the residual reproductive value of each clam relative to its pre-transplant value. Transplanting undersize clams may be more advantageous because they are more likely to spawn at least once before harvest. The experiments demonstrate that spawner transplants may strengthen *S. solida* populations and can be used in stock-enhancement programmes which, in conjunction with effective management measures, can contribute to the sustainability of the *S. solida* fishery.

6.1 Introduction

Many bivalve stocks around the world have collapsed as a consequence of a combination of commercial fishing effort, recreational and commercial watercraft activities, recruitment failure, mass mortality, and habitat degradation (Arnold, 2001). These impacts not only affect potential fishery yields, but also may compromise the productive potential of ecosystems. Management efforts to limit stock collapses have been implemented in many bivalve fisheries, but even when harvesting pressure is removed or when habitat loss is reversed, there is no assurance that affected populations will rebound. When population density decreases below a threshold level, recruitment may fail and population recovery may be constrained by depensatory effects, rendering the population effectively sterile (Stoner and Ray-Culp, 2000). As a result, natural recovery of the population may be delayed, and active intervention may be necessary to restore stocks to reproductive viability. According to Bell et al. (2005), such

"restocking" can involve releasing cultured juveniles into the wild to rebuild the spawning-stock biomass of the depleted stocks to a level where the fishery can once again provide regular harvests. On the other hand, when the natural supply of juveniles fails to reach the carrying capacity of the habitat, recruitment may be inadequate to increase the productivity of an operational fishery. This situation can be redressed if stock rebuilding effort is implemented to augment the biomass of spawning adults (Bell et al., 2005; Lorenzen, 2005). Bivalve restocking or enhancement (for definitions, see Bell et al., 2005) programmes have been implemented worldwide. Exemplary approaches include habitat rehabilitation (Luckenbach et al., 1999), stock management programmes with seeding efforts (Arnold et al., 2005), direct release of larvae (Preece et al., 1997; Arnold et al., 2002), and the introduction of cultured juveniles or adults (Peterson et al., 1996; Arnold et al., 2002, Bell et al., 2005).

The white clam (*Spisula solida*) is a characteristic bivalve of the Portuguese coast and constitutes the basis of an important fishery in the coastal waters there. However, in the past decade, *S. solida* populations have become depleted through environmental and anthropogenic factors including overfishing, endangering the sustainability of the fisheries that depend on the stocks. To reverse this negative trend, it is necessary both to adjust fishing effort via modifications to the management regulations and to rebuild depleted populations of *S. solida*. Fishery management measures, including license limitation, minimum mesh size, temporal closures, size limits, and daily catch quotas, have been applied, but no effort has yet been made to rebuild *S. solida* populations. We report here the results of a study designed to assess the feasibility of transplanting individuals of two size classes (<25 mm SL and ≥25 mm SL) into an area closed to harvest. This technique was designed to increase the local abundance of mature clams, so increasing the density of broodstock and enhancing fertilization success and the resultant supply of larvae (Arnold, 2001).

6.2 Material and methods

The effort to rebuild the reproductive viability of *S. solida* populations was initiated in June 2003 in a closed area off Vale do Lobo in southern Portugal bounded by latitudes 8º03′00″W and 8º04′50″W and depths of 0–10 m. This closed area comprised a historically important fishing ground for the target species which has been severely

overfished in recent years. The pretransplant density of *S. solida* in the area was 1 clam m^{-2}. Within this area, two 50 m^2 (10 m×5 m) plots were identified, the corners of each located using DGPS and marked with 80 kg concrete weights, and each further subdivided into 1 m^2 grid squares. A total of 4000 *S. solida* was captured from the adjacent fishing grounds with the assistance of local dredge fishers and subdivided into two shell length groups: legal-sized (LS) clams (29.2±1.52 mm SL) exceeded the 25 mm SL minimum harvest size, but undersized (US) clams (24.95±1.50 mm SL) were less than the LS. Clams were planted into their respective plots by scuba divers at a density of 40 clams m^{-2}.

At 2 weeks and again at 3 months after transplantation, five of the 1 m^2 subplots from each LS- and US-transplanted area were sampled by scuba divers. Subplots were randomly sampled without overlap. Sampling consisted of hand-raking all the clams from each subplot. All harvested clams were counted and a random sample of ten per subplot was collected for analysis. Simultaneously, samples of LS and US clams from the adjacent natural beds were taken for a control comparison. Initially, it was planned to apply the same sampling strategy 1 year after transplantation. However, the corner markers of the experimental areas and the transplanted individuals were displaced during two storms that hit the area after the 3-month sampling had been completed. Therefore, 1 year after transplantation, the whole experimental area was dredged and all surviving clams retrieved and counted. The reproductive stage of the clams in the sample was compared with clams from an adjacent area.

In the laboratory, clams were placed in seawater filtered through 0.45 mm at 20ºC for 24 h to purge their stomachs in preparation for condition index (CI), histological, and biochemical analyses. For each treatment group, the CI was calculated for 20 clams using the ash-free dry weight (AFDW)/dry shell weight ratio (Walne and Mann, 1975). A total of 20 specimens from each treatment group was examined histologically to determine the gametogenic stage of both sexes. Each animal was then assigned to one of six stages of reproductive development following Gaspar and Monteiro (1998): Stage 0, inactive; Stage I, early active; Stage II, late active; Stage III, ripe; Stage IV, partially spawned; Stage V, spent.

Finally, the meat of five clams from each treatment group was frozen and stored at -20ºC for biochemical analysis. For each, protein content was determined using the modified Lowry method (Shakir et al., 1994), lipids were extracted from fresh homogenized material in chloroform/methanol (Folch et al., 1957), total lipid was estimated spectrophometrically after charring with concentrated sulphuric acid (Marsh and Weinstein, 1966), and glycogen content was determined from dried (80ºC for 24 h) homogenate using the anthrone reagent (Viles and Silverman, 1949). Always, the results were the mean of duplicate determinations and are expressed as a percentage of AFDW. The calorific content of protein, lipid, and carbohydrate in tissues was calculated using the factors 17.9 kJ g^{-1} (Beukema and De Bruin, 1979), 33 kJ g^{-1} (Beninger and Lucas, 1984), and 17.2 kJ g^{-1} (Paine, 1971), respectively.

Statistical analyses were performed using a t-test to study the effect of size (US vs. LS) on clam survival during sampling (2 weeks vs. 3 months after transplanting). For each CI, protein, total lipid, and total energy content analyses, a three-factor analysis of variance (ANOVA) was used to investigate differences between size (US vs. LS), treatment (control vs. transplant) and sampling date (2 weeks vs. 3 months after transplanting). Analyses of interactive effects were included in the ANOVA process, and data were initially arcsine-transformed to normalize variance. Whenever the assumptions of ANOVA were breached, a non-parametric Kruskal–Wallis test was performed, and multiple pair comparisons among means were performed using apost hoc Tukey test. The statistical analyses were carried out with the SIGMASTAT 3.11 statistical package.

6.3 Results

Immediately after transplanting, most clams had burrowed into the sediment. Two weeks after transplanting, survival appeared to be greater for US (80%) than for LS clams (65%). After 3 months, survival had decreased to 60% and 52% for US and LS, respectively (Figure 6.1). However, the difference (t-test: $p>0.05$) in survival between US and LS clams 2 weeks and 3 months after transplanting was not significant. One

year later, 45% of the clams we initially transplanted (US and LS combined) were recovered from the plots.

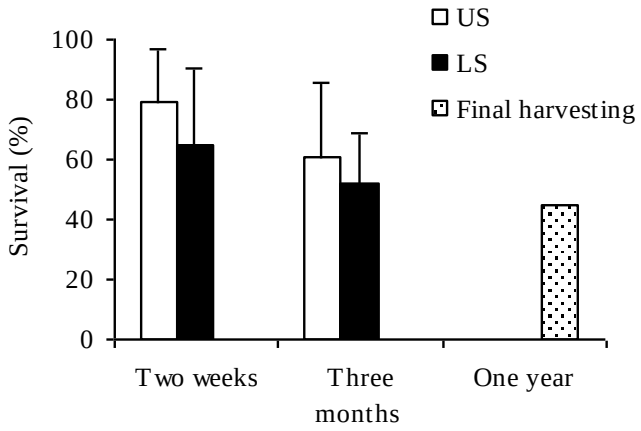

Figure 6.1 Percentage of survival (± SD) of transplanted undersize (US) and legal size (LS) S. solida clams, during the experimental period.

Values of the condition index (CI) varied between 5.33±0.82 and 7.78±0.85 (Table 6.1). The mean CI of all transplanted and control clams decreased 2 weeks after transplanting, but recovered 3 months later. Generally, mean values of the CI were similar among treatments; we detected no significant differences (ANOVA: $p>0.05$) between control and transplanted clams, for either US or LS.

Table 6.1 Mean values (±SD) of ash-free dry weight (mg), proteins, total lipids, glycogen (µg mg^{-1} AFDW) and total energy (kJ g^{-1} AFDW) in transplanted and control undersize (US) and legal size (LS) *S. solida* clams.

	Initial	Two weeks		Three months	
		Transplanted	Control	Transplanted	Control
			LS		
CI	6.3±1.1	5.8±0.6	5.7±1.0	6.4±0.8	7.4±1.4
AFDW (mg)	220.0±60.0	249.9±55.3	260.7±55.3	274.8±70.1	362.1±80.1
Proteins (µg.mg^{-1} AFDW)	460.0±90.2	520.6±44.1	514.3±47.2	430.4±95.7	429.7±66.1
Total lipids (µg.mg^{-1} AFDW)	56.1±17.9	42.6 ±13.6	35.0±14.9	27.5 ±11.0	27.7±5.0
Glycogen (µg.mg^{-1} AFDW)	22.2±6.2	15.0±6.6	61.5±17.8	61.2±26.2	144.6±28.4
Total energy (kJ.g^{-1}AFDW)	10.5	11.0	11.4	9.7	11.1
			US		
CI	6.3±0.9	5.9±1.0	5.3±0.8	6.5±1.0	7.8±0.8
AFDW (mg)	160.2±36.8	151.4±31.2	153.4±26.5	157.2±38.5	233.1±52.9
Proteins (µg.mg^{-1} AFDW)	443.2±52.6	480.0±64.4	516.0±65.6	482.1±72.2	434.5±144.30
Total lipids (µg.mg^{-1} AFDW)	20.4±8.2	31.1±9.6	51.4±25.7	16.7±8.0	47.7±11.2
Glycogen (µg.mg^{-1} AFDW)	143.3±50.1	91.5±34.7	52.0±39.9	147.8±120.8	88.3±12.3
Total energy (kJ.g^{-1}AFDW)	11.1	11.2	11.8	11.7	10.9

At the beginning of the experiment, most clams (~90%) were in an inactive stage (stage 0) and the rest spent. Stage 0 continued to dominate control and transplant clam samples collected after 2 weeks and 3 months, as well as at the end of the study (June 2004). Changes during the experimental period in the biochemical composition of clams (protein, total lipid, and glycogen) and the total energy content for US and LS animals are shown in Table 6.1. Protein was the dominant constituent of clams (430–521 µg mg^{-1} AFDW), followed by glycogen (15–148 µg mg^{-1}AFDW) and total lipid (17–56 µg mg^{-1} AFDW).

Protein and total energy content showed the least variation during the study, and the statistical analysis revealed that, generally, protein and total energy values were similar and that any small differences between control and transplanted individuals either for US and LS clams were not significant (ANOVA: $p>0.05$). However, there were significant differences in glycogen (Kruskal–Wallis, $p <0.001$) and total lipid (ANOVA, $p <0.001$) content between LS and US clams (initial mean values of glycogen 22.2±6.2 and 143.3±50.1 µg mg^{-1} AFDW, respectively). For both the LS and the US clams, mean glycogen content decreased 2 weeks after transplanting then increased up to the 3-month sample. However, there was an exception to this, in the LS control in the 2-week

sample, for which mean glycogen increased relative to that at the start. Transplanted LS clams contained significantly less glycogen than control clams (Kruskal–Wallis, $p<0.001$), and transplanted US clams contained significantly more glycogen than control clams (Kruskal–Wallis, $p<0.05$).

The total lipid content of transplanted and control LS clams decreased during the experiment. For US clams in both control and transplant samples, however, the total lipid content increased during the first 2 weeks, and then decreased up to 3 months after transplanting. Despite the differences in mean total lipid content of the initial sample between LS and US clams (56.1±17.9 and 20.4±8.2 μg mg^{-1} AFDW, respectively), the differences in total lipid content of these two groups were not significant (ANOVA, $p>0.05$) either 2 weeks or 3 months after transplantation. There was no significant difference in the total lipid content of control and transplanted LS clams (ANOVA, $p>0.05$), but transplanted US clams had significantly (ANOVA, $p<0.001$) less total lipid than control ones.

6.4 Discussion

The success of any restoration effort may be site-specific and will likely depend on the same factors that contributed to the population decline originally (Arnold et al., 2005; Bell et al., 2005). A lot of effort has been devoted to attempting to restore depleted bivalve populations, generally without success because the conditions that originally led to the demise of the populations had not been ameliorated. We therefore chose a spawner transplant strategy for our attempt, because the principal cause of *S. solida* depletion in Algarve coastal waters was the synergetic action of overfishing and recruitment failure. The technique was designed to rebuild local high densities of mature clams and therefore to enhance fertilization success and the resulting larval supply (Arnold, 2001). The choice of an area that was an important fishing ground originally, but that had become overfished, ensured that the substratum habitat was appropriate for the work. Moreover, the clams to be transplanted were collected from an adjacent natural population and the experiments were undertaken in a closed area, contributing to the validity and applicability of the results.

Survival was satisfactory when measured against earlier studies: 60% and 52% of US and LS, respectively, remained alive 3 months after transplantation, and 45% of the transplanted clams were still alive 1 year after transplantation. These results suggest that, for the particular situation in which the experiment was carried out, the spawner-density-enhancement technique can contribute to a substantial increase in local abundance of mature clams. In other bivalve-transplantation projects, the mortality associated with the transplant event was greater (Arnold et al., 2002, 2005). Most of the transplanted clams were buried within a few minutes of transplantation, probably contributing to the good rate of survival 2 weeks later. Arnold et al. (2002) reported a mortality of >50% for adult hard clams 2 weeks after transplantation which he attributed to a failure of the transplanted clams to burrow quickly into the sediment. Other authors have shown too that stock enhancement tends to be unsuccessful because there is an inverse relationship between size and mortality, expressed as prey size refuge (Arnold, 1984; Peterson et al., 1995). Size did not affect survival in our study, possibly because the transplant density we selected (40 clams m^{-2}) was similar to the mean density observed in adjacent natural beds. Although we did not test the influence of density on survival, Peterson et al. (1995) and Goodsell et al. (2006) said that a low-density replanting approach provides a more viable method of reducing predation and other density-dependent losses.

The physiological condition of clams was not affected by the method of transplantation. The range in CI values (from 5.33±0.82 to 7.78±0.85) agreed with that of Gaspar and Monteiro (1999) for *S. solida* from the same latitude and period of the year. The Walne and Mann (1975) CI is appropriately applied in reproductive studies because it follows the gametogenic cycle of the species. Several authors have shown that this CI is related to de novo synthesis of lipid during gametogenesis (Costa Muniz et al., 1986; Massapina et al., 1999), so it increases before spawning through gametogenic development, and decreases thereafter. The samples taken in June and September 2003 corresponded to post-spawning *S. solida* undergoing physiological recovery and accumulating reserves to be used for future gametogenesis (Gaspar and Monteiro, 1999). Our histological results confirmed that >90% of both transplanted and control clams were sexually inactive and that this result was independent of clam size.

Therefore, the main features of the reproductive cycle of transplanted clams remained unchanged from those of their area of origin, even 12 months after transplantation.

Many studies of marine invertebrates have shown that the reproductive cycle and environmental conditions are reflected in the biochemical composition (Costa Muniz et al., 1986; Massapina et al., 1999). The total lipid and protein contents of eggs are the most important factors determining larval viability (Massapina et al., 1999), so biochemical composition can be a good diagnostic on which to compare physiological condition of transplanted and control clams. The relative quantities of protein (430–521 µg mg^{-1} AFDW), glycogen (15–148 µg mg^{-1} AFDW), and total lipid (17–56 µg mg^{-1} AFDW) measured in *S. solida* were similar to those of other bivalves (Robert et al., 1993; Marin et al., 2003). The patterns of biochemical constitution and total energy were similar for transplanted and control clams, both LS and US, further supporting our conclusions from the histological analyses that transplantation had little effect on the reproductive status of transplanted clams.

The role of somatic protein as an energy reserve may extend to situations of extreme nutritional stress and energy imbalance (Beninger and Lucas, 1984). Relative to clams that were not transplanted, the protein content of US or LS white clams does not appear to have been affected by transplant stress.

Glycogen was lower in the initial sample of LS clams, compared with US clams, which may be related to recent spawning. According to Brown and Russel-Hunter (1978), glycogen depletion increases with age (or size) as well as with successive breeding periods. The relatively small reserves of glycogen recorded from LS clams may make recovery from transplantation stress more difficult, and that is reflected in the low values of glycogen in transplanted LS clams relative to control clams of similar size. This explanation also lends support in explaining the relatively poor survival of transplanted LS clams.

Glycogen loss is synchronous with lipid accumulation and represents an important metabolic reserve to maintain energy and to support gametogenesis, and lipid loss accompanies spawning (Robert et al., 1993). According to Marin et al. (2003), a simultaneous decrease in total lipid and glycogen energy content suggests different sources of physiological stress related to environmental conditions. During our

experiment, the lipid content of *S. solida* was inversely related to glycogen content for both US and LS clams. Further, although we found the lowest relative values of lipids in transplanted US clams compared with control clams of similar size, our overall results suggest that transplantation stress had no significant effect on the physiology of transplanted clams, because glycogen and lipid content varied inversely. Similar inverse relationships between lipid and glycogen content have been reported for *Ruditapes decussatus* (Beninger and Lucas, 1984; Robert et al., 1993) and *Mytilus edulis* (Gabbott, 1983).

The survival rates we observed show that transplanting either LS or US *S. solida* can be a feasible and successful strategy for enhancing collapsed populations in Portuguese coastal waters and elsewhere. The resultant increase in local abundance of mature clams may lead to increased fertilization success and hence increased residual reproductive value of each clam relative to its pre-transplant value. Similar results have been reported for hard clams (*Mercenaria mercenaria*) and abalone (*Haliotis rubra*), attesting to the general applicability of this approach for rebuilding populations of marine molluscs (Peterson et al., 1995; Goodsell et al., 2006). However, we did note some differences in the apparent physiological health of transplanted US clams compared with transplanted LS animals, supporting the case for smaller *S. solida* to be used for transplantation. Additionally, transplanting US clams may be advantageous because they have the opportunity to spawn at least once before they are harvested, so increasing the likelihood that they will contribute to larval production and potential repopulation of adjacent areas.

Our study was conducted on a small scale for experimentation only, but larger scale experiments will be necessary to realize a significant contribution to future *S. solida* year-class success. The study did, however, allow us to conclude that transplantation can be an effective bivalve stock-enhancement strategy which, in conjunction with management measures that control harvest within reasonable estimates of sustainable yield, can contribute to a *S. solida* fishery that is economically and biologically sustainable in Portuguese coastal waters.

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Chapter 7

Conclusions

Experimental Bivalve Hatchery of IPMA

Chapter 7.1

General Conclusions

The synergistic action of fishing pressure coupled with the rapid growth rate, short lifespan and high variability in recruitment of *S. solida* and *C. gallina* leads to large inter-annual fluctuations in stock abundance and periodic recruitment failure that endanger the sustainability of fisheries (Gaspar, 1996). As a result, in some years the abundance of these species decreases dramatically, threatening the biological and economic sustainability of the fisheries. On the other hand, presently *V. corrugata* populations also declined in Ria de Aveiro (northern Portugal) due to overfishing and recruitment failure (Maia, et al., 2006) and in Ria Formosa lagoon (south coast of Portugal) where populations of this species declined dramatically (Massapina and Arrobas, 1991).

The main objective of the present work was to increase knowledge on the biological processes and hatchery production of *C. gallina*, *S. solida* and *V. corrugata*, to support future restocking and stock enhancement programs for wild stock management as well as to promote the target species as candidates for the shellfish aquaculture industry, encouraging the diversification in this activity. The work performed focused on the introduction of new species in aquaculture, the best founder population and the optimal reproductive time of the target species for artificial spawning induction, as well as, the increase on productivity and on reducing costs in hatchery production. Furthermore, the success of a stock enhancement strategy was tested but only for *S. solida*.

The broodstock for aquaculture production of *C. gallina* and *S. solida* was obtained from wild populations that will be the target of future restocking programs. According to Alarcón et al. (2004) the wild populations represent the primary source of genetic variability for aquaculture stocks, and therefore broodstock should be obtained from the natural population to be restocked or from one genetically close population. The broodstock availability of *V. corrugata* from Ria Formosa population revealed to be insufficient due to the severe overfishing. Thus, Ria de Aveiro population was a viable alternative since it was showed in this study that, on one hand, genetic plasticity of this population is high and on the other hand, both population presented genetic similarity. In addition to the favorable genetic factors, the similarities between Ria Formosa and Ria de Aveiro in terms of habitat (both shallow water mesotidal lagoon with a large intertidal area covered and with similar semidiurnal tidal regimes, salinity, and sediment) can also positively influence the success of a restocking program. This

information on the genetic structure of two Portuguese populations of the pullet carpet shell can also contribute to the appropriate broodstock management, minimizing the deleterious effects in the *V. corrugata* progenies produced in hatchery and enhancing the creation of positive outcomes from restocking.

Furthermore, the information obtained on the reproductive activity of the three target species as well as in its nutrient storage and exploitation strategy provides a valuable insight into their biology, essential for assessing the potential of these species for aquaculture and for the sustainable management of wild stocks. This information provided the optimal reproductive time for artificial spawning induction. The detailed knowledge of the gametogenic and spawning periods and consequent energy storage showed that the three target species have different reproductive strategies. For example, *S. solida* can adopt a flexible strategy in terms of storage and exploitation of energy and modify the reproductive response when abnormal temperature oscillations occur. This reproductive suppleness can have interesting implications for the implementation of profitable aquaculture, since broodstock may be susceptible to manipulation via conditioning. *C. gallina* is an opportunistic species while *V. corrugata* is more conservative; however both species have an extended natural spawning period and high gonadal development rate that, in the wild populations, provide larvae over a large period of the year implying relatively easy manipulation of broodstock without extensive and expensive conditioning. Moreover, the results obtained on both the biochemical composition of oocytes and the larval viability of *V. corrugata* during the spawning season revealed that the producers can invest on "wild" broodstock, in two distinct periods of the year: in February/May, with more chance to obtain a higher number of oocytes, and in late May/July with a weaker response to the spawning stimulation, however with more successful larval hatching. This opportunity has interesting implications for the implementation of profitable aquaculture of the species, since it allows the management of its production with low costs. On the other hand, the great capacity for gonadal regeneration of *V. corrugata*, coupled with its high gonadal development rate also constitutes an added value in terms of broodstock manipulation, since it allows extending the broodstock conditioning in order to achieve multiple qualities spawning in the same year. All the results obtained on reproductive activity showed that there are no reproductive impediments to produce larvae of good quality from the three target species.

Moreover, the knowledge of the reproductive strategies of the target species obtained in this study could also provide important insights for future management actions of the resources. According to several authors, fisheries regulatory measures require information on the biology of exploited species to guarantee the promotion of bivalve species recruitment (Gaspar and Monteiro, 1999; Delgado, et al., 2013; Vasconcelos, 2007). In this study, small differences inherent to environmental factors were found in *S. solida*, *C. gallina* and *V. corrugata* reproductive cycle comparatively with previous studies performed with the same populations (Gaspar and Monteiro, 1999; Gaspar and Monteiro, 1998; Maia et al., 2006). However, these small discrepancies can be the difference between being successful or failing in stocks preservation, if regulatory fisheries measures were not adapted to cover these changes. Furthermore, since the global climatic changes foreseen for the future can give rise to significant alterations in the reproductive cycle pattern of bivalves (García-Domínguez et al., 2011) we suggest that the study of the reproductive cycle should be conducted regularly in order to adjust management regulations if necessary.

After the selection of the best broodstock and of the optimal reproductive time of the three target species, the next step was to develop/improve the rearing techniques to produce larvae and juveniles of good quality in artificial conditions. The introduction of the new species *C. gallina* and *S. solida* in aquaculture was based on the existing technology (static seawater method - Batch system) for traditional species. The results of this study showed that the two studied species *C. gallina* and *S. solida* have potential to be produced artificially with the traditional techniques used for other bivalves and fed with microalgae species whose production is common in bivalve hatcheries. There were no constrains that prevented their production; however the results showed that *C. gallina* can be more attractive for aquaculture than *S. solida*. The possibility to obtain gametes of *S. solida* both by thermal shock and stripping is an advantage for the beginning of the process, since both techniques allow the incubation control, however the low fecundity of the species can be an obstacle. The long larval period can favour the appearance of problems during this phase, increasing larval mortality and thus, making the rearing process less successful. Nevertheless, the high percentage of *S. solida* metamorphosed larvae achieved can be a great advantage if it is considered the restock of natural populations with larvae ready to settle. This restock technique was already efficaciously used with other species (Preece et al., 1997; Arnold et al., 2002).

Concerning *C. gallina*, although the succeeded method to obtain embryos did not allow the full control of spawning, the short larval period of the species is undoubtedly a great advantage in aquaculture. This reduces significantly the appearance of problems during the rearing, increasing larval success, making the all process less expensive. Other advantage found was the great contribution of yolk reserves of *C. gallina* during the larval period. The low mortality in the first days of larvae life and the high percentage of metamorphosed larvae observed even in the unfed treatment are major advantages for aquaculture production and for restocking programs. Indeed, it allows the release of early larvae or larvae ready to settle in depleted areas in periods where food availability is poor, because larvae may rely on other sources of energy for the maintenance of their metabolism. The high larval growth rate obtained with ordinary produced microalgae (T-iso) in bivalve hatcheries also benefits the production of this species. The possibility to produce juveniles in hatchery in an inexpensive and easy way makes *C. gallina* also an interesting candidate to alternative or complementary products for the shellfish aquaculture industry. Finally, the absence of juvenile mortality provides the possibility to perform this life cycle phase of *C. gallina* in off-shore long-lines systems. This opportunity would minimize production costs as well as some of the adversities inherent to the adaptation of juveniles to the natural beds, namely predation, before being used in restocking programs by seeding the natural beds of Algarve coast.

Since *V. corrugata* larvae production had been previously established by traditional aquaculture technologies (Cerviño-Otero, 2011), the feasibility of rearing *V. corrugata* larvae with an optimized laboratory-scale recirculating aquaculture system (RAS) was evaluated and compared with the traditional larval rearing system (Batch). The results of this study showed that the larval rearing of *V. corrugata* could be performed at higher larval stoking densities in a RAS system, without decreasing their survival. Furthermore, the RAS system achieved higher larval growth rates and lower larval rearing time until the settling stage than the Batch system. All these constitute a significant reduction in the operating costs to produce the pullet carpet shell in hatchery. Thus, the RAS system allows mass production of inexpensive and good quality larvae that can be used for the implementation of more realistic restocking programs based on hatchery produced settled larvae, namely through its seed in the depleted subtidal areas of the Ria Formosa lagoon. These restocking actions could contribute to rebuild the

stocks of this species in Ria Formosa by ensuring the productive potential of the ecosystems and consequently reviving this species fishery in this coastal lagoon.

Moreover, aquaculture in Portugal is greatly supported by the European clam (*Ruditapes decussatus*) production in Ria Formosa which represents 66% of the national shellfish aquaculture (DGRM, 2012). However, this culture is clearly limited by the availability of seed since this production proceeds exclusively from natural recruitment (Matias et al., 2009). In the last few years, recruitment failures also clearly compromised the productive potential of the ecosystem, creating some instability in the national market. This situation exposed the fragility of this monospecific dependent economy. The advantages of RAS system benefit the pullet carpet shell as a strong candidate for the shellfish aquaculture industry in Ria Formosa as in Ria de Aveiro, encouraging the diversification in this activity. *V. corrugata* can be reared in the deeper parts of bivalve commercial parks.

As mentioned above, the low fecundity of *S. solida* was found to be problematic for hatchery production, which weakened the possibility of rearing larvae and/or juveniles artificially, for restocking programs. Faced with this constraint, it was therefore decided to go further in an effort to contribute to rebuild relatively high-density patches of *S. solida*, and a pilot experiment was designed to determine the feasibility of a stock-enhancement plan that consisted of transplanting individuals from natural clam beds to a closed fishing area. The survival rates observed in this study showed that transplanting either legal-sized (LS - exceeded the 25 mm) or undersized (US – less than the LS) clams can be a feasible and successful strategy for enhancing the populations in Algarve coastal waters. The resultant increase in local abundance of mature clams may lead to increased fertilization success and hence increased residual reproductive value of each clam relative to its pre-transplant value. However, we did observe differences in the apparent physiological health of transplanted US clams compared with transplanted LS animals, supporting the transplantation of smaller *S. solida*. Additionally, transplanting US clams may be advantageous because they have the opportunity to spawn more than once before they are harvested, so increasing the likelihood that they will contribute to larval production and potential repopulation of adjacent areas. The study allowed us to conclude that transplantation can be an effective bivalve stock-enhancement strategy which, in conjunction with management measures that control harvest within reasonable

estimates of sustainable yield, can contribute to the sustainable exploitation of *S. solida* stocks along the Algarve coast.

Finally, the results obtained in this thesis highlight the importance of the progress of a sustainability science approach to develop aquaculture and encouraging the diversification in this activity to promote the target species for the shellfish aquaculture industry as well as to support future restocking and stock enhancement programs for wild stock management. We expect that this knowledge can be widely used in fisheries restoration and can have significant contributions to the yields, sustainability, and resilience of the target species fisheries.

Chapter 7.2

Future research needs

As in any research work some unanswered questions persist, others require further studies and new topics to be addressed in future studies arose. The present study highlighted the need for continuous research studies linking fundamental and applied approaches to examine the complex biological processes and provide innovative technology, in order to improve *C. gallina, S. solida* and *V. corrugata* hatchery production.

This study began by drawing attention that the knowledge of population genetic structure is pivotal in efficient management of bivalve fisheries, namely if restocking/stock enhancement programs of wild stock will be based on hatchery produced larvae or seed. According to Gaffney (2006), three primary genetic concerns should be taken into consideration before implementing shellfish restoration projects: (1) identification and use of the correct genetic material for producing hatchery lines; (2) maintenance of genetic variability in hatchery stocks; and (3) maintaining Ne in the wild population. The safety measures performed in this study to minimize the deleterious genetic effects of the hatchery production, *i.e.* the genetic selection of broodstock, *per se* is not enough to ensure the larvae or seed genetic variability. When designing future restocking actions based on aquaculture production, effective breeding programs should be design, ensuring the preservation of the genetic variability in the hatchery stocks to protect the genetic diversity of the target populations and to maintain the effective size (Ne) of the wild population to be restocked or enhanced.

Additionally, the results achieved in this thesis can effectively contribute to the implementation of the target species hatchery culture. Notwithstanding, many challenges still remain and other studies can be performed to improve production methodologies and thus maximize the success of larval and post-larval culture. They mainly concern broodstock management, methods for larval rearing, improvement of settlement, quality of seed in terms of immunity, genetic diversity and sanitary status, among others. Improvements of knowledge in these areas will lead to better hatchery methodology, which can be transferred to a commercial scale, ensuring mass production of reliable quality larvae and seed. In this context, the new technology for bivalve larval production studied, the RAS, can promote the mass production of inexpensive but quality larvae of the pullet carpet shell. This system achieved higher larval growth rates and lower larval rearing time until the settling stage than the traditional Batch system,

resulting in a significant reduction in the operating costs. However, further work is required to maximize the larval production in the RAS system, such as achieve the control and stabilization of water quality from a physical, chemical and microbiological point of view, aiming at increasing the survival of larvae and the definition of the optimal larval stoking density. Furthermore, it is also important to make use of the knowledge of the ecophysiological processes of larvae and hatchery production acquired in this thesis to test the RAS technology in the two other target species, *C. gallina* and *S. solida*, since the mass production of larvae is an important requirement for the implementation of successful restocking programs based on hatchery production.

Finally, it is imperative to apply all the knowledge acquired in this work in the promotion of restocking and/or stock enhancement programs of the depleted wild populations of the target species with larvae, larvae ready to settle or seed produced in hatchery and consequently contribute to the best ecosystems management. Nevertheless, it is necessary to perform previous pilot experiments to test the most appropriate restocking technique for each species.

In this thesis, the stock-enhancement pilot experiment performed, that consisted of transplanting individuals from natural clam beds to a closed fishing area, was conducted on a small scale for experimentation only, but larger scale experiments will be necessary to ensure a future *S. solida* year-class success, which, in conjunction with management measures that control harvest within reasonable estimates of sustainable yield, can also contribute to an economical and biological sustainable fishery in the Algarve coast.

According to Costa-Pierce et al. (2013) in the new era of human history called the "Anthropocene", capture fisheries, aquaculture, ecosystems and communities cannot continue to be planned and managed separately. New, more integrated "seafood ecosystems" that restore and protect marine ecosystems and revive social ones need to be implemented by using the ecosystem approach to aquaculture to redevelop the world's working waterfronts.

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