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# Study of broodstock conditioning and determination of markers of gamete quality in the european clam

## *Ruditapes decussatus*

Joana Sousa

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**Joana SOUSA**

Préparée à Institut Français de Recherche  
pour l'Exploitation de la Mer

et

Instituto Português do Mar e da Atmosfera

**Study of broodstock  
conditioning and  
determination of markers  
of gamete quality in the  
European clam  
*Ruditapes decussatus***

**Thèse soutenue le 10 juillet 2014**

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## List of Abbreviations

ADAMs: A Disintegrin and A Metalloprotease domain

ADAR: RNA-specific adenosine deaminase

ADCY: Adenylate Cyclases

Anub1: AN1 zinc finger + ubiquitin

APA: Agência Portuguesa do Ambiente

APC/C: anaphase promoting complex/cyclosome

$\beta$ 3Gal-T:  $\beta$ 1,3-galactosyltransferase

$\beta$ 4Gal-T:  $\beta$ 1,4-galactosyltransferase

Birc: Baculoviral IAP repeat

BP: Biological process

CaM: Calmodulin family

Cam-PDE1C: Calcium/calmodulin-dependent 3',5'-cyclic nucleotide phosphodiesterase 1

CAN: Calcineurin A

CC: Cellular component

CCNB1: Cyclin B1

CCNB1: G2/mitotic-specific cyclin-B1

Ccnb1ip1: Human Enhancer of Invasion 10 - Hei10

CCNB2: Cyclin B2

CCNE2: cyclin E2

CDA: Cytidine Deaminase

cdc: cell division cycle

CDK2: Cyclin-Dependent Kinase 2

cdk2: Cyclin-dependent kinase 2

cDNA: Complementary DNA

CEP57 - centrosomal protein 57kDa

Cg-GPH: Glycogen phosphorylase

Cg-GYS: Glycogen synthase

CRISPRs: Clustered Regularly Interspaced Short Palindromic Repeats

DAVID: Database for Annotation, Visualization and Integrated Discovery

DGRM: Direção-Geral de Recursos Naturais

Dmc1: Meiotic recombination protein

dsRNA: double-stranded RNA

dynll2: dynein light chain 2

FC: Fold Change

FDR: False discovery rate

Fj: four jointed protein

Foxl2: Forkhead box protein L2

FucT: fucosyltransferase

FUT10:  $\alpha$ 1,3-fucosyltransferase

FUT8:  $\alpha$ 1,6-fucosyltransferase

GOI: Gonadal occupation index

GVBD: Germinal Vesicle Break Down

HORMAD1: HORMA domain containing protein 1

Hsp70: Heat Shock 70 kDa protein

HSP90B1: Heat shock protein 90kDa beta member 1

InsP6: Inositol hexakisphosphate

IP3: Inositol triphosphate

IPMA: Instituto Português do Mar e da Atmosfera

ISH: *in situ* hybridization

KIF: kinesin family

Mad211 - mitotic arrest deficient protein 2

Mad211: Mitotic arrest deficient-like 1

MAPK1: Mitogen-Activated Protein Kinase 1

MF: Molecular function

MHR: Medium Hatching Rate

MOSPD2: Motile Sperm Domain Containing Protein 2

MPF: Maturation-Promoting Factor

NGS: Next Generation Sequencing

Oyvlg: Oyster vasa-like gene

PAHs: polycyclic aromatic hydrocarbons

PCA: Principal Component Analysis

PCBs: polychlorinated biphenyls

PDI: Protein Disulfide Isomerase

PGCs: Primordial Germ Cells

PNRF: Ria Formosa Natural Park

PSA: Proteasome Subunit Alpha

PTM: Pavlidis template matching

RAD1: Cell cycle checkpoint protein

RNAi: RNA interference

SAM: Significance Analysis of Microarray

SCA: Spermatogenesis and Centriole Associated

SCP-2: Synaptonemal Complex Protein 2

scp2: Synaptonemal Complex Protein 3

scp3: Synaptonemal Complex Protein 3

skp: S-phase kinase-associated protein

Sp: Sperm surface Protein

SPAG: Sperm associated antigens

SPEF: Sperm flagellar proteins

SRA: Sequence Read Archive

SSRI: Selective Serotonin Reuptake Inhibitor

SST: Sea surface temperature

TNFR: Tumor Necrosis Factor Receptor

TSSK: Testis-specific serine/threonine-protein kinase

Vg/VTG: Vitellogenin

XYLT:  $\beta$ 1,2-xylosyltransferase

5-HT: Serotonin

## Preamble

The present thesis was realized in the context of the European project REPROSEED “REsearch to improve PROduction of SEED of established and emerging bivalve species in European hatcheries”, funded by the seventh framework program: Cooperation Work Programme Theme 2 from 01 April 2010 – 31 March 2014. The overall objective of REPROSEED was to link pure and applied approaches to examine complex biological processes and provide innovative technology in order to improve bivalve seed production in Europe. Between the four economically important marine bivalve species chosen for this project, the European clam *Ruditapes decussatus* was the object of the present work. Improvement of knowledge on broodstock management and gamete quality were the main objectives and are key steps to improve production of seed of emerging bivalve species, like *R. decussatus*.

This work was performed with the collaboration of two different partners of REPROSEED project: IPMA - Instituto Português do Mar e da Atmosfera, Olhão, Portugal, where I did my first year for sampling and histology analysis, and IFREMER - Institut français de recherche pour l'exploitation de la mer, Plouzané, France, where I spent the last two years. In addition, during the course of this thesis, I did a 3 month stay at the University of Padova, Legnaro, Italy (another partner of REPROSEED project) dedicated to microarray analysis. IPMA was in charge of my salary and Ifremer was in charge of all costs of my thesis.



# General Introduction

## I. *Ruditapes decussatus*: Biology and Ecology

### 1.1. Taxonomy and Distribution

The current classification of the European clam, *Ruditapes decussatus* according to the World Register of Marine Species (WoRMS 2014), is as follows:

Kingdom: Animalia

Phylum: Mollusca

Class: Bivalvia

Subclass: Heterodonta

Infraclass: Euheterodonta

Order: Veneroida

Superfamily: Veneroidea

Family: Veneridae

Genus: *Ruditapes*

Species: *Ruditapes decussatus* (Linnaeus, 1758)

Globally, *R. decussatus* is distributed along the Atlantic coast, from Norway to Congo, passing through the North Sea, being also in the Mediterranean and Red Sea, where this species migrated through the Suez Canal, Egypt (Fig.1) (Parache 1982). In Portugal, it is present in “Ria de Aveiro”, “Lagoa de Óbidos”, estuaries of Tejo, Sado and Arade, “Ria de Alvor” and “Ria Formosa” (Vilela 1950). It is also present in São Jorge Island, Açores more precisely in “Lagoa da Fajã de Santo Cristo” (Jordaens et al. 2000).

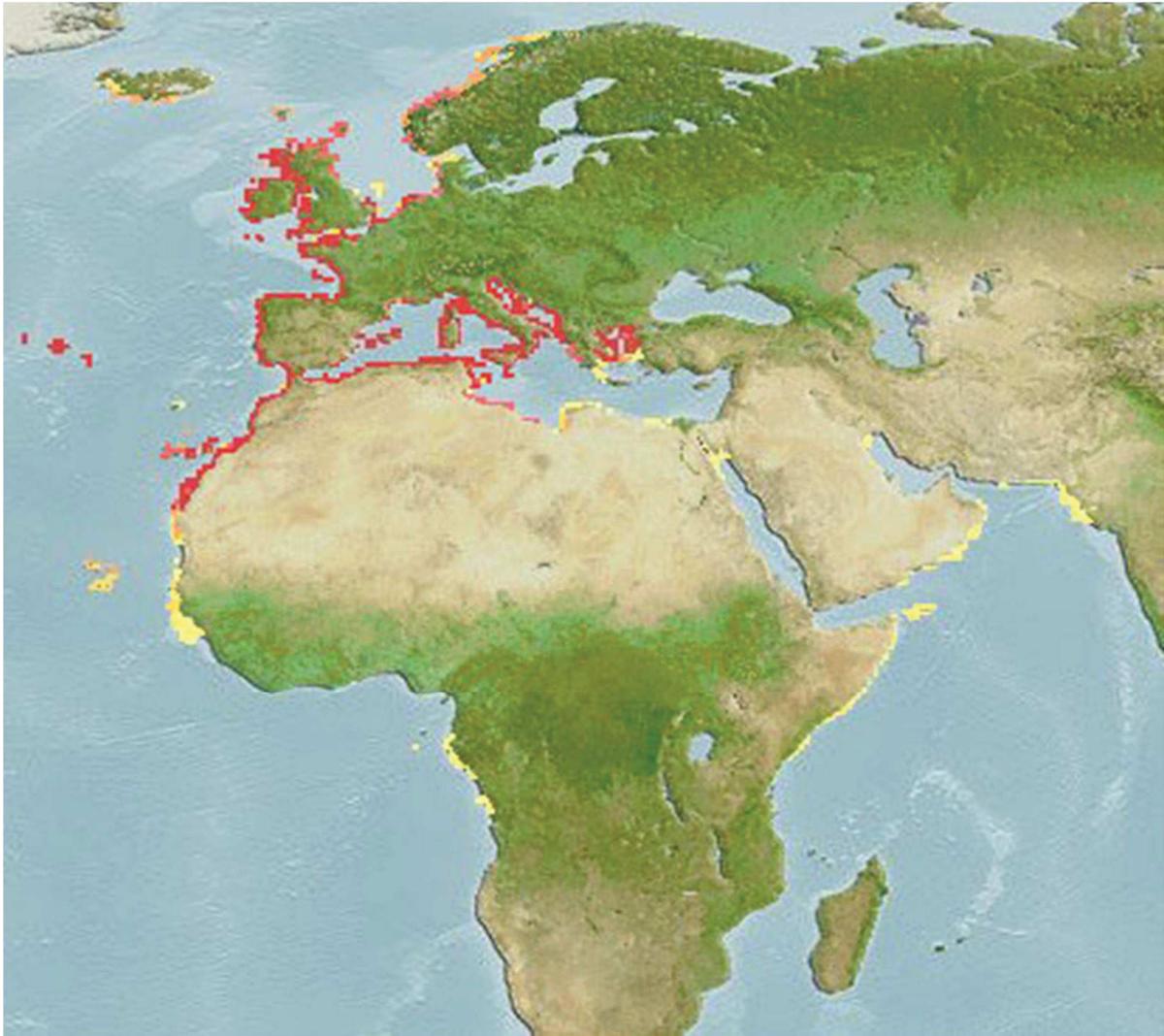


Figure 1 - Geographical distribution of *Ruditapes decussatus* (Computer Generated Map for *Ruditapes decussatus* (un-reviewed). [www.aquamaps.org](http://www.aquamaps.org). Aug. 2010).

## 1.2. Habitat

Clams, like most filter feeders living in the intertidal zone, take advantage of the tidal movement in estuaries: the water currents generated by the tides continuously supply a much larger quantity of food than the amount produced locally, being of critical interest for primary production (Gutiérrez 1991). Mid-estuarine areas usually consist of sand or sandy silt, often suitable for clam cultivation. *R. decussatus* lives burrowed in sand and gravel or silty mud but prefers stony sandy sediments. This species lives in the first littoral zone or intertidal zone and is buried 15-20 cm deep in the sand from the middle of the intertidal zone to a depth of a few meters (FAO 2013).

### 1.3. External Morphology

The species *R. decussatus* has a solid equivalve shell, with an oval shape more or less elongated with radial and concentric streaks (Fig.2) (Banha 1984). The dorsal side of the valves corresponds to the umbo (point of convergence of radial streaks) and the ventral side is the opposite. The external valves coloration is highly variable. Depending on the colour of the sediment, it can be white, yellow, orange, light or dark brown (Valence and Peyre 1989). Each valve presents three cardinal teeth: the central one in the left valve and the central and the posterior in the right are bifid. Pallial line and adductor scars are distinct (Poppe and Gotō 1991).

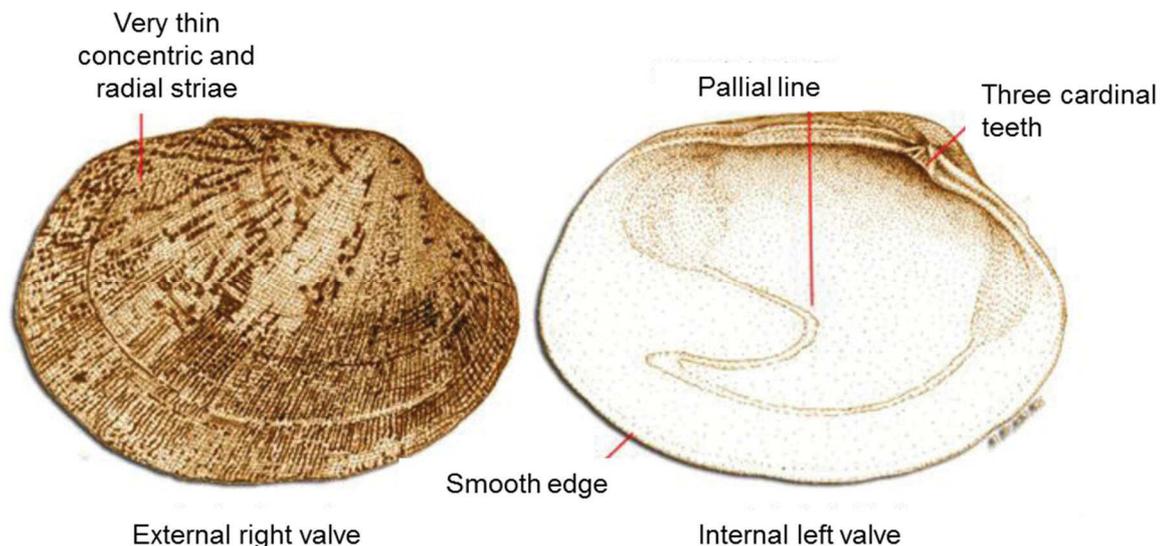


Figure 2 - External and internal view of *Ruditapes decussatus* shell morphology. (www.ictioterm.es).

### 1.4. Internal Morphology

In the internal body of *R. decussatus* we can observe the following parts: mantle, siphons, gills, labial palps, foot, adductor muscle, the digestive system, the circulatory system, the nervous system and the urogenital system (Fig. 3).

The **mantle** consists of a fold with two lobes which segregates and supports both the valves. The main function of the mantle is to secrete the shell. However, this structure also has a sensory function and can initiate closure of the valves in response to unfavorable environmental conditions. It can also control inflow of water into the body chamber and, in addition, has a respiratory function (Banha 1984).

The **two siphons**, one inhalant and other exhalant are long, separated and pigmented in their entire length (Parache 1982).

The **gills** are improved organs with cilia and allow the animals to obtain their basic requirements: respiration and partly for filtering food from the water. Two pairs of gills are located on each side of the body (Banha 1984).

The **labial palps** are fixed in the body walls and promote the food particles passage to the mouth (Banha 1984).

The **foot** is located at the base of the visceral mass. It is a well-developed organ that is used to burrow into the substrate and anchor the animal in position (Cesari and Pellizzato 1990).

The **two adductor muscles** are located near the anterior and posterior margins of the shell valves and have a cylindrical shape, which contraction allows the valves movement (Vilela 1950). The muscle(s) close the valves and act in opposition to the ligament and resilium, which spring the valves open when the muscles relax (Cesari and Pellizzato 1990).

The **digestive system** is simple. The large gills filter food from the water and direct it to the labial palps, which surround the mouth. The food is taken into the labial palps to the mouth, which is connected to the stomach through a short esophagus. From the stomach, the food continues to the following digestive diverticulum and intestine. The intestine ends at the anus, which is located in the cloacal chamber, near the adductor muscle (Cesari and Pellizzato 1990).

The **circulatory system** is simple and an open type one, consisting of veins, arteries, heart, pericardium, and tissue, with circulating hemolymph (Cesari and Pellizzato 1990).

The **nervous system** is quite simple and essentially consists of three pairs of ganglia (cerebral, pedal and visceral) (Cesari and Pellizzato 1990).

Finally, the **urogenital system** with sexes generally separated (dioecious), with an unusual and extremely infrequent manifestation of hermaphroditism already observed (Delgado and Pérez-Camacho 2002). The gonads occupy a major portion of the visceral mass and are generally only evident during the breeding season. Microscopic examination of the gonad is required to determine the sex of the animal. The renal system is difficult to observe (Cesari and Pellizzato 1990).

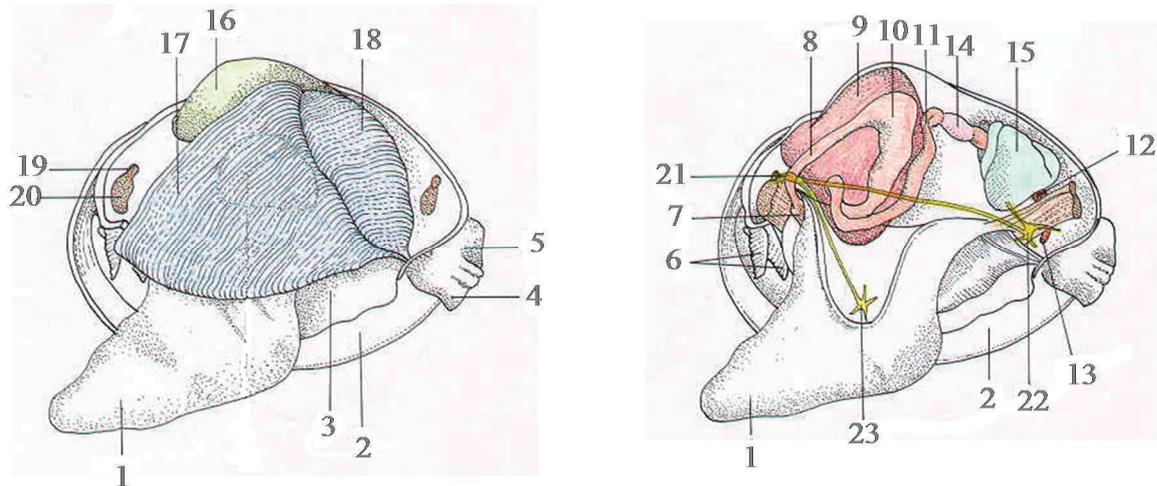


Figure 3 - *Ruditapes decussatus* internal morphology. 1.Foot 2.Mantle 3.Paleal Cavity 4.Inhalant siphon 5.Exhalant siphon 7.Mouth 8.Esophagus 9.Digestive gland 10.Stomach 11.Intestine 12.Recto 13.Anus 14.Heart 15.Diverticulum filter 16.Gonad 17.Gills 18.Gills 19.Foot retractor muscle 20.Adductor muscle 21.Cerebral ganglion 22.Visceral ganglion 23.Pedal ganglion (Adapted from Universe, 2006).

### 1.5. Development and Growth

*R. decussatus* may have the first spawning in its first years of benthic life with about 20mm (Matias 1991). The natural spawning period of *R. decussatus* is confined to a particular time of the year (between May to October in temperate zones) (Banha 1984). After the spawning the individuals present a consequent weakness, followed by a period of sexual rest until February, which is characterized by an accumulation of reserves and a decrease in growth (Banha 1984). During the spawning, eggs and sperm are released into the water where fertilization occurs. The presence of sperm in the water will frequently trigger spawning in other animals. Sperm is discharged in a thin, steady stream through the exhalent opening or exhalent siphon. Discharge of eggs is more intermittent and they are emitted in clouds from the exhalent opening or exhalent siphon (Cesari and Pellizzato 1990). The gametes are expelled into the water column, through the exhalant siphon and the fertilization occurs externally. About 24 hours after fertilization, the trochophore larvae appear (passing through the multicelled blastula and gastrula stages) and gradually are transformed in D-larvae (about 48 hours after fertilization). This larva is characterized by the appearance of the shell (Barnes 1980). The subsequent shelled larva is called a veliger larva because of its velum with which it swims and eats. *R. decussatus* veliger stage lasts for about three weeks, during which it grows to

approximately 230  $\mu\text{m}$ , depending on the environmental conditions, mainly food abundance (Pérez-Camacho et al. 1994). At this point it becomes a pediveliger and both crawls with its foot and swims with its velum looking for a suitable habitat for adult growth. At the end of larval period, with an average duration of 2-4 weeks, the larvae reaches between 300-400 micrometers and the metamorphosis starts giving them the final form, allowing the sediment fixation (Chícharo 1996). The animal passes from a pelagic to its benthic final phase and they are then referred as post-larvae, juveniles or seed (Fig. 4).

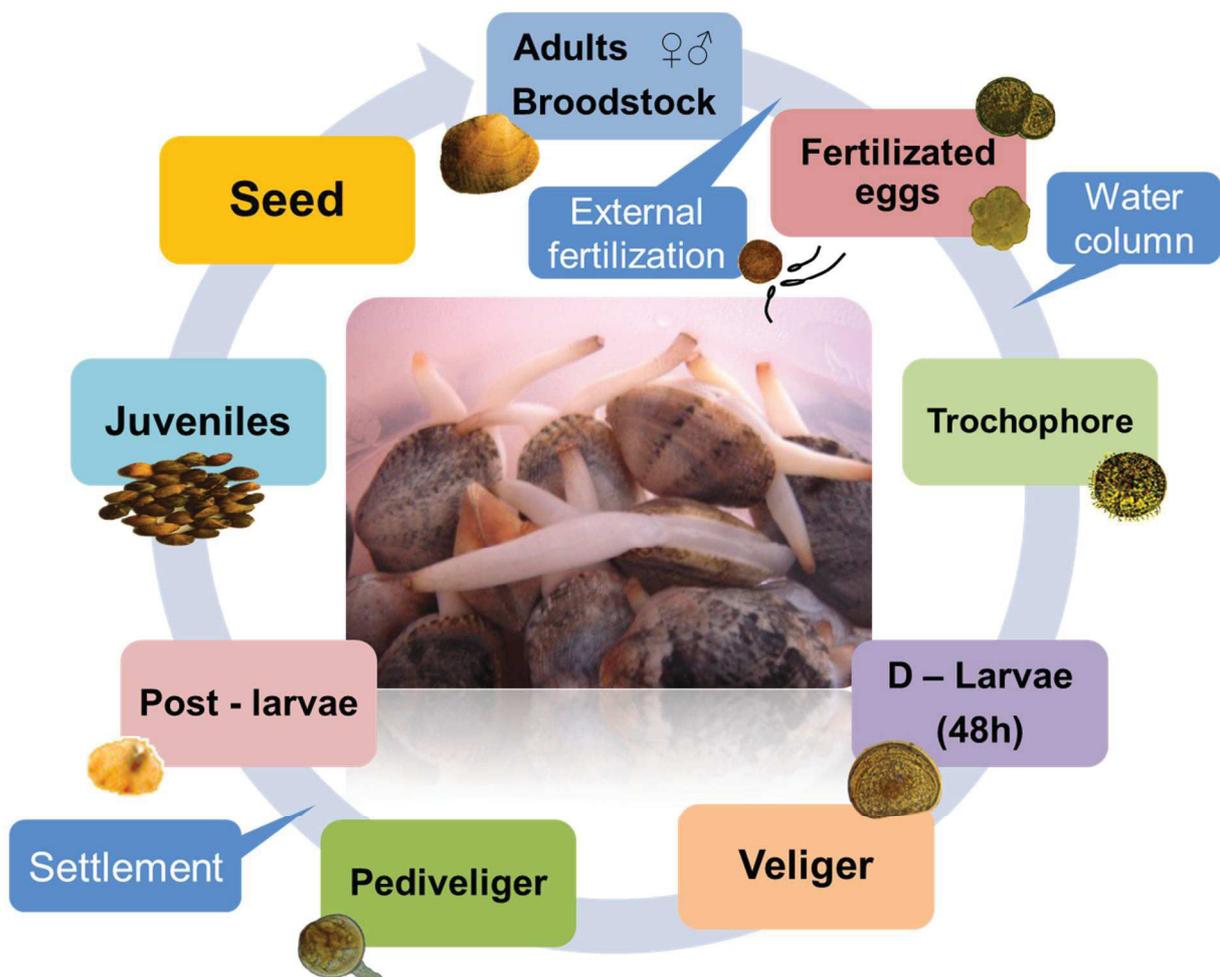


Figure 4 - Schematic *Ruditapes decussatus* life cycle.

The benthic development is conditioned by food and temperature, but other environmental factors, such as salinity, sediment composition or the dissolved oxygen concentration may also have a major influence (Parache 1982).

*R. decussatus* starts their benthonic phase, settling on muddy or sandy sediment, in intertidal areas. The burrowing depth is of approximately 10 to 12 cm, depending on various factors, such as consistency of the sediment, population density, physiological state of individuals and the size of their siphons. This species tolerates salinities of 20 to 40 and the optimal temperature for growth is 20-22 °C. The clam stops their growth below 12 °C (Vilela 1950). Growth of juveniles greatly depends of geographic localization, however a seasonal trend could be describe: growth is generally fast during spring and summer when food is abundant and water temperatures are warmer and it virtually ceases in winter, resulting in an annual growth line in the shell (Matias 2007). The growth in bivalves, as in fish, only ends with death. Their growth rates decrease gradually as the animals get old, being the size limit determined by the environmental conditions (Banha 1984). *R. decussatus* can grow up to 7.5 cm in length, however commercial size (3.5 cm) are attained in two years. As written before, food availability and temperature have been considered the main factors affecting growth in *R. decussatus* and in all bivalve species (Aranda-Burgos et al. 2014; Camacho et al. 2003). Normally, growth is related to the age of the animal but is a very plastic phenotype. Rates of growth amongst individuals are highly variable, and much of this is genetically controlled (Dittman et al. 1998; Hedgecock et al. 1996). In natural populations of adult bivalves, growth rates are positively correlated with the degree of multi-locus heterozygosity (Koehn and Shumway 1982). More recently, a negative correlation between growth rate and aneuploidy (cytogenetic phenomenon known as an abnormal diploid chromosome number) was also put in evidence in *R. decussatus* (de Sousa et al. 2011), as well as in the Pacific *Crassostrea gigas* (e.g. Leitão et al. 2001; Thiriot-Quievreux et al. 1998; Zouros 1996) and the Portuguese *Crassostrea angulata* oysters and interspecific hybrids (Batista, Leitão et al. 2007b). The study of this last phenomenon is of particular importance, since the variability of growth rate is one of the biggest problems faced by bivalve producers.

### **1.6. Reproduction and gametogenesis**

*R. decussatus* sexual maturity is dependent on size, rather than age and geographic distribution, and is generally attained when the clams are about 20 mm (Ojea et al. 2004). A comprehensive anatomical and histological study of this species

has previously shown that there is generally no coexistence of male and female germinal lines in the same specimen, thus confirming its gonochorism (Vilela 1950). However, as described before, an unusual and extremely infrequent manifestation of hermaphroditism in *R. decussatus* was observed with developing female germinal lines in follicle walls, and groups of spermatozoa in the lumen (Delgado and Pérez-Camacho 2002). Given the presence of the mixed structures and the degree of maturity reached by the gametes (female gametes in the early stages of gametogenesis and fully ripe male gametes) it is possible that there was a change of sex, from male to female, which is common in other species of bivalve molluscs, such as oysters (Asif 1979), *Mytilus edulis* (Sugiura 1962), *Mytilus galloprovincialis* (Lubey 1959) and *Ruditapes philippinarum* (Devauchelle 1990; Ponurovsky and Yakovlev 1981).

The gonad regresses at the end of the annual reproductive cycle, usually by the end of summer in temperate regions, and regenerates at the beginning of the following one by the end of winter. The gonad is composed of many-branched, ciliated ducts from which numerous sacs and termed follicles, open. Gametes arise by proliferation of germinal cells that line the follicle wall. The gonad undergoes continuous development until it becomes fully mature but this development has been divided into several stages for convenience, e.g. sexual rest, initiation of gametogenesis, advanced gametogenesis, maturation, partial spawning and post-spawning and gonadal regression (adapted from Delgado and Pérez Camacho 2005). When the gonads or gonadal tissue are fully mature they are very evident and form a significant portion of the soft parts of the animal. Gonaducts that will carry the gametes to the body chamber develop, enlarge and are readily observed in the gonad. At this time the animal is frequently referred to as being spawn (Serdar et al. 2010; Shafee and Daoudi 1991). In terms of fecundity, Matias et al. (2009) observed a very high variability in the recorded values in this species, which ranged from 0.02 million eggs/female to 3 million eggs/female released.

During the initial stage of gametogenesis, period of **sexual rest (I)**, gonadal follicles are absent and connective and muscular tissues occupies the entire zone from the digestive gland to the foot. There is no evidence of gonadal development and sex determination is not possible. In the stage of **initiation of gametogenesis (II)**, follicles and gonadal acini begin to appear in females and males respectively. They increase in size and appear filled with oocytes in the growth phase in the

females and with immature gametes (spermatogonia and spermatocytes) in the males. During **advanced gametogenesis (III)**, the follicles occupy a large part of the visceral mass. The presence of muscular and connective tissue is reduced. At the end of this stage, characterized by intense cellular growth in females, the oocyte protrudes to the center of the lumen, remaining attached to the wall via the peduncle. The abundance of free oocytes equals those attached to the wall of the follicle. In males, the majority of the acini are full of spermatids and spermatozooids. In the **maturation period (IV)**, corresponding to the maturity of the majority of gametes, the rupture of the peduncle of mature oocytes occurs, and the oocytes consequently occupy the follicular interior. In males, the gonadal acini mainly contain spermatozooids. Throughout this period partial spawning may occur, and it concludes with the emission of gametes. After spawning clams should progress to an inactive stage, However, in this species it is normal the occurrence of a non-continuous gonad development, with microscopic examinations of the gonadal tissues showing simultaneous spawning and recovery of the gonad, called **partial spawning (V)**. In this phase the gametes are discharged and 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. In males, lines of spermatogonias are found in the follicles and in females, ovogonias attached to follicle walls are observed. Finally, during the **post-spawning and gonadal regression (VI)**, half or more than the half of the follicles are empty. The oocytes are sometimes stretched or torn and the development of small oocytes is visible on the wall of the acini (Reproductive scale for *R. decussatus* gonad development, adapted from Delgado and Pérez Camacho (2005) (Fig. 5).

Temperature has traditionally been assigned a major role in the evolution of the reproductive cycle, although the existence of sufficient nutritional reserves in the animal and/or the abundance of food are also essential (Camacho et al. 2003). Therefore, a way to induce sexual maturation in bivalves is the manipulation of their physical and nutritional environments (Gallager and Mann 1986). This process of artificially obtaining sexually mature individuals is called broodstock conditioning (described posteriorly). Temperature is normally responsible for initiating the process, whereas food supply determines the extension of the reproductive process (Lubey 1959). In order to clarify this, several studies have been done, where the influence of food (Delgado and Pérez Camacho 2005), temperature (Delgado and Pérez-

Camacho 2007; Marshall et al. 2012; Matias et al. 2009) and the interaction of both parameters (Galbraith and Vaughn 2009; Hamida et al. 2004a) in bivalve gamete development, was observed. Reproduction is a process requiring abundant energy for gonad development. In *R. decussatus* the energy from ingestion is mainly channeled to reproductive processes, especially when food is restricted. The amount of food is directly related to the rate of the gonadal development and with the total quantity of gonad generated. Additionally, a food restriction limits gonadal recovery after spawning episodes (Delgado and Pérez Camacho 2005). In terms of temperature, the rate of gonadal development is directly related to the increase of this parameter. In broodstock conditioning the occurrence of advanced gametogenesis and reproduction period phases are also dependent on the conditioning temperature, since higher temperature accelerated gametogenesis development (Matias et al. 2009). Within the same species, the geographic origin can also play an important role in gametogenesis (Chryssanthakopoulou and Kaspiris 2005; Drummond et al. 2006; Matias et al. 2009; Urrutia et al. 1999).

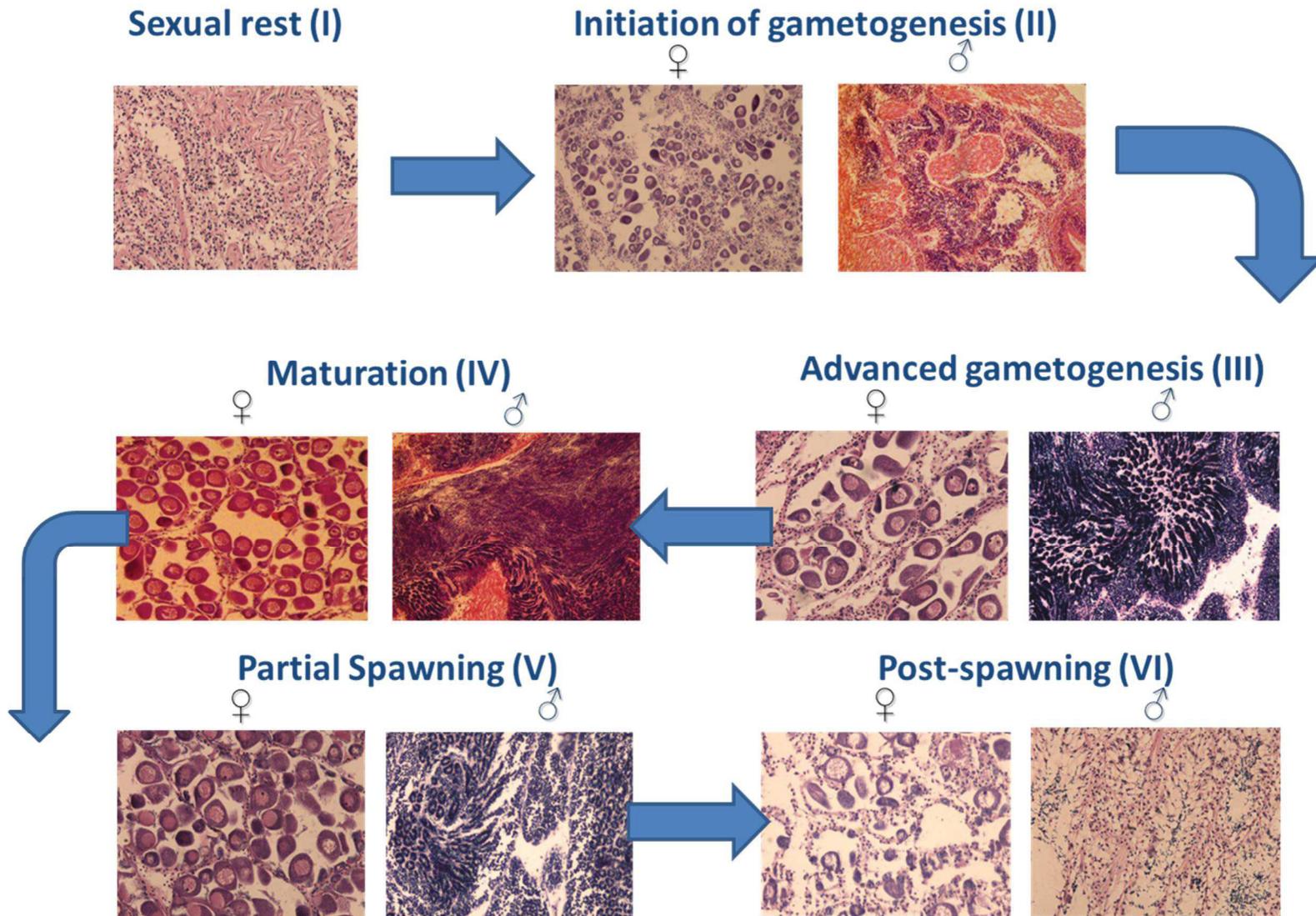


Figure 5 - Photomicrographs showing stages in the development of *Ruditapes decussatus* female and male gonad.

### 1.6.1. Molecular mechanisms of reproduction in bivalves

While molecular mechanisms of reproduction are pretty well studied in model organisms, information is still limited in marine bivalves. A large and exhaustive approach is required for investigating the molecular bases at the origin of the variability of reproductive success. Some dedicated studies have however already been made in this area and several candidate genes appeared of great interest for studying reproduction processes. Useful molecular markers in the reproduction of *C. gigas* have been reported lately. For example, the expression of glycogen phosphorylase (Cg-GPH) and synthase (Cg-GYS) driven by the oyster's reproductive cycle, reflected the central role of glycogen in energy storage and gametogenic development in *C. gigas* (Bacca et al. 2005). Another example is a germline specific gene, the vasa gene, a key determinant for germline formation in eukaryotes. This gene, highly conserved through evolution, encodes a RNA helicase protein member of the DEAD-box family (Linder et al. 1989). In the oyster *C. gigas* a vasa-like gene (Oyvlg) was isolated and characterized. It was observed that its expression was restricted to germline cells both in males and females, including germinal stem cells and auxiliary cells, suggesting the role of Oyvlg in germline development (Fabioux et al. 2004b). Later, this gene was used as a molecular marker to establish the developmental pattern of germline cells during oyster ontogenesis, where it was suggested that the germline of *C. gigas* is specified at early development by maternal cytoplasmic determinants, including Oyvlg mRNAs, in putative PGCs (primordial germ cells) that would differentiate into germinal stem cells in juvenile oysters (Fabioux et al. 2004a). Still with the Oyvlg, the technique RNA interference (RNAi), which can be a quick and efficient technique for determining the loss-of-function phenotype of a gene, was performed (Fabioux et al. 2009). The results showed a knockdown phenotype corresponding to germ cell underproliferation and prematurely arrested meiosis throughout the gonad, appearing to be essential particularly for mitotic proliferation and early meiosis (Fabioux et al. 2009). The vasa-like gene was also studied in the mussel, *M. galloprovincialis* with similar results (Obata et al. 2010).

## II. Production of *Ruditapes decussatus*

The production of bivalve molluscs is a strategic activity since it contributes significantly to the preservation of local economies and generates capital and employment on the littoral areas (Matias 2013). The European clam is considered a high value seafood product and one of the most economically important bivalve species in Southern European countries like Italy, Spain and Portugal (Matias et al. 2009). *R. decussatus* is extensively produced and harvested in Portugal, where clam farming represents an important economic sector. This species is central to aquaculture's revenue, representing 27% of the total seafood cultured in Portugal and 64 % of shellfish production (DGPA 2012). In this country, the main production areas of this species are the Ria de Aveiro (40°42'N 08°40'W), the Ria de Alvor (37°07'N 08°36'W) and the Ria Formosa Lagoon (37° 01'N; 07° 49'W) (Fig. 6). The culture of *R. decussatus* in Ria Formosa Lagoon represents 90 % of the national production and it is central to the socioeconomic framework involving, directly or indirectly more than 4500 people (INE 2007).



Figure 6 - Ria de Aveiro, Ria de Alvor and Ria Formosa Lagoon location.

Historical records show that *R. decussatus* was one of the major aquaculture species in Europe, but due to overfishing, recruitment failures and some outbreaks of bacterial infection and parasitism, producers started to substitute this species for a closer clam from the same family, the manila clam *R. philippinarum*. The introduction of this species, with a faster growing rate and believed to be more resistant to some diseases, originated a progressive replacement of the native clam (Leite et al. 2013). However, in Portugal, *R. decussatus* is commercially more important and more appreciated by consumers than *R. philippinarum*. Moreover, the culture of this last one is interdict in Portugal, since the Manila clam is considered, in the Portuguese legislation, an exotic species (Matias et al. 2009). Despite the fact that its culture decreased considerably in other countries such as France, Italy and Spain, there is some desire to increase *R. decussatus* production.

*R. decussatus* is reared in plots in the intertidal zone exploited by clam farmers, usually organized in professional associations. Clam farmers pay an annual rent to the state, which gives them the exclusive right to cultivate clams on the leasehold (Matias 2013). The seasonal environmental changes and the localization of the ground plots have a great influence on the bivalve population development, namely in survival, growth and reproduction, for example the quality and availability of food, water quality and pollution, are factors of great importance to the bivalve ground plot productivity (Abalde et al. 1990; Kraeuter and Castagna 1989; Royo 1986).

The artificial production of bivalve juveniles allows the selection of the characteristics of the seed (size and weight) that will be more suitable for future stages of farming, by a precise control of the diet and the environmental conditions, allowing the attainment of optimum growth rates and healthy juveniles (Matias 2013).

## 2.1. *Ruditapes decussatus* production phases

### 2.1.1. Broodstock conditioning

The European clam production still relies mainly on the collection of wild seed, which production depends on some basic principles that follow successive biological steps (Gosling 2003; Helm and Bourne 2004). First, broodstock is brought into the hatchery for conditioning (i.e. the activation and acceleration of gametogenesis) and are kept in the best possible conditions of high water flow, temperature and food availability (Fig. 7). Cultured marine microalgae species are used as the principal food supply during conditioning. *R. decussatus* will require 4 to 8 weeks of conditioning to reach fully maturity and spawning readiness during late winter and early spring (Delgado and Pérez Camacho 2005; Joaquim et al. 2008). At the end, bivalves are induced to spawn in a controlled environment, normally by thermal stimulation (Joaquim et al. 2008a). Finally, gametes are mixed to perform fertilization with gamete quality being crucial for the success of this step.

As mentioned before, broodstock conditioning is the first phase in hatchery husbandry. Artificial reproduction of bivalves requires the use of animals that have reached optimal sexual condition, which, according to Kennedy et al. (1996), depends on the synergistic effects of both internal and external factors. Survival of larvae is partly dependent on stored energy reserves derived from the eggs. Performance of the larval rearing system, reflected in the proportion of oocytes which survive to the early-juvenile stage, may be substantially improved by managing broodstock conditioning to maximize the proportion of viable gametes (Lannan 1980). According to Massapina et al. (1999), inherent physiological variability among bivalve gamete and larvae can always be expected in hatchery, and understanding the source of this variability is crucial in reducing it in culture situations. Therefore, the knowledge of the natural reproductive cycle of bivalves is fundamental to establish successful hatchery based production, especially for the broodstock conditioning step.

As mentioned before, the spawning of European clam occurs from May up to October along Portuguese coasts. The larval rearing can be extended from these periods since the complete maturation is often achieved before natural spawning. The same process was done in *C. gigas*, with the production of gametes throughout the year (including in the autumnal resting period), by appropriate modulation of

temperature and photoperiod that drive the reproductive internal clock of *C. gigas*, in particular for the regulation of gonial proliferation and germ cell maturation, both essential steps in oyster reproduction (Fabioux et al. 2005). However, authors did not describe the effects on gamete quality and subsequent effects on larval development. The results depend on the quality of maturation process. The determination of quality indices could contribute to the knowledge of the *R. decussatus* hatchery reproduction potential.

Moreover, the establishment of optimal molluscan broodstock diets has had an important effect on their reproduction and the quality of oocytes (Andersen and Ringvold 2000; Utting and Millican 1997), varying between species. In *M. edulis*, the work by Pronker et al. (2008) on the impact of different micro-algae mixtures on broodstock reproductive performance, provided useful information for mussel conditioning. Although, despite several targeted studies (Delgado et al. 2004; Delgado and Pérez-Camacho 2007; Hamida et al. 2004a; Matias et al. 2009), we still lack an effective and robust European clam broodstock conditioning methodology, in order to overcome several issues, like spawning induction success and gamete quality variability.



Figure 7 - Broodstock conditioning system. (Copyrights: Sandra Joaquim, 2014).

### 2.1.2. Spawning and fertilization

In hatchery production, spawning is the procedure by which conditioned bivalves are induced to liberate their mature gametes in response to applied stimuli. Commonly, mature clams are placed in a spawning tank and spawning is induced by thermal shocks between 5 °C to 28 °C, at one hour interval (Joaquim et al. 2008b). The number of cool/warm cycles that are required to induce spawning depends on the state of maturity of the gametes and the readiness of the adults to spawn. Generally, if the adults do not respond within a 3 to 4 h period they are returned to the conditioning tanks. Adults may start spawning on either the cool or warm part of the cycle, most commonly the warm (Fig. 8). Additional stimuli can be provided in the form of stripped eggs or, preferentially, sperm from an opened individual. The first adults to spawn are almost always males. As each male and female begin to spawn it is necessary to remove it from the spawning tank and transfer it to an individual spawning beaker with filtered seawater at the same temperature (Joaquim et al.

2008b). The batches of oocytes and sperm are pooled separately and gently washed into a clean glass. Small volumes of the pooled sperm suspension are mixed with eggs during gentle agitation, aiming to obtain around 10 spermatozooids per oocyte in a microscopic view (Cesari and Pellizzato 1990; Joaquim et al. 2008b).

Among the major difficulties reported in hatchery production of this species, spawning control and gamete quality are the most significant.



Figure 8 - Mature clams placed in a spawning tank in the warm part of the cycle. (Copyrights: Ana Margarete Matias, 2013).

#### 2.1.2.1. Meiotic maturation and spawning induction

As previously mentioned, bivalve gametes are usually obtained by applying thermal shocks to adult clams (Le Pennec 1978). However, an alternative method of “stripping” exists, which allows to obtain mature gametes easier and faster, by dissecting the gonad. As the name demonstrates, this procedure involves removal of gamete from gonad tissue. This practice is widely used to collect oocytes before natural egg emission in some bivalve species whose eggs can be fertilized directly after the dissection, such as the Pacific oyster *C. gigas*. In the Pacific oyster oocytes

collected from the gonads are blocked in prophase I. GVBD (Germinal Vesicle Break Down) can be induced by oocyte incubation in seawater or in different chemicals such as serotonin (Osanai 1985). However, at this phase, the oocytes of *R. decussatus* are not ready to be fertilized (Colas and Dubé 1998; Hamida et al. 2004b).

Molluscs and other lower invertebrates belonging to the protostome group exhibit special features in their ionic regulation of meiotic maturation. According to Colas and Dubé (1998), there are two types of mollusc oocytes:

**Class I oocytes** – These oocytes are fertilized at the prophase of the first division of meiosis and meiotic maturation is induced to proceed uninterruptedly to completion, followed by syngamy of male and female haploid pronuclei, directing further mitotic divisions. *Barnea* and *Spisula* are examples of bivalve genus with oocytes class I.

**Class II oocytes** – These oocytes are held in the ovaries at prophase of the first division of meiosis and development reinitiates during spawning through hormonal stimulation or other stimuli, undergoing GVBD. A second barrier to development occurs at metaphase I, prior to extrusion of the first polar body, until fertilization releases this metaphase arrest and allows further stages of maturation to take place (Fig. 9). *Ruditapes*, *Crassostrea* and *Mytilus* are examples of bivalve genus with oocytes class II.

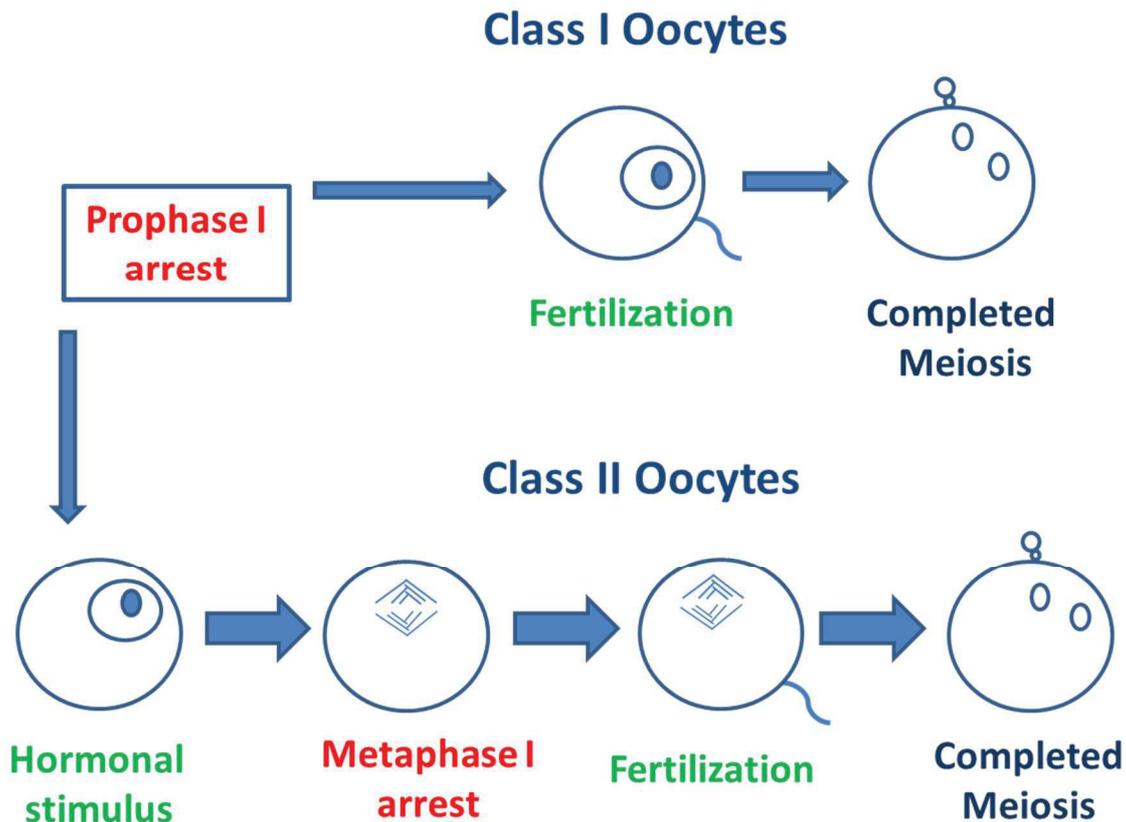


Figure 9 - Meiotic maturation in mollusc oocytes. Class I and class II oocytes.

The molecular cascade, and the underlying genes involved in this phenomenon have been little studied in bivalves, showing mainly the crucial role of  $\text{Ca}^{2+}$  signaling pathway during oocyte maturation (e.g. Abdelmajid et al. 1993; Colas and Dubé 1998; Deguchi and Morisawa 2003; Leclerc et al. 2000; Stricker and Smythe 2001; Zhang et al. 2009). In terms of release of prophase I arrest, regardless the type of oocytes, Colas and Dubé (1998) showed that an early  $\text{Ca}^{2+}$  influx is a prerequisite to allow activation of prophase I-arrested oocytes in most, if not all, protostome species. In *Ruditapes* during the course of maturation, functional voltage-operated  $\text{Ca}^{2+}$  channels are expressed between prophase and metaphase I. Results obtained using pharmacological tools and direct binding of specific dihydropyridines strongly suggest that these channels are dihydropyridine-sensitive calcium channels (Leclerc et al. 2000). In *Ruditapes* they become functional after hormonal stimulation, increasing their number before GVBD. In *Crassostrea* the dihydropyridine-sensitive calcium channels are already present at prophase stage and their density is constant from prophase to metaphase I. Taken together this data suggest that the absence of dihydropyridine-sensitive  $\text{Ca}^{2+}$  channels in the membrane of prophase I arrested

oocytes of *Ruditapes* may account for their inability to be fertilized at this stage, while the presence of dihydropyridine-sensitive  $\text{Ca}^{2+}$  channels in prophase I arrested oocytes of *Crassostrea* may explain their fertilizability (Leclerc et al. 2000).

Additionally, the mechanisms by which, depending on the species, initially activated prophase I oocytes either stop at metaphase or go on to completion of meiosis, are not totally resolved. *Ruditapes* is one of the genera where the processes of prophase I arrest followed by metaphase I arrest and release by fertilization has been best analyzed as this is a convenient model to study both sequences of events (Colas and Dubé 1998). It has been suggested that short lived proteins continuously synthesized were responsible for maintaining metaphase arrest in some molluscs' oocytes, with cyclin regulatory proteins representing good candidates since their disappearance, due to protein synthesis inhibition, triggered metaphase/anaphase transition and polar body extrusion (Colas and Dubé 1998). In *Ruditapes* and other molluscs, calcium may act via a calcium calmodulin-kinase since calcium calmodulin antagonists were able to prevent the metaphase I release and cyclin degradation upon fertilization (Abdelmajid et al. 1993; Colas and Dubé 1998; Leclerc et al. 2000; Whitaker 1996; Zhang et al. 2009).

Spawning induction has been achieved in bivalves with more or less success depending on the species. Induction of spawning of ripe scallops, with oocyte maturation process along the genital ducts (similar to *decussatus*), has previously been made by various researchers employing chemical and/or physical stimuli (Velasco et al. 2007). Among the physical stimuli applied were changes in temperature, exposure to water currents, desiccation and application of electrical shocks. Among the chemical stimulants administered as potential inducers of spawning, intragonadal injections of neurotransmitters such as serotonin, dopamine, noradrenalin and/or hormones such as prostaglandin, have been tested (Velasco et al. 2007). Additional studies were made in order to assess possible roles of sex steroids in bivalves (Croll and Wang 2007). Varaksina and Varaksin (1991) and Varaksina et al. (1992) reported that injections of estradiol, progesterone and testosterone stimulated both oogenesis and spermatogenesis in adult *Yezo* scallops *Mizuhopecten yessoensis*. In the coot clam *Mulinia lateralis*, administration of methyltestosterone accelerated sex maturation and resulted in an increase in spawning frequency (Moss 1989). Moreover, injections with estradiol stimulated vitellogenesis in the oyster *C. gigas* (LI et al. 1998; Osada et al. 2003). More recently,

an exploratory approach to identify novel genes differentially expressed in the maturation process and the estrogen response in the marine bivalve, *M. edulis* was performed, providing evidence that mussels may be impacted by exogenous estrogen exposure (Ciocan et al. 2011).

Even though, serotonin (5-HT) is believed to be the natural inducer of oocyte maturation in bivalves (Deguchi and Osanai 1995). This neurohormone is a regulator synthesized from L-tryptophan that allows GVBD and the completion of meiosis. Its intervention prompts an increase in intracellular pH and the expulsion of the first polar body (Colas and Dubé 1998; Kyojuka et al. 1997). This technique was used for *Spisula*, *Barnea*, *Crassostrea* and *Ruditapes* isolated prophase I oocytes (Leclerc et al. 2000) and it was shown that the oocytes of *R. philippinarum* species possess a single class of original 5-HT receptors coupled with G-proteins in order to be functional (Gobet et al. 1994). For *R. decussatus*, serotonin also improved fertilization of stripped oocytes. However, the percentage of fertilized oocytes and development to D larvae remain low, and abnormalities are frequent (Hamida et al. 2004b).

#### 2.1.2.2. Sperm – egg interaction

Fertilization is the process by which the gametes from two individuals of the same species fuse together to give rise to a new individual. Mechanisms operate at many levels to ensure the outcome of this process. However, the moment when the gametes recognize each other, by the interaction between glycoproteins of the extracellular matrix of the egg and cell surface receptors on the sperm, is certainly one of the most important (Rosati and Focarelli 1990; Wassarman et al. 2001). In terms of knowledge of sperm-egg recognition no specific studies were done in the European clam *R. decussatus*. Indeed, in invertebrates, most of the studies in this subject are limited to sea urchins (Mengerink and Vacquier 2001) and the mollusc bivalve *Unio elongatulus* (Rosati et al. 2000). In species like bivalves, with external fertilization, sperm-egg recognition can be achieved by contemporary spawning of the gametes and/or release of chemoattractants from eggs, which also increase sperm motility and respiration (Rosati et al. 2000). This interaction triggers an acrosome reaction in the spermatozoon that enables the gamete to cross the extracellular matrix and reach the oocyte plasma membrane, where fusion between the two cells occurs (Focarelli et al. 2003). The acrosome reaction is triggered by

increases in intracellular  $\text{Ca}^{2+}$  and pH and results in exocytosis of the acrosomal vesicle (an organelle in the sperm head). Following the acrosome reaction, secondary binding events occur, and the membrane exposed by the AR fuses with the egg plasma membrane. Protein–carbohydrate interactions play a critical role in this complex process (Mengerink and Vacquier 2001). Metz (1985) reported that the oocyte of the marine bivalve *M. galloprovincialis* is generally enclosed by an extracellular coat and another acellular, or cellular envelope, while the apical region of the sperm cell is characterized by an acrosomal apparatus which undergoes a series of changes on interaction with the oocyte. These changes enable the sperm to cross the external layer and fuse with the oocyte.

In the mollusc bivalve *U. elongatulus*, the egg is peculiar because it is highly polarized (Focarelli et al. 1988). In this organism, sperm only binds in a defined area of the vitelline coat at the vegetative pole. Two glycoproteins with masses of 180 kDa (gp180) and 273 kDa (gp273) constitute the vitelline coat in *Unio* (Focarelli and Rosati 1993). By means of polyclonal antibodies raised against the two purified glycoproteins, it has been shown that the two components are localized differently with gp273 prevalent in the binding area. It has been demonstrated that the ligand activity is exerted through O-linked oligosaccharides and that fucose is crucial in this function (Focarelli and Rosati 1995).

### 2.1.2.3. Gamete quality

A high variability in reproductive success is commonly observed in most bivalve hatcheries. It has been shown to be partly attributable to gamete quality, sperm–egg interaction and differential larval survival (Boudry et al. 2002). Egg quality can be defined as the ability of the egg to be fertilized and to subsequently develop into a normal embryo (Kjørsvik et al. 1990). Similarly, sperm quality can be defined as its ability to successfully fertilize an egg and subsequently allow the development of a normal embryo. Therefore, gamete quality has received increasing attention and many studies have been made related to egg or sperm quality of different species (e.g. Bobe and Labbé 2010; Corporeau et al. 2012; Galbraith and Vaughn 2009; Lango-Reynoso et al. 2000; Lonergan et al. 2003; Rime et al. 2004; Suquet et al. 2010). Gamete quality is influenced by many factors explaining most often its variability. In marine species such as bivalves and fishes, the main factors that

apparently affect gamete quality are nutrition (availability of food, algal consumption and essential fatty acids), abiotic environmental factors (temperature, photoperiod and salinity), husbandry practices (spawning induction, egg postovulatory ageing and gamete handling post-stripping), stress and pollutants (e.g. Angel-Dapa et al. 2010; Bobe and Labbé 2010; Cannuel and Beninger 2005; Delgado and Pérez Camacho 2005; Galbraith and Vaughn 2009; Migaud et al. 2013) as well as genetic factors (Brooks et al. 1997). Considering these influences, the origin of different populations have to be considered in hatchery operations (Devauchelle and Mingant 1991; Lannan 1980), being a great study interest in a context of improvement of aquaculture production.

In terms of predictors of oocyte quality in bivalves, several criteria were tested, such as gonad color (Mason 1958) and mean oocytes diameter, as a direct consequence of nutrition (Cannuel and Beninger 2005). Likewise, oocyte organic matter and lipid content are important predictors since a significant relationship was observed between these biochemical parameters and broodstock condition index and hatching rate (Cannuel and Beninger 2005; Massapina et al. 1999). Indeed, most articles have focused on vitelline reserves (lipids, proteins, mRNA, vitamins) required to embryo and larval development. Finally, the egg size and shape was also reported, with large eggs surviving better than small eggs (Baynes and Howell 1996; Kraeuter et al. 1981). However, as these indicators appear not consistently to correlate with 'quality' in all species, it remains that the most reliable indicators used by hatcheries are fertilization success, hatching rate and survival, three criteria of interest in aquaculture (Migaud et al. 2013). Gamete quality was determined in *C. gigas* based on D-larval yields (Corporeau et al. 2012; Massapina et al. 1999). Alternatively, time to settlement was also considered as an oocyte quality marker in *C. gigas* (Cannuel and Beninger 2005).

### 2.1.3. Larval development, settlement and metamorphosis

*R. decussatus* fertilized eggs are incubated at 20 °C in conical tanks with filtered and ultraviolet treated seawater with slight aeration. Embryo stocking densities are of 100,000 per liter. After 48h of incubation, newly developed D-shaped larvae are drained to a sieve battery and then measured for shell length and survival evaluation. The water is changed every 2 days, using 1-µm sand-filtered, ultraviolet

sterilized seawater. After these operations larvae are transferred to a tank at a larval density of 10,000 larvae per liter (da Costa et al. 2012; Joaquim et al. 2008b). Larvae are now at the stage where they need feeding with unicellular cultured microalgae. Prior to this time, energy for respiration and development was derived from reserves laid down during egg development (oogenesis) by the maturing females. Bivalve larvae swim freely in the water column for much of the larval phase. As they develop towards the end of the larval phase, less food is consumed, and larvae spend an increasing period on the bottom of the tank (Matias 2013). This occurrence marks the beginning of metamorphosis. Larval culture ends when over 50% of the population is newly-settled post larvae that are transferred to containers for settlement (da Costa et al. 2012).

#### **2.1.4. Post-larvae and juvenile**

Post larvae from settling size to approximately 2 mm shell-height are reared in flow-through system indoor conditions and fed with cultured microalgae (Matias 2013).

Thereafter seed is transferred outdoors into the natural environment until it reach saleable size for future stages of farming (Fig. 10) (Helm and Bourne 2004). The main objective is to rapidly grow seeds to commercial size in order to make the operation economically attractive. Afterwards it is usual to consider a growth phase, from March to October, and a resting growth phase, from November until February, depending on the hydrological conditions at the precise location of the ground plot (Matias 2007). Clams are usually left to grow in the ground plots for 1.5 to 2 years after transplantation, depending on the initial size of the seed. Ground plots are maintained and cleaned during this on-growing phase. Ground plots are harvested periodically, in a rotation system, in order to have two intense periods of harvest: winter (December and January) and summer (July, August and September). The common technique for harvesting the clams consists on digging and tilling the sediment with a modified knife with a large blade (Fig. 11) (Matias 2013).



Figure 10 - Clam farmer seeding in Ria Formosa Lagoon. (Copyrights: Carlos Sousa, 2005).



Figure 11 - Clam farmers harvesting in Ria Formosa Lagoon. (Copyrights: Carlos Sousa, 2005).



### III. Genomics in marine bivalves

In order to solve certain issues that have not been elucidated using “standard” approaches, molecular biology and genomics can bring significant progresses.

The study of marine bivalves by genomic approaches is very recent and it has been developing mainly due to the recent advances in sequencing technologies and the substantial reduction in the associated costs (Suárez-Ulloa et al. 2013). The economic importance of some marine bivalves has motivated a great deal of biological research, which provides the most direct scientific rationales for developing genomic resources and tools. Strong rationales for studying the clam genome also come from its phylogenetic position in the Lophotrochozoa, an understudied clade of bilateral animals. Bivalves play an important role in estuarine and coastal marine habitats, where the majority of humans live. In these ecosystems, environmental degradation is substantial, and bivalves suffer intense mortalities from disease and stress. The most immediate applications of a wide variety of sequences and genomic tools from marine bivalves fall under three headings: (1) functional genomics, in which bivalves permit a phylogenetic contrast in studies of genome function, and diversity, (2) comparative and evolutionary genomics, in which bivalves belonging to the Lophotrochozoa help to shed new lights on mechanisms of evolutionary biology, speciation in the sea and the evolution of sexuality, and (3) environmental genomics, in which bivalves are a model for understanding the adaptation through genetic and physiological bases of complex traits (*e.g.* growth, reproduction and survival) that are strongly impacted by environmental change and stresses, and of great interest for aquaculture purpose. However, the characterization of bivalve genomes is still in progress given the lack of reference assemblies as well as the presence of specific sequence features such as high density of repetitive regions and very high levels of polymorphism (Suárez-Ulloa et al. 2013), with the only bivalve genome considered complete belonging to the Pacific oyster *C. gigas* (Zhang et al. 2012). Although the great commercial and biological importance of bivalves, their genome size (*C. gigas* genome is approximately 558 Mb in size) and repetitive organization of the non-coding fraction represents the main difficulties for their *de novo* sequencing and for the efficient assembly of these repeated regions (Suárez-Ulloa et al. 2013). Indeed, a ~40-fold and a ~100-fold sequencing coverage was needed to produce the draft

genome of the pearl oyster *Pinctada fucata* (Takeuchi et al. 2012) and the complete genome of *C. gigas* (Zhang et al. 2012), respectively.

The development of cDNA and Suppression-subtractive hybridisation (SSH) libraries has led to a significant increase in the number of Expressed Sequence Tags (ESTs) in databases, constituting powerful tools for the study of differential gene expression and identification of genes involved in specific biological functions, constituting the basis for DNA microarray technology (Romero et al. 2012). Nowadays, the combination of microarray and NGS technologies is significantly speeding up *de novo* gene discovery and microarray design, allowing transcriptomic analyses of, for example, hemocytes, gills, mantle and gonad of several bivalve species (Suárez-Ulloa et al. 2013). Repositories such as the Mytibase (Venier et al. 2009) represent useful resources for the transcriptomic study of the mussel *Mytilus*, providing large-scale ESTs with critical relevance for developing microarray platforms aimed to the biomonitoring of marine pollution. ESTs have been also put together for other bivalve species such as the clams *R. philippinarum* in the RuphiBase, where a database consisting of 32,606 unique transcripts was constructed (Milan et al. 2011), *Chamelea gallina* in the ChamaleaBase (Coppe et al. 2012), the mussel *Bathymodiolus azoricus* in the DeepSeaVent database (Egas et al. 2012), as well as the Pacific oyster *C. gigas* in the GigasDatabase (Fleury et al. 2009). In *R. decussatus*, before Reproseed project, only 4667 ESTs were available in NCBI.

Sequencing, notably under experimental design dedicated to RNAseq approach, and data mining of ESTs are essential steps for the comparative identification of molecules and related pathways of response to specific stimuli. This task is greatly facilitated by the availability of high throughput sequencing technologies yielding unprecedented amounts of sequence data.

### **3.1. Genomic approaches in reproduction**

For complex traits, as reproductive features, molecular biology and genomics are powerful tools in order to answer complex questions. For example, NGS technique was used successfully in rainbow trout to identify a master sex-determining gene, sdY, for sexually dimorphic on the Y chromosome, based on the assumption that such a gene would be expressed in a specific fashion during early testicular differentiation (Yano et al. 2012).

The first time that the expression of several specific genes, involved in reproduction mechanisms, was described at the molecular level in a marine bivalve, was in the scallop *Argopecten purpuratus* (Boutet et al. 2008). A cDNA library was made from gonad tissue from where after sequence analysis, 7 genes involved in reproduction mechanisms were characterized. Some of them showed a sex- and maturation-specific expression pattern that helped studying the mechanisms of induction of maturation of gonad in this commercially important mollusk. Later, Banni et al. (2011), through a medium-density cDNA microarray, described the temporal variation in gene expression patterns in sexes and gonadal development stages in *M. galloprovincialis*. The data showed a clear temporal pattern in transcriptomic profiles of mussels sampled over an annual cycle, establishing gene expression rhythms that authors would thereafter test in response to thermal variation, food availability and reproductive features across months.

In *C. gigas*, the gonad transcriptome was described and lists of genes of interest specific to each reproductive stage and sex were established. This work by Dheilly et al. (2012) showed a significant divergence in gene expression patterns of males and females coinciding with the start of gonial mitosis. By comparing the transcriptome of stripped oocytes and somatic tissues, the expression of genes was localized in either germ cells or somatic cells of the mature gonad helping at knowledge molecular in this complex gonadic tissue wich is a mixture of germ cells, storage tissue, smooth muscle fibers and circulating hemocytes surrounding the digestive system. This work provided new highly valuable information on genes specifically expressed by mature spermatozoids and mature oocytes, such as female-specific genes including foxL2, defining relevant candidates for further functional studies.

As written before, the recently released draft genome of the pearl oyster *P. fucata* (Takeuchi et al. 2012) provided a novel and powerful platform for obtaining structural information on the genes and proteins involved in bivalve reproduction. In this context the pearl oyster draft genome was analysed to screen reproduction-related genes (Matsumoto et al. 2013). As a result, more than 40 gene models were identified with high accuracy, including vasa, nanos, doublesex- and mab-3-related transcription factor, 5-hydroxytryptamine (5-HT) receptors, vitellogenin, estrogen receptor, and others.

Moreover, genes expressed during spermatogenesis in the functional hermaphrodite scallop *Nodipecten subnodosus*, were identified (Llera-Herrera et al. 2013). A large number of genes for which the annotation suggested their involvement in different steps of the spermatogenesis process were isolated and annotated, including checkpoint genes (*rad1*, *hormad1*, *dtl/cdt2*), important to understand meiosis blockage associated with total sterility in triploid *N. subnodosus*, and sex-differentiation genes with one of the most conserved genes at the bottom of the sex-determination cascade (*dmrt1*) in this functional hermaphrodite *Pectinidae* species. A number of genes from transposable elements and microsatellite loci were also discovered, evidencing that transposition is an active process during spermatogenesis in *N. subnodosus* (Llera-Herrera et al. 2013). Actually, in marine molluscs displaying hermaphroditic features, data on sexual determinism and differentiation including molecular sex determining cascade are scarce. In order to overcome this gap, in a recent study, a gonad transcriptomic analysis of the blacklip pearl oyster *Pinctada margaritifera*, a protandrous hermaphrodite species, was performed, allowing identifying potential markers of sex and gametogenesis useful for future researches on reproduction. The study also put in relief the importance of *dmrt1*, *fem-1* and *foxl2* as starting points for further functional studies on sex determining cascade in the pearl oyster (Teaniniuraitemoana et al. submitted). Finally, in *R. philippinarum* some genes potentially involved in sex determination were also found. Three transcripts (*psa*, *birc*, and *anubl1*) with specific sex and family biases in this species were analysed and their localization in gametogenic cells was confirmed by *in situ* hybridization (Milani et al. 2013).

### **3.2. Genomic approaches in other mechanisms: Environmental factors**

Physical environmental factors, such as prolonged periods of high/low temperatures, extreme salinities and industrial and domestic pollution as well as the increase in bacterial and viral pathogens in seawater leads to mortality at each developmental stage (Romero et al. 2012). When the genomic approach was first initiated in bivalves, the focus was on the effects of environmental pollution (Romero et al. 2012). Compounds present in the sea as a direct consequence of human activities still represent an issue of great concern, especially in heavily anthropized

coastal regions. These compounds include pesticides and drugs, polycyclic aromatic compounds, heavy metals and many other chemicals resulting from industrial and urban settlements, conveyed in wastewaters and rivers and finally entering the oceans (Suárez-Ulloa et al. 2013). The development of studies based on genomic approaches could improve the evaluation of the impact of most chemical compounds on marine organisms and communities, and provide a more robust basis for biomonitoring programs (Suárez-Ulloa et al. 2013). Marine bivalves of the genus *Mytilus* are intertidal filter-feeders commonly used as biosensors of coastal pollution. Due to their relatively tolerance to a wide range of environmental changes, they are often used as sentinels in ecotoxicological investigations (Venier et al. 2011). For this reason, microarrays have been primarily used in mussels to study the large-scale transcriptional response to different environmental stress factors like okadaic acid exposure (Manfrin et al. 2010), sublethal concentrations of mercury (Dondero et al. 2006), mixtures of heavy metals or organic contaminants (Venier et al. 2006), heat stress (Lockwood et al. 2010) and salinity stress (Lockwood and Somero 2011). Other example of transcriptomic approaches to the study of anthropic compounds includes the expression profile of the hepatopancreas (digestive gland) of *R. philippinarum* by using an Agilent Oligo Microarray platform (Milan et al. 2013a). In this study, Manila clams were collected in different periods of the year and in distinct areas of the Venice Lagoon, characterized by different levels of pollutants, in order to investigate the effects of seasonality and water pollution on the digestive gland transcriptome (Milan et al. 2013a). In the same species, the effect of the pharmaceutical ibuprofen was also tested using microarrays. This class of environmental contaminants is also continuously entering aquatic environments and this genomic approach was able to suggest that ibuprofen can interfere with various signaling pathways in clams, such as arachidonic acid metabolism, apoptosis, peroxisomal proliferator-activated receptors and nuclear factor-kappa B, helping the elucidation of the putative mechanisms of action of ibuprofen in non-models species (Milan et al. 2013b).

In bivalves, most of genomic studies have focused on analyzing the immune system with the aim of identifying the molecular basis of the most common pathologies (Saavedra et al. 2009). The high mortality of bivalves in the larval stage, juveniles and adults is a main problem in bivalve cultures. Mortalities decrease the global bivalve population and cause dramatic economic losses (Romero et al. 2012).

Thus, an understanding of the interactions among different biotic and abiotic factors influencing survival is a high priority for bivalve aquaculture. A global analysis of transcriptome responses to different conditions, offers unprecedented opportunities to achieve this objective. Indeed, transcriptomic studies have helped identify specific groups of genes involved in the response and adaptation of bivalves to external conditions (Suárez-Ulloa et al. 2013). In this way, the new genomic technologies clearly “open the door” for the directed manipulation of bivalves to improve the modern intensive aquaculture systems (Romero et al. 2012).

In mussel, the first systematic production and annotation of ESTs was performed in *M. galloprovincialis* by Venier et al. (2003). These ESTs were combined with other cDNA and SSH libraries (Pallavicini et al. 2008) from bacteria-stimulated mussels. Venier et al. (2009), constructed, sequenced and annotated 17 cDNA libraries from different Mediterranean mussel tissues challenged with toxic pollutants, temperature variations and potentially pathogenic bacteria, representing a valuable platform for expression studies, marker validation and genetic linkage analysis for investigations in the biology of Mediterranean mussels. In the same species, Craft et al. (2010) pyrosequenced expressed genetic transcripts from both male and female mussel tissues, generating novel observations on expression of different tissues, mitochondria and associated microorganisms.

In the oyster, the first published library was constructed in *C. virginica* with the aim of identifying genes to be used as bioindicators of exposure to environmental pollutants, toxins and infectious agents (Jenny et al. 2002). Gueguen et al. (2003) performed a similar study in *C. gigas* and described genes involved in defense mechanisms after exposure to four pathogenic strains of *Vibrio* sp. This approach allowed the characterization of sequences that have potential immune function. Later, in order to understand why the protozoan pathogen *Perkinsus marinus* provokes a lethal disease in *C. virginica* but not in *C. gigas*, the SSH method was employed to characterize genes up-regulated during parasite challenge in both hemocytes and gills from these two species (Tanguy et al. 2004), highlighting some differences in gene expression in response to this infection.

In clams, the first cDNA library was generated in *R. philippinarum* infected with *Perkinsus olseni* with 1,850 clones sequenced and 29 ESTs shown to be related to immune genes (Kang et al. 2006). SSH libraries have also been constructed in *R. decussatus*, one after bacterial stimulation of the clams (Gestal et al. 2007) and a

second one after *Perkinsus olseni* infection (Prado-Alvarez et al. 2009). Finally, Tanguy et al. (2008) reported 124 contigs and 1,814 singletons for *R. decussatus*, including immune genes with homologies to lectins and ferritins.

The construction of several libraries described has led to an increase in the number of ESTs in databases, essential to design probes in microarrays. In oysters, the first cDNA microarray produced contained 4460 sequences derived from *C. virginica* and 2320 from *C. gigas* (Jenny et al. 2007) and was applied to study the response of selected families of *C. gigas* to heat stress challenge (Lang et al. 2009) and hypoxia (Sussarellu et al. 2010). Additionally, several studies suggested that genes involved in defense and innate immune response play an essential role as determinants of the resistance to summer mortality in *C. gigas* (Chaney and Gracey 2011; Fleury et al. 2010; Fleury and Huvet 2012; Huvet et al. 2004). In the same species, an oligo-microarray covering a total of 31,918 contig sequences was constructed to survey variation in gene expression across multiple oyster tissues, providing experimental evidence for gene function assignments and identifying gene clusters related to tissue-specific processes (Dheilly et al. 2011).

For *R. decussatus* and *R. philippinarum*, ESTs resources available in public databases were examined in order to increase the knowledge on genes related with the immune function in these animals. As a result, 13 immune-related ESTs were selected and studied to compare the immune response of both species following a *Vibrio alginolyticus* challenge, where the immune response seemed to be faster in *R. philippinarum* than that in *R. decussatus* (Moreira et al. 2012). Recently, the next-generation sequencing technologies have been applied to the study of different processes in molluscs. In bivalves, this technology has been applied to investigate different physiological processes, such as shell deposition and repair in the Antarctic clam *Laternula elliptica* (Clark et al. 2010) and biomineralisation in *Pinctada margaritifera* (Joubert et al. 2010). The developmental process has been analysed at different larval stages in the clam *Meretrix meretrix* (Huan et al. 2012). In an attempt to identify genes potentially involved in bivalve innate immunity, high-throughput sequencing was performed in the deep-sea hydrothermal vent mussel *Bathymodiolus azoricus* (Bettencourt et al. 2010) and in *R. philippinarum* using in vitro immune-stimulated hemocytes (Moreira et al. 2012), producing more than 700,000 sequencing reads.



# **Aims and outlines of the thesis**

The production of bivalve molluscs is a strategic activity since it contributes significantly to the preservation of local economies and generates capital and employment on the littoral areas (Matias 2013). The increasing market demand, the unpredictability of bivalve production and the concurrent impoverishment of the wild sets generated a great interest in hatchery-produced, a solution which could meet spat requirements in the bivalve industry, and could be applicable to the production of high unit value species such as clams, oysters and scallops. Noteworthy, seed supply is already crucial for some species like the hard clam *Mercenaria mercenaria*, for which farmers have to rely entirely on hatcheries, because unlike most bivalves, large quantities of their seed cannot be easily harvested in the wild (FAO 2013). Other species such as *C. gigas*, *R. philippinarum* and *Argopecten irradians* are routinely produced in hatcheries throughout the world and rely only partially on wild spatfall. While hatchery technology is relatively well developed for these species, other bivalves such as the European clam *R. decussatus*, *Pecten maximus*, *Placopecten magellanicus* and *Ostrea edulis* proved more difficult to rear a routine basis (Pauletto 2014).

In Portugal, the production of bivalves is one of the most important social and economic activities, with a great growing potential as a fisheries subsector, due to the edaphic-climatic and geographic conditions. Artisanal production of bivalve molluscs is mainly based on the culture of the European clam, *R. decussatus* and oysters (*Crassostrea* spp) (Matias 2013). The European clam production still relies on the collection of wild seed. Although this traditional practice has allowed the development of *R. decussatus* production, seed collection can have a negative environmental impact or be a limiting factor for the development of this activity. Indeed, since the two last decades, the European clam production has suffered a decrease due to several constrains, namely recruitment failures, excessive pressure on the capture of juvenile on natural banks, and abnormal mortalities associated to environmental degradation and pathogens (e.g. *Perkinsus olseni*) (Matias 2013).

To address this situation in *R. decussatus*, artificial spawning and larval rearing programs were developed to provide an alternative source of seed (Matias et al. 2009), but still need improvement to gain robustness of seed production. The first and crucial step is to know and manipulate the natural gonadal cycle and spawning period of the clams so that adults can be induced to spawn earlier or later than in the natural environment (Ojea et al. 2008). Despite the on-growing know-how in bivalves

hatchery, some biological aspects remain unknown or poorly understood, such as bivalve feeding requirements, being crucial during broodstock conditioning for successful reproduction and emphasizing the larval stages the most critical stages in the life cycle of bivalves (Helm and Bourne 2004; Rico-Villa et al. 2006).

Production of *R. decussatus* seed in hatcheries is a relatively new industry for which most methods have been developed using empirical approaches, adapting methods across species and measuring the resulting effects in terms of growth, reproduction and survival. Hatchery production is still affected by several limitations, which are often species specific and are encountered at different stages in the biological cycle of molluscs. The main bottlenecks involve (i) broodstock management and gamete quality, (ii) appropriate methods for larval rearing, (iii) metamorphosis synchronisation and improvement of settlement, (iv) quality of seed in terms of immunity, genetic diversity and sanitary status (Pauletto 2014).

Applied research on biochemistry and physiology could allow a better understanding of the effect of biotic (micro-algal diet, bacterial communities, etc.) and abiotic (temperature, salinity, etc.) factors (e.g. Magnesen et al. 2006; Nicolas et al. 2004). However, most of these studies only had a limited impact on the bivalve industry. Recent resources that are expected to affect the bivalve aquaculture in the coming years are those based on “omic” studies. In the last decade, it has been demonstrated that “omic” characterization can be of great help for the identification of the genes and proteins which are involved in different processes of interest. Such approaches are already known to be very powerful in aquaculture fish species to control and improve reproduction, by studying the impact on the egg transcriptome of several manipulations performed to control the timing of ovulation or ovulatory process induction (Bonnet et al., 2007). However, until now in bivalves, these applications are mostly notable for pollution biomonitoring (Suárez-Ulloa et al. 2013). Indeed, these promising resources have been poorly exploited in the “emerging bivalve species” like *decussatus*, where hatchery based production of seed is still limited. Thus, the purpose of the present thesis is to improve the knowledge on the reproduction of the European clam *R. decussatus* through cellular and genomic approaches, with two major applications: broodstock conditioning and gamete quality, aiming to answer two major questions: What are the processes affecting gamete quality and what are the mechanisms and major factors involved at each stage of their maturation?

This work will be divided in three main chapters:

- **Chapter 1** – Comparison of the natural reproductive cycle of two Portuguese populations of *Ruditapes decussatus*: Ria de Aveiro and Ria Formosa Lagoon.
- **Chapter 2** – A Microarray-Based Analysis of Gametogenesis in Two Portuguese Populations of the European Clam *Ruditapes decussatus*.
- **Chapter 3** – A microarray-based analysis of oocyte quality in the European clam *Ruditapes decussatus*.

# 1

## **“Comparison of the natural reproductive cycle of two Portuguese populations of *Ruditapes decussatus*: Ria de Aveiro and Ria Formosa Lagoon”<sup>1</sup>**

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<sup>1</sup> Part of this study is published in “Aquaculture” by Matias et al. (2013) (Appendix 1), in which I am co-author.

Due to the scarcity of information on *R. decussatus* production, the main objective of this chapter is the evaluation of the reproductive cycle of two Portuguese populations of the main production areas of this species in Portugal: Ria de Aveiro and Ria Formosa lagoons. Besides their importance, it was observed a difference in the spawning induction success between the two populations, with the southern clam's population (Ria Formosa) presenting the worst results, and consequently, making this study more opportune for the improvement of aquaculture production.

This evaluation was made through the comparison of the gonadal development, gonadal area and oocyte diameter of both populations, by histological analysis performed during 12 months (July 2011 - June 2012).

## I. Introduction

To be able to establish and improve rearing programs for *R. decussatus*, a detailed knowledge of the species natural reproductive cycle and spawning periods is crucial.

As reported before, two of the main production areas of this species in Portugal are the Ria de Aveiro (40°42'N; 08°40'W) in Western coast of Portugal and the Ria Formosa Lagoon (37° 01'N; 07° 49'W) in Southern Portugal. Broodstock conditioning experiments carried out in the experimental bivalve hatchery of the Portuguese Institute of Sea and Atmosphere (IPMA) in Tavira, Portugal, with these populations, have systematically shown more difficulties concerning the induction of artificial spawning in the southern clam's population (unpublished data). Indeed, further studies have suggested that the differences in gonadal cycles and conditioning optima in different populations have to be considered in hatchery operations (Devauchelle and Mingant 1991; Lannan 1980). Regarding the European clam, it has been reported that the ecotype *decussatus* living in different areas, even at the same latitude, could strongly differ in terms of their fecundity levels and biochemical compositions (Shafee and Daoudi 1991; Trigui-El Menif et al. 1995). These differences between populations are of great interest to study in a context of improvement of aquaculture production.

Previous works have compared the reproduction of *R. decussatus* from different populations in several locations. For instance in Greece, where the reproductive cycle of two natural populations were determined by monthly examination of gonads (Chryssanthakopoulou and Kaspiris 2005); in Tunisia, by describing and comparing the gametogenic activity *in situ* and in hatchery (Hamida et al. 2004b) and by comparing two different populations through gonadal index, fecundity and biochemical composition of eggs (Trigui-El Menif et al. 1995) and finally, in two Morocco lagoons, where aspects of gametogenesis were studied (Shafee and Daoudi 1991). All these studies suggested that the main differences between populations are mainly due to environmental aspects like salinity, temperature and chlorophyll. In Portuguese populations of this species, only the gametogenic cycle has been determined in former studies of Vilela (1950) and Pacheco et al. (1989).

Histological techniques provide general information on gonad development. The seasonal variations in the different stages of gametogenesis have been followed through histological examination allowing the identification of any phenomenon liable to affect reproductive activity in bivalves (Lango-Reynoso et al. 2000). Several researchers have correlated the developmental stages to certain cytological features generally recognized in all bivalve mollusks. Unfortunately, the determination of these stages is often subjective and there is little agreement in order to establish the number of stages that should be included in the classification scale (Barber and Blake 1991). Barber and Blake (1991) mentioned that the most complete approach would consist in applying at least two methods, each of them being either quantitative or qualitative. They also underlined that histology is always required to verify reproductive events concerning gamete development. Therefore, the present study aims to characterize the reproductive cycle of these two European clam populations, by comparing the gonadal development, gonadal area and oocyte diameter, with the help of image analysis and histology.

The information taken from this chapter will be important for the establishment of a successful hatchery-based production of *R. decussatus*.

## **I. Materials and Methods**

### **1.1. Sample collection**

Twenty clams were hand-collected, monthly, at the same location in Ria de Aveiro and Ria Formosa Lagoon, during 12 months (July 2011 - June 2012). Both areas are shallow water mesotidal lagoons with semidiurnal tidal regimes that constitute the major hydrodynamic forces (Mahowald et al. 2000) (Fig. 12). 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 (Falcao 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 of the lagoon is 3.2 in a spring tide and 1.0 in a neap tide. The freshwater

inputs are almost negligible and salinity remains close to 36 all year long (Águas 1986; Falcao and Vale 1990). 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 (Mahowald et al. 2000). This lagoon has an important freshwater input coming from the Vouga and the Antuã rivers (Mahowald et al. 2000; Moreira et al. 1993) and salinity ranged between 31 and 36. These two ecosystems are currently used for clam production and fish aquaculture ponds.

In the laboratory, clams were placed in 0, 45 µm-filtered seawater at 20 °C for 24 h to purge their stomachs before histological analyses.

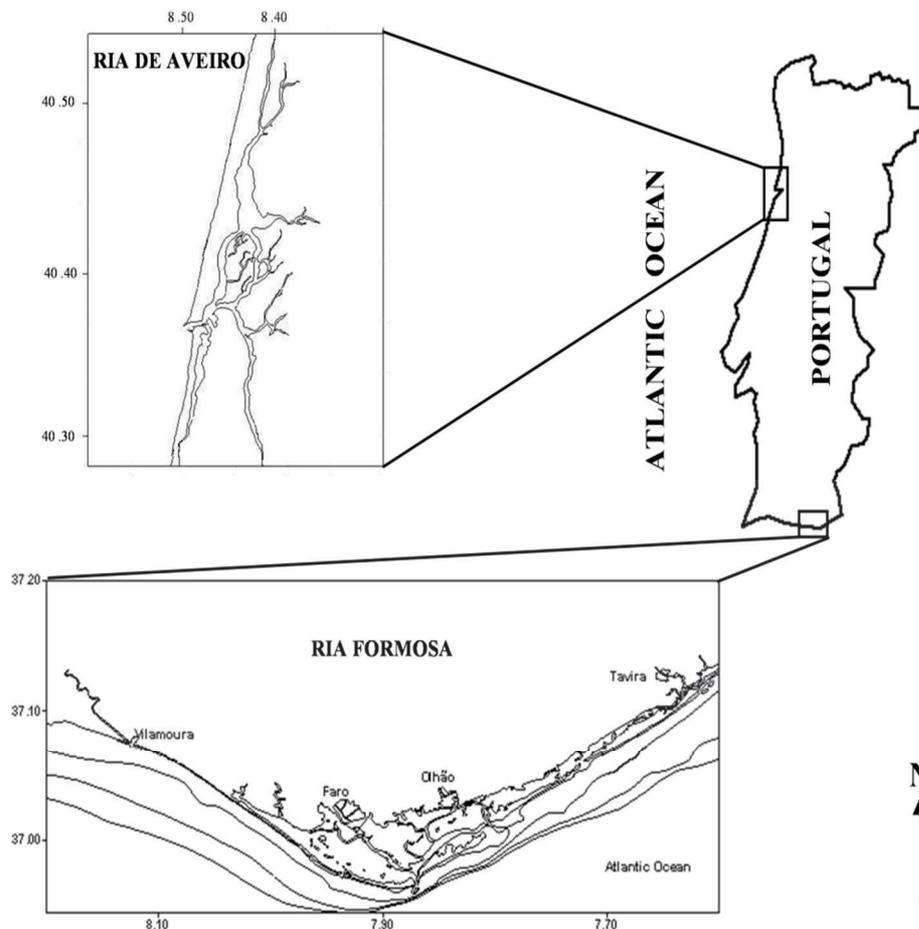


Figure 12 - Ria de Aveiro and Ria Formosa Lagoon location.

## 1.2. Histology

At every month, between July 2011 and June 2012, the gonads of twenty clams collected from each population were immediately dissected and a 3-mm transversal section of the visceral mass of each sample was excised using a microtome cutter in front of the pericardic region (Fig.13) and immediately fixed in 10 to 20 ml modified Davidson's solution (Latendresse et al. 2002) at 4°C for 48 h. After, the modified Davidson's solution was discarded and 10 to 20 ml 70% ethanol were added and kept at 4°C. Sections were dehydrated in ascending ethanol solutions, cleared with a xylene substitute named Microclearing (Diapath, Italy), and embedded in paraffin wax. Sections of 5 mm were cut, mounted on glass slides and stained with Harry's hematoxylin-Eosin Y (Martoja and Martoja-Pierson 1967). Slides were then examined under a light microscope and sex and stages were determined according to a scale development based on (Delgado and Pérez Camacho 2005) (Table 1). 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.

Percentage areas of gonadic tubules, connective tissue and digestive gland were then determined on each histological section. Slides were scanned with a digital scanner (hp scanjet 7400 c) and the images saved in \*.TIFF format. Tissue areas were then measured using image analysis software (Image J version 1.4.3.67). Gonadal area percentage was estimated as pixel number, from gonad / pixel number on total sections, as described in Fabioux et al. (2005).

In order to measure the oocytes diameter, histological sections were also examined under the microscope Olympus BX51 equipped with camera Olympus DP 71 using image analysis software (Cell B version 2.7). The surface of each oocyte was measured by drawing its perimeter on the computer screen to calculate an area in «pixels» (S) that was transformed into a theoretical diameter (D) expressed in  $\mu\text{m}$  using the relation  $D = \sqrt{(4S/\pi)}$  established from a previous calibration (Lango-Reynoso et al. 2000). The measurements were theoretical conducted only in the oocytes that displayed a well-defined germinal vesicle to ensure that each section passed through the center of the gamete. This operation was carried out on 100 randomly chosen

oocytes for each slide. Thus, the mean oocyte diameter corresponds to the average of the diameters measured on each female individual sampled.

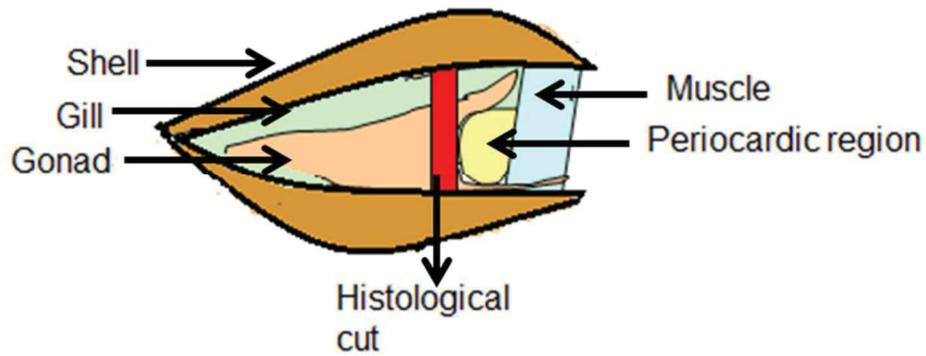


Figure 13 - The 3-mm cross section schema of the visceral mass for histological analysis.

**Chapter 1 - Comparison of the reproductive cycle of two Portuguese populations of *Ruditapes decussatus*: Ria de Aveiro and Ria Formosa Lagoon**

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Table 1 - Reproductive scale for *Ruditapes decussatus* adapted from Delgado and Pérez-Camacho (2005).

Stage	Histologic description
Period of sexual rest (phase I)	Gonadal follicles are absent and connective and muscular tissue occupies the entire zone from the digestive gland to foot. There is no evidence of gonadal development and sex determination is not possible.
Initiation of gametogenesis (phase II)	Follicles and gonadal acini begin to appear in females and males. They increase in size, and appear covered with oocytes in the growth phase in the females and with immature gametes (spermatogonia and spermatocytes) in the males.
Advanced gametogenesis (phase III)	The follicles occupy a large part of the visceral mass. The presence of muscular and connective tissue is reduced. At the end of this stage, characterised by intense cellular growth in females, the oocyte protrudes from the centre of the lumen, remaining attached to the wall via the peduncle. The abundance of free oocytes equals those attached to the wall of the follicle. In males, majority of the acini were full of spermatids and
Ripe (phase IV)	Corresponding to the maturity of the majority of gametes. In the mature oocytes the rupture of the peduncle occurs, and the oocytes consequently occupy the follicular interior. In males, the gonadal acini mainly contain spermatozoids.
Partially spawned (phase V)	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 (phase VI)	Abundant interfollicular connective tissue. Occasional residual sperm or oocytes resent.

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### 1.3. Data analysis

For comparisons of sex and gametogenic stage distributions between conditions, we used Fisher's exact test taking into account the small data sets.

Differences in percentage of gonadal area were analyzed using one-way analysis of variance (ANOVA) after angular transformation. In order to compare the mean of males' and females' gonadal area at each stage in both populations, Fisher's Least Significant Difference (LSD) test at a significance level of  $p=0.05$ , was used (STATGRAPHICS Centurion XV.II).

Differences in oocyte diameter were analyzed using one-way analysis of variance (ANOVA). Normality of the dataset was tested using *Shapiro–Wilk*. Since normality was not achieved, Student-Newman-Keuls test, at a significance level of  $p=0.05$ , was used to compare the mean of oocyte diameter of each population at each stage (STATGRAPHICS Centurion XV.II).

The Pearson correlation coefficient was used to determine the degree of association between parameters, at a significance level of  $p=0.05$  (STATGRAPHICS Centurion XV.II).

## II. Results

### 2.1. Gonadal development

The sexes were clearly separated and no hermaphrodites were found. Both sexes showed synchronism in gonadal development in each population, separately, with the evolution of the gametogenic stages occurring simultaneously (*vide* Matias et al. 2013 – Appendix 1). Additionally, the reproductive cycle of *R. decussatus* was characterized by a seasonal pattern in both populations, with a ripe stage in spring followed by spawning that began in late spring and extended during summer and a period of sexual rest beginning in early autumn and extended during winter, in both populations (Fig. 14).

Although the onset of the gametogenic cycle occurred in February 2012 for both populations, we observed that the maturation process began with a higher

number ( $p=8.3e-5$ ) of individuals in the northern population reaching stage II in this month (17 individuals in Ria de Aveiro against 2 individuals in Ria Formosa) (Fig. 15). The individuals from Ria de Aveiro Lagoon reached its peak of reproductive effort in March, with 17 individuals in stage III against 5 individuals from Ria Formosa) ( $p=0.0007$ ). Ria Formosa lagoon reached its peak of reproductive effort in May-June. Also spawning began earlier, in April ( $p=4.5e-6$ ), for the population of Ria de Aveiro, with 6 individuals in stage IV against 0 individuals in Ria Formosa at this stage. Spawning only began in May-June for Ria Formosa.

Nevertheless, during this period and in spite of the seasonal pattern, *R. decussatus* did not show a continuous gonad development since after spawning clams did not progress to an inactive stage. Indeed, in the microscopic examinations of the gonadal tissues, all clams showed simultaneous spawning and recovery of the gonad. So, we considered this stage of the reproductive cycle of *R. decussatus* as partially-spawned (stage V-Table 1). This phenomenon occurred for both males and females of the two populations (Fig. 16). In August the majority of the individuals of Ria de Aveiro Lagoon were already in stage VI contrasting with the Ria Formosa ones ( $p=0.008$ ). In September the majority of clams from both populations had already spawned, being in an inactive phase and remaining in this stage during approximately six months, until the next onset of gametogenesis.

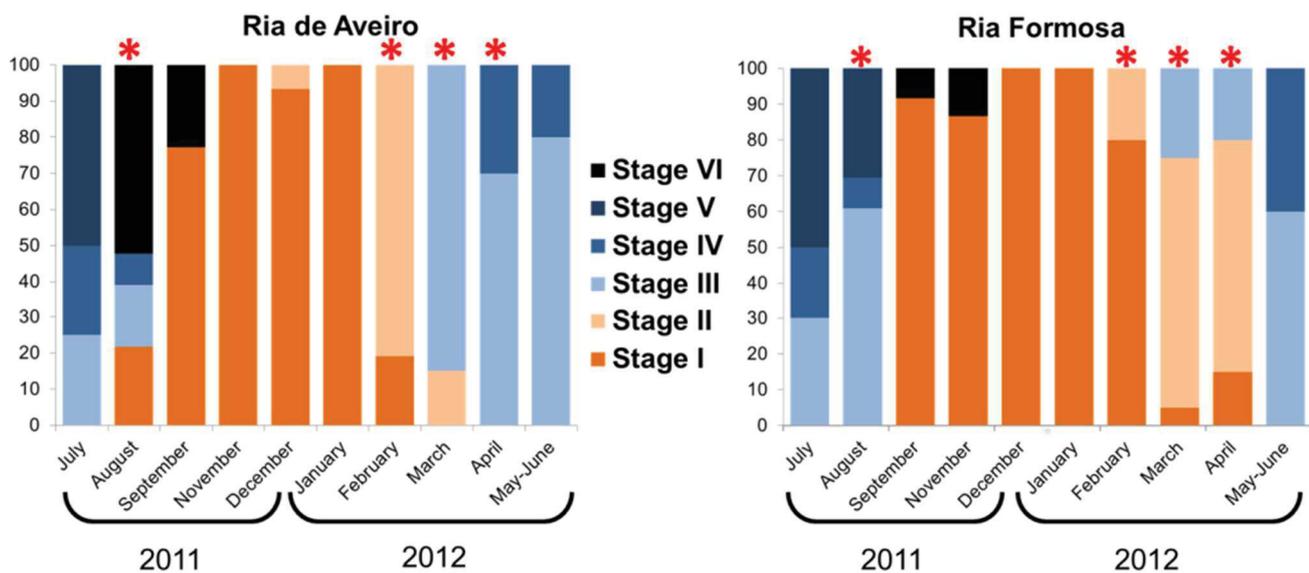


Figure 14 - Monthly variations in gonadal development of *Ruditapes decussatus* populations from Ria de Aveiro and Ria Formosa Lagoon, from July 2011 to June 2012. Significant differences between populations in the months of August (2011) and February, March and April (2012).

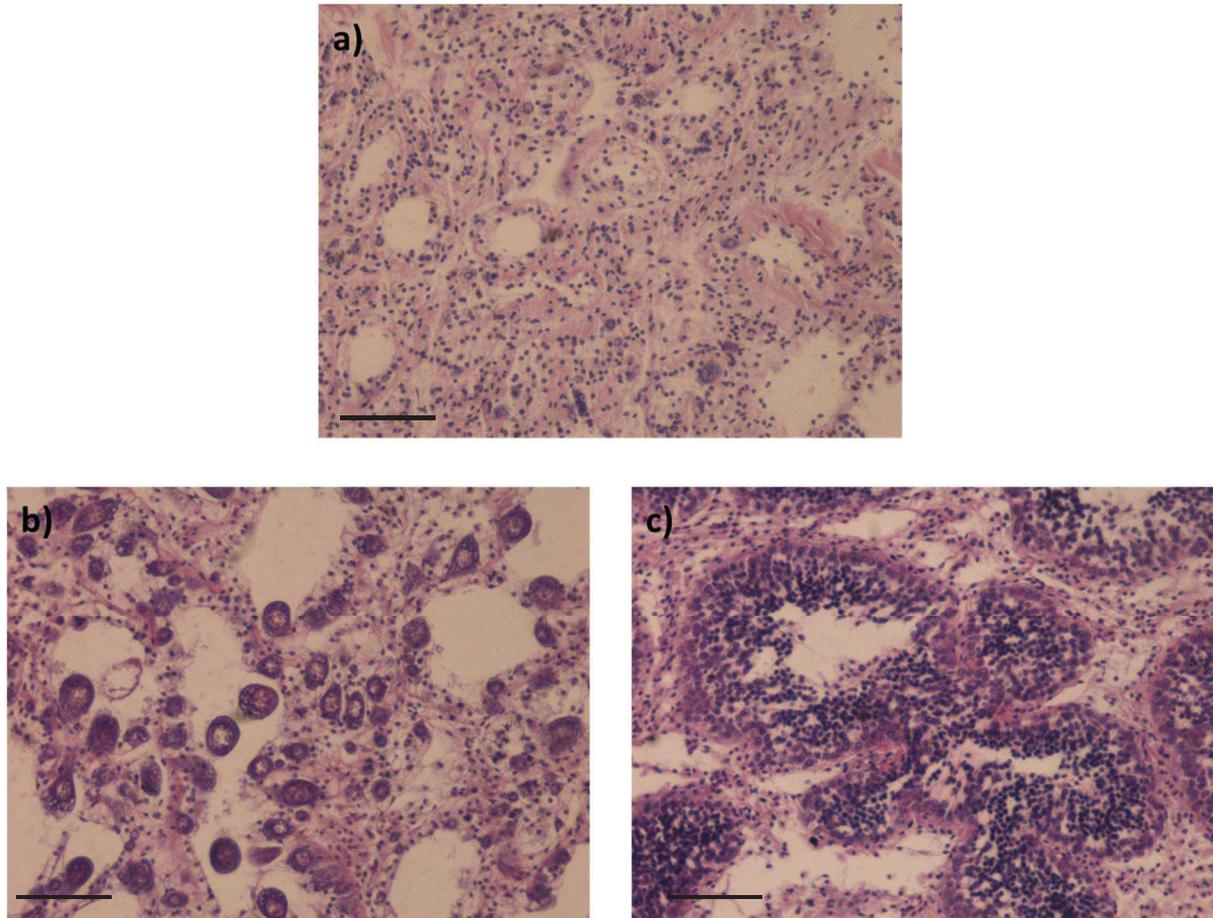


Figure 15 - Photomicrographs showing the development of *Ruditapes decussatus* gonad at the same month (February 2012) of a) Ria Formosa - stage I, b) Ria de Aveiro females - stage II and c) Ria de Aveiro males - stage II. Scale bar: 200  $\mu$ m.

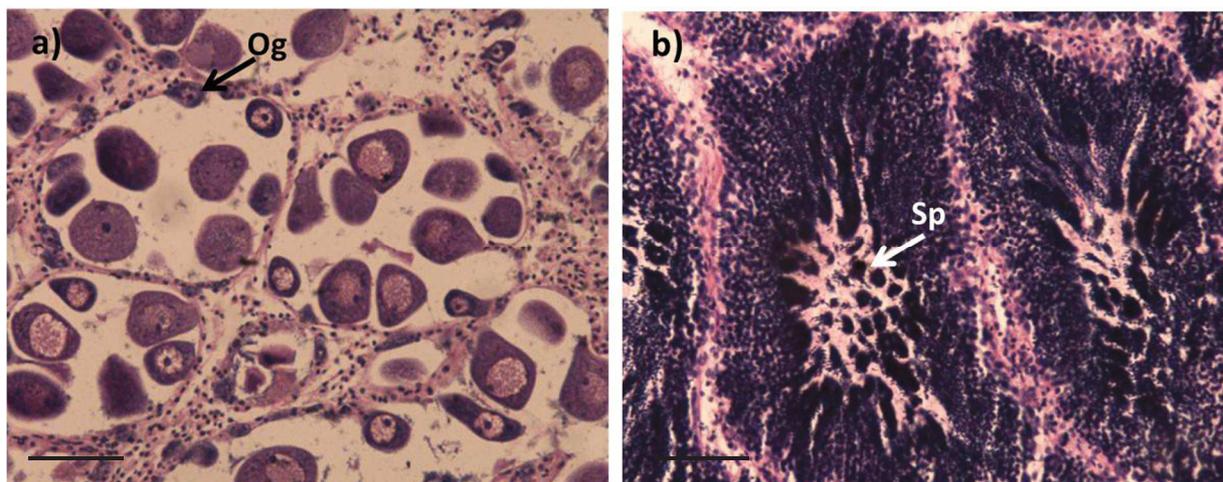


Figure 16 - Photomicrographs showing examples of partial spawning in a) females and b) males of *Ruditapes decussatus*. Og – Ovogonia. Sp – Spermatozoa. Scale bar: 200  $\mu$ m.

## 2.2. Gonadal area

In general, between the two populations, the gonadal area followed the same pattern as the gonadal development with no significant differences between populations, regardless of the sex and gametogenic stage. The two populations reached the highest values of gonadal area at stages III ( $33.7 \pm 7.5$  % for females and  $41.1 \pm 8.4$  % for males from Ria de Aveiro and  $25.4 \pm 4.6$  % for females and  $34.0 \pm 4.4$  % for males from Ria Formosa), IV ( $37.9 \pm 9.8$  % for females and  $48.2 \pm 7.4$  % for males from Ria de Aveiro and  $29.3 \pm 7.8$  % for females and  $43.2 \pm 7.2$  % for males from Ria Formosa) and V ( $36.4 \pm 17.7$  % for females and  $48.6 \pm 5.3$  % for males from Ria de Aveiro and  $28.4 \pm 6.5$  % for females and  $36.3 \pm 5.3$  % for males from Ria Formosa). On the other hand, the two populations reached the lowest values of gonadal area at the stage II ( $22.5 \pm 6.0$  % for females and  $14.5 \pm 4.1$  % for males from Ria de Aveiro and  $18.0 \pm 1.7$  % for females and  $20.8 \pm 4.8$  % for males from Ria Formosa) (Fig.17).

No significant differences in gonadal area were found between populations ( $P > 0.05$ ). The only differences observed were between gametogenic stages within each population ( $P < 0.05$ ). In the females from Ria Formosa population the main differences of gonadal area were observed between stage II ( $18.0 \pm 1.7$  %) and stages IV ( $29.3 \pm 7.8$  %) and V ( $28.4 \pm 6.5$  %), with a significant increase of the gonad. In the males the main differences of gonadal area were observed between stage II ( $20.8 \pm 4.8$  %) and stages III ( $34.0 \pm 4.4$  %), IV ( $43.2 \pm 7.2$  %) and V ( $36.3 \pm 5.3$  %). In the females from Ria de Aveiro population the differences of gonadal area were also observed between stage II ( $22.5 \pm 6.0$  %) and stages IV ( $37.9 \pm 9.8$  %) and V ( $36.4 \pm 17.7$  %). Finally, in the males the main differences of gonadal area were observed, as well, between stage II ( $14.5 \pm 4.1$  %) and stages III ( $41.1 \pm 8.4$  %), IV ( $48.2 \pm 7.4$  %) and V ( $48.6 \pm 5.3$  %).

In both populations and sexes the maximum of gonadal area was achieved at stage IV. On the other hand, and as expected, in both populations and sexes the minimum of gonadal area was achieved at stage II.

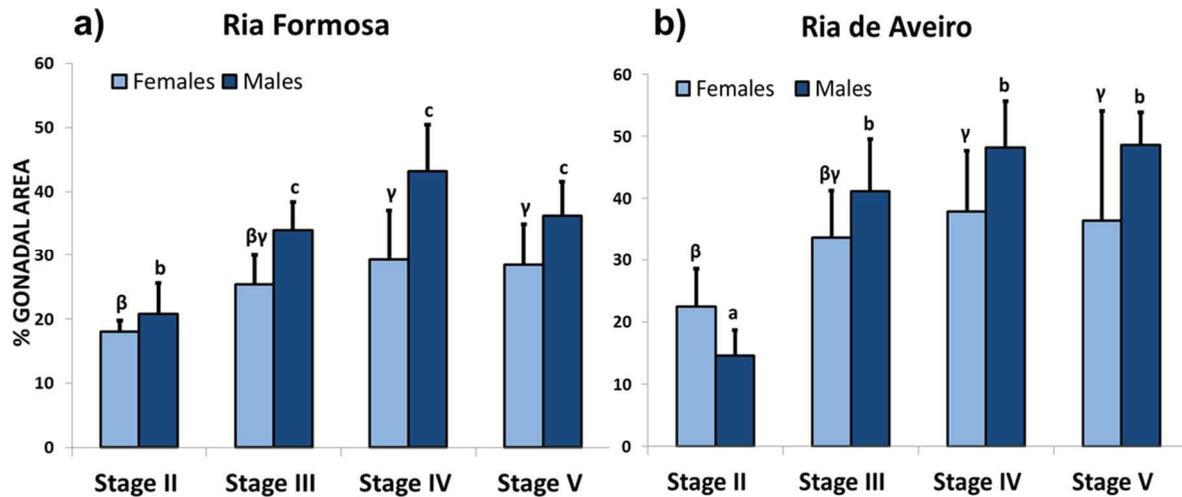


Figure 17 – Gametogenic stages variation in gonadal area of *Ruditapes decussatus* populations from a) Ria Formosa and b) Ria de Aveiro. In females from both populations significant differences were found between stages II and stages IV and V. In males from both populations significant differences were found between stages II and stages III, IV and V. Roman and Greek letters designate significant differences indicated by Fisher's Least Significant Difference (LSD) test, at a significance level of  $p=0.05$ .

### 2.3. Oocyte diameter

In terms of oocyte diameter no significant differences were found between populations ( $P>0.05$ ) (Fig.18). The proliferation of oocytes started at the beginning of gametogenesis (stage II) and continued to grow until stage IV and V when they reached their maturation. The only differences observed were between gametogenic stages ( $P<0.05$ ), with stage II ( $11.1\pm 1.7 \mu\text{m}$  for Ria Formosa and  $9.5\pm 2.3 \mu\text{m}$  for Ria de Aveiro) being significantly different from stage III ( $19.1\pm 1.2 \mu\text{m}$  for Ria Formosa and  $19.1\pm 1.5 \mu\text{m}$  for Ria de Aveiro). Although, between stages IV ( $26.3\pm 1.1 \mu\text{m}$  for Ria Formosa and  $27.6\pm 0.8 \mu\text{m}$  for Ria de Aveiro) and V ( $26.7\pm 1.5 \mu\text{m}$  for Ria Formosa and  $30.0\pm 1.8 \mu\text{m}$  for Ria de Aveiro), no significant differences were found.

The surface percentage occupied by the gonad was directly linked to the size of oocytes produced ( $P>0.05$ ) ( $R^2=0.98$  for Ria Formosa and  $R^2=0.95$  for Ria de Aveiro).

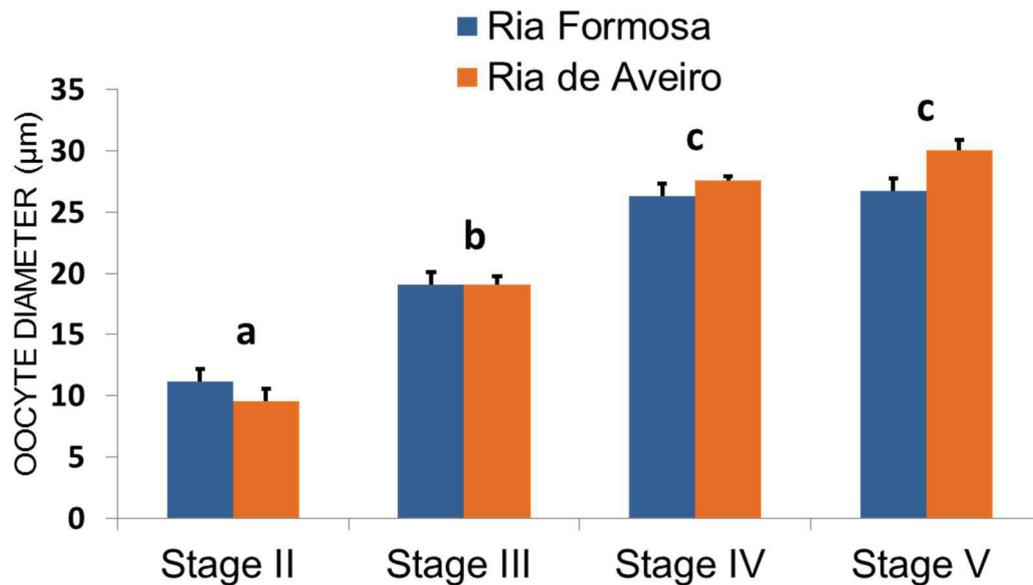


Figure 18 - Gametogenic stages variation in oocyte diameter of *Ruditapes decussatus* populations from Ria de Aveiro and Ria Formosa Lagoon. In both populations significant differences were found between stage II and stage III and between stage III and stages IV and V. Roman letters designate significant differences indicated by the Student-Newman-Keuls test, at a significance level of  $p=0.05$ .

### III. Discussion and Conclusion

The reproductive activity of bivalves, which includes a sequence of events from gametogenesis to spawning, is controlled by the interaction between endogenous and exogenous factors, mainly food availability and temperature (Camacho et al. 2003; Enríquez-Díaz et al. 2009; Fabioux et al. 2005; Normand et al. 2008). Temperature is closely linked to the geographical locations affecting indirectly the availability of food and/or consequently the timing and duration of the reproductive cycle and number of spawning per year. Indeed, for *R. decussatus* a different number of annual spawns has been described within Europe (da Costa et al. 2012). Seawater temperature and chlorophyll patterns in the two studied populations were reported by Matias et al. (2013) (Appendix 1). These two parameters were typical from temperate climates. In both locations, temperature increased during spring, stabilized in summer, decreased during autumn and remained low in winter. Also both lagoons were characterized by several phytoplanktonic blooms in spring/summer. Nevertheless, Ria de Aveiro was distinguished by relatively lower SST values (around less  $2.95 \pm 1.31$  °C) and higher chlorophyll values (around more  $3.49 \pm 3.37$  mg m<sup>-3</sup>) than Ria Formosa Lagoon (Matias et al. 2013). In addition, genetical effects can also influence the reproductive activity of bivalves (Boudry et al. 2002). Indeed, genetic diversity was also previously studied between Ria Formosa

and other Portuguese population of *R. decussatus*, Ria de Alvor. This genetic study was performed by Random Amplified Polymorphic DNA (RAPD), however, a higher variation within populations (65%) than between populations (35%) was observed, despite the different geographic origin (Pereira et al. 2011). The high levels of genetic variation within populations suggest that these bivalves are phenotypically plastic and may respond adequately to changing environments. A high degree of genetic homogeneity among populations has also been described for populations of other marine invertebrates, such as *Concholepas concholepas* (Gallardo and Carrasco 1996), *Ostrea chilensis* (Toro and Aguila 1996), *Venus antique* (Gallardo et al. 1998) and *Venerupis senegalensis*, coming from the same lagoons described in the present work (Joaquim et al. 2010).

The sequence of gametogenic stages showed that the reproductive cycle of these two populations of *R. decussatus* follow a seasonal cycle, as has been previously reported for this and several other bivalve species (e.g. Albentosa et al. 2007; Bayne 1976; Xie and Burnell 1994). Largely, the reproductive cycle of *R. decussatus*, from both studied populations, comprised a ripe stage in spring followed by spawning that began in late spring and extended during summer until early autumn in both populations. A similar reproductive cycle was described in the studies by Breber (1980) and Ojea et al. (2004) for populations of *R. decussatus* from Venice (Italy) and from Galicia (Spain), respectively. However, other authors have shown the occurrence of two major periods of spawning, in spring and then in summer or early autumn in different populations of this species (Borsa and Millet 1992; Chryssanthakopoulou and Kaspiris 2005; Laruelle et al. 1994; Shafee and Daoudi 1991), including in populations from the Ria Formosa Lagoon (Pacheco et al. 1989; Vilela 1950). The differences observed between populations have been frequently associated to the influence of the geographical location and consequently by the inherent environmental factors as salinity, temperature and chlorophyll abundance (da Costa et al. 2012). Despite a generally common reproductive cycle, some specific significant differences (in certain months) were observed between gonadal development in the two geographically distinct populations (between July 2011 and June 2012), with the maturation process beginning earlier in the northern population (Ria de Aveiro). These differences concerning the onset of the gametogenic cycle and the proliferation of gonias were probably associated to the

low levels of chlorophyll observed in Ria Formosa, especially from spring to summer months (*vide* Matias et al. 2013 – Appendix 1).

Beside the fact that the seasonal pattern presented a spawning period well-defined in time, clams of the two studied populations exhibited an advantageous reproductive strategy for the species ensuring a consistent supply of gametes during the whole spawning period. Indeed, histological analyses showed simultaneously gonias, maturing gametocytes and variable proportions of fully matured gametes in the same individual, both in males and females. This high capacity for gonadal regeneration had also been previously observed in *Venerupis senegalensis* from Ria de Aveiro (Joaquim et al. 2011). Nevertheless, and despite the intra-individual asynchrony, the maintenance of a synchronized gonadal development observed between males and females ensures the reproductive success of the species since sperm and oocytes will be expelled into the water column simultaneously during the spawning period, increasing the probability of fertilization. This synchronism had previously been reported by Laruelle et al. (1994) and Ojea et al. (2004) for this species. Both populations showed a long reproductive rest phase that was extended by a period of approximately six months, during autumn and winter. The onset of the gametogenic cycle with the proliferation of gonias seems to be associated with the rise of SST and chlorophyll in late winter/early spring for both populations and the development of gametes intensified quickly until the attainment of the ripe stage late spring (Matias 2013). These results were generally consistent with the previous findings by Chryssanthakopoulou and Kaspiris (2005) and Shafee and Daoudi (1991) in other populations of this species, although, as previously mentioned, these authors reported another onset of gametogenesis in July/August.

Previous studies showed that the gonadal area is representative of reproductive effort in the oyster *C. gigas* (Royer et al. 2008). Indeed, when examining the median part of the visceral mass, the percentage of surface occupied by the gonad is directly linked to the number and/or size of gametes produced, being representative of reproductive effort which corresponds to the proportion of energy allocated to reproduction (Normand et al. 2009; Todd and Havenhand 1983). In the present study, both gonadal area and oocyte diameter followed the same pattern as the gonadal development with their progression beginning at stage II in late winter/early spring, reaching its maximum size at stages IV and V during spring, with

no differences between the two studied populations. In terms of evolution of oocyte diameter, other studies showed similar results in this species (Hamida et al. 2004b; Smaoui-Damak et al. 2006; Delgado and Pérez-Camacho 2007), although with greater diameter values. Indeed, oocyte size is a highly variable trait both within and among species of marine invertebrates, being highly influenced by environmental factors, such as temperature, salinity, and toxicants (Moran and McAlister 2009). The similar values of both gonadal area and oocyte diameter at stages IV and V confirm that *R. decussatus* has a partial spawning period, during which occurs successive and simultaneous production of gametes and spawning.

Even if a little difference between populations were observed, it would be interesting to produce progenies from the two parental populations, Ria Formosa and Ria de Aveiro, and condition the offspring in a common garden, in order to, posteriorly, measure the genetic effects of certain reproductive traits. Moreover, with the aim to determine if environmental factors influence certain reproductive characters, reciprocal transplants among sites could be performed as well. The global information obtained in this study and in the one performed by Matias et al. 2013 – (Appendix 1) on the gametogenic cycle, will allow the determination of the optimal reproductive time for artificial spawning induction for aquaculture production of this species.



# 2

## “A microarray-based analysis of gametogenesis in two Portuguese populations of the European clam *Ruditapes decussatus*”<sup>2</sup>

### Article 1

de Sousa JT, Milan M, Bargelloni L, Pauletto M., Matias D., Joaquim S., Matias AM,  
Quillien V., Leitão L., Huvet A. (2014). PLoS ONE 9:e92202.

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<sup>2</sup> “PLOS ONE” version (Appendix 2)

In the first chapter of this thesis, with the exception of specific timings of gonadal development dynamics, the histological approach did not show any significant differences concerning the gametogenesis (intensity) between the two Portuguese populations of the European clam *R. decussatus*. Since the causes of the differences between the two populations, concerning spawning induction success remained unknown, the gonad transcriptome of the individuals sampled in these two populations was established to better understand the molecular cascade of the gametogenesis of *R. decussatus*, and to identify transcriptomic differences between these two populations that can originate their main reproductive behavior.

In order to improve transcriptomic information on *R. decussatus* gonad by means of Next Generation Sequencing techniques (RNA sequencing), a large amount of cDNA sequences were obtained through the sequencing by 454 Roche and Illumina platforms, thus providing a great number of transcripts for further “omic” studies in this species, especially for reproductive aspects. A microarray platform (8x60,000) for gene expression analysis was then constructed and a dataset of 64 individual gonad samples from both populations with different gametogenic stages and sexes, was analysed.

## I. Introduction

The European clam, *Ruditapes decussatus* (Linnaeus, 1758) is a bivalve mollusc of the family Veneridae, native to the European Atlantic and Mediterranean coastal waters. The European clam, despite a relatively low European production (8200 tons/year) (FAO 2013), is considered a high value seafood product and one of the most important bivalve species economically in Southern European countries like Italy, Spain and Portugal (Matias et al. 2009).

The culture of *R. decussatus* is clearly limited by the availability of seed. Its production is almost exclusively based on natural recruitment, which is subject to high annual fluctuations due to pollution and other environmental factors. To address this situation, artificial spawning and larval rearing programs were developed to provide an alternative source of seed (Matias et al. 2009), but still need improvement to gain robustness of seed production.

*R. decussatus* is a gonochoric molluscan species (possessing separate sexes) that reproduces annually (Vilela 1950). Generally, the gonad regresses at the end of the annual reproductive cycle, which is the end of summer in temperate regions, and regenerates at the beginning of the following one (the end of winter). During the initial stage of gametogenesis, period of sexual rest (I), gonadal follicles are absent and connective and muscular tissue occupies the entire zone from the digestive gland to the foot. There is no evidence of gonadal development and determination of sex is not possible. During the second stage of gametogenesis (II) follicles and gonadal acini begin to appear in females and males, respectively. They increase in size, and appear filled with oocytes in the growth phase in the females and with immature gametes (spermatogonia and spermatocytes) in the males. During advanced gametogenesis (III) the follicles occupy a large part of the visceral mass and it's possible to observe the first signs of partial emission of gametes when it occurs. The maturation period (IV) corresponds to the maturity of the majority of gametes. Throughout this period partial spawning may occur, and it concludes with the total emission of gametes (Delgado and Pérez Camacho 2005).

Currently, the knowledge about the molecular mechanisms of reproduction in marine bivalves is very limited, but is expected to increase rapidly, due to the advent of high throughput genomic approaches (Dheilly et al. 2012). Here, a large and exhaustive approach is proposed for investigating the molecular bases of variability in

reproductive success and, more generally, the reproductive mechanisms in *R. decussatus*. Some specific studies have recently explored this topic. The gonadal transcriptome was described and lists of genes of interest for reproductive stage and sex were established for the alternative and irregular protandrous hermaphrodite, the Pacific oyster, *Crassostrea gigas* (Dheilly et al. 2012) and for the functional hermaphrodite scallop *Nodipecten subnodosus* (Llera-Herrera et al. 2013). Most importantly, cross-referencing these lists of genes allowed the identification of potential markers of early sex differentiation and new highly valuable information on genes specifically expressed by mature spermatozooids and mature oocytes. Additionally, dedicated studies have been made in *Argopecten purpuratus*, *C. gigas* and *Mytilus galloprovincialis* emphasizing specific reproductive processes (Boutet et al. 2008; Fabioux et al. 2004a; Fabioux et al. 2004b; Fleury et al. 2008).

In *R. decussatus*, apart from a few specific gene expression analyses (see Moreira et al. 2012) and a first version of DNA microarray designed to unravel host-parasite interactions (Leite et al. 2013), very little information is available on genome-wide expression profiling for physiological, environmental or aquaculture questions.

For the present work, cDNA libraries of oocytes, larval stages and different gonadal maturation stages were sequenced on the Illumina platform, and a custom oligonucleotide microarray representing 51,678 assembled contigs was designed and used to characterize the transcriptomic bases of reproduction in *R. decussatus*. Microarray analyses were performed in four gonadal maturation stages of two Portuguese wild populations, Ria Formosa in Southern Portugal (37°01'N; 07°49'W) and Ria de Aveiro in Western coast of Portugal (40°42'N; 08°40'W). These populations were already characterized by different responses to spawning induction, which is of great interest to study in a context of improvement of aquaculture production (Matias et al. 2009; Matias et al. 2013).

The present study provides new highly valuable genomic information for the understanding of reproduction of this species and emphasizes some candidate genes as possible starting points for further functional studies, for instance on spawning and gamete quality with the aim of elucidating how and to what extent environmental factors affect relevant reproductive-gene expression.

## II. Material and Methods

### 2.1. Ethics statement

The European clam is not considered as an endangered or protected species in any Portuguese or international species catalog, including the CITES list ([www.cites.org](http://www.cites.org)). The European clams from Ria de Aveiro (40°42'N; 08°40'W) and Ria Formosa (37°01'N; 07°49'W) were produced and captured with the permission of DGRM (Direção-Geral de Recursos Naturais, Segurança e Serviços Marítimos), APA (Agência Portuguesa do Ambiente) and PNRF (Parque Natural da Ria Formosa - National park defined by the Ramsar Convention).

### 2.2. Animal sampling for RNA-sequencing

Clams were collected in Ria de Aveiro (40°42'N; 08°40'W), and conditioned in a common garden setting to accelerate their gonad development from October 2011 to January 2012 in the experimental bivalve hatchery of Portuguese Institute of Sea and Atmosphere (IPMA) in Tavira, Portugal. Food regimes consisted of different algal mixtures containing *Isochrysis galbana* (clone T-ISO) and *Skeletonema costatum* (Ria Formosa autochthone clone). Microalgae were cultured in 80 L bags with f/2 medium (Guillard 1975), in a temperature-controlled room at  $20 \pm 2^\circ\text{C}$  under continuous illumination (9900 lux) and aeration. Gonad samples were collected and immediately dissected: a transversal section of the gonadal area was fixed for histological examination and the rest of the gonads were frozen immediately in liquid nitrogen. Later, the gonads were crushed into a fine powder at  $-196^\circ\text{C}$  with a Dangoumau mill and stored in liquid nitrogen until RNA extraction. For the histological analysis, a 3-mm cross section of the visceral mass of each sample was excised using a microtome cutter in front of the pericardic region and immediately fixed in 10 to 20ml modified Davidson's solution (Latendresse et al. 2002) at  $4^\circ\text{C}$  for 48 h. After, the modified Davidson's solution was discarded and 10 to 20ml 70% ethanol was added and kept at  $4^\circ\text{C}$ .

Sections were dehydrated in ascending ethanol solutions, cleared with a xylene substitute named Microclearing (Diapath, Italy), and embedded in paraffin

wax. Sections of 5  $\mu\text{m}$  were cut, mounted on glass slides and stained with Harry's hematoxylin-Eosin Y (Martoja and Martoja-Pierson 1967). Slides were then examined under a light microscope and sex and stages were determined according to the 4 stages previously described (see Introduction). Percentage areas of gonadic tubules, connective tissue and digestive gland were then determined on each histological section. Slides were scanned with a digital scanner (hp scanjet 7400c) and the images saved in \*.TIFF format. Tissue areas were then measured using image analysis software (Imaq Vision Builder, National Instruments Corp.). Gonad area percentage was estimated as pixel number, from gonad / pixel number on total sections, as described in Fabioux et al. (2005). 35 individuals corresponding to five gonad samples of each reproductive stage and sex were chosen for RNA-sequencing.

### **2.3. Animal sampling for microarray analysis**

Twenty clams from two wild populations - Ria Formosa (South of Portugal) and Ria de Aveiro (Northern of Portugal) - were sampled monthly between July 2011 and July 2012. At each sampling date the gonads of the collected clams were immediately dissected for RNA extraction and a transversal section of the gonadal area fixed for histological analysis, as described above. Gene expression analysis was done on 64 individual gonads representative of all the reproductive stages and sexes from both populations, chosen on the basis of histological data (Table 2; Fig. 19; Fig. 20).

Table 2 - Number and sex of the 64 individuals of each gonad developmental stage from the two studied populations used in the microarray analysis.

Gonad developmental stage	Sex	Ria Formosa	Ria de Aveiro
I		5	5
II	F	5	5
	M	5	5
III	F	5	5
	M	3	7
IV	F	5	3
	M	3	3

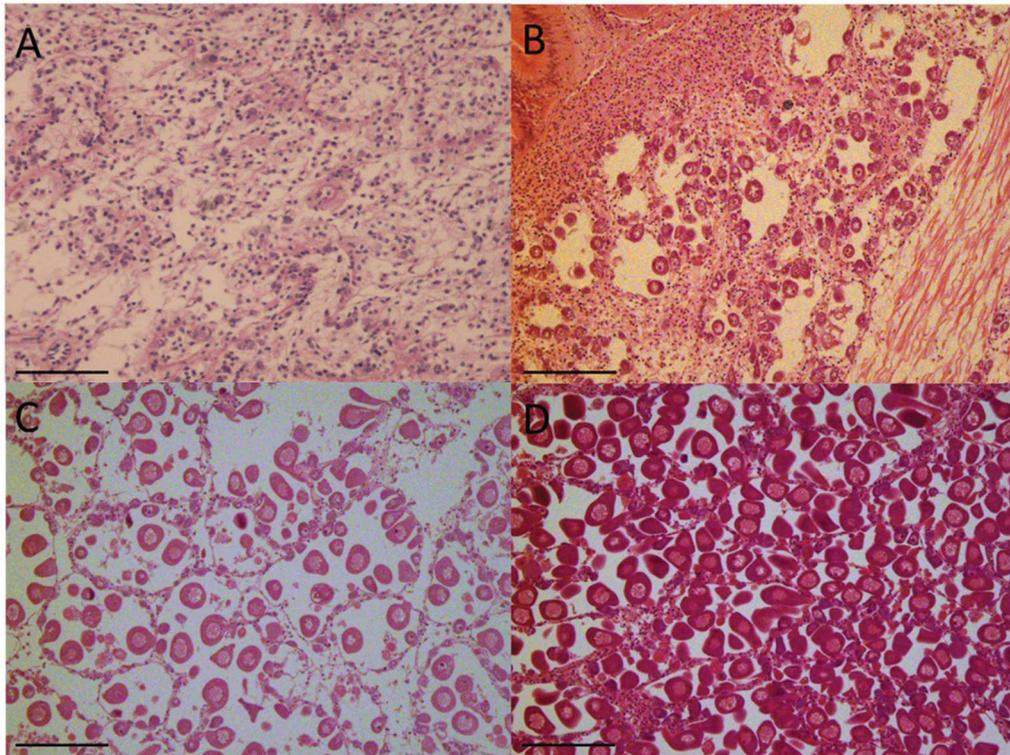


Figure 19 - Photomicrographs showing stages in the development of *Ruditapes decussatus* female gonad. A. Sexual rest. B. Initiation of gametogenesis; C. Advanced gametogenesis; D. Maturation. Scale bar: 200  $\mu$ m.

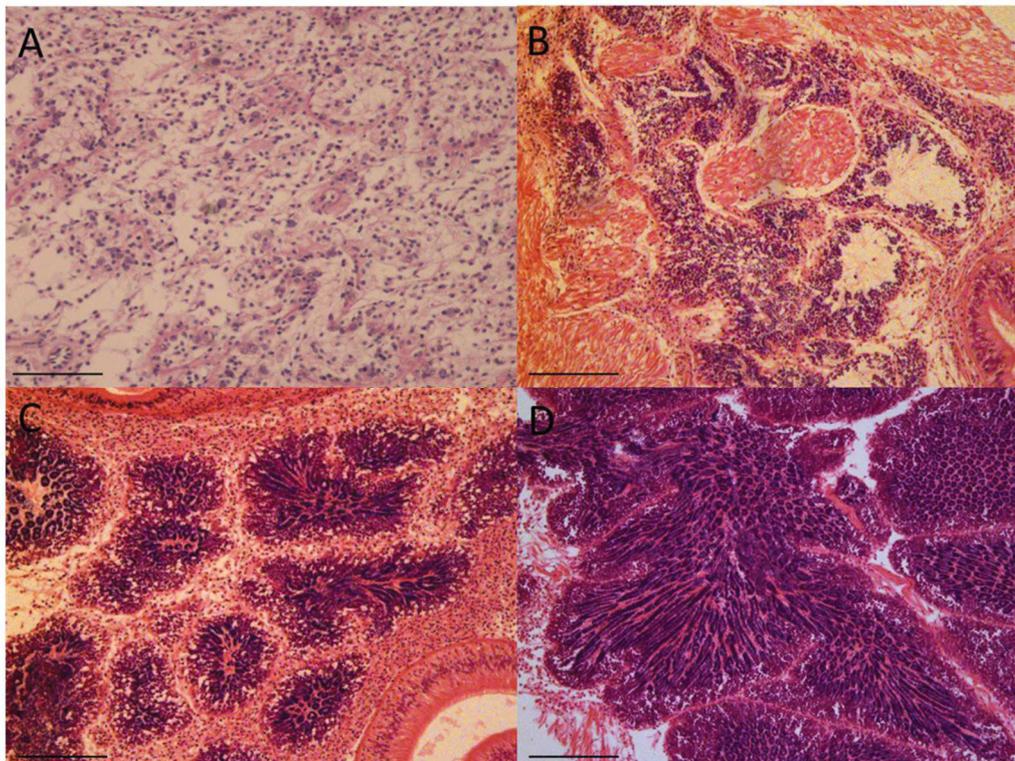


Figure 20 - Photomicrographs showing stages in the development of *Ruditapes decussatus* male gonad. A. Sexual rest. B. Initiation of gametogenesis; C. Advanced gametogenesis; D. Maturation. Scale bar: 100  $\mu$ m in A, B, C; 200  $\mu$ m in D.

## 2.4. RNA extraction

For RNA-sequencing and microarray analysis, total RNA was isolated using Extract-all (Eurobio) procedure. RNA quality and integrity were analysed on the Agilent bioanalyzer using RNA nanochips and Agilent RNA 6000 nanoreagents (Agilent Technologies, Waldbronn, Germany). RNA concentrations were measured at 260 nm using a ND-1000 spectrophotometer (Nanodrop Technologies) using the conversion factor 1 OD = 40 mg/mL total RNA. Samples were stored at -80°C until further use.

## 2.5. cDNA library construction and sequencing

Sequencing of gonadal maturation stages was performed through a multiplexing strategy on the Illumina platform HiSeq2000. A total of 7 cDNA tagged libraries from gonads of 5 individuals, of each developmental stage (sexual rest stage I and stages II/III/IV in both sexes), were prepared on a 7-plex paired-end sequencing lane (2x100bp). cDNA libraries were constructed, amplified and sequenced at BGI Tech (Shenzhen, China). All the Illumina reads were analyzed with FastQC software in order to assess the sequence quality. Illumina reads have been deposited in GenBank (SRA) with the accession numbers SRR974930, SRR974931, SRR974932, SRR97493, SRR974934, SRR974935, SRR974936.

## 2.6. Assembly and transcriptome annotation

To obtain a wider *R. decussatus* transcriptome representation, Roche 454 and Illumina reads have been added to the Illumina reads produced by a 7-plex paired-end sequencing lane (Table S1). Specifically, additional Illumina reads have been obtained through the sequencing of stripped and spawned oocytes, larvae at 48 hours post-fertilization, and larvae at 17, 21 and 30 days post-fertilization cDNA libraries (Pauletto et al. unpublished data; personal communication). In addition, reads obtained by the sequencing by 454 Roche of hemocytes, oocytes and larval stages cDNA libraries have been also considered for the assembly (Novoa et al., unpublished data; personal communication). These libraries have been produced

within the European project REPROSEED and will be public with the complete transcriptome of *R. decussatus* (Pauletto et al, Novoa et al. unpublished data). All these data were merged also with publicly available Sanger sequences and nearly 500,000 reads obtained with 454 sequencing of larvae, gonads and several adult tissues (MGE011 and cDN18; SRA058431 (Leite et al. 2013). A summary of the origin of *R. decussatus* sequences and assembly information are shown in Table S1.

Assembly was performed separately for each library by using MIRA3 or CLC Genomic Workbench for 454 and Illumina reads, respectively. A total of 990,111 contigs were then merged to reduce the redundancy by using CAP3, resulting in a total of 503,705 contigs (unpublished data). 454 and Illumina reads have been assembled by CLC Genomic Workbench, MIRA 3 and CAP3 with default parameters.

A preliminary annotation was obtained for 141,001 contigs through Blast similarity searches conducted against several protein databases (e-value  $1 \times 10^{-5}$ , see Table S2).

## 2.7. Microarray design

All databases used for the annotation step (see Table S2) were considered to reduce the redundancy in annotated contigs. A total of 44,333 contigs found non-redundant (with a unique annotation) in at least one reference database have been considered for microarray design. Putative sense-strand orientation was inferred from the matching protein-coding gene in reference data bases. For 915 contigs that showed ambiguous orientation two probes with opposite orientations (sense and antisense) were designed. For the remaining 43,418 contigs with putatively unambiguous orientation a single (sense) probe was designed.

Since the microarray format could accommodate approximately 60,000 probes, non-annotated contigs that showed the highest expression in gonads based on RNA-seq data (see below) were included in the microarray design. In total 7,376 non-annotated contigs were added, and for each of them, two probes with opposite orientation (sense and antisense) were designed. Probe design was carried out using the Agilent eArray interface (<https://earray.chem.agilent.com/earray/>), which applies proprietary prediction algorithms to design 60-mer probes. A total of 59,951 out of 60,000 probes were successfully obtained, representing 51,709 different

*R. decussatus* contigs. The sequences of the 51,709 contigs representing in *R. decussatus* DNA microarray platform have been provided in Table S3. The percentage of annotated transcripts represented on the microarray was 85.7%. In Table S4 has been reported the annotation of each probe represented in DNA microarray platform. Probe sequences and other details on the microarray platform can be found in the GEO database (<http://www.ncbi.nlm.nih.gov/geo/>) under accession number GPL17766.

## 2.8. Labeling and microarray hybridization

Sample labeling and hybridization were performed according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol with the Low Input Quick Amp Labeling kit. Briefly, for each sample 100 ng of total RNA was linearly amplified and labeled with Cy3-dCTP. A mixture of 10 different viral poly-adenylated RNAs (Agilent Spike-In Mix) was added to each RNA sample before amplification and labeling to monitor microarray analysis work-flow. Labeled cRNA was purified with Qiagen RNeasy Mini Kit, and sample concentration and specific activity (pmol Cy3/ $\mu$ g cRNA) were measured in a NanoDrop® ND-1000 spectrophotometer. A total of 600 ng of labeled cRNA was prepared for fragmentation by adding 5 $\mu$ l 10X Blocking Agent and 1 $\mu$ l of 25X Fragmentation Buffer, heated at 60°C for 30 min, and finally diluted by addition with 25 $\mu$ l 2X GE Hybridization buffer. A volume of 40 $\mu$ l of hybridization solution was then dispensed in the gasket slide and added to the microarray slide (each slide containing eight arrays). Slides were incubated for 17 h at 65°C in an Agilent hybridization oven, subsequently removed from the hybridization chamber, quickly submerged in GE Wash Buffer 1 to disassemble the slides and then washed in GE Wash Buffer 1 for approximately 1 minute followed by one additional wash in pre-warmed (37°C) GE Wash Buffer 2.

## 2.9. Data acquisition, correction and normalization

Hybridized slides were scanned at 2 $\mu$ m resolution using an Agilent G2565BA DNA microarray scanner. Default settings were modified to scan the same slide twice at two different sensitivity levels (XDR Hi 100% and XDR Lo 10%). The two linked

images generated were analyzed together and data was extracted and background subtracted using the standard procedures contained in the Agilent Feature Extraction (FE) Software version 10.7.3.1. The software returns a series of spot quality measures in order to evaluate the quality and the reliability of spot intensity estimates. All control features (positive, negative, etc.), except for Spike-in (Spike-in Viral RNAs), were excluded from subsequent analyses.

Raw gene expression data of all 64 analyzed individual gonads (Table 2) were deposited in the GEO database under accession number GSE51150.

Spike-in control intensities were used to identify the best normalization procedure for each dataset. After normalization, spike intensities are expected to be uniform across the experiments of a given dataset. Normalization procedures were performed using R statistical software. Quantile normalization always outperformed cyclic loess and quantile-normalized data were used in all subsequent analyses. Normalized data were deposited in GEO archive under accession number GSE51150.

## 2.10. Data analysis

Differences in percentage gonad area were analysed using one-way analysis of variance after angular transformation. Comparisons of sex and gametogenic stage distributions between lines were made using Chi-square tests.

A principal component analysis (PCA) using the TMeV 4.5.1 (TIGR MULTIEXPERIMENT VIEWER) (Saeed et al. 2003) was applied to assess the distribution of the studied groups. Statistical tests implemented in the program Significance Analysis of Microarray (SAM) were used to identify differentially expressed probes between the two clam populations. The same approach was used to identify differentially expressed probes in both populations between males and females. A SAM quantitative correlation analysis was also performed to identify genes with a correlation between mRNA levels and gonad area, after angular transformation. A Pavlidis template matching (PTM) analysis was implemented using the TMeV 4.5.1 in order to verify whether the expression of specific genes increased during gonad development. A one-way ANOVA parametric test was used to identify the probes whose expression changed during gonad development in the two

populations, using a p-value cut-off of 0.01 and an adjusted Benjamini & Hochberg correction using R software v2.15.2. Hierarchical clustering was performed using TMeV on statistically significant transcripts, to group experimental samples together based on similarity of the overall experimental expression profiling.

A more systematic and functional interpretation for significant gene lists was obtained using an enrichment analysis with the Database for Annotation, Visualisation, and Integrated Discovery (DAVID) software (Huang et al., 2009). “KEGG Pathway”, “Biological process” (BP), “Molecular function” (MF) and “Cellular component” (CC) analyses were carried out by setting the gene count equal to 2 and the ease equal to 0.1. Because the DAVID database contains functional annotation data for a limited number of species, it was necessary to link *R. decussatus* transcripts with sequence identifiers that could be recognized in DAVID. This process was accomplished using dedicated Blast searches performed with Blastx against zebrafish Ensembl proteins. Finally, *Danio rerio* Ensembl Gene IDs were obtained from the corresponding Ensembl protein entries using the BIOMART data mining tool (<http://www.ensembl.org/biomart/martview/>). *D. rerio* IDs corresponding to differentially transcribed clam genes as well as all of the transcripts that were presented on the array were then used to define a “gene list” and a “background” in DAVID, respectively.

### III. Results

#### 3.1. Prevalent Gene Expression Patterns

A Principal Component Analysis (PCA) was applied to the entire gene expression database (59,951 probes) for the 64 clams (Fig. 21).

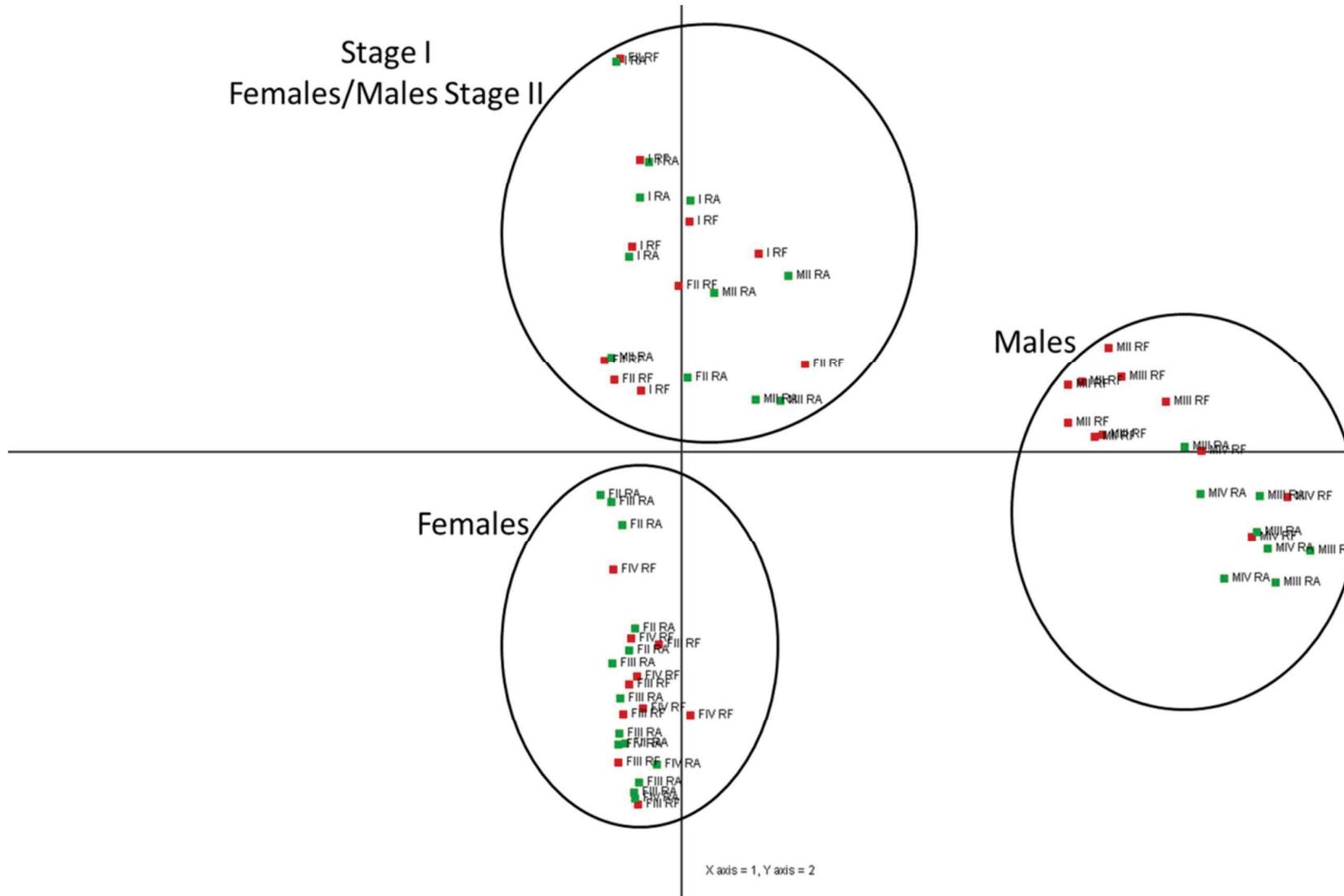


Figure 21 - Principal Component Analysis. PCA applied to all 59,951 transcripts of the 64 individual clam gonads sampled from Ria de Aveiro (RA) and Ria Formosa (RF). I = Stage I (sexual rest); FII = Female stage II (gametogenesis); FIII = Female stage III (advanced gametogenesis); FIV = Female stage IV (maturation); MII = Male stage II (gametogenesis); MIII = Male stage III (advanced gametogenesis); MIV = Male stage IV (maturation).

We observed a clear clustering of the different sexes and gametogenic stages determined by histological methods. Along the first axis, PC1, which explained 35% of the variation, male and female individuals were discriminated. Gonad developmental stages were organized along the second axis, PC2 (15%). A significant divergence in expression patterns between male and female gonads was observed in stages III and IV (PC2). At these later stages, males and females predictably possess the most distinctive expression profiles, while at stages I and II this separation is not as clear along the second axis.

### **3.2. Two-unpaired class Significance Analysis of Microarray (SAM) between sexes**

In order to compare gene expression profiles between sexes in *R. decussatus*, independently from stage and population of origin, a two-unpaired class Significance Analysis of Microarray (SAM) test was carried out on normalized data (FDR=5%; FC>1.5). A list of 16,996 significant probes, corresponding to 11,641 unique transcripts, was obtained (Table S5).

Hierarchical clustering using Pearson's correlation for the set of 16,996 differentially expressed probes identified three main clusters: Stage I, Females and Males (Fig. 22). However, there are some exceptions to this hierarchical clustering. Indeed, a stage I individual appeared on the same branch as females.

A total of 7,145 differentially expressed probes were up-regulated in the gonads of males with a FC ranging from 1.5 to 255, while a total of 9,751 differentially expressed probes were up-regulated in the females gonads with a FC ranging from 1.5 to 374.

A putative annotation with zebrafish Ensembl Gene IDs was obtained for 6,506 probes that were differentially expressed between the two sexes. These annotated transcripts were used to define a gene list for functional annotation with DAVID. Enrichment analysis showed 9 KEGG, 95 BP terms, 22 CC terms, and 45 MF terms to be significantly over-represented (Table S6).

"Cell cycle" (dre04110), "oocyte meiosis" (04114) and "Progesterone-mediated oocyte maturation" (dre04914) were the most represented in the enriched KEGG pathway terms, with 49, 45 and 38 genes respectively. Cyclin B1 (CCNB1) and cyclin



exhibited a significant change in expression over the reproductive cycle of male and female gonads (Table S7). A putative annotation with *D. rerio* Ensembl Gene IDs was obtained for 6,451 probes that were differentially expressed between gametogenic stages. Enrichment analysis showed 9 KEGG, 20 BP terms, 12 CC terms, and 40 MF terms to be significantly over-represented (Table S8).

“Focal adhesion” (dre04510) and “Regulation of actin cytoskeleton” (dre04810) were the most represented within enriched KEGG pathway terms, with 74 and 62 genes respectively. “Microtubule-based process” (GO:0007017) was one of the enriched biological processes with the highest representation.

In addition, we decided to emphasize the expression progression of certain well-known reproduction-related genes in the animal kingdom, in order to verify whether their expression increased during gonad development. Vitellogenin (VTG), a female specific glycoprotein, was identified in our female samples with an increase in expression along the gametogenic cycle, from stage I to stage IV, with the higher values achieved in stage IV (maturation period). It was also observed that the females from Ria Formosa presented higher expression values of VTG. Another female specific gene, Condensin-2, was also over-expressed in females during gonad development, with the same trend as VTG. In terms of male specific genes, the motile sperm domain containing protein 2 (MOSPD2) and the synaptonemal complex protein 2 (SCP-2) were identified, showing an increase in expression along the spermatogenesis, from stage I to stage IV.

### **3.4. Identification of genes with a correlation between mRNA levels and gonad area**

Quantitative correlations between microarray data and values of gonad area of stages II, III and IV from males and females, separately, after angular transformation, were evaluated via a SAM quantitative correlation analysis (FDR=5%). Females showed a total of 10,415 probes significantly (direct or inverse) correlated with the gonad areas (Table S9). Of these probes, 7,226 were annotated by similarity and associated with a known protein. We noticed the presence of positively correlated genes coding for Forkhead box protein L2 (Foxl2), VTG, condensin 2, Mitotic apparatus protein p62 and Cep57. In relation to male gonads, a total of 18,730

probes showed a significant (direct or inverse) correlation with gonad area (Table S10). A putative annotation was obtained for 12,843 probes. Genes expressed included dpy-30, sperm associated antigens 6 (SPAG6), 16 (SPAG16) and 17 (SPAG17), MOSPD2, sperm surface protein Sp17 and sperm flagellar proteins 1 and 2. Additionally, functional analysis of significant transcripts showed that different KEGG pathway terms, such as “Cell cycle” (dre04110), “oocyte meiosis” (04114) and “Regulation of actin cytoskeleton” (dre04810), were influenced by gonad area as well.

### 3.5. Comparison between populations using two-unpaired class Significance Analysis of Microarray (SAM)

In order to compare gene expression between the two sampled populations of *R. decussatus*, considering all stages and sexes, a two-unpaired class Significance Analysis of Microarray (SAM) test was carried out on normalized data, enforcing a False Discovery Rate (FDR) of 5% and a Fold Change threshold of 1.5. A list of 2,900 significant probes, corresponding to 2,228 unique transcripts, was obtained.

A putative annotation with *D. rerio* Ensembl Gene IDs was obtained for 1,777 probes out of the 2,228 differentially expressed transcripts between the two populations (Table S11). Enrichment analysis revealed a total of 2 KEGG pathways, 23 BP terms, 15 CC terms, and 25 MF terms significantly over-represented among the differentially expressed probes (Table S12). “N-Glycan biosynthesis” (dre00510) term was the most represented in the enriched KEGG pathway terms, with 9 genes.

Hierarchical clustering, using Pearson’s correlation, was performed considering the set of 2,900 differentially expressed probes. As reported in Fig. 23, with some exceptions, three main clusters were identified: Ria Formosa, Ria de Aveiro and Female Stage III/IV from Ria de Aveiro.

In order to support the results obtained by hierarchical clustering, several comparisons between populations in each reproductive stage and sex were performed. The comparison between stage IV females from both populations was the one that presented the highest number of differentially expressed probes. For this analysis, a two-unpaired class Significance Analysis of Microarray (SAM) test was carried out on normalized data (FDR=5%; FC>1.5). A list of 6,765 significant probes, corresponding to 5,133 unique transcripts, was obtained (Table S13). A putative

annotation with *D. rerio* Ensembl Gene IDs was obtained for 3,229 probes. An enrichment functional annotation analysis was performed with DAVID, revealing 11 KEGG pathways, 88 BP terms, 29 CC terms, and 51 MF terms significantly over-represented (Table S14). “Cell cycle” (dre04110), “Spliceosome” (dre03040), “Ubiquitin mediated proteolysis” (dre04120) and “Oocyte meiosis” (dre04114) terms were the most represented in the enriched KEGG pathway terms, with 45, 32, 30 and 28 genes respectively. Among the genes differentially expressed between females stage IV of the two populations, the great majority were common to “Oocyte meiosis” (dre04114) and “Cell cycle” (dre04110) pathways, like the S-phase kinase-associated protein 1 (skp1) and the Mad2l1. Other important differentially expressed genes were also identified, such as the cdc16, cdc20, cdc27. Genes like CCNB1, cyclin E2 (CCNE2), and cdk2 were also present in both pathways.

Another comparison that revealed an important number of differentially expressed probes, at the population level, was between stage II males. A list of 5,801 significant probes, corresponding to 3,588 unique transcripts was obtained (Table S15). A putative annotation with *D. rerio* Ensembl Gene IDs was obtained for 3,181 probes. An enrichment functional annotation analyses has been performed by DAVID, revealing 14 KEGG pathways, 56 BP terms, 23 CC terms, and 50 MF terms significantly over-represented (Table S16). “Insulin signaling pathway” (dre04910) was the most represented in the enriched KEGG pathway terms. Important differentially expressed genes were found, such as, the Mitogen-Activated Protein Kinase 1 (MAPK1). However, for the remaining comparisons performed, no additional significant results were observed between populations.



To better understand the different reproductive behaviors observed between northern and southern Portuguese populations, gonads of clams collected in Ria Formosa (Southern Portugal) and Ria Aveiro (Western coast of Portugal) were analyzed (Fig. 24).

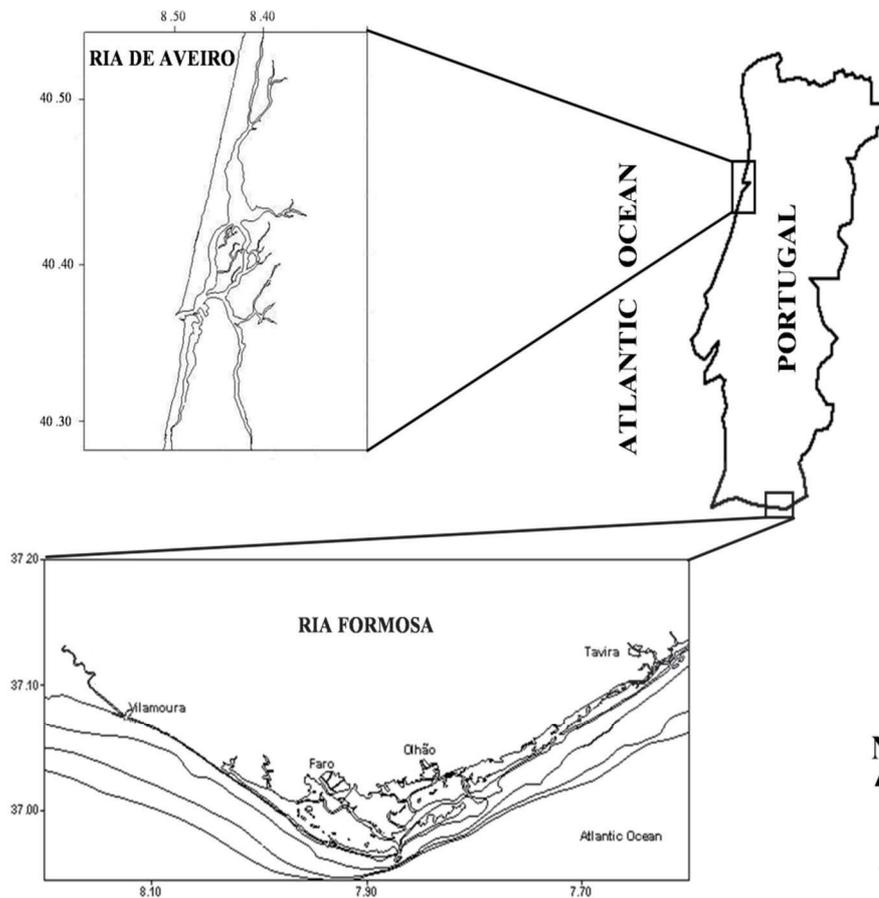


Figure 24 - Ria de Aveiro and Ria Formosa Lagoon location. Ria Formosa (37°01'N; 07°49'W) in Southern Portugal and Ria Aveiro (40°42'N; 08°40'W) in Western coast of Portugal.

#### 4.1. Sex explains most of the variance in gonad gene expression

Although the preliminary analysis using PCA (Fig. 21) did not discriminate populations, a clear clustering of the different sexes and gametogenic stages was observed. The main sex effect on gonadic gene expression was also reported for an alternative and irregular protandrous hermaphrodite marine bivalve, the Pacific oyster (Dheilly et al. 2012). The differences between males and females increased over time, following the continuous gametogenesis from stage II to stage IV gonads. Considering gene expression pattern, there is no evidence of sex determination at

stage I based on the PCA results, showing that, with this analysis, clam sex is undetermined at stage I.

In the hierarchical clustering performed on the probes differentially expressed between sexes (Fig. 22), a stage I individual was detected clustered on the same branch as females. Although the sex of this clam could not be determined histologically, we suggest that this individual had already initiated sex differentiation, being possibly in a later stage I, and could therefore be classified as female according to its gene expression pattern. Indeed, through Pavlidis template matching (PTM) analysis by TMeV, we observed that this specific sample over expressed VTG, a female specific glycoprotein identified as being necessary for building up the oocyte in oysters (Dheilly et al. 2012), among other species. Oysters, at an undetermined sex stage, were found to express male- or female-specific genes, suggesting that sex differentiation already took place at the first stage of gametogenesis, even if it could not be visualized histologically (Dheilly et al. 2012). As a result, in some cases of individuals in later stage I, gene expression patterns could indicate sex determination without histological information.

Following the strong sex effect on gene expression reported above, with 16,996 probes differentially expressed between sexes, 9 KEGG pathways were revealed. Among these pathways, “oocyte meiosis” (dre04114) and “Progesterone-mediated oocyte maturation” (dre04914) were the most represented with 45 and 38 genes, respectively, as well as “Cell cycle” (dre04110) with 49 genes. These pathways are characteristic of females, certainly related to the higher transcription rate in female germ cells compared to male germ cells. Indeed, oocytes provide most of the information for the development of zygotes (Watson 2007). Among the genes involved, we found the Mad211, a component of the spindle-assembly checkpoint that prevents the onset of anaphase until all chromosomes are properly aligned at the metaphase plate, and the CaM family, which belongs to one of the two main groups of calcium-binding proteins. Calmodulins are supposed to be very important in the oogenesis of *R. decussatus*, since  $Ca^{2+}$  increases are recognized as essential for the oocytes to be released from the metaphase I arrest (Hidaka 1985; Gobet et al. 1994; Deguchi and Morisawa 2003; Tosti 2006). The oocytes of the European clam (classified as class II, due to their meiotic maturation distinct from other bivalve species) are held in the ovaries at prophase of the first division of meiosis and their

development reinitiates during spawning, by hormonal stimulation or other stimuli. A second barrier to development occurs at metaphase I, prior to extrusion of the first polar body, until fertilization releases this metaphase arrest, allowing further stages of maturation to take place (Colas and Dubé 1998). Furthermore, cyclins found differentially expressed (CCNB1, CCNB2 and CDK2), also trigger metaphase/anaphase transition and further progress through meiosis by disappearing, due to protein synthesis inhibition (Colas and Dubé 1998). In order to better understand the differences between sexes in each reproductive stage, the same analysis was performed in each one. The number of differentially expressed probes increased as the reproductive stages developed, with stage IV presenting the highest number of differentially expressed probes, confirming the results from PCA analysis. Speriolin, a novel centrosomal protein present in the connecting piece region of mouse and human sperm (Goto et al. 2010), was present in the list of differentially expressed probes between males and females at stage IV, with a FC value of 332, as well as TSSK-5 with a FC value of 249. mRNA expression of these genes is highly testis-specific, with a very low level detected in other tissues of the marine bivalve *Argopecten purpuratus* (Boutet et al. 2008).

#### 4.2. Differentiation of gonadal developmental stages

From the 13,218 probes that exhibited a significant change in expression over the reproductive cycle of male and female gonads, 9 KEGG pathways appeared notably represented. “Focal adhesion” (dre04510) and “Regulation of actin cytoskeleton” (dre04810), were the most represented, with 74 and 62 genes, respectively. In sea star oocytes, the cortical actin cytoskeleton was noticed to play critical roles in modulating  $Ca^{2+}$  release during meiotic maturation, as well as in regulating cortical granule exocytosis (Kyojuka et al. 2008). Additionally, “microtubule-based process” (GO:0007017), one of the enriched biological processes with the highest representation, participates in several important events in spermatogenesis, including nuclear elongation, cytoplasmic redistribution and reduction and development of the flagellum (Kyojuka et al. 2008). Within the genes involved in this pathway, we found the dynein light chain 2 (dynll2a and dynll2b), several kinesin family members (KIF11, KIF17, and KIF7). These genes were also

found highly expressed in male gonads in *C. gigas* and may be involved in flagella and cilia structure, locomotion and control (Dheilly et al. 2012).

VTG, a female specific glycoprotein previously described and Condensin-2, expressed in oocytes and involved in maintaining the rigidity of chromosomes in prophase were identified in our female samples with increase in expression as oogenesis progressed. In terms of male specific genes, we identified the MOSPD2 and the SCP-2, involved in the meiotic phase of spermatogenesis, also with an increase in expression during progression of spermatogenesis. All these genes also increased in expression along the gametogenic cycle in male and female gonads of *C. gigas* (Dheilly et al. 2012). Scp2 and scp3, genes involved in synaptonemal complex formation, were recently found to be highly expressed in the maturing testis of the scallop *Nodipecten subnodosus* (Llera-Herrera et al. 2013). This synaptonemal complex is a meiosis-specific structure essential for chromosome pairing since disruption of the localization of scp2 and scp3 during early recombination resulting in aneuploidy (Shin et al. 2010). Our results indicate that synaptonemal complex formation, and therefore meiosis I, was occurring from early to late testis maturation. This results is similar to what was found in *N. subnodosus*, for which the authors concluded that the expression pattern of scp2 and scp3 might serve as future markers for the meiotic stage and may prove helpful in differentiating primary and secondary spermatocytes, which is not morphologically possible (Llera-Herrera et al. 2013).

A quantitative correlation between microarray data and values of gonad area considered as a proxy of the reproductive effort (e.g. in oyster (Royer et al. 2008), were evaluated separately for females and males. The clam gonad undergoes continuous development until it becomes fully mature. At that moment, the gonads or gonadal tissue form a significant portion of the soft parts of the animal. Gonaducts that will carry the gametes to the body chamber develop, enlarge and are readily observed in the gonad. For this reason, in males as in females, the gonadal area increases with the evolution of the reproductive stages and is considered as a proxy of the reproductive effort (Serdar and Lok 2010). Reproductive effort and the underlying mechanisms are of great interest for many marine bivalve species that have a very high fecundity, a characteristic of the “r” demographic strategy. Indeed, gametogenesis has a major impact on several physiological functions, generating

phenotypic and genetic trade-offs with growth and survival (as established in oysters (Ernande et al. 2004), meaning that reproductive effort improves at the expense of survival. We identified 7,226 annotated probes correlated with female gonad area and therefore, potentially, with the number and/or the size of the gametes produced. These probes included *Foxl2*, an evolutionarily conserved female-specific transcription factor, which is conserved from the sponge *Suberites domuncula* to humans (Lee et al. 2005); VTG; condensing 2, expressed in oocytes and involved in maintaining the rigidity of chromosomes in prophase; Mitotic apparatus protein p62 that binds to condensed chromosomes at prophase of meiosis I; and *Cep57*, a centrosomal protein required for microtubule attachment to centrosomes. These female-specific genes, which were positively correlated with gonad areas, were also identified as potentially involved in oogenesis in *C. gigas* (Dheilly et al. 2012). In males, genes expressed included the *SPAG6*, *SPAG16* and *SPAG17*, *MOSPD2*, sperm surface protein *Sp17*, sperm flagellar proteins 1 and 2 and *dpy-30*, all involved in spermatogenesis. *dpy-30* is involved in male development and described as an attractive new candidate gene for the regulation of sex differentiation in oysters (Dheilly et al. 2012). This gene was also identified and characterized as an essential component of the dosage compensation machinery for sex determination in *Caenorhabditis elegans* (Hsu and Meyer 1994).

### 4.3. Differential gene expression between populations

In *R. decussatus*, as in other bivalves, the period of spawning in natural populations differs with geographic location and, once reproductive maturity is reached, it may be triggered by several environmental factors including temperature, chemical and physical stimuli, water currents or a combination of these and other factors (Matias et al. 2013). The presence of gametes in the water provides an additional stimulus that generates a spawning response in broodstock, due to the presence of hormones (pheromones) that will act as recognition signals between gametes (Parwadani Aji 2011). Indeed, a surface glycoprotein was identified as the mate recognition signal in the caridean shrimp *Palaemonetes pugio* where glucosamine was considered a pheromone functioning in mate recognition (Caskey et al. 2009). Although it had a low statistical significance value for enrichment in

DAVID ( $P = 0.09$ ), “N-Glycan biosynthesis” (dre00510) was found among the enriched KEGG pathways with the highest number of gene entries. N-glycans have an important role acting as recognition signals and binding between the extracellular coat of the egg and components of the sperm plasma membrane, generally termed “primary binding” (Di Patrizi et al. 2001). Taking into account that “N-Glycan biosynthesis” (dre00510) pathway was down-regulated in the population of Ria Formosa, we propose that, in some way, this recognition process can explain some of the differences in spawning induction success observed between the two Portuguese populations. Indeed, it was previously demonstrated that environment can strongly affect reproduction of *R. decussatus*, notably in terms of fecundity level (Matias et al. 2013). Additionally, among the genes involved in N-Glycan biosynthesis, we found fucosyltransferase (FucT) differentially expressed with a Fold Change of 2.23. This enzyme is implicated in species-specific binding of sperm to eggs in mammals (Wassarman et al. 2001; Chiu et al. 2007; Venditti et al. 2010). In addition, ADAMs family genes (A Disintegrin and A Metalloprotease domain) as well as several integrin ligands appeared differentially expressed between the two populations. Several studies have indicated that these molecules are involved in cell adhesion and sperm-egg interaction (e.g. Evans 2001, Fard Jahromi and Shamsir 2013; Eto et al. 2002), supporting the hypothesis that “primary binding” could be responsible for the main differences in the reproductive behavior of the two populations.

Recent histological examinations of these two populations showed significant differences in the dynamics of gametogenesis (Matias et al. 2013): the maturation process begins earlier in the northern population probably due to contrasted environmental factors mainly temperature, which is already characterized as the main source of variation in the timing of gametogenesis (e.g. Fabioux et al. 2005). Sea surface temperature (SST) was relatively lower compared to Ria Formosa lagoon, though with higher chlorophyll values (Matias et al. 2013). Nonetheless, similar values of oocyte diameter and gonadic area were reported, suggesting similar investment in reproduction in the two populations. In terms of gene expression, hierarchical clustering using Pearson’s correlation, identified three main clusters: Ria Formosa, Ria de Aveiro and Female Stage III and IV from Ria de Aveiro (Fig. 23), suggesting that the biggest differences originate from the females in the later stages

of gametogenesis, and may be due to a difference in gamete maturity that may be implicated in the differences in response to spawning induction. These differences can originate from environmental differences and/or some genetically based variation between the two populations. Among all the comparisons made between populations for each reproductive stage and sex, the one performed with stage IV females presented the highest number of differentially expressed probes (5,133 probes; Table S13). In this comparison, “Cell cycle” (dre04110), “Spliceosome” (dre03040), “Ubiquitin mediated proteolysis” (dre04120) and “Oocyte meiosis” (dre04114) terms were the most represented in the enriched KEGG pathway terms with 45, 32, 30 and 28 genes, respectively. Due to their specific environments, these populations might adopt different reproductive strategies: the Ria de Aveiro population retrieves its energy reserves immediately after spawning, however, the same is not verified in clams from Ria Formosa Lagoon, with their consequent decline. Considering these facts, it is likely that, at some point, the process of oogenesis diverged between females of the two populations. Among the genes differentially expressed, the SKP1 and the Mad2l1 previously described, were present. The SKP1 is an essential component of the SCF (SKP1-CUL1-F-box protein) ubiquitin ligase complex, which mediates the ubiquitination of proteins involved in cell cycle progression, signal transduction and transcription. Other important differentially expressed genes were found, such as *cdc16*, *cdc20*, *cdc27*, which are components of the anaphase promoting complex/cyclosome (APC/C), a cell cycle-regulated E3 ubiquitin ligase controlling progression through mitosis and the G1 phase of the cell cycle. In addition, genes like *CCNB1* and *CCNE2* were present and are essential in controlling the progression of cells through the cell cycle, by activating Cdk enzymes. Cyclin-encoding transcripts represent maternal mRNAs, known to be stocked in oocytes during oogenesis and maternally transmitted to embryos before the start of embryonic transcription (Wada et al. 2012).

A total of 3,588 differentially expressed probes were also obtained in the comparison made between males of stage II. “Insulin signaling pathway” (dre04910) was the most represented in the enriched KEGG pathway terms. In addition to its function in somatic cells, it was demonstrated that insulin signaling plays an essential role in cell proliferation and growth during male *Drosophila* gametogenesis where sperm production is regulated by hormonal control via insulin-like peptides (Ueishi et

al. 2009). Moreover, the level of some mRNA in insulin pathway was also identified as correlated with gamete quality in fishes (Aegerter et al. 2004; Bobe et al. 2003). Additionally, the MAPK1 was found differentially expressed. Studies have shown that male reproductive function is modulated via the MAPK cascade. The MAPK cascade is involved in numerous male reproductive processes, including spermatogenesis, sperm maturation and activation, capacitation and acrosome reaction, before fertilization of the oocyte (Li et al. 2009). Considering that no additional significant results were observed for the remaining comparisons performed, we suggest that it is in the later stages of oogenesis (stage IV) and in the earlier stages of spermatogenesis (stage II), that the major differences between the two studied populations occur.

## V. Conclusions

In this study, we identified several differentially expressed probes, which led us to a specific pathway involved in the recognition signals and binding between the oocyte and components of the sperm plasma membrane. We believe that these results can be useful to *R. decussatus* hatchery production program management. Furthermore, as a possible starting point for further research devoted to reproduction and spawning improvement of this species, we identified genes specifically expressed in either males or females that showed increasing expression over the course of gametogenesis.

The designed custom oligonucleotide microarray can be useful for future studies into the molecular mechanisms involved in the European clam differentiation and development. Additionally, we produced lists of relevant pathways and candidate genes that helped to improve our understanding of gametogenesis and that are essential in addressing physiological, environmental or aquaculture questions concerning the European clam *R. decussatus*.





**“A microarray-based analysis of  
oocyte quality in the European clam  
*Ruditapes decussatus*”**

**Article 2**

**de Sousa JT**, Milan M, Pauletto M, Bargelloni L, Joaquim S, Matias D, Matias AM,  
Quillien V, Leitão L, Huvet A (2014). Submitted to Marine Biotechnology

In the previous chapter, regarding the comparison between the two Portuguese populations of *R. decussatus*, the presence of certain genes differentially expressed (FucT and ADAMs family) in addition to the enriched KEGG pathway “N - Glycan biosynthesis”, suggested that sperm-egg interaction could have a major influence on the differences concerning spawning induction between the two populations. As reported in this thesis, the moment when the gametes recognize each other by the interaction between glycoproteins of the extracellular matrix of the egg and cell surface receptors on the sperm, is certainly one of the most important in the fertilization process (Rosati and Focarelli 1990; Wassarman et al. 2001). Despite the fact that several studies pointed out a few interesting factors driving meiosis progression in bivalves, mainly the fact that *R. decussatus* oocytes cannot be fertilized at prophase I (e.g. Colas and Dubé 1998; Deguchi and Osanai 1995; Gobet et al. 1994; Osanai 1985), the reason of this phenomenon remains unidentified and little is known in cell signaling during gamete maturation in *R. decussatus*.

In order to better understand the molecular processes leading to female gametes maturation and competence and deepen the genomic study made in chapter 2, expression profiles characterizing poor and good quality oocytes with different competence levels were evaluated in the present chapter, using the same array previously described.

Moreover, in the context of the Reproseed project, another PhD thesis (Pauletto 2014) was accomplished. Gene expression studies were performed to improve the knowledge on the major biological mechanisms regulating *R. decussatus* oocytes maturation and in *P. maximus* as well, by comparing stripped and spawned oocytes. Regarding the molecular comparison of stripped and spawned oocytes of *R. decussatus*, an article by Pauletto et al. (Unpublished data) (Appendix 3), in which I participated, is in progress, with me as one of the co-authors. Main data and conclusions of this study will be debated and related with this chapter in the general discussion of the present thesis.

## I. Introduction

Today, the European clam, *Ruditapes decussatus*, is considered one of the most economically important bivalve species in Portugal and in other Southern European countries where it is extensively produced and harvested. The European clam constitutes the majority of revenue from aquaculture in Portugal, representing 76% of the national annual shellfish (DGPA 2012). The production of this species and many other marine bivalves in Europe is dependent upon the production and collection of wild seed, which has been compromised in recent years due to recruitment failure and severe mortalities (Matias et al. 2009). To address this issue, artificial spawning and larval rearing programs were developed to provide an alternative source of seed (Matias et al. 2009), but improvements are still needed to achieve robust and reproducible seed production in captivity. In hatcheries, bivalve gametes are usually obtained by applying thermal shocks to adults. Although an alternative method of gonad dissection, or “stripping”, exists, which is an easier and faster method of accessing gametes. This technique can only be used in species where oocytes can be fertilized directly after dissecting the gonad, such as the Pacific oyster *C. gigas*. Unfortunately, the oocytes of *R. decussatus* are not ready to be fertilized at this stage, representing a further problem for the production of this species (Colas and Dubé 1998; Hamida et al. 2004a).

Reproduction is often one of the most vulnerable time periods for organisms, making them particularly sensitive to changes in environmental conditions (Galbraith and Vaughn 2009). High variability in reproductive success is commonly observed in most bivalve hatcheries and it has been shown to be partly attributable to gamete quality, sperm–egg interaction and differential viability of genotypes (Boudry et al. 2002). Therefore, gamete quality has received increasing attention and several studies have focused on egg or sperm quality in different species (e.g. Bobe and Labbé 2010; Corporeau et al. 2012; Galbraith and Vaughn 2009; Lonergan et al. 2003; Rime et al. 2004; Suquet et al. 2010).

Gamete quality is influenced by many environmental and biological factors. In marine species such as bivalves and fishes, the main factors that appear to affect gamete quality are nutrition (availability of food, algal consumption and essential fatty acids), abiotic environmental factors (temperature, photoperiod and salinity), husbandry practices (spawning induction, egg postovulatory ageing and gamete

handling post-stripping), stress and pollutants (e.g. Angel-Dapa et al. 2010; Bobe and Labbé 2010; Cannuel and Beninger 2005; Delgado and Pérez Camacho 2005; Galbraith and Vaughn 2009; Migaud et al. 2013).

Oocyte quality, defined as the egg's potential to produce viable offspring, can be characterized by embryo development yields (Kjorsvik et al. 1990) and was determined in marine bivalves based on D-larval yields (e.g. Corporeau et al. 2012; Massapina et al. 1999). In terms of predictors of oocyte quality in bivalves, several criteria have been tested, such as gonad color (Mason 1958) and mean oocyte diameter as a direct consequence of nutrition (Cannuel and Beninger 2005). Egg size and shape have also been tested, with large eggs surviving better than small eggs (Baynes and Howell 1996; Kraeuter et al. 1981). Finally, oocyte organic matter and lipid content have been described as important predictors of oocyte quality since a significant relationship was observed between these biochemical parameters, broodstock condition index, and hatching rate (Cannuel and Beninger 2005; Massapina et al. 1999). However, not all indicators consistently correlate with 'quality' in all bivalve species, the most reliable indicators being fertilization success, D-larval yields and survival, three criteria of interest in aquaculture (Migaud et al. 2013).

In the present study, we conducted genome-wide expression profiling of clam oocytes sampled from 25 females displaying variability in subsequent D-larval yields obtained from experimental conditioning and fertilization. We employed a custom oligonucleotide microarray containing 51,678 probes representing unique contigs described and validated in de Sousa et al. (2014). The main objectives of the present work were to analyze molecular features of *R. decussatus* oocytes and to define candidate genes, markers of clam oocyte quality.

## **II. Material and Methods**

### **2.1. Oocyte sampling**

Broodstock of *R. decussatus* (> 35 mm shell length) were collected from Ria de Aveiro (40°42'N; 08°40'W) (western coast of Portugal) and conditioned to accelerate their gonad development in the experimental bivalve hatchery of the Portuguese Institute of Sea and Atmosphere (IPMA) in Tavira, Portugal. Fifty-five

individuals were placed in experimental 30-L tanks in a flow through system at  $20 \pm 1$  °C with a daily phytoplankton supply equivalent to 4% of the dry weight of the clams' soft tissue (Utting and Millican 1997). Food regime consisted of algal mixtures containing a volume of 1/3 *I. aff galbana*, 1/3 *C. calcitrans* and 1/3 *Skeletonema costatum* (Ria Formosa autochthone clone). The water was enriched with this mixed diet and distributed to the tanks at a flow rate of 0.6 to 0.8 L min<sup>-1</sup>. Microalgae were cultured in 80 L bags with seawater (salinity =  $36 \pm 1$  g L<sup>-1</sup>) filtered (0.45 µm), UV treated, and enriched with sterilised f/2 medium (Guillard 1975), in a temperature-controlled room at  $20 \pm 2$  °C under continuous illumination (9900 lux). For diatom culture, sodium metasilicate (molar concentration in final medium -  $1.06 \times 10^{-4}$  M) was added as a silica source in seawater microalgae culture at a concentration of 1mL L<sup>-1</sup>. A continuous aeration was provided to enhance growth and prevent the algae from settling.

After 2 to 4 weeks, clams were induced to spawn on four different sampling dates by thermal stimulation, with temperature alternating between 5 to  $28 \pm 1$  °C, during 30min and 1h, respectively. To avoid uncontrolled fertilization, females once identified were stored in individual containers for spawning. From each female, 20 000 oocytes were taken and rinsed with iso-osmotic ammonium formate (3 % w/v) to remove salt. For total RNA isolation each sample was homogenized in Extract-all (Eurobio) and stored in liquid nitrogen until further total RNA extraction. The remaining oocytes were fertilized by addition of a mixture of sperm from 6 to 7 males (different mixture for each sampling time), to provide a ratio of ten spermatozoa per oocyte in microscope yield. To assess the percentage of oocytes of each female that was successfully fertilized and developed through the early embryo stages, embryos from each treatment were incubated in triplicate 500 ml tanks, with 1-µm filtered and UV-irradiated seawater, maintained at  $20 \pm 2$  °C, at a density of 75 eggs per ml. After 48 h of incubation, the D-larvae were collected on a 30-µm mesh screen and the veliger rate (% of D-larvae) relative to the initial number of eggs was calculated, based on three 1 ml aliquots.

## 2.2. RNA extraction

For microarray analysis, RNA quality and integrity were analyzed on the Agilent bioanalyzer using RNA nanochips and Agilent RNA 6000 nanoreagents

(Agilent Technologies, Waldbronn, Germany). RNA concentrations were measured at 260 nm using a ND-1000 spectrophotometer (Nanodrop Technologies) using the conversion factor 1 OD = 40 mg/mL total RNA. Samples were stored at -80°C until further use.

### **2.3. *Ruditapes decussatus* DNA microarray**

Gene expression analyses of all the oocytes from the 25 females analyzed were performed using an array composed of 59,951 out of 60,000 probes, representing 51,709 different *R. decussatus* contigs, as described by de Sousa et al. (2014). The percentage of annotated transcripts represented on the microarray was 85.7%. Probe sequences and other details on the microarray platform can be found in the GEO database (<http://www.ncbi.nlm.nih.gov/geo/>) under accession number GPL17766.

### **2.4. Labeling and microarray hybridization**

Sample labeling and hybridization were performed according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol with the Low Input Quick Amp Labeling kit. Briefly, for each sample 100 ng of total RNA was linearly amplified and labeled with Cy3-dCTP. A mixture of 10 different viral poly-adenylated RNAs (Agilent Spike-In Mix) was added to each RNA sample before amplification and labeling to monitor microarray analysis work-flow. Labeled cRNA was purified with Qiagen RNeasy Mini Kit, and sample concentration and specific activity (pmol Cy3/μg cRNA) were measured in a NanoDrop® ND-1000 spectrophotometer. A total of 600 ng of labeled cRNA was prepared for fragmentation by adding 5μl 10X Blocking Agent and 1μl of 25X Fragmentation Buffer, heated at 60°C for 30 min, and finally diluted by addition with 25μl 2X GE Hybridization buffer. A volume of 40μl of hybridization solution was then dispensed in the gasket slide and added to the microarray slide (each slide containing eight arrays). Slides were incubated for 17 h at 65°C in an Agilent hybridization oven, subsequently removed from the hybridization chamber, quickly submerged in GE Wash Buffer 1 to disassemble the slides and then washed in GE Wash Buffer 1 for approximately 1 minute followed by one additional wash in pre-warmed (37°C) GE Wash Buffer 2.

## 2.5. Data acquisition, correction and normalization

Hybridized slides were scanned at 2µm resolution using an Agilent G2565BA DNA microarray scanner. Default settings were modified to scan the same slide twice at two different sensitivity levels (XDR Hi 100% and XDR Lo 10%). The two linked images generated were analyzed together, data were extracted and background subtracted using the standard procedures contained in the Agilent Feature Extraction (FE) Software version 10.7.3.1. The software returns a series of spot quality measures in order to evaluate the quality and the reliability of spot intensity estimates. All control features (positive, negative, etc.), except for Spike-in (Spike-in Viral RNAs), were excluded from subsequent analyses.

Raw gene expression data of all the oocytes from the 25 females analyzed were deposited in the GEO database under accession number GSE54954. Spike-in control intensities were used to identify the best normalization procedure for each dataset. After normalization, spike intensities are expected to be uniform across the experiments of a given dataset. Normalization procedures were performed using R statistical software. Quantile normalization always outperformed cyclic loess and quantile-normalized data were further adjusted for the known between-experiments batch effects by implementing the parametric Combat in R, to avoid bias due to different batches of the oligonucleotide microarray (Johnson et al. 2007). Normalized data were deposited in GEO archive under accession number GSE54954.

## 2.6. Data analysis

One-way ANOVA parametric tests were used to investigate the significance of the factor time on the D-larval yield after angular transformation (STATGRAPHICS Centurion XV.II) and on the gene expression, after a base 10 logarithmic transformation (TMeV 4.5.1 - TIGR MULTIEXPERIMENT VIEWER), using a p-value cut-off of 0.05.

Results of D-larval yields were expressed as mean  $\pm$  standard deviation (SD). Percentages were square-root transformed and statistical analysis were performed using Student's t-test at a significance level of  $p=0.05$  in order to compare good and poor quality oocytes of *R. decussatus*. T-Tests ( $p$ -value  $<0.01$ ) were computed to compare gene expression between good and poor quality oocytes of *R. decussatus*

(TMeV 4.5.1). The resulting gene lists were then filtered, and only those probes with Fold Change (FC)>1.5 were considered differentially transcribed.

A principal component analysis (PCA) using TMeV 4.5.1 (Saeed et al. 2003) was applied to assess the distribution of the studied groups. Hierarchical clustering was performed using TMeV 4.5.1 on statistically significant transcripts to group experimental samples basing on similarity of the overall experimental expression profiling.

A quantitative correlation analysis was performed by SAM (Significance Analysis of Microarray) (FDR=5%) to identify genes with a correlation between mRNA levels and D-larval yield.

### **III. Results and Discussion**

#### **3.1. D-larval yield**

Oocytes of 25 females were analyzed in this study. High variability between individuals was observed for D-larval development, ranging from 0 to 95%.

Although fertilization of oocytes was carried out with a different mixture of sperm in each of the four sampling dates, we still observed oocyte quality variation within each date (0 to 41% in day 1; 4 to 46% in day 2; 22 to 95% in day 3 and 5 to 88% in day 4). Additionally, a one-way ANOVA ( $p>0.05$ ) revealed that there was no statistically significant difference among the D-larval yields across the four sampling times showing that there was no noticeable effect of sperm pools on D-larval yield. Considering such statistical results and since the environmental conditions and experimental procedures were kept uniform across oocyte batches, we assume that this variation was most likely to be due to the intrinsic quality of oocytes of each female. Such individual variability is commonly observed in molluscs (Corporeau et al. 2012; Massapina et al. 1999) as well as in fishes (Salze et al. 2005) and may originate from biochemical and molecular composition of oocytes and broodstock nutrition. From the 25 studied females, 18 were split into two groups, between which there is a significant difference for early developmental success of fertilized oocytes ( $p<0.001$ ): a good quality group of 8 samples with a D-larval yield superior to 40% (mean  $\pm$  SD =  $57 \pm 22\%$ ) and a poor quality group of 10 samples with a D-larval yield

inferior to 20% (9 ±5 %). The remaining 7 females presented an intermediary D-larval yield (27 ±6 %) and were not chosen for gene expression analysis (Fig. 25). Sampling times were randomly distributed in these two groups.

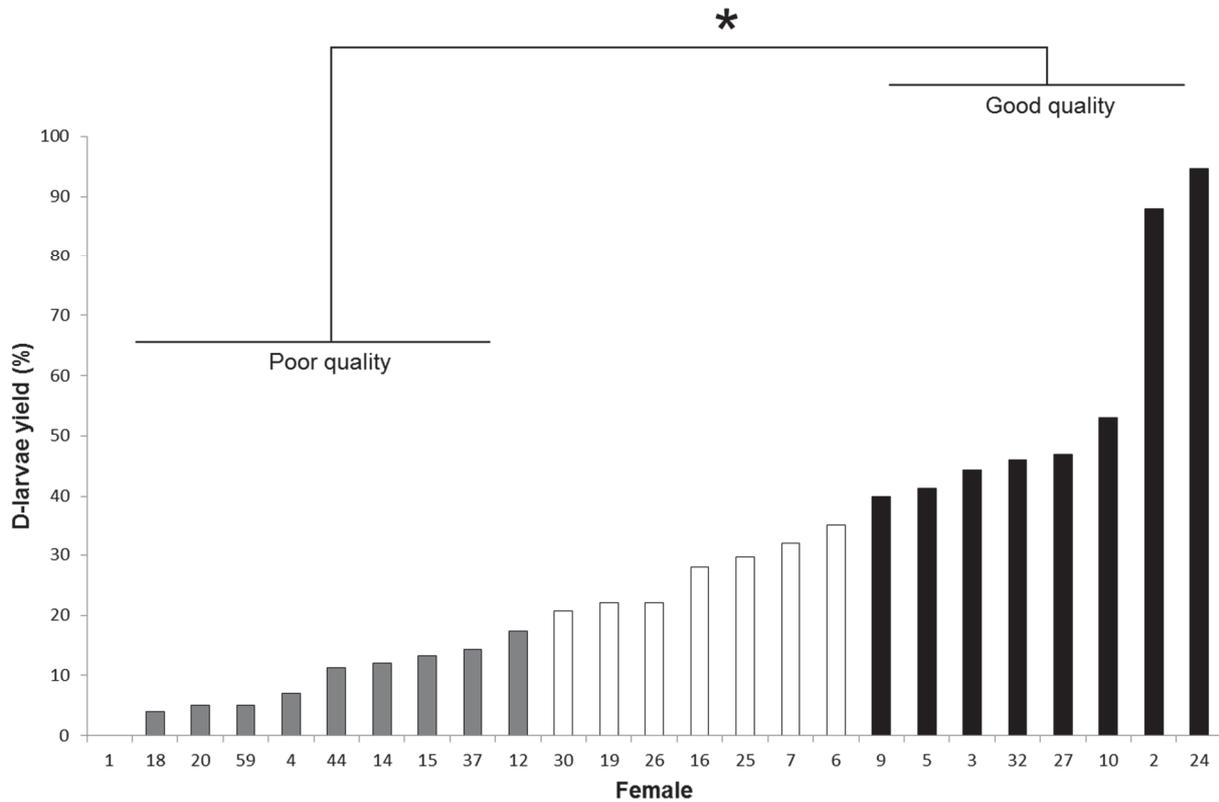


Figure 25 - Individual D-larval yield (mean ± SD) obtained from 25 females of *Ruditapes decussatus*. The females were divided into three groups: Good quality (black bars), intermediate quality (white bars) and poor quality (grey bars) oocytes. Results are expressed as the percentage of oocytes that reached D-larval stage 48h after fertilization.

### 3.2. Identification of Differentially Expressed Genes between good and poor quality oocytes

In order to compare gene expression between good and poor quality oocytes, we carried out student's T tests ( $p < 0.01$ ) on the 10 samples with the lowest D-larval yield versus the 8 samples with the highest D-larval yield mentioned before (with a minimum  $FC > 1.5$ ). This analysis identified a list of 526 significant probes, corresponding to 359 unique transcripts (Online Resource 1). A Principal Component Analysis (PCA) was applied to the set of the 526 differentially expressed probes on the oocytes from the 18 females analyzed (Fig. 26). We observed a divergence in

expression patterns between good and poor quality oocytes, determined by D-larval yield, along the first axis PC1, which explained 83% of the variation.

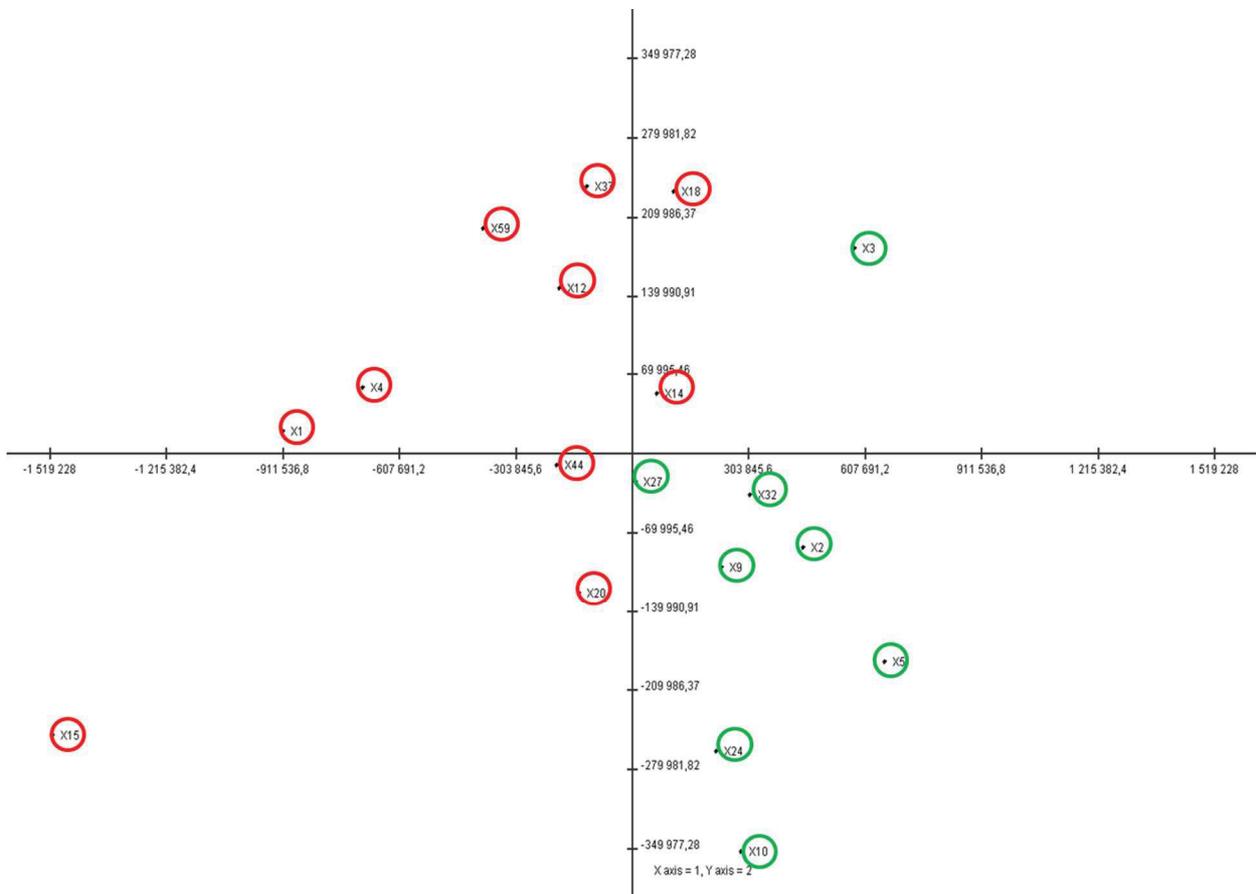


Figure 26 - Principal Component Analysis (PCA) applied to the set of the 526 differentially expressed probes on the oocytes from the 18 females of *Ruditapes decussatus* analyzed. The 10 samples with the lowest D-larval yield (red circles) and the 8 samples with the highest D-larval yield (green circles) are highlighted.

Hierarchical clustering, using Pearson's correlation, was also performed on the same samples, considering the set of 526 differentially expressed probes. As reported in Fig. 27, two main clusters were identified: good and poor quality oocytes. A total of 361 differentially expressed probes were more abundant in good quality oocytes with a FC ranging from 1.5 to 11.6, while a total of 165 differentially expressed probes were more abundant in poor quality oocytes with a FC ranging from 1.5 to 10.2.

To check if time of sampling had limited or no effect, and consequently no sperm effect on gene expression analysis, a one-way ANOVA ( $p > 0.05$ ) was performed revealing that 11% of genes (6415) were differentially expressed between

sampling times. These genes were likely influenced by sperm mixture or maturing process that evolved over time. However, all the 526 probes that appeared differentially expressed between good and poor quality oocytes fall within the remaining 89% of the genes (53522) that were not statistically different between sampling dates.

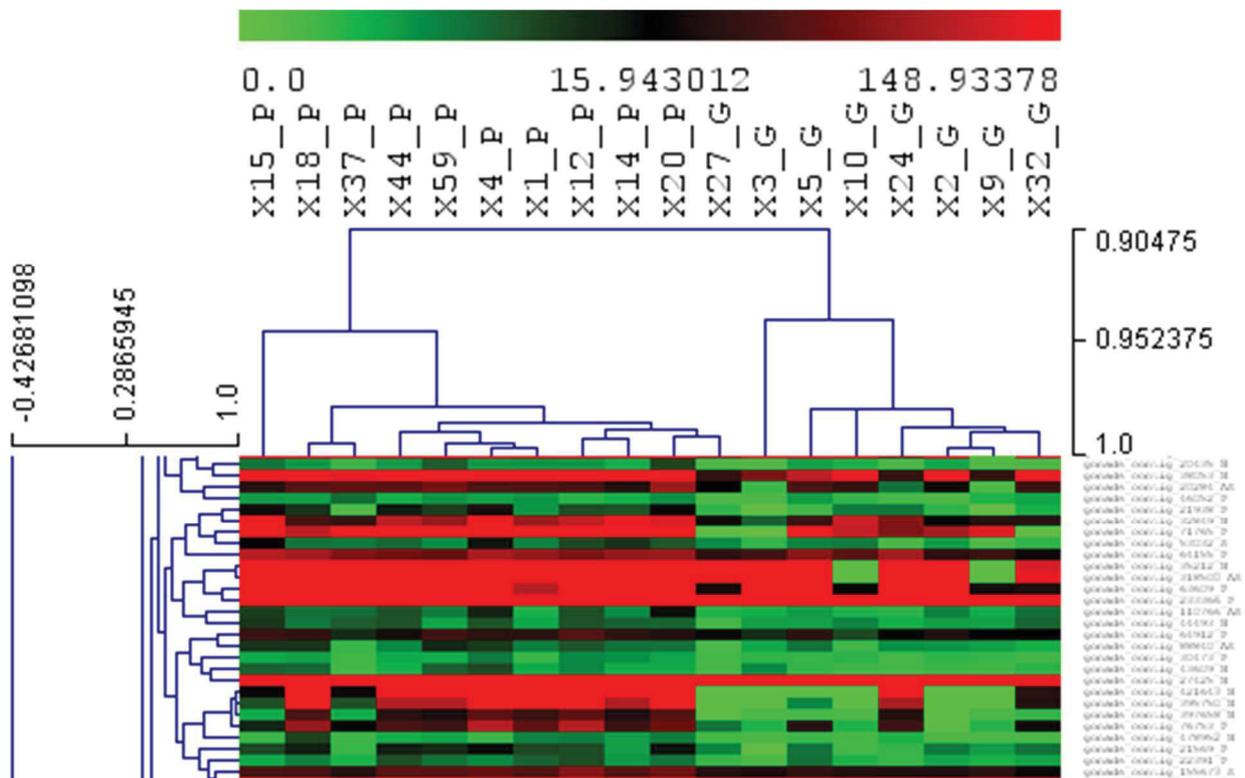


Figure 27- Hierarchical clustering obtained using Pearson's correlation considering the set of 526 differentially expressed probes between the 10 females with the lowest D-larval yield and the 8 females of *Ruditapes decussatus* with the highest D-larval yield analyzed. Heat map of oocyte quality specific genes (P – Poor quality oocytes / G – Good quality oocytes).

### 3.3. Chaperone proteins are main determinants of good quality oocytes in *Ruditapes decussatus*

Among the genes that had higher abundance in good quality oocytes, we noticed the presence of chaperone molecules that we consider to be potential markers of oocyte quality, confirmed by a literature review. Chaperones are proteins that assist the non-covalent folding or unfolding of other proteins and are widespread in cells across taxonomic groups. Protein folding is a key step for normal protein synthesis, but is also severely affected by stressors such as high/ low temperature or

oxidative stress. In fact, several chaperones are over-expressed under cellular stress conditions to counteract the potential damage caused by protein misfolding (Macario 2007). In the present study, chaperones were found to be more abundant in good quality oocytes. Such evidence might be related, at least partially, with the method used to induce clam spawning, i.e. thermal shock, with temperature alternately increasing from 5 to  $28 \pm 1$  °C. It is possible that chaperone proteins play a major role in protecting oocytes from thermal shock or other cellular stresses induced by manipulation. Good quality eggs might be those that are able to mount a more efficient response to the challenge inherent in thermal induction of spawning. Our results are based on evidence at the mRNA level and will require confirmation at the protein level. However, with the exception of DnaJ/HSP40, it is very remarkable to observe that all chaperones revealed in the present study were recently found to be associated, at the protein level, with high quality oocytes in another bivalve species, the Pacific oyster *C. gigas*, with some of them being maternal mRNAs essential for fertilization, first cleavage and embryonic genome activation (Corporeau et al., 2012).

DnaJ (Hsp40) homolog subfamily C member 27-A, Heat Shock 70 kDa protein 12A (Hsp70), Cyclophilin B and Protein Disulfide Isomerase family A (PDI) were the chaperone proteins highlighted as differentially expressed between oocyte quality groups. DnaJ (Hsp40) homolog was the most abundant chaperone at the mRNA level in good quality oocytes (FC=5.3). DnaJ contains the J domain through which the chaperone binds to Hsp70s to determine their activity by stabilizing their interaction with substrate proteins (Fan et al. 2003; Qiu et al. 2006). In turn, Hsp70 (FC=1.7) binds to nascent polypeptides to facilitate correct folding and prevents the aggregation of non-native and misfolded (Corporeau et al. 2000; Delelis-Fanien et al. 1997). Hsp70 is a well-known stress related protein already described in several bivalve species, including *C. gigas* (Corporeau et al. 2012; Tanguy et al. 2004), the Mediterranean mussel *Mytilus galloprovincialis* (Cellura et al. 2007), the manila clam *Ruditapes philipinarum* (Kang et al. 2006; Moreira et al. 2012) and the European clam *R. decussatus* (Gestal et al. 2007; Moreira et al. 2012). Indeed, in *C. gigas*, it was suggested that greater abundance of the protein HSP70 could be related to better protein folding in two-cell stage embryos and better protection of oocytes and early embryos directly exposed to oxidative stress, temperature and pH fluctuations in the sea water (Corporeau et al. 2012). It was also reported that the chaperone function of Hsp70 is required for protection against stress-induced apoptosis (Mosser

et al. 2000). Thus, we can similarly suggest that this higher expression of DnaJ (Hsp40) homolog and Hsp70 in good quality oocytes might be implicated in the protection and maintenance of developing oocytes in *R. decussatus*. Finally, the extensive set of Hsp70 genes (88 putative homologs) in the *C. gigas* genome, which corresponds to the expansion of “stress genes” recently reported in the oyster genome (Zhang et al. 2012) suggests a complexity of the chaperone responses in marine bivalves that merits further investigation.

Cyclophilin B (FC=1.5) was also more highly expressed in good quality oocytes. Cyclophilins are known to catalyze protein folding in cooperation with heat-shock proteins (Corporeau et al. 2012). Such proteins are collectively known as immunophilins and it was suggested that cyclophilins have a diverse array of additional cellular functions, including roles as chaperones and in cell signaling (Wang and Heitman 2005). The functions reported for cyclophilins in model organisms imply that these proteins could have a crucial role in oocyte viability and immune protection, thus explaining their higher mRNA expression in gametes that have higher D-larval yields.

A last chaperone molecule, found more abundant in good quality oocytes, is Protein Disulfide Isomerase family A (PDI) (FC=1.5). A few studies have suggested that PDIs are involved in oocyte development (Ohashi et al. 2013), probably by mediating the formation of disulfide bonds in proteins, particularly Vitellogenin (Liao et al. 2008). Furthermore, several studies suggested an essential role of PDI for the sperm–egg fusion at fertilization (Calvert et al. 2003; Ellerman et al. 2006), leading us to suggest that sperm-egg interaction could also be one of the reasons to explain the high individual variability in oocytes quality when estimated by D larval yields.

### **3.4. Prophase I block and meiotic maturation in *Ruditapes decussatus* oocytes**

With the exception of some cases like oysters, where oocytes can achieve completion of meiosis in seawater after stripping (Osanai 1985), oocytes of most marine bivalve species like *R. decussatus* are arrested at prophase of the first meiosis and spend their entire growth phase in a state analogous to the G2/M transition of a mitotic cell cycle (Colas and Dubé 1998). To release this block, hormonal stimulation or other stimuli are needed for the oocytes to pass through

metaphase, which is why oocytes cannot be fertilized directly after dissecting the gonad (Colas and Dubé 1998; Hamida et al. 2004). Spawning induction has been achieved in bivalves with variable success depending on the species, using various stimuli including temperature shock, addition of gametes to the water over the broodstock, injection with neurotransmitters such as serotonin, dopamine, noradrenalin and hormones such as prostaglandin (Velasco et al. 2007). In addition, in some bivalve species, a second barrier to development occurs at metaphase I, prior to extrusion of the first polar body, until fertilization releases this metaphase arrest and allows further stages of maturation to take place (Colas and Dubé 1998). The molecular cascade, and the underlying genes involved in this phenomenon, has been little studied in bivalves showing mainly the crucial role of  $Ca^{2+}$  signaling pathway during oocyte maturation (e.g. Abdelmajid et al. 1993; Colas and Dubé 1998; Deguchi and Morisawa 2003; Leclerc et al. 2000; Stricker and Smythe 2001; Zhang et al. 2009). It has been suggested that continuously synthesized, short-lived proteins are responsible for maintaining metaphase arrest in some molluscan oocytes, with cyclin regulatory proteins as good candidates since their disappearance triggered metaphase/anaphase transition and polar body extrusion (Colas and Dubé 1998). In *Ruditapes* and other molluscs, calcium may act via a calcium calmodulin-kinase since calcium calmodulin antagonists were able to prevent metaphase I release and cyclin degradation upon fertilization (Abdelmajid et al. 1993; Colas and Dubé 1998; Leclerc et al. 2000; Whitaker 1996; Zhang et al. 2009). Indeed, in this work we found the enzyme Calcium/calmodulin-dependent 3',5'-cyclic nucleotide phosphodiesterase 1C (Cam-PDE1C) (FC=1.7) among the genes more abundant in good quality oocytes, confirming the importance of this pathway in the maturation process of oocytes of this species.

Among the genes more abundant in poor quality oocytes, we observed Guanine nucleotide-binding protein subunit beta-like protein 1 (G protein) and Histone-lysine N-methyltransferase PRDM9. Since these genes are involved in oocyte maturation and in the transition of oocytes through meiosis (Voronina and Wessel 2004; Williams et al. 1996; Wu et al. 2013), we could suggest that their overexpression is implicated in this important cell process.

Guanine nucleotide-binding protein subunit beta-like protein 1 (G protein) (FC=2.2) was present in the list of more abundant genes in poor quality oocytes. There are several studies that indicate that G proteins are involved in oocyte growth

and maturation (Voronina and Wessel 2004; Williams et al. 1996). G-proteins may also be involved in releasing the oocytes from an arrest inherent within the oocyte. For example, in starfish, progression of an oocyte into meiotic divisions is dependent upon activation of subunits of G-proteins (Chiba and Hoshi 1995; Jaffe et al. 1993; Thomas et al. 2002). Although G proteins are involved in several different processes, based on these examples, it is possible that the overexpression of this protein deregulates the oocyte maturation of *R. decussatus*. More precisely, the initiation of meiosis and the transition of the oocytes through metaphase I may be impacted. Moreover, it was reported that these proteins could also be involved in the important process of sperm-egg interaction (Fard Jahromi and Shamsir 2013; Leclerc and Kopf 1999; Lee et al. 2005) as the chaperone molecule PDI mentioned before.

Finally, Histone-lysine N-methyltransferase PRDM9 appeared as a relevant candidate to oocyte quality in *R. decussatus* as it displayed the highest differential (FC=10.2) between the two groups of clam oocytes. This gene is thought to be essential for proper meiotic progression in species in which it was documented. Actually, it has been proposed that PRDM9 binds to specific sites in the genome of oocytes and spermatocytes, where its methyltransferase activity leads to a local enrichment of histone H3 peptide dimethylated on lysine 4 (H3K4me2) and recruits the meiotic recombination machinery (Wu et al. 2013). During meiosis, homologous recombination occurs preferentially at defined hotspots. In mammals, the fast-evolving DNA-binding domain of PRDM9 has been identified as a major hotspot determinant that may explain the rapid rates of hotspot redistribution during evolution (Hochwagen and Marais 2010). Although the PRDM genes are not well studied in invertebrates, their importance in the cell cycle already described in vertebrates (Baudat et al. 2010) led us to suggest that PRDM9 could play an important role in the meiotic progression of *R. decussatus* oocytes and therefore in their maturation. Further gene expression studies of these candidates are needed to compare spawned and stripped oocytes and follow the processes of gamete maturation along the genital ducts.

### 3.5. Cell cycle control, Stress and Apoptosis

Among the genes that were more abundant in poor quality oocytes, some appeared to be implicated in cell cycle and cell responses to stress. DNA polymerase

$\alpha$  (FC=2.3) was one of these genes. This enzyme is required for cell-cycle checkpoint control, DNA repair (Oda et al. 1996) and efficient chromosomal DNA replication in *Xenopus* genus oocytes, as well as in eukaryotic cells in general (Waga et al. 2001). This enzyme is involved in purine synthesis. The inhibitory effects of purines on meiotic progression are well documented in the mouse and are thought to play a role in inhibiting the hydrolysis of cAMP (high intra-oocyte levels of cAMP are associated with meiotic arrest) (Sutton et al. 2003). Synthesis of purines and phosphoinositides is involved in spontaneous and progesterone-induced nuclear maturation in *Bufo arenarum* oocytes, while maturation in mouse oocytes is induced in vivo when purine metabolism is altered. Besides, in *C. gigas*, two enzymes involved in de novo purine synthesis were also more abundant in low quality oocytes of this species (Corporeau et al. 2012). Taken together, these results suggested that purine metabolism has an essential role in the oocyte maturation in animals such as clams.

In addition, we also noticed that genes belonging to apoptosis pathway and p53 signaling pathway were over expressed in poor quality oocytes. It is known that the p53 superfamily proteins originally evolved to mediate programmed cell death of damaged germ cells or to protect germ cells from genotoxic stress (Vilgelm et al. 2010; Walker et al. 2011). Thus, we can hypothesize that the greater abundance of genes in the p53 signaling pathway in poor quality oocytes could be due to a stress suffered by the cells, thus affecting fertilization success.

In the list of more abundant genes in poor quality oocytes we also observed the presence of Tumor necrosis factor receptor (TNF) (FC=2.4). TNF is one of the factors that activate the death receptor-dependent pathway by binding to cell surface death receptors. The activation of this pathway results in the activation of Caspase-8 (Trounson et al. 2013). The presence of this factor, together with the presence of the genes belonging to apoptosis pathway, corroborates the hypothesis of the high incidence of programmed cell death in oocytes with lowest D-larval yield.

Considering the entire dataset composed of 25 females, quantitative correlations between microarray data and D larval yields were evaluated via a SAM quantitative correlation analysis (FDR=5%). The results showed a total of 6 probes significantly correlated with the D-larval yield (Online Resource 2). Of these 6 probes, 5 were negatively correlated with the D-larval yield and only 2 probes were annotated by similarity and associated with known proteins: Caspase-8 and Transcription factor E2F5 (Fig 28). The role of Caspase-8 which correlated negatively with D-larval yield,

is very interesting since caspases represent a family of intracellular cysteine proteases linked both to the initial and final stages of apoptosis in numerous types of cells (Johnson and Bridgham 2002). Particularly, there are several studies that show the importance of caspases in the death by apoptosis of unfertilized ovulated oocytes (Johnson and Bridgham 2002; Papandile et al. 2004; Reynaud and Driancourt 2000; Trounson et al. 2013). In bivalves and specifically in *R. decussatus*, the gonad passes through several developmental stages from sexual rest to ripe and spent (Delgado and Pérez Camacho 2005; Matias et al. 2013). In these last stages mature oocytes are spawned while those that are not released begin their resorption in the gonad. In this study all the oocytes were spawned by induction and were expected to be mature and ready to be fertilized. However, based on our results, it could be possible that over-expression of apoptosis-related genes is triggered as a consequence of unsolved cell stress, like thermal stress, affecting negatively the fertilization success. Lastly, the negative correlation of E2F5 with the D-larval yield suggests the deregulation of the cell cycle and the normal development of gametes by this protein.

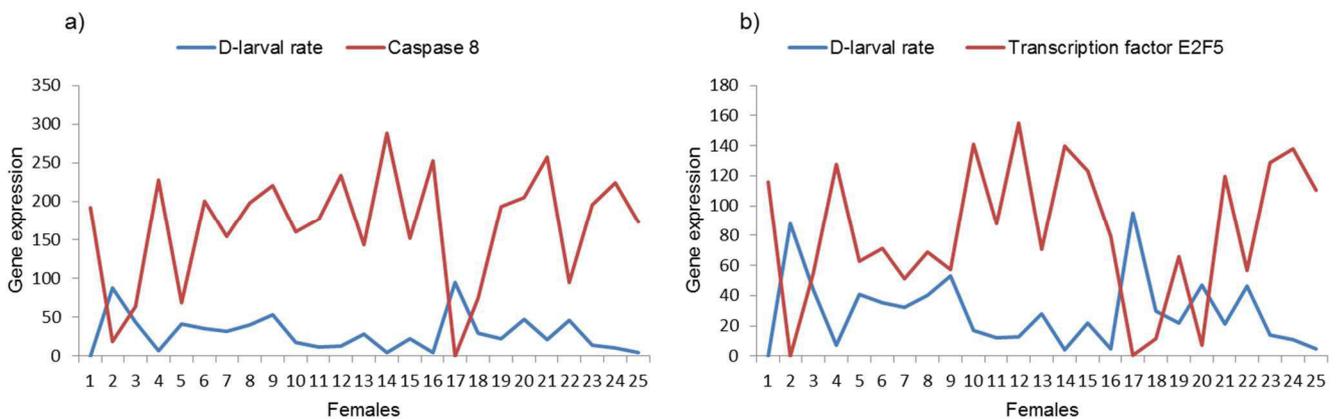


Figure 28 - Negative correlations between gene expression values of a) Caspase 8 and b) Transcription factor E2F5 with the D-larval yield of *Ruditapes decussatus*.

## IV. Conclusion

Individual variability in oocyte quality is commonly observed in marine molluscs and is one of the biggest problems in establishing a successful hatchery-based production of several marine species (e.g. Bobe and Labbé 2010; Boudry et al. 2002; Corporeau et al. 2012; Massapina et al. 1999). Using a microarray-based analysis, we identified several genes that were differentially linked with oocyte quality, which we consider to be potential markers of oocyte quality in *R. decussatus*. Genes coding for the chaperone proteins are suggested to be involved in important roles for the oocyte protection. Apoptosis appeared as one important pathway that we found more abundantly represented in poor quality oocytes and negatively correlated with the D-larval yield, noticeable by the presence of caspase-8, linked to programmed cell death in numerous types of cells. Among the candidate genes, DnaJ (Hsp40) homolog, tumor necrosis factor receptor and caspase 8 appeared to be candidates of particular interest for more specific and precise individual functional investigation in relationship with reproductive success of *R. decussatus*. This study is particularly relevant since poor and variable gamete quality is one of the major bottlenecks hampering the development of commercial aquaculture of the European clam.

# General Discussion

Bivalves are the main component of the benthic fauna of many marine and estuarine areas. In the last centuries, due to their high economic importance, its production has increased due to the tendency of man to cultivate it. However, until now, most of the bivalve production in Europe still relies on the collection of wild seed. Consequently bivalve production is highly dependent on overall environmental conditions that influence growth, survival and reproduction, and juvenile natural recruitment, the basis of production (Matias 2013). One significant example is the European clam *R. decussatus*, targeted in this thesis, a species with a high social and economic importance in many Southern European countries. In order to overcome the random availability of seed, the development of *R. decussatus* rearing programs and the development of hatchery technology for the production of this species, seems essential. However, until know, only very few studies have been performed on this subject (e.g. Albentosa et al. 1996; Albentosa et al. 1999; Delgado and Pérez Camacho 2005; Delgado and Pérez-Camacho 2007; Fernandez-Reiriz et al. 1999; Ojea et al. 2004; Ojea et al. 2008), mainly concerning the evaluation of the gametogenic cycle, biochemical composition and the effects of food and temperature on spat and broodstock conditioning.

*R. decussatus* seed production relies on basic principles that follow successive biological steps (Gosling 2003, Helm and Bourne 2004; Joaquim et al. 2008b). Therefore, the present thesis aimed to evaluate and improve the knowledge on the reproduction of *R. decussatus* at the broodstock conditioning phase, as well as determinate relevant markers of oocyte quality. Overall, the ultimate objective of this study was to participate in the contribution to improve production programs of this species.

## I. Gametogenesis in *Ruditapes decussatus*: Histological and genomic approaches

Investigations on reproductive cycle of bivalves have been carried out using several methods that can be classified in direct and indirect methods. Direct methods, such as biochemical analysis and condition indexes are based on gonadal tissue analysis and they are more reliable and commonly used by scientists and oyster farmers (Bodoy et al. 1986), with some of them being a good way to estimate the quantity of produced and spawned gametes. Indirect methods are based on detecting larvae in plankton or estimating the subsequent post-larval settlement or seed recruitment, which could provide information about the spawning period (da Costa et al. 2012). Macroscopic observation of the gonad, another direct method, has been a method widely used due to its easiness, with the observation of the relative size, shape and color of the gonads (Aracena et al. 2003; Mazé and Laborda 1990; Remacha-Trivino and Anadon 2006). However, the information provided is scarce and difficult to interpret. Histological preparation of gonad tissue is time consuming and costly, but allows gathering detailed information about the reproductive cycle at a structural level (da Costa et al. 2012).

Up to now, the studies performed on the reproduction of *R. decussatus* focused in the evaluation of mean gonad weight (Ojea et al. 2004), reproductive cycle and gonad development (e.g. da Costa et al. 2012; Delgado and Perez-Camacho 2003; Delgado and Pérez Camacho 2005; Matias et al. 2013; Serdar and Lok 2009; Serdar et al. 2010; Xie and Burnell 1994), biochemical composition and condition index (e.g. Aníbal et al. 2011; Camacho et al. 2003; Hamida et al. 2004b; Ojea et al. 2004; Serdar and Lok 2009), external factors affecting the gametogenic cycle (e.g. Camacho et al. 2003; Delgado and Pérez Camacho 2005; Delgado and Pérez-Camacho 2007; Matias et al. 2009; Ojea et al. 2008; Smaoui-Damak et al. 2006), oocyte diameter (e.g. Delgado and Pérez-Camacho 2007; Hamida et al. 2004b; Laruelle et al. 1994; Ojea et al. 2008; Smaoui-Damak et al. 2006; Xie and Burnell 1994) and gonadal index, which consist in assigning numerical values to each developmental stage (i.e. each of the stages of a gametogenic scale) allowing a calculation of a histological index for each sample (Matias et al. 2013 - Appendix 1).

The commonest method to analyse histological preparations is a qualitative method consisting of ascribing each preparation to a previously established

gametogenic scale, as it was performed in Chapter 1 of this thesis. The gametogenic cycle of the two main Portuguese populations of *R. decussatus* (Ria Formosa and Ria de Aveiro) were studied through histological analyses of male and female gonads. The reproductive cycle of *R. decussatus* from both populations followed an annual cyclicity and comprised a resting phase during the period of coldest seawater temperatures (October to January), a ripe stage in spring followed by an extensive spawning period that began in late spring and extend during summer until early autumn. This extended and continuous spawning period, during which occurs successive and simultaneous production of gametes and spawning may be an advantageous strategy for the species because it ensures a continuous supply of gametes. However, based in histological analysis made between July 2011 and June 2012, significant differences were observed in terms of dynamics of gametogenesis in the two geographically distinct studied populations. The maturation process began earlier in the northern population (Ria de Aveiro), coinciding with the first phytoplanktonic bloom, which is typical of an opportunistic strategy. On the other hand, in Ria Formosa population the gametogenesis took place when there was low food availability, thus allowing the first spawning release when seawater temperature increased (August), which is typical of a conservative strategy (vide Matias et al. 2013 - Appendix 1). Although several authors considered *R. decussatus* as a conservative species (Ojea et al. 2004; Urrutia et al. 1999), Aníbal et al. (2011) concluded that *R. decussatus* from Ria Formosa Lagoon exhibited an intermediate strategy, corroborating with the biochemical analysis made by Matias et al. (2013).

Indeed, gametogenic cycles in bivalves are affected by the geographical location and consequently by the environmental factors. Various authors have indicated the existence of one single period of gamete release during summer in *R. decussatus* in Spain (Figueras 1957; Perez-Camacho 1980; Villalba et al. 1993), Italy (Breber 1980) and Ireland (Xie and Burnell 1994). Other authors reported two major periods of spawning, in the spring and again in summer, in France (Borsa and Millet 1992; Laruelle et al. 1994) and Morocco (Shafee and Daoudi 1991), with the first one being a partial spawning, as occurred with the two Portuguese populations studied. Additionally, some differences can be observed when we compare, for example, the reproductive cycle of *R. decussatus* in South of Tunisia (Hamida et al. 2004b). In this population gametogenesis started in January and February, with the gonadic activity increasing from March to November-December. Spawning events

occurred irregularly from June to December (Hamida et al. 2004b). The reproductive cycle of the two European clam populations studied in the present work, were similar to those made in other bivalves from temperate regions. According to Lango-Reynoso et al. (2000), in the Japanese oyster *C. gigas*, the early gametogenesis stage was observed from October/November to March for three populations living along the French Atlantic shoreline, which corresponds to winter months when water temperature is low. This is the time when germinal cells produce young oocytes that remain in a “stand-by” stage until the temperature increases and there is a greater availability of food at the beginning of spring. The growing stage was observed in April–June which corresponds to the highest gametogenic activity. In this stage, there was a re-activation of the young oocytes development. Similar observations were made in several temperate Pectinidae like *Argopecten irradians* in which the primary germ cells and gonial cells develop in winter and early spring (Sastry 1970).

A more objective determination of the degree of maturation is provided by methods that measure the area occupied by gametes. In the present study no significant differences were observed between the two studied populations of *R. decussatus*. The same method was used by Fabioux et al. (2005) to measure the gonad area in *C. gigas*, however, in this species, the gonad develops around the digestive gland contrasting with *R. decussatus*, where the anatomic features of the gonad make it difficult to separate from the digestive system. In both species the gonad area increases during the course of the gametogenic cycle, from activation of the gonad through gametogenesis and spawning, decreasing in the subsequent recession. When examining the median part of the visceral mass, the surface percentage occupied by the gonad was directly linked to the size of oocytes produced ( $R^2=0.98$  for Ria Formosa and  $R^2=0.95$  for Ria de Aveiro) and is therefore representative of reproductive effort (Royer et al. 2008) which corresponds to the proportion of energy allocated to reproduction (Todd and Havenhand 1983; Normand et al. 2009).

Other way of quantifying the gonad by histology involves measuring mean oocytes diameter, which as well, clearly reflects the gametogenic cycle. Oocytes gradually increase in size as they mature, reaching their maximum size prior to spawning. Then, mean oocyte diameter decreases sharply after spawning due to the release of the larger and mature female gametes (da Costa et al. 2012). In the present study, no significant differences were observed between populations,

regarding oocyte diameter. Although, comparing with previous studies in the same species (e.g. Delgado and Pérez-Camacho 2007; Hamida et al. 2004b; Laruelle et al. 1994; Ojea et al. 2008; Smaoui-Damak et al. 2006; Xie and Burnell 1994), we observed a decrease in the oocyte size in these Portuguese populations. Indeed, oocyte size is a highly variable trait both within and among species of marine invertebrates, being highly influenced by environmental factors, such as temperature, salinity, and toxicants (Moran and McAlister 2009). The first chapter of this thesis had a major importance in order to better understand the natural reproductive cycles of the two European clam populations. Indeed, the manipulation of the gonadal cycle and spawning period of the clams (so that adults can spawn earlier or later than occurs in the natural environment) is crucial (Ojea et al. 2004).

In the subsequent chapter, the transcriptome of the gametogenesis of *R. decussatus* was performed with interesting results. For developing genomic approaches, a new microarray platform specific to this species was set up. cDNA libraries of oocytes, larval stages and different gonadal maturation stages were sequenced on the Illumina platform, and a custom oligonucleotide microarray representing 51,678 assembled contigs was designed and then applied to identify the transcripts whose expression changed during gonad development and to identify differential gene expression between the gonads of the two Portuguese populations of this species. In the third chapter, gene expression analyses of oocytes coming from 25 females of *R. decussatus* from Ria de Aveiro were performed, using the same array described before, in order to determinate relevant markers of oocyte quality. These data substantially expanded genomic information on *R. decussatus* (only 4667 total reads were available in NCBI before Reproseed project) and are crucial for constructing comprehensive DNA microarrays in order to accelerate the development of both genomics and genetics in this commercially important species. Besides, until the present study, apart from a few specific gene expression analyses (Moreira et al. 2012) and a first version of DNA microarray designed to unravel host-parasite interactions (Leite, Milan et al. 2013), very little information was available on genome-wide expression profiling for physiological, environmental or aquaculture questions in this species.

Genes like *Foxl2*, an evolutionarily conserved female-specific transcription factor and vitellogenin (VTG), a female specific glycoprotein, were identified in females' gonads and increased in expression as oogenesis progressed. These

female-specific genes, with VTG also positively correlated with gonad area, were also identified as potentially involved in oogenesis in *C. gigas* (Corporeau et al. 2012; Dheilly et al. 2012) and in the scallop, *Patinopecten yessoensis* (Osada et al. 2004). Similarly in the scallop, VTG showed a high amount during the growing stage and the level was retained until spawning stage. The yolk protein or vitellin (Vn) accumulated in the oocytes of oviparous animals is generally synthesized outside the oocytes as a yolk protein precursor or vitellogenin (Vtg), which is called hetero-synthetic vitellogenesis (Osada et al. 2004). Indeed, *in situ* hybridization demonstrated the expression of Vtg mRNA in the auxiliary cells closely associated with growing oocytes of the scallop (Osada et al. 2004). In relation to Foxl2, this is one of the most conserved genes involved in the early events of the cascade leading to ovary differentiation and, similarly to VTG, also identified as potentially involved in oogenesis in *C. gigas*, not only in female gonadal differentiation, but also in maintaining its differentiation throughout the adult life (Dheilly et al. 2012; Naimi et al. 2009; Santerre et al. 2012). Regarding males, dpy-30 and the motile sperm domain containing protein 2 (MOSPD2) were identified in the gonads, increasing in expression as spermatogenesis progressed. dpy-30 is known to be involved in male development and described as an attractive new candidate gene for the regulation of sex differentiation in oysters (Dheilly et al. 2012).

All these genes helped to improve our understanding of gametogenesis in *R. decussatus*, being important for a possible starting point for further researches devoted to reproduction and spawning improvement.

## **II. Spawning induction in *Ruditapes decussatus*: A challenging task**

As already reported, one of the major objectives of the present thesis was to better understand the causes of the differences between the two wild populations of *R. decussatus*, concerning spawning induction responses, with the population of the north (Ria de Aveiro) presenting more satisfactory results (Matias et al. 2009; Matias et al. 2013). This difference has a great study interest since the low percentages of artificial spawners and their randomness represents a big problem in the aquaculture production of the European clam, mainly due to the fact that *R. decussatus* oocytes cannot be fertilized directly after gonad dissection (Colas and Dubé 1998; Hamida et

al. 2004b). Spawning induction allows production in hatchery facilities to be planned and also enables controlled fertilization to be carried out. However, the lack of efficient artificial stimuli to induce spawning still hinders the controlled development of several bivalve species (da Costa et al. 2012). Spawning induction has been achieved in bivalves with more or less success depending on the species. When a spawning method is chosen several factors must be considered: effectiveness, repeatability, ease, price, whether or not it causes mortalities in broodstock after spawning induction, possible effects on the viability of the gametes and subsequently during embryonic and larval development. Thermal stimulation successfully induced spawning in several clam species, being a cheap, easy and widely-used method to induce spawning (da Costa et al. 2012). Salinity is other environmental factor influencing reproduction of estuarine and marine invertebrate. Long-range seasonal fluctuations in salinity are important to the synchronization of the gametogenic cycle, reproductive pattern and stimulate spawning (Stephen 1980). In addition, fluoxetine, a commonly prescribed antidepressant (Prozac), is a selective serotonin reuptake inhibitor (SSRI). SSRIs block the reuptake of serotonin (5-HT) from the pre-synaptic nerve cleft resulting in an increased 5-HT neurotransmission in humans. As such, they can mimic the action of 5-HT (Lazzara et al. 2012). Fluoxetine has been already tested in marine bivalves like *Macoma balthica* (Honkoop et al. 1999) and zebra mussel *Dreissena polymorpha* (Fong 1998; Fong et al. 2003; Fong and Molnar 2008; Lazzara et al. 2012). On the other hand, hydrogen peroxide application to induce bivalves spawning was first reported by Morse et al. (1977). The addition of hydrogen peroxide to alkaline seawater provides an inexpensive, simple, and reliable method for the control and synchronous induction of broadcast spawning in males and females of certain bivalve and gastropod molluscs (Morse et al. 1978). Enzymology and physiological studies indicate that spawning may result from a peroxide-induced stimulation of the endogenous enzymatic synthesis of potent hormone-like prostaglandin molecules (Morse et al. 1978). Nevertheless, less success was achieved in Indian pearl oysters *P. fucata* with H<sub>2</sub>O<sub>2</sub> treatment (Alagarwami et al. 1982). These conversely results indicate H<sub>2</sub>O<sub>2</sub> concentration must be optimized for any cultured species, sometimes in combination with other treatments. Morse (1984) has been able to identify optimal conditions for gametogenesis and spawning of green (*H. fulgens*), pink (*H. corrugata*), and black abalone (*H. cracherodii*) using peroxide method.

Spawning induction of ripe clams has already been tested in several studies employing chemical and/or physical stimuli. Among the physical stimuli, the thermal stimulation and the administration of doses of microalgae food were previously applied (e.g. Matias et al. 2009; Ojea et al. 2008). Among the chemical stimulants administered as potential inducers of spawning, intragonadal injection of neurotransmitters such as serotonin (5-HT) (Hamida et al. 2004a) and the addition of gametes to the water, were also tested in this species (e.g. Matias et al. 2009; Ojea et al. 2008).

Although the percentage of spawning induction success is normally higher in the Ria de Aveiro population (Matias et al. 2009; Matias et al. 2013), we still face major difficulties in obtaining artificial spawners from this wild population of *R. decussatus* (Chapter 3). The Table 3 below demonstrates the difficulty in successfully obtaining spawning in these individuals. Indeed, to accomplish the microarray-based analysis of oocyte quality in chapter 3, a total of 17 spawning inductions were performed during the year of 2013.

Table 3 – Percentage of clams spawning at each day by thermal stimulation (TS), fluoxetine 2mg/L (F), hydrogen peroxide 1 ml/L (HP) and three different salinities (S30, S34, S38).

Spawning induction day	Spawning Stimuli	Response	% of spawners	% of female spawners	% of male spawners
28/01/2013	TS + F	No	–	–	–
13/02/2013	TS + F	No	–	–	–
14/02/2013	TS + S30	No	–	–	–
14/02/2013	TS + S34	No	–	–	–
14/02/2013	TS + S38	No	–	–	–
18/02/2013	TS + HP	No	–	–	–
04/03/2013	TS	No	–	–	–
08/04/2013	TS + F	No	–	–	–
09/04/2013	TS + F	No	–	–	–
17/04/2013	TS	Yes	4	–	4
18/04/2013	TS	Yes	39	11	28
23/04/2013*	TS	Yes	62	19	43
07/05/2013	TS	Yes	57	23	44
29/05/2013*	TS	Yes	72	21	51
12/06/2013	TS	Yes	66	18	48
03/07/2013	TS	Yes	21	2	19
10/07/2013	TS	Yes	40	26	14

\* These animals spawned twice

All broodstock lots were induced to spawn by thermal stimulation (with temperature alternating between 5 to  $28 \pm 1$  °C, during 30min and 1h, respectively). In eight tentative of spawning induction, this traditional stimulus was applied combined with different salinities (30, 34, 38) or with two different chemical stimuli, fluoxetine 2mg/L (F) and hydrogen peroxide 1 ml/L (HP). However, these inducers were often not effective to induce spawning, with several negative answers. In contrast, the traditional thermal stimuli *per se*, showed to be the most effective one, with males responding much more often than females, as similarly observed by Matias et al. (2009). The oocytes used for this work were the ones from 18/04/2013, 23/04/2013, 29/05/2013 and 12/06/2013. Interestingly, the broodstock from 23/04/2013 and 29/05/2013 was the same one, spawning twice. Since there was no individual tracking and no histological analysis performed in this broodstock, several hypotheses for this phenomenon can be suggested. Firstly, the individuals that spawned in the second date (29/04/2013) could have been different from the ones that spawned on the first attempt (23/05/2013). Secondly, the individuals that spawned on the first attempt could have recovered their entire gonad during broodstock conditioning, or thirdly, due to partial spawning. Indeed, frequently after spawning, clams do not progress to an inactive stage. As already reported in chapter 1, in the microscopic examinations of the gonadal tissues all clams from the natural environment showed simultaneous spawning and recovery of the gonad. However, the fact that this population spawned twice it did not affect the results, since oocyte quality variation within each date was still observed. Actually, although spawning induction and fertilization of oocytes were carried out in four different sampling dates and with a different mixture of sperm, no statistically significant difference was found among the D-larval yields. Considering such statistical results, it was assumed that this variation was most likely to be due to the intrinsic quality of each female.

### **III. Sperm-egg interaction: The “culprit” for the differences between oocyte quality and between the two populations?**

Fertilization is the process by which two gametes belonging to the same species encounter one another and fuse to create a new individual. Species use different pathways to ensure that their gametes arrive correctly at the meeting point (Rosati et al. 2000). In species with external fertilization, like bivalves, this goal can

be achieved by contemporary spawning of the gametes and/or release of chemoattractants from eggs, which also increase sperm motility and respiration (Hardy et al. 1994). The egg surface, with the property to first recognize and bind sperm, is composed by a glycoprotein coat surrounding the egg. In both invertebrates and vertebrates, the coat consists of a fibrous network whose supramolecular organization and thickness vary slightly from species to species (Rosati et al. 2000).

Glycosylation plays an important role in several types of recognition processes associated with fertilisation and development and in the last decades considerable efforts have been made to identify molecules and pathways utilized during gamete interaction, and the details of the process are beginning to be understood (Rosati et al. 2000; Staudacher et al. 2009).

In the gene expression comparison between the two sampled populations, considering all stages and sexes (Chapter 2), the pathway “N-Glycan biosynthesis” (dre00510) was found among the enriched KEGG pathways, with the highest number of gene entries. There are strong evidences that this pathway could be involved in the recognition signals and binding between the oocyte and components of the sperm plasma membrane in several species. Indeed, it was reported that N-glycans have an important role in “primary binding” for example in the human (Clark 2013), mouse (Clark and Dell 2006; Clark 2011), chicken (Robertson et al. 2000), snail (Staudacher et al. 2009) and in the bivalve, *Unio elongatulus* (Di Patrizi et al. 2001; Focarelli and Rosati 1995; Rosati et al. 2000). The structure of protein-linked glycans determines recognition events, decides on binding or not and influences the speed or quality of biological processes. Differences within the oligosaccharide structures appear not only between different species but also between individuals of the same species (Staudacher et al. 2009), which could be the case of the European clam individuals from the two studied populations. Furthermore, the current glycosylation pattern highly depends on the developmental and physiological status of the cell. N-Glycosylation starts in the endoplasmatic reticulum by formation of a lipid linked precursor, containing two GlcNAc residues, nine mannoses and three glucoses, which are transferred *en bloc* onto the growing polypeptide chain. While the first steps of the biosynthetic pathway of N-glycans seem to be similar in nearly all kinds of organisms, the final modification steps are highly heterogeneous and lead to the wide range of various glycan structures. The key enzyme for the formation of complex glycans is the GlcNAc-transferase I, which forms the GlcNAcMan<sub>5</sub>GlcNAc<sub>2</sub>-

structure (Staudacher et al. 2009). After previous action of GlcNAc transferase I, the glycan is a suitable substrate for a number of further modifying enzymes such as core  $\alpha$ 1,6-fucosyltransferase, core  $\alpha$ 1,3-fucosyltransferase,  $\beta$ 1,2-xylosyltransferase,  $\beta$ 1,2-GlcNAc-transferase II and  $\beta$ 1,3 – or  $\beta$ 1,4-galactosyltransferase which build up larger and more complex glycan structures. Most of the known modifying glycosyltransferases depend on the previous action of GlcNAc-transferase I. In some tissues the glycans are also further modified by additional non-sugar structural features, such as methylation, sulphation, phosphorylation or others to create even more variations (Staudacher et al. 2009).

The glycosylation pathway of a specific cell or organism is usually elucidated on one hand by the analysis of the glycan structures and on the other hand by the determination of the enzymes which are responsible for the formation of these glycans (Staudacher et al. 2009). In fact, the enzymes  $\alpha$ 1,3-fucosyltransferase (FUT10) and  $\alpha$ 1,6-fucosyltransferase (FUT8), involved in the biosynthetic glycosylation pathway, were found differentially expressed in the comparison between the two populations, considering all stages and sexes, with a Fold Change of 2.2 and 1.9, respectively. Fucosylation is often the final process in glycan biosynthesis and is involved in many cell adhesion and cell–cell interactions, like fertilization (Lunau et al. 2013). The enzymes  $\beta$ 1,2-xylosyltransferase (XYLT) and  $\beta$ 1,3-galactosyltransferase ( $\beta$ 3Gal-T) were also differentially expressed in the comparison between the two populations, considering all stages and sexes, with a Fold Change of 1.5 and 2.3, respectively. Both the enzymes are involved in glycosylation in gastropods (Staudacher et al. 2009). Interestingly, we also found the enzyme or  $\beta$ 1,4-galactosyltransferase ( $\beta$ 4Gal-T) more abundant in good quality oocytes with a Fold Change of 1.6 (Chapter 3). However, several studies revealed that this enzyme is found on the sperm of mammals, mediating fertilization by binding oligosaccharide residues in the egg coat, or zona pellucida, and that the ability of the zona pellucida to bind sperm is conferred by oligosaccharides of the ZP3 glycoprotein (Miller et al. 1992; Tengowski et al. 2001).

Still in Chapter 3, other important gene involved in sperm – egg interaction observed in the list of differentially expressed probes, was the chaperone molecule Protein Disulfide Isomerase family A (PDI), more abundant in good quality oocytes, with a fold change of 1.5. This is a peptidylprolyl isomerase that belongs to the immunophilin family, being also advantageous for the viability and immune protection

of eggs and early embryos of Pacific oyster (Corporeau et al. 2012). As reported before, several studies suggested an essential role of PDI for the sperm–egg fusion at fertilization (Calvert et al. 2003; Ellerman et al. 2006). Moreover, another interesting result was that the same gene was found down regulated in the individuals of Ria Formosa lagoon, with a Fold Change of 1.5. Since, Ria Formosa is the population where spawning induction is less successful, this finding supports the hypothesis that sperm–egg interaction could be the origin of the differences between populations and oocyte quality. Furthermore, a partial characterization of PDI family A indicates a corresponding ORF of 423 amino acids with a significant identity of 66% with the hypothetical protein LOTGIDRAFT\_151365 of the giant owl limpet (*Lottia gigantea*) (Gasteropoda) as well as a significant identity of 67% with the Protein disulfide-isomerase A6 of the Pacific oyster *C. gigas* (Bivalvia). Two putative conserved domains of the thioredoxin family have been detected. Actually, a number of eukaryotic proteins contain domains evolutionary related to thioredoxin, most of them being protein disulfide isomerases (PDI) (Holmgren 1985).

A list of chaperone proteins were also reported as main determinants of good quality oocytes in *R. decussatus* (Chapter 3). Molecular chaperones represent a diverse family of proteins that are ubiquitously expressed in virtually all living organisms. Originally, identified on the basis of their ability to confer cellular resistance to various environmental stress conditions, members of the molecular chaperone family have been variously referred to as ‘cell stress response’ or heat shock proteins (Hsps) (Nixon et al. 2005). Besides their possible role in oocyte protection, it has been reported that chaperone proteins can also be involved in sperm – egg interaction (Gething 1997; Nixon et al. 2005). For example in the sea urchin, the egg receptor for sperm is a unique cell surface glycoprotein that mediates sperm – egg interaction in fertilization. Much of the egg receptor for sperm contains high sequence identity to the HSP70 subfamily (Gething 1997), that was found more abundant in good quality oocytes, with a Fold Change of 1.7. Another chaperone protein, Heat shock protein 90kDa beta member 1 (HSP90B1), known also as endoplasmic reticulum chaperone was present in the list of differentially expressed probes, more abundant in good quality oocytes, with a Fold Change of 1.7, as well. This protein was already found on the surface of mature mouse oocytes (Nixon et al. 2005).

Such data reinforce the notion that “primary binding” could be the cause of the differences between the two European clam populations, observed by Matias et al. (2009), regarding spawning induction.

#### **IV. Meiotic maturation and oocyte quality in *Ruditapes decussatus*: An additional bottleneck in hatcheries**

A factor that strongly limits the development of *R. decussatus* hatcheries is the impossibility to obtain fertile gametes by gonadal stripping. This practice is widely used to collect oocytes, before natural egg emission, in some bivalve species (e. g. in oyster), whose eggs can be fertilized immediately after. As already mentioned in this thesis, meiotic progression in germ cells is not regulated in the same manner across bivalve species. In bivalves such as *Spisula* or *Barnea* spawned oocytes are arrested at prophase I and fertilization occurs at this stage leading to meiosis reinitiation (Colas and Dubé 1998). However, oocytes of *Ruditapes* and *Crassostrea* exit from prophase I and undergo germinal vesicle breakdown (GVBD) after spawning, being further blocked at the first metaphase (metaphase I) (Osanai and Kuraishi 1988). The release from metaphase I is naturally triggered by fertilization or can be artificially induced, however in both cases, it seems that an increase in intracellular  $Ca^{2+}$  has a major role in meiosis reinitiation (e.g. Abdelmajid et al. 1993; Guerrier et al. 1993; Moreau et al. 1996). Though, *Ruditapes* and *Crassostrea* meiotic progression is not regulated in the same way. In *Crassostrea*, isolated oocytes from ovaries (stripped) can be fertilized at prophase I (Osanai, 1985), whereas *Ruditapes* oocytes cannot be fertilized at this stage (Colas and Dubé 1998).

The role that external calcium, through voltage-gated channels, might play in the induction of GVBD was first reported in molluscs that are fertilized at the prophase I stage (Allen 1953; Deguchi and Osanai 1994; Dubé 1988), or undergo the second arrest in metaphase I (Dubé and Guerrier 1982). Although, it was after recognized that also the intracellular calcium increase plays a crucial role in almost all the species studied, independently from their meiotic arrest (Deguchi and Osanai 1994; Guerrier et al. 1993; Juneja et al. 1994). In particular, the interplay between external and internal calcium currents is evident in *Ruditapes*, where a serotonin-induced surge of intracellular calcium was shown to trigger maturation even in the absence of external calcium (Guerrier et al. 1993). It has been suggested that short

lived proteins continuously synthesized were responsible for maintaining metaphase arrest in some molluscs' oocytes, with cyclin regulatory proteins representing good candidates since their disappearance, due to protein synthesis inhibition, triggered metaphase/anaphase transition and polar body extrusion (Colas and Dubé 1998).

Considering that in *R. decussatus* the gametogenesis process only ends when oocytes pass through the oviduct, another gene expression study on this species was performed by Pauletto et al. (Unpublished data) (collaborative study in the REPROSEED context - Appendix 3). Using the same microarray platform (8x60,000) designed for the present thesis, a dataset of 15 oocytes samples were analyzed. Expression profiles characterizing stripped oocytes (that didn't complete meiosis and therefore were not ready to be fertilized) and spawned oocytes, with different competence levels (depending on the D-larval rates), were evaluated in order to shed light on the molecular processes leading to female gametes maturation and competence. Released oocytes were obtained from 10 of the same females used for the study made in chapter 3 of the present thesis. In addition, gonads from five females were dissected and oocytes were collected through a practice known as "gametes stripping". Gene expression analysis and evaluation of DEGs between oocytes at different maturation stages and quality pointed out significant results and provided a first overview on the transcriptome changes underlying stripped oocyte infertility and oocyte competence. Among the 198 annotated DEGs between stripped and spawned oocytes, several transcripts were implicated in the regulation of cell cycle.

During hormone-induced maturation, animal oocytes require an increase in active MPF (maturation-promoting factor) to complete GVBD (Stricker and Smythe 2001). MPF is a key G2/M phase regulator in eukaryotic cells and is composed by cdc2 kinase (known also as cdk1) and cyclin B (Nurse 1990). MPF is induced during the meiosis resumption and its activity is regulated by phosphorylation of cdc2 kinase. Cdc2 kinase is activated when M-phase inducer phosphatase (cdc25) dephosphorylates threonin-14 and tyrosine-15 sites, leading, in turn, to MPF activation and meiosis resumption (Pauletto et al. Unpublished data). In the study by Pauletto et al. (Unpublished data), six probes coding for cdc25 or cdc25-like proteins were reported to be more abundant in released oocytes, suggesting a prominent role of these phosphatases in the resumption of meiotic cell cycle progression in

*R. decussatus*. The controlling function of cdc25 phosphatases in the meiosis I progression has been proposed in a wide range of species, like *Caenorhabditis elegans* (Kim et al., 2010), *Drosophila* (Alphey et al., 1992), starfish (Kishimoto, 2011), mouse (Oh et al. 2010), *xenopus* (Gaffrè et al., 2011) and the Surf clam *S. solidissima* (Yi et al. 2002).

As already reported in this thesis, in *Ruditapes* and other bivalve molluscs, calcium may act via a calcium calmodulin-kinase since calcium calmodulin antagonists were able to prevent the metaphase I release and cyclin degradation upon fertilization (Abdelmajid et al. 1993; Colas and Dubé 1998; Leclerc et al. 2000; Whitaker 1996; Zhang et al. 2009). Indeed, the differential expression of several transcripts involved in calcium ( $\text{Ca}^{2+}$ ) signaling was very common in the present work and in the work performed by Pauletto et al. (Unpublished data) (Appendix 3). In the identification of differentially expressed genes between oocyte quality (Chapter 3), the enzyme Calcium/calmodulin-dependent 3',5'-cyclic nucleotide phosphodiesterase 1C (Cam-PDE1C) was found among the genes more abundant in good quality oocytes, with a Fold Change of 1.7, confirming the importance of this pathway in the maturation process of oocytes of this species. Additionally, in the identification of differentially expressed genes between *R. decussatus* populations (Chapter 2), several genes of the Calmodulin family (CaM) were found among the differentially expressed genes between sexes and between populations. Moreover, in the work made by Pauletto et al. (Unpublished data), a putative sodium/calcium exchanger 3 was reported to be more abundant in the released oocytes and homologs of *C. gigas* regucalcin and calmodulin showed higher expression levels in ovarian oocytes. Regucalcin, as calmodulin is a calcium binding protein supposed to contribute to the meiosis regulation and its mRNA variations suggest an involvement in maintaining the calcium homeostasis in immature oocytes. Interestingly, the regucalcin mRNA was expressed at lowest level in the Medium Hatching Rate (MHR) oocytes, thus indicating that low levels of this transcript could be related to higher quality eggs (Pauletto et al. Unpublished data). Still in the study by Pauletto et al. (Unpublished data), the analysis of the expression profiles of Low Hatching Rate and Medium Hatching Rate oocytes showed important variations occurring in the abundance of transcripts encoding proteins regulating the oocyte meiosis, such as adenylate cyclases (ADCY), IP3r-1, IP3r-2 and the catalytic subunit alpha isoform of protein phosphatase 2B. ADCYs are membrane-associated enzymes that catalyse the

formation of the secondary messenger cyclic adenosine monophosphate (cAMP) which is thought to have a pivotal role in the meiotic maturation in several species (Richard, 2007). Indeed, in the analysis performed for the present thesis, the enzyme cAMP-specific 3',5'-cyclic phosphodiesterase was found more abundant in good quality oocytes. It is generally accepted that an almost universal decrease in oocyte cAMP is sufficient to trigger the release of oocytes from prophase I arrest or at least positively affects it, although, contrasting data show that high levels of cAMP may only transiently block GVBD or may even release the oocyte from meiotic arrest (Tosti 2006). In the study by Pauletto et al. (Unpublished data), the mRNA transcripts encoding ADCY type 5 and 6 were more abundant in oocytes with a lower developmental competence. Thus, despite cAMP levels are regulated also by other factors, such as progesterone and mPRs, more likely ADCYs have a key role in cAMP balancing and meiotic progression in *R. decussatus*. Moreover, the variations in the mRNA levels reported for IP3 receptors by Pauletto et al. (Unpublished data) provide interesting information concerning the bivalve oocyte maturation. IP3r are membrane glycoprotein complexes acting as  $Ca^{2+}$  channels and are activated by inositol triphosphate (IP3), which has been strongly implicated in the conversion of external stimuli to intracellular  $Ca^{2+}$  signals (Berridge and Taylor 1988; Ciapa and Whitaker 1986; H Ji 1990; Yoshida and Imai 1997). Another member of the inositol polyphosphate kinase family, Inositol hexakisphosphate (InsP6), was also found to be more abundant in good quality oocytes in the gene expression analysis performed in the present work. The family of Inositol polyphosphate kinase has been shown to have a predominant role also in oocytes, in both the formation and propagation of  $Ca^{2+}$  waves at fertilization (Kume et al. 1993) and it has been demonstrated that intracellular injections of IP-3 triggered GVBD in *Spisula sp.* (Bloom et al. 1988) and *Ruditapes sp.* (Guerrffir et al. 1996). In the work made by Pauletto et al. (Unpublished data), putative orthologs of the mammalian IP3r-1 and IP3r-2 were differentially expressed in relation to the oocyte competence. In particular the IP3r-1 was more abundant in the MHR oocytes, while IP3r-2 expression decreased as the oocyte quality increased. The opposite trend of expression reported for the two IP3r types allowed to think that in the European clam oocytes, at least two IP3r are expressed and that not only the presence of this receptor is crucial in determining a complete oocyte maturation and fertilization, but most probably also the isoform identity. These preliminary results suggest the importance of inositol polyphosphate kinase family

during the oocyte maturation phases in *R. decussatus*. Despite little is known about the molecular regulation of intracellular  $\text{Ca}^{2+}$  occurring during oocyte maturation in bivalves, these preliminary results pointed out a few important genes possibly involved in such a complex mechanism.

Another important molecular process, that could differentiate stripped and spawned oocytes, was proposed for a few members of the WNT signaling pathway (GO:0016055) (Pauletto et al. Unpublished data). Recently, in mammals WNT pathway signaling has been reported to be implicated in ovarian development, oogenesis, and early development (Wang et al. 2004; Zheng et al. 2006). The expression levels of three probes putatively encoding a WNT proteins receptor, frizzled-8, were more abundant in released oocytes (Pauletto et al. Unpublished data). This data might suggest that frizzled-8 expression supports the WNT signaling pathway activation by favoring the recognition of WNT proteins. Moreover, the results reported that a putative ortholog of *C. gigas* WNT4 was less abundant in the female gametes extracted from mature gonads, in comparison with the released oocytes, and its mRNA expression tended to increase with the eggs quality. These findings suggest that not only WNT4 transcript could be implicated in the *R. decussatus* oocyte development but it could also be an important transcript whose abundance affects oocyte quality, like it has been proposed in mouse (Wang et al. 2004).

However in the study made by Pauletto et al. (Unpublished data), a putative four jointed protein (Fj) encoding transcript was found significantly more expressed in spawned oocytes than in stripped ones. Interestingly, in the gene expression analysis made for the present thesis, the same transcript was found more abundant in good quality oocytes of *R. decussatus*. The role of Fj has been poorly investigated in both vertebrates and invertebrates. However, a few studies focusing on this gene have been performed in *Drosophila melanogaster*, where it has been demonstrated that this protein directly interacts with WNT4 (also found significantly more expressed in spawned oocytes), being a regulator of the Hippo signaling pathway. Hippo signaling influences planar cell polarity during early development, controls organ size by regulating cell proliferation and apoptosis (a process further discussed in the next division), and is an important regulator of tumorigenesis (Tagliabracci et al. 2013). Fj is conserved but restricted to higher vertebrates and only a limited range of invertebrates, including *Drosophila* and *Anopheles* (Tagliabracci et al. 2013). Considering the lack of functional information concerning Fj, it is difficult to propose a

specific role of this gene in *R. decussatus*, nevertheless it can be suggested that its expression in oocyte maturation is probably linked to the oocyte competence on fertilization and larval development.

Finally, among the genes with higher abundance in good quality oocytes, the presence of the chaperone molecules DnaJ (Hsp40) homolog subfamily C member 27-A, Heat Shock 70 kDa protein 12A (Hsp70), Cyclophilin B and Protein Disulfide Isomerase family A (PDI) was observed. These chaperone proteins play a major role in protecting oocytes from thermal shock or other cellular stresses induced by manipulation. Interestingly, most of the chaperones revealed in the present study were recently found to be associated, at the protein level, with high quality oocytes in another bivalve species, the Pacific oyster *C. gigas*, with some of them being maternal mRNAs. Indeed, besides yolk proteins, the ovulated oocyte (unfertilized egg) contains many other components such as maternal mRNAs, proteins, vitamins and hormones. Maternally inherited mRNAs and proteins accumulate in the oocyte throughout oogenesis. After fertilization, those maternal factors support early embryonic development until activation of zygotic transcription and thus play a pivotal role during early embryogenesis (Bobe and Labbé 2010). For example, the direct modulation of oocyte signaling pathways using pharmacological compounds or hormones can affect fertilisation success and developmental potential (Corporeau et al. 2012). In conclusion, since these chaperone proteins were more abundant in good quality oocytes, it can be suggested that they play an important role in the early embryonic development (D-larvae) of *R. decussatus*.

The gene expression analysis performed in both studies allowed identifying a few important mechanisms which could have a key role in the process of bivalve oocyte maturation and competence acquisition. The transcripts which seemed to play a major role in the female gametes maturation and competence acquisition were those encoding proteins involved in the cell cycle progression, calcium regulation, WNT signaling and oocyte protection.

## **V. Apoptosis: Programmed cell death in *Ruditapes decussatus* oocytes**

Apoptosis is an intrinsic, genetically controlled process, responsible for destroying redundant, dysfunctional or damaged cells (Motta et al. 2013). Tumor

Necrosis Factor Receptor superfamily (TNFR) was observed in the list of more abundant genes in poor quality oocytes. Interestingly, the same superfamily of cell membrane receptors was found in the list of differentially expressed probes between populations (gene expression analysis performed for gametogenesis in the two populations), being up-regulated in the individuals of Ria Formosa lagoon, where spawning induction responses are lower. This superfamily includes FAS, CD40, CD27, and RANK, receptors for Tumor Necrosis Factor  $\alpha$  (TNF $\alpha$ ), an inflammatory cytokine produced by macrophages/monocytes during acute inflammation and responsible for a diverse range of signaling events within cells, leading to necrosis or apoptosis (Idriss and Naismith 2000). Additionally, quantitative correlations between microarray data and D larval yields were evaluated in spawned oocytes with caspase-8 being negatively correlated. Caspases represent a family of intracellular cysteine proteases linked both to the initial and final stages of apoptosis in numerous types of cells, with several studies showing the importance of caspases in the death by apoptosis of unfertilized ovulated oocytes (Johnson and Bridgham 2002; Papandile et al. 2004; Reynaud and Driancourt 2000; Trounson et al. 2013).

Moreover, in the gene expression study, made by Pauletto et al. (Unpublished data) between stripped and spawned oocytes of *R. decussatus*, at least three enzymes involved in the ceramide metabolism were found differentially expressed: a ceramide synthase, less abundant in ovarian oocytes, a ceramidase and a sphingomyelinase, both less abundant in released oocytes. The enzymes controlling the metabolism of ceramide in oocytes have been poorly studied in molluscs and only recently, sequences of genes associated with ceramide metabolism and signaling have been investigated in the Pacific oyster, concluding that ceramide and sphingolipid metabolism may be involved in the oyster's stress and/or immune responses (Timmins-Schiffman and Roberts 2012). Conversely, in vertebrates quite a few studies have been focused on the role of ceramide in oocytes and two main hypotheses have been suggested (Coll et al. 2007). First, it has been proposed that the generation of ceramide is a part of the signal transduction pathway activated in response to progesterone and that the increase in ceramide is likely to be functionally important in the resumption of the meiotic cycle (Buschiazzo et al. 2011; Morrill and Kostellow 1998; Strum et al. 1995). Second, it has been recently demonstrated that ceramide induces the default apoptosis process in oocytes and it has a central role in the age-related decrease of eggs quality (Miao et al. 2005; Perez et al. 2005).

Follicular atresia, a term used to describe the breakdown and resorption of gametes, was recently been emphasized together with the programmed cell death, apoptosis, which is recognized as being a golden key to maintain the tissue-level homeostasis in multicellular organisms (García-Gasca et al. 2010; Kennedy 2002). Follicular atresia could be a normal event in female gonads in spawning and post-spawning stages, but not in gametogenic or mature gonads since these are proliferative stages (García-Gasca et al. 2010).

Like atresia, apoptosis can also be induced by factors such as cytokines, hormones, viruses, xenobiotics, radiation, oxidative stress and hypoxia (Üçüncü and Çakıcı 2009). In bivalves, the end of the spawning season is usually marked by gamete atresia (degeneration and phagocytosis) within the follicles. The occurrence of this atresia appears to be a common phenomenon linked to the reproductive strategy (Kandeel et al. 2013). Darriba et al. (2004) enumerated three possible reasons for oocyte atresia: (1) a limited capacity of the follicle and a control mechanism covering cell number; (2) a self-cleaning process at the end of gametogenic cycle in preparation for the next cycle; and (3) a response to environmental or contaminating stress condition. Indeed, it was shown that apoptosis in the mussel *Mytella strigata* is a mechanism of follicular atresia with apoptotic index being a useful indicator of environmental stress in coastal ecosystems (García-Gasca et al. 2010).

As already reported in the transcriptomic study of spawned oocytes, since all the oocytes were spawned by induction and expected to be mature and ready to be fertilized, it can be possible that over-expression of apoptosis-related genes could be triggered as a consequence of unsolved cell stress, like thermal stress (since thermal shock was the method used to induce clam spawning), affecting negatively the fertilization success.



# Conclusion and Perspectives

## Conclusion

The present study allowed the improvement of the knowledge available on the European clam production, more particularly on some relevant aspects of gametogenesis and oocyte quality. The experiments performed on the reproductive cycle of the two most important populations of *R. decussatus* in Portugal, Ria de Aveiro and Ria Formosa, showed that, with the exception of specific timings of gonadal development dynamic, no significant differences concerning the gametogenesis (intensity) were found between them.

Transcriptomic analysis were performed using combined Next Generation Sequencing (NGS) technologies in order to improve transcriptomic information on *R. decussatus*, thus providing a great number of transcripts for further “omic” studies in this species. This thesis described the development of a new microarray platform for the European clam and the application of this technology for gene expression profiling. This microarray platform (8x60,000) was then used here through the study of the gonads of each developmental stage and sex from both studied populations (Chapter 2), as well as different oocyte quality (Chapter 3) transcriptomes. The microarray platform was applied for identifying differential gene expression between the two populations of *R. decussatus*, identifying genes involved in clam reproduction features (e.g. investment), as well as a list of sex and stage specific markers. Lastly, the same microarray platform was applied for determining relevant markers of oocyte quality in this species. During the course of this study, several important differentially expressed probes and pathways were identified. Some transcripts that were mainly identified and seemed to play a major role in the gametogenesis were those encoding proteins involved in the sperm-egg interaction, oocyte protection, calcium regulation and apoptosis. The gene coding for Protein Disulfide Isomerase family A (PDI) and the genes of the Calmodulin family (CaM) are very interesting candidates in order to perform functional studies in the future, due to their potential involvement in important reproductive pathways like sperm-egg interaction and oocyte protection and maturation.

We believe that these results will be very useful and open perspectives for future studies into the molecular mechanisms involved in the European clam differentiation, as well as for the development of hatchery production program management in this species.

## Perspectives

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 *R. decussatus* seed production. Since spawning success and oocyte quality are the most important remaining bottlenecks in the culture of *R. decussatus*, the performance of multidisciplinary studies with zootechnical, genomic and proteomic approaches that could allow the establishment of a consistent methodology to obtain gametes more regularly and with higher quality, seems essential.

Besides the D-larval rate, other parameters could be tested as potential gamete quality indicators in *R. decussatus*. For example, counting the thermal stimulations, or the time needed to obtain a true spawning by individual or the timing between the stimulus onset and the first effective spawning by individual, could be considered. Moreover, in order to verify if these characters have a genetic basis and are heritable, crosses between individuals presenting good quality and those presenting bad quality parameters could be also an interesting approach. Through the evaluation of the suggested parameters, it could be possible to assess if gamete quality in *R. decussatus* was also originated from the spawning induction success. In addition, the identification of the genes, proteins and molecular cascades that may correlate with these characters, as well as their modulation under environmental effects, could be another relevant perspective.

Furthermore, since the results obtained in this study were only based at the mRNA level, confirmation at the protein level should be performed. Indeed, it was very remarkable to observe that all chaperones more abundant in good quality oocytes in chapter 3 were recently found to be associated, at the protein level, with high quality oocytes in another bivalve species, the Pacific oyster *C. gigas*, with some of them being maternal mRNAs essential for fertilization, first cleavage and embryonic genome activation (Corporeau et al. 2012). For this reason, the performance of a similar study will be important in order to find out which maternal protein factors could be related to oocyte quality in *R. decussatus*, through a proteomic study using two-dimensional electrophoresis (2-DE).

Another proteomic approach that could be performed in order to elucidate the role of certain pathways and genes found during the course of the present thesis is

the stimulation of specific targets by mimicking certain molecules. This method was accomplished, for example, by Guévelou (2012), where a functional *in vivo* pharmacological approach was performed using AICAR injections to modulate signal AMPK (AMP-activated protein kinase) in *C. gigas*. Moreover, an “opposite” approach by inhibiting specific proteins through injection of antibodies could also be appropriate. This method was done, for example in oyster, by Corporeau et al. (2011), where *in vivo* functional inactivation of mature og TGF- $\beta$  using antibody injection was performed to study the function of og TGF- $\beta$  in the gonad of *C. gigas*. These methods could be applied in important genes identified in the present study, like the ones involved in sperm-egg interaction and calcium pathway, or, conversely in genes which function are still unknown, like the putative four jointed protein (Fj), with the aim of forcing phenotype modifications in individuals.

Knowledge of spatiotemporal or stimulus-dependent expression patterns of a given gene represents valuable information to assign this gene a putative function. Therefore, the use of *in situ* hybridization (ISH) for visualizing gene transcripts in eukaryotic cells could be an important complement to this expression profiling study, in response to different stimuli and factors like spawning inducers (fluoxetine and hydrogen peroxide) as well as abiotic and biotic factors, known as being important for gametogenesis (temperature and food). Moreover, ISH is a rapid and reliable technique for chromosomal investigations that is presently used for a large diversity of molecular cytogenetic studies, such as chromosome identification, gene mapping, localization of gene expression, and analysis of chromosome rearrangements in a wide variety of genomes. In bivalves it represents the great majority of recent studies in chromosomal banding (Leitão and Chaves 2008). The use of this technique could be useful, since the individual identification of chromosomes and the establishments of precise karyotypes are essential, for instance, in studies on the economical important phenomena of aneuploidy, triploidy, and tetraploidy. Indeed, several studies in bivalves have shown that phenotypic changes occur due to quantitative changes in the gene expression pattern, caused by aneuploidy, (e.g. Leitão et al. 2001; de Sousa et al. 2011; de Sousa et al. 2012; Thiriou-Quievreux et al. 1998; Zouros 1996). The possible target genes for this methodology could be the ones coding for the chaperone proteins (Hsp40, Hsp70 and PDI), since they are over-expressed under cellular stress conditions like thermal shock or others. The physical

location of those key genes would require, however the previous characterization of the full length cDNA and a significant part of the gene (introns and exons).

Gene function is primarily assessed on the basis of altered phenotypes associated with gene disruption. However, in the last decade, reverse genetic and “omic” tools, most of all transcriptomic, have been widely exploited to investigate several fields in bivalves. Thus, additional promising techniques that could help understanding or confirming the specific functions of certain genes, are the RNA interference (RNAi) or the recently developed “gene editing” strategy called “CRISPRs” (Clustered Regularly Interspaced Short Palindromic Repeats) (Jansen et al. 2002). RNAi is a highly evolutionarily conserved process of posttranscriptional gene silencing by which double-stranded RNA (dsRNA), when introduced into a cell, cause sequence-specific degradation of homologous mRNA sequences (Almeida and Allshire 2005). It was firstly described in plants and *C. elegans* (Fire et al. 1998; Jorgensen 1990) but also recognized later as process existing in fungi and animals (Elbashir et al. 2001; Romano and Macino 1992). Following these observations, RNAi-based applications have generated substantial enthusiasm and became a powerful tool for reverse genetic studies, emerging as a powerful alternative technique for determining the loss-of-function phenotype of a gene (Elbashir et al. 2001). Despite that, in molluscs, this technique is still scarcely used and it remains a technical challenge (Béguet et al. 2013; Fabioux et al. 2009; Huvet et al. 2012; Suzuki et al. 2009). Conversely, RNAi has been widely used in vitro and in vivo in vertebrate and invertebrate species (Berns et al. 2004; Dash et al. 2008; Robalino et al. 2004). The results of the present study suggests that the most suitable genes for RNAi could be the ones involved in oocyte maturation (Calmodulin family) and apoptosis (caspase 8), conducted in adult individuals, in order to observe their effects in the gametes produced. However this type of functional study can also be performed directly in the gametes using lipofection (Rivière G, Personal Communication).

Finally, since the main objective of the present thesis was to better understand the differences between the two wild Portuguese populations of *R. decussatus* in terms of spawning induction success, complementary studies could also be performed in this subject in order to know if other environmental effects, like pollutants, are in the origin of this phenomenon. Several hotspots of pollution have been identified in the Ria Formosa Lagoon. The contaminants already known to be

present in the water, sediments and biota from specific sites of the lagoon, include metals, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and organotin compounds (Matias 2013). Therefore, an approach in terms of marine environmental stressors through gene expression analysis could be done, as it was performed by Milan et al. (2013a) with the Manila clam *R. philippinarum* from four locations within the Venice Lagoon. Since bivalves are sedentary, filter-feeding organisms, they tend to accumulate metals and other pollutants in their tissues (mainly the gills and digestive gland). Therefore, there is increasing interest in the study of the physiology of marine bivalves (e.g. reproduction) and their response to environmental stimuli (e.g. Chapman et al. 2009; Milan et al. 2011; Venier et al. 2006).

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# Appendices

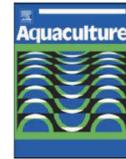
## Appendix 1

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## The reproductive cycle of the European clam *Ruditapes decussatus* (L., 1758) in two Portuguese populations: Implications for management and aquaculture programs



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## ABSTRACT

The reproductive cycle, as well as its nutrient storage and utilization, of two populations of *Ruditapes decussatus* from the main production areas of this species: Ria de Aveiro and Ria Formosa Lagoon (Portugal) were studied here over a 24 month period (May 2010–April 2012). The reproductive cycle of both populations of *R. decussatus* followed an annual cyclicality that comprised a ripe stage in spring followed by a spawning period that began in late spring and extended throughout summer until early autumn. This extended and continuous spawning period may be an advantageous strategy for this species by ensuring a continuous supply of gametes. Moreover, *R. decussatus* can adopt different reproductive strategies depending on the geographical origin. The results of both cycle of nutrients stored and nutrients utilization showed that clams of both populations present a high reproductive effort that almost depletes its energy reserves. Nevertheless, while Ria de Aveiro population retrieves them immediately after spawning, the same is not verified in clams from Ria Formosa Lagoon with their consequent debilitation. Also, based on the glycogen pattern it was possible to infer that the Ria Aveiro population is an opportunistic one, while the Ria Formosa Lagoon population exhibited an intermediate strategy. However, both populations could be considered as viable broodstock for intensive hatchery production of juveniles and the observed extended spawning periods presents interesting implications for the implementation of profitable aquaculture. Moreover, this species presented a great capacity for gonadal regeneration, which coupled with its high gonadal development rate would provide larvae during most of the year without extensive and expensive broodstock conditioning.

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## 1. Introduction

The European clam *Ruditapes decussatus* is widely distributed along the coastal and estuarine areas of Europe and Northern Africa and represents an important resource income due to its high commercial value (Matias et al., 2009). *R. decussatus* is extensively produced and harvested in Portugal, where clam farming represents an important economical sector. This species is central to aquaculture's revenue, indeed in 2009, the national annual production reported reached 2 metric tons (representing 27% of the total seafood cultured in Portugal) (DGPA, 2011). The main production areas of this species are the Ria de Aveiro (40°42'N; 08°W) and the Ria Formosa Lagoon (37°01'N; 07°49'W). In these production areas, clams are reared in plots in the intertidal zone. Clam farming involves seeding juveniles, collected

from natural beds, into plots maintained in tidal flats and harvesting commercial size animals. The culture of *R. decussatus* in Ria Formosa Lagoon represents 90% of the national production and it is central to the socioeconomic framework. However, during the last two decades, the European clam production has suffered an important decrease due to several constraints, namely recruitment failures and excessive pressure on the capture of juveniles on natural banks and severe clam mortalities.

To address this situation, artificial spawning and larval rearing programs could provide an alternative source of spat.

To be able to establish and improve rearing programs for *R. decussatus*, a detailed knowledge of the species reproductive cycle and spawning periods is crucial. Effectively, the differences in gonadal cycles and conditioning optima in different populations have to be considered in hatchery operations (Devauchelle and Mingant, 1991; Lannan et al., 1980). There is also evidence that responses also vary between different geographical populations of the same species, as has been found for *Mytilus galloprovincialis* (Iglesias et al., 1996) and *Argopecten purpuratus*

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(Avendaño and Le Pennec, 1997). In the case of the European clam, in natural conditions, it has been reported that the ecotype *decussatus* living in different areas, even at the same latitude, could strongly differ in terms of their fecundity levels and biochemical compositions (Shafee and Daoudi, 1991; Trigui-El-Menif et al. 1995).

A relationship between the reproductive cycle and energy storage and utilization cycles has also 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). The energy storage and utilization cycles translate into a seasonal pattern of biochemical composition that can vary according to species and geographical origin (Albentosa et al., 2007; Matias et al., 2009). 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). Food and temperature are the main factors that regulate the timing and rate of energy storage in bivalves (Joaquim et al., 2011). The effect of these variables is complex and depends specifically on acquisition and expenditure of energy (Pérez Camacho et al., 2003). The most common model consists of an accumulation of energy during the periods where food is abundant. This energy is then used for the gametogenic synthesis and latter released during the spawning process (Albentosa et al., 2007). Proteins are mainly used in structural functions and represent an energy reserve in adult bivalves, particularly during gametogenesis and in situations of low glycogen levels, or severe energy equilibrium (Beninger and Lucas, 1984). Carbohydrates are assumed to constitute the most important bio-energy reserve in bivalve molluscs and, because of their hydro-solubility, are available for immediate use; being glycogen the main component for supplying energy demands (Fernández-Castro and Vido-de-Mattio, 1987) and reproductive cycle (e.g. Newell and Bayne, 1980; Pazos et al., 2005). Lipids, due to their large calorific contributions per structural unit, account for a greater proportion of the energy reserves in bivalves than carbohydrates or proteins (Ojea et al., 2004). They play an important role in the gamete formation and are the main reserve of oocytes and bivalve larvae (Matias et al., 2009, 2011).

Although previous works have studied the natural reproduction of *R. decussatus* and its biochemical composition (Beninger and Lucas, 1984; Chryssanthakopoulou and Kaspiris, 2005; Hamida et al., 2004; Laruelle et al., 1994; Ojea et al., 2004; Rodríguez et al., 1993; Serdar and Lök, 2009; Shafee and Daoudi, 1991; Trigui-El-Menif et al., 1995; Xie and Burnell, 1994), in Portuguese populations of this species only the gametogenic cycle has been determined in former studies of Vilela (1950) and Pacheco et al. (1989). Therefore, the present study aims to characterize the reproductive cycle of two populations of *R. decussatus* from the main production areas of this species: Ria de Aveiro and Ria Formosa Lagoon, and also include patterns of nutrient storage and utilization. This information would be essential for the establishment of a successful hatchery-based production.

## 2. Materials and methods

### 2.1. Sample collection

Generally, samples of *R. decussatus* were hand-collected, monthly, at the same location in Ria de Aveiro and Ria Formosa Lagoon, during 24 months (May 2010–April 2012). Both areas are shallow water mesotidal lagoons with semidiurnal tidal regimes that constitute the major hydrodynamic forces (Dias et al., 2000; Nobre et al., 2005) (Fig. 1). 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, 2003; 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 of the lagoon is 3.2 in a spring tide and 1.0 in a neap tide. The freshwater inputs are almost negligible and salinity remains close to 36 all year long (Águas, 1986; Falcão and Vale, 2003). 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 (Dias et al., 2000; Moreira et al., 1993) and salinity ranged between 31 and 36. These two ecosystems are currently used for clam production and fish aquaculture ponds.

Monthly data on sea surface temperature (SST) and chlorophyll during the study period 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).

### 2.2. 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.

#### 2.2.1. Histology

Ten individuals of each sex from each monthly sample and each population were examined histologically to determine the gametogenic stages in both sexes. The visceral mass was separated from siphons and gills and fixed in Davison 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 hematoxylin 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 development based on Delgado and Pérez-Camacho (2005) (Table 1). 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):  $GI = [(\sum \text{ind. each stage} \times \text{stage ranking}) / \text{total ind. each month}]$ .

For each of the stages a numerical ranking was assigned as follows: Period of sexual rest (0); initiation of gametogenesis (3); advanced 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).

#### 2.2.2. Condition index

The dry meat and shell weight of 10 clams, from each monthly sample and from each population, 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):  $[\text{ash free dry weight (AFDW) of meat (g)/dry shell weight (g)}] \times 100$ .

#### 2.2.3. Biochemical composition

The meat of ten clams from each monthly sample of the two populations was frozen and stored at –20 °C for biochemical analyses. For each specimen, protein was determined using the modified Lowry

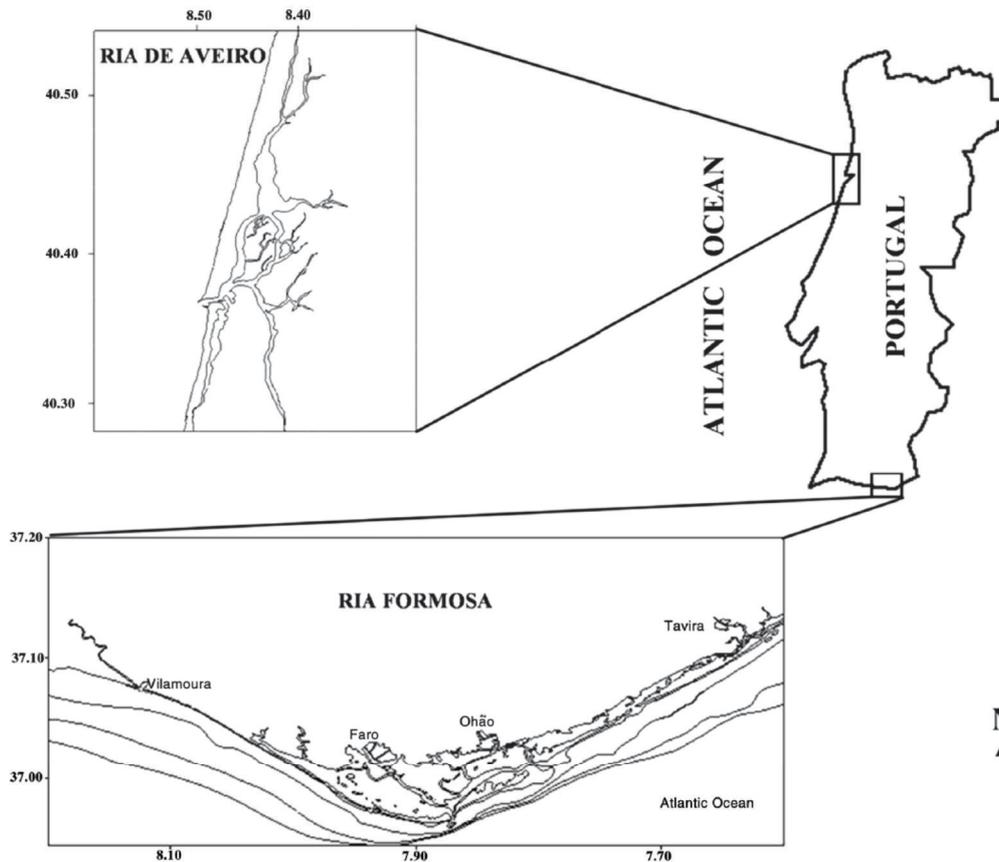


Fig. 1. Ria de Aveiro and Ria Formosa Lagoon location.

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 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 proteins, lipids and carbohydrates in tissues was calculated using the factors 17.9 KJ g<sup>-1</sup> (Beukema and De Bruin, 1979), 33 KJ g<sup>-1</sup> (Beninger and Lucas, 1984) and 17.2 KJ g<sup>-1</sup> (Paine, 1971), respectively.

### 2.3. Statistics

Seasonal variations in condition index, biochemical composition and histological parameters 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 Pearson 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. Results

### 3.1. Temperature and chlorophyll

The evolution of the monthly SST and chlorophyll during the experimental period in Ria de Aveiro and Ria Formosa Lagoon are presented in Fig. 2. Ria de Aveiro presented lower temperature values than Ria Formosa Lagoon (around less  $2.95 \pm 1.31$  °C). A seasonal cycle in SST was observed in the two geographical locations studied, and the monthly means ranged between 19.49 °C in September 2010 and 12.99 °C in February 2012 for Ria de Aveiro and 24.01 °C in August 2010 and 15.03 °C in February 2012 in Ria Formosa Lagoon. The evolution of the chlorophyll during the experimental period showed seasonal variations in geographical locations with several phytoplanktonic blooms in spring for Ria Formosa Lagoon and in spring/summer and autumn in Ria de Aveiro. Ria Formosa Lagoon presented lower values than Ria de Aveiro (around less  $3.49 \pm 3.37$  mg m<sup>-3</sup>) and the average of chlorophyll was  $4.12 \pm 3.19$  mg m<sup>-3</sup> and  $0.63 \pm 0.60$  mg m<sup>-3</sup> for Ria de Aveiro and Ria Formosa Lagoon, respectively.

### 3.2. Gametogenic cycle

The sexes were clearly separated and no hermaphrodites were found. Both sexes showed synchronism in gonadal development. The reproductive cycle of *R. decussatus* was characterized by a seasonal

**Table 1**  
Reproductive scale for *Ruditapes decussatus* adapted from Delgado and Pérez-Camacho (2005).

Stage	Histologic description
Period of sexual rest (phase I)	Gonadal follicles are absent and connective and muscular tissue occupies the entire zone from the digestive gland to foot. There is no evidence of gonadal development and sex determination is not possible.
Initiation of gametogenesis (phase II)	Follicles and gonadal acini begin to appear in females and males. They increase in size, and appear covered with oocytes in the growth phase in the females and with immature gametes (spermatogonia and spermatocytes) in the males.
Advanced gametogenesis (phase III)	The follicles occupy a large part of the visceral mass. The presence of muscular and connective tissue is reduced. At the end of this stage, characterized by intense cellular growth in females, the oocyte protrudes from the center of the lumen, remaining attached to the wall via the peduncle. The abundance of free oocytes equals those attached to the wall of the follicle. In males, majority of the acini were full of spermatids and spermatozooids.
Ripe (phase IV)	Corresponding to the maturity of the majority of gametes. In the mature oocytes the rupture of the peduncle occurs, and the oocytes consequently occupy the follicular interior. In males, the gonadal acini mainly contain spermatozooids.
Partially spawned (phase V)	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 (phase VI)	Abundant interfollicular connective tissue. Occasional residual sperm or oocytes resent.

pattern in both populations (Fig. 3), however, no significant correlations were found between SST and GI and in chlorophyll and GI in both populations (Table 2). The onset of the gametogenic cycle occurred in February in 2011 for males and females of both populations, in March in 2012 for males and females of Ria de Aveiro population and in April and March in 2012 for males and females of Ria Formosa Lagoon

population, respectively. Despite the fact that no correlations were observed between SST and GI and chlorophyll and GI in both populations, the onset of the gametogenic cycle coincided with the increase of SST and with phytoplanktonic blooms. The development of gametes intensified quickly during the following month. The two populations reached its peak of reproductive effort between May and June, represented by the highest values of GI (Ria de Aveiro: females = 4.4 in May and males = 3.5 in June; Ria Formosa Lagoon: females = 4.2 in May and males = 3 in June) (Fig. 4). Spawning began in late spring for both populations; in 2010, generally in June (except for males of Ria Formosa Lagoon population that began in May) and in March and May 2011 for Ria Formosa Lagoon and Ria Aveiro populations, respectively. Spawning of *R. decussatus* intensified during summer as SST increased, and continued until early autumn in both populations. Nevertheless, during this period and in spite of the seasonal pattern, *R. decussatus* did not show a continuous gonad development, after spawning clams did not progress to an inactive stage. Indeed, in the microscopic examinations of the gonadal tissues, all clams showed simultaneous spawning and recovery of the gonad. So, we considered this stage of the reproductive cycle of *R. decussatus* as partially-spawned (stage V – Table 1). This phenomenon occurred in all studied years and for both males and females of the two populations. In October the majority of clams had already spawned and was inactive and remained in this stage during approximately six months (which coincided with the decrease of SST and chlorophyll) until the next onset of gametogenesis. The gonadal index followed the same pattern as the gonadal development; no significant differences in GI were found between populations or between sexes (ANOVA,  $P > 0.05$ ).

3.3. Condition index

Condition index exhibited statistically significant differences between populations (K–W.,  $H = 7.683$ , d.f. = 1,  $P = 0.006$ ), especially when clams were in the inactive stage, between September and October 2010 and August and November 2011. These differences between populations were also relevant between May and July 2010 (ANOVA,  $F = 26.61$ , d.f. = 7,  $P < 0.001$ ). In Ria de Aveiro population, CI was positively correlated with SST (Pearson,  $r = 0.74$ ,  $P < 0.001$ ), however no correlation was observed between these two parameters in Ria Formosa Lagoon. No correlations were observed between CI and chlorophyll for both populations. In 2010, the CI of the Ria de Aveiro population generally trended upwards until September following SST increase (with an exception in July, with a GI contribution) and phytoplanktonic bloom (August), when the highest value ( $11.96 \pm 1.38$ ) of the sampling period was registered (Fig. 5), coinciding with the end of the reproductive cycle of the species. In the following month the CI decreased coinciding with the end of spawning. However, no relationship was observed between GI and CI for the Ria de Aveiro population (Table 2). In the Ria Formosa Lagoon population the CI in 2010 remained high and relatively stable in the first three months of sampling when the majority of clams were in late activity and ripe stage, decreasing sharply from July with the evolution of spawning and consequent rest period. This decline was also coincident with the lowest values of chlorophyll. The lowest CI value ( $4.00 \pm 0.80$ ) of the sampling period was registered in October 2010 in the Ria Formosa Lagoon population. In 2011, Ria de Aveiro population showed two peaks in the CI, the first one in May and another one in October. In contrast, Ria Formosa Lagoon population only showed one peak in May/June which correspond to the maximum GI value of this year achieved with the contribution of the ripe stage of the most clams. The decline of Ria Formosa population CI coincided with the rest period of clams. The CI of the Ria Formosa Lagoon population was positively correlated with the GI (Pearson,  $r = 0.87$ ,  $P < 0.001$ ) (Table 2). Condition index of both populations increased slightly from February to April 2012, following the trend of 2011 (Fig. 4).

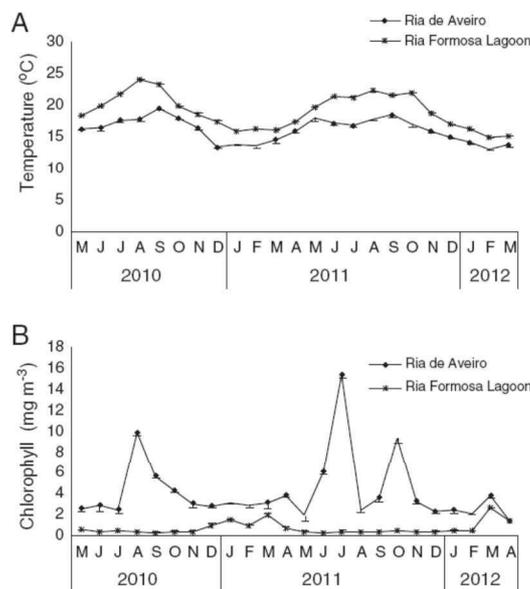


Fig. 2. Monthly mean values of sea surface temperature (SST) (A) and chlorophyll (B) in Ria de Aveiro and Ria Formosa Lagoon from May 2010 to April 2012.

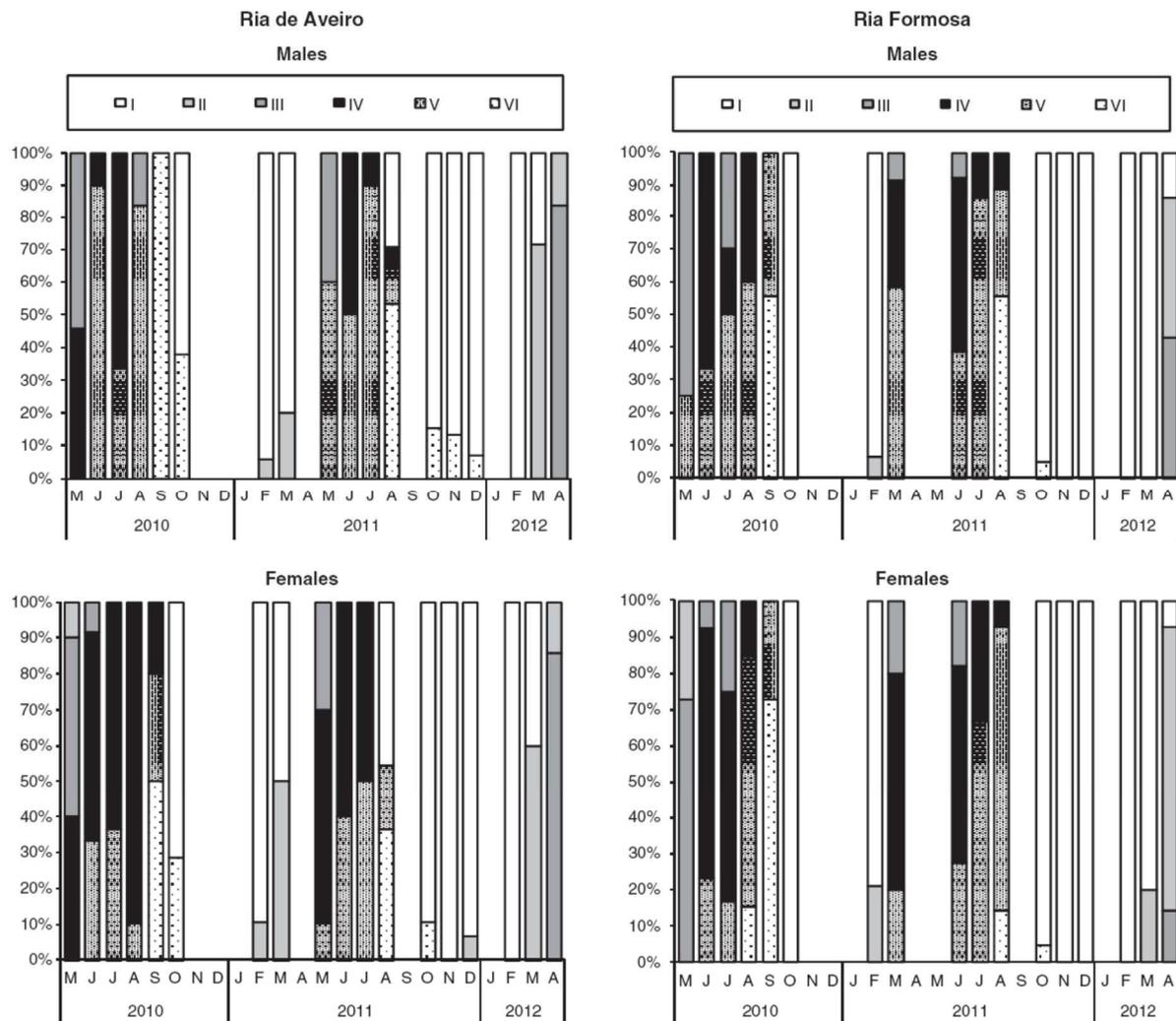


Fig. 3. Monthly variations in gonadal development of *Ruditapes decussatus* populations from Ria de Aveiro and Ria Formosa Lagoon, during May 2010 to April 2012. Males (top) and females (bottom).

**Table 2**  
Results of Pearson correlation between studied parameters (*r*, correlation coefficient, *P*, *P* value, n.c., no correlation was found).

	Ria de Aveiro							Ria Formosa Lagoon						
	Temperature glycogen (SST)	Gonadal index (GI)	Condition index (CI)	Proteins	Total lipids	Glycogen	Total energy	Temperature (SST)	Gonadal index (GI)	Condition index (CI)	Proteins	Total lipids	Glycogen	Total energy
Chlorophyll	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.	n.c.
Temperature (SST)		n.c.	$r = 0.55$ $P < 0.001$	n.c.	n.c.	n.c.	n.c.		n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
Gonadal index (GI)			n.c.	n.c.	n.c.	n.c.	n.c.			$r = 0.87$ $P < 0.001$	n.c.	n.c.	$r = 0.60$ $P = 0.01$	n.c.
Condition index (CI)				n.c.	n.c.	n.c.	n.c.				n.c.	n.c.	$r = 0.78$ $P < 0.001$	n.c.
Proteins					$r = -0.52$ $P < 0.01$	n.c.	$r = 0.87$ $P < 0.001$					n.c.	n.c.	$r = 0.91$ $P < 0.001$
Total lipids						n.c.	n.c.					n.c.	n.c.	n.c.
Glycogen							n.c.						n.c.	n.c.

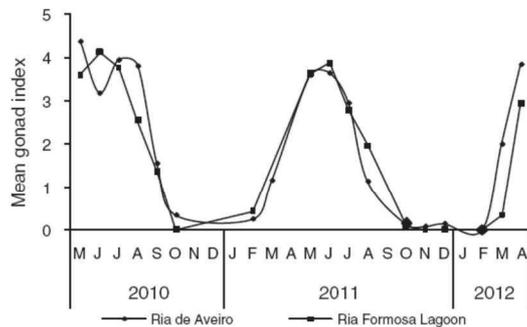


Fig. 4. Monthly variations in gonad index (GI) (mean, n = 20) of *Ruditapes decussatus* populations from Ria de Aveiro and Ria Formosa Lagoon, during May 2010 to April 2012.

3.4. Biochemical composition

Proteins were the predominant dry tissue constituent of the clams followed by total lipids and glycogen (Table 3). The highest protein content values were recorded in May 2010 ( $531.7 \pm 180.0 \mu\text{g mg}^{-1}$  AFDW) and October 2010 ( $520.8 \pm 123.5 \mu\text{g mg}^{-1}$  AFDW) and the lowest in September 2010 ( $128.3 \pm 32.6 \mu\text{g mg}^{-1}$  AFDW) and February 2011 ( $142.2 \pm 23.6 \mu\text{g mg}^{-1}$  AFDW) for Ria de Aveiro and Ria Formosa Lagoon populations, respectively. Significant differences were found between populations (ANOVA,  $F = 4.04$ ,  $d.f. = 1$ ,  $P = 0.045$ ), especially in July, August and September 2010 and February 2011. Glycogen content between clams of the two populations showed significant differences (ANOVA,  $d.f. = 1$ ,  $F = 58.89$ ,  $P < 0.001$ ), especially when clams were inactive in terms of reproduction (Period of sexual rest). In this period, the reserves of glycogen were considerably higher in the Ria de Aveiro clams than in the ones from Ria Formosa. This content generally decreased in both populations until August 2010. In September 2010, the highest ( $53.7 \pm 22.9 \mu\text{g mg}^{-1}$  AFDW) and the lowest ( $7.2 \pm 2.4 \mu\text{g mg}^{-1}$  AFDW) values were recorded for Ria de Aveiro and Ria Formosa Lagoon populations, respectively. After that, the glycogen contents showed opposing trends until February 2011, when the values of the two populations approached. The lowest glycogen values of clams from Ria de Aveiro ( $9.1 \pm 5.8 \mu\text{g mg}^{-1}$  AFDW) were observed in June 2011. Glycogen was positively correlated with CI (Pearson,  $r = 0.79$ ,  $P < 0.001$ ) and GI (Pearson,  $r = 0.60$ ,  $P = 0.01$ ) in Ria Formosa Lagoon population (Table 2). The lowest (Ria de Aveiro:  $35.0 \pm 9.8 \mu\text{g mg}^{-1}$  AFDW; Ria Formosa:  $27.2 \pm 7.3 \mu\text{g mg}^{-1}$  AFDW) and the highest (Ria de Aveiro:  $118.1 \pm 20.5 \mu\text{g mg}^{-1}$  AFDW; Ria Formosa:  $112.1 \pm 15.1 \mu\text{g mg}^{-1}$  AFDW) total lipid values were reached in October 2010 and June 2010 and April 2012 and July 2011, respectively. No significant differences were found between populations for total lipids (ANOVA,  $P > 0.05$ ). Total lipids of clams from Ria de Aveiro population were negatively correlated with proteins (Pearson,

$r = -0.52$ ,  $P < 0.039$ ). Proteins contributed the most to the total energy content (Ria de Aveiro: Pearson,  $r = 0.87$ ,  $P < 0.001$ ; Ria Formosa Lagoon: Pearson,  $r = 0.91$ ,  $P < 0.001$ ) (Table 2). Significant differences were observed between populations for total energy (K-W,  $H = 3.899$ ,  $d.f. = 1$ ,  $P < 0.05$ ), especially in July and August 2010 and February 2011 and 2012.

4. Discussion

The reproductive activity of bivalves, which includes a sequence of events from gametogenesis to spawning, is controlled by the interaction between endogenous (Enrriquez-Diaz et al., 2009; Normand et al., 2008) and exogenous factors, mainly food availability and temperature. Moreover, temperature is closely linked to the geographical locations affecting indirectly the availability of food and/or consequently the timing and duration of the reproductive cycle and number of spawning per year. For *R. decussatus* a different number of annual spawns has been described within Europe (da Costa et al., 2012).

Seawater temperature and chlorophyll patterns, in this study, were common for both studied lagoons and were typical from temperate climates. In both lagoons, temperature increased during spring, stabilized in summer, decreased during autumn and remained low in winter. Also both lagoons are characterized with several phytoplanktonic blooms in spring/summer. Nevertheless, Ria de Aveiro was distinguished by relatively lower SST values and higher chlorophyll values than Ria Formosa Lagoon.

Despite no significant correlation was found between SST and the gonadal index, the sequence of gametogenic stages showed that the reproductive cycle of these two populations of *R. decussatus* follow a seasonal cycle, as has been previously reported for this and several other bivalve species (e.g. Albentosa et al., 2007; Gabbott, 1976; Xie and Burnell, 1994). The reproductive cycle of *R. decussatus* comprised a ripe stage in spring followed by spawning that began in late spring and extended during summer until early autumn in both populations. A similar reproductive cycle was described in the studies by Breber (1980) and Ojea et al. (2004) for a population of *R. decussatus* from Galicia (Spain) and from Venice (Italy), respectively. However, other authors have shown the occurrence of two major periods of spawning, in spring and then in summer or early autumn in different populations of this species (Borsa and Millet, 1992; Chryssanthakopoulou and Kaspiris, 2005; Laruelle et al., 1994; Shafee and Daoudi, 1991) including in populations from the Ria Formosa Lagoon (Pacheco et al., 1989; Vilela, 1950). The differences observed between studies have been frequently associated to the influence of the geographical location and consequently by the inherent environmental factors (vide da Costa et al., 2012). Nevertheless, in our study, no significant differences were observed between the reproductive cycles of the two geographically distinct populations studied.

Beside the fact that the seasonal pattern presented a spawning period well-defined in time, clams of the two studied populations exhibited an advantageous reproductive strategy for the species ensuring a consistent supply of gametes during the whole spawning period. Indeed, histological analyses showed simultaneously gonias, maturing gametocytes and variable proportions of fully matured gametes in the same individual, both in males and females. This high capacity for gonadal regeneration had also been previously observed by our team in *Venerupis senegalensis* from Ria de Aveiro (Joaquim et al., 2011). Nevertheless, and despite the intra-individual asynchrony, the maintenance of a synchronized gonadal development observed between males and females ensures the reproductive success of the species since sperm and oocytes will be expelled into the water column simultaneously during the spawning period, augmenting the probability of fertilization. This synchronism had previously been reported by Laruelle et al. (1994) and Ojea et al. (2004) for this species. Both populations showed a long reproductive rest phase that was extended by a period of approximately six months, during autumn and winter.

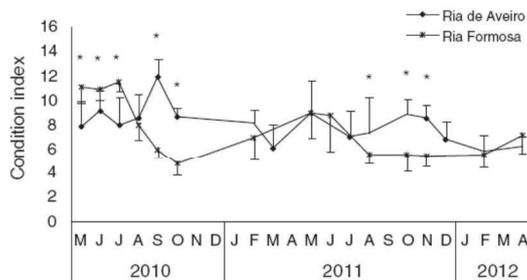


Fig. 5. Condition index (mean  $\pm$  SD) of *Ruditapes decussatus* populations from Ria de Aveiro and Ria Formosa Lagoon, during May 2010 to April 2012. (\*statistically significant differences,  $P < 0.05$  found between populations).

**Table 3**  
Mean values ( $\pm$ sd) of proteins, glycogen, total lipids ( $\mu\text{g mg}^{-1}$  AFDW) and total energy ( $\text{kJ g}^{-1}$  AFDW) of *Ruditapes decussatus* during the experimental period.

Year	Month	Ria de Aveiro				Ria Formosa Lagoon				
		Protein ( $\mu\text{g mg}^{-1}$ AFDW)	Glycogen ( $\mu\text{g mg}^{-1}$ AFDW)	Total lipids ( $\mu\text{g mg}^{-1}$ AFDW)	Total energy ( $\text{kJ mg}^{-1}$ AFDW)	Protein ( $\mu\text{g mg}^{-1}$ AFDW)	Glycogen ( $\mu\text{g mg}^{-1}$ AFDW)	Total lipids ( $\mu\text{g mg}^{-1}$ AFDW)	Total energy ( $\text{kJ mg}^{-1}$ AFDW)	
2010	May	531.7 $\pm$ 180.0	45.0 $\pm$ 10.6	46.1 $\pm$ 9.7	12.0	406.3 $\pm$ 113.6	42.0 $\pm$ 14.9	51.6 $\pm$ 18.1	9.9	
	Jun	482.0 $\pm$ 98.0	37.7 $\pm$ 12.9	41.9 $\pm$ 25.0	10.8	388.5 $\pm$ 78.2	45.0 $\pm$ 9.3	27.2 $\pm$ 7.3	8.7	
	Jul	465.4 $\pm$ 77.8*	32.0 $\pm$ 15.0	38.9 $\pm$ 9.6	10.3*	206.2 $\pm$ 105.3*	38.7 $\pm$ 9.3	37.4 $\pm$ 13.1	5.7*	
	Aug	349.5 $\pm$ 101.0*	18.3 $\pm$ 4.2	45.6 $\pm$ 10.6	8.2*	157.4 $\pm$ 40.0*	28.6 $\pm$ 11.5	42.2 $\pm$ 8.9	4.8*	
	Sep	128.3 $\pm$ 32.6*	53.7 $\pm$ 22.9*	53.5 $\pm$ 11.7	5.1	274.7 $\pm$ 67.5*	7.2 $\pm$ 2.4*	40.2 $\pm$ 9.1	6.5	
	Oct	342.02 $\pm$ 37.2	34.6 $\pm$ 12.3*	35.0 $\pm$ 9.8	8.0	520.8 $\pm$ 123.5	13.3 $\pm$ 12.2*	41.3 $\pm$ 16.7	11.1	
	Nov	–	–	–	–	–	–	–	–	
	Dec	–	–	–	–	–	–	–	–	
	2011	Jan	–	–	–	–	–	–	–	–
		Feb	407.8 $\pm$ 69.4*	25.5 $\pm$ 7.0	47.3 $\pm$ 11.9	9.4*	142.2 $\pm$ 23.6*	22.1 $\pm$ 7.4	45.6 $\pm$ 11.0	4.5*
		Mar	301.2 $\pm$ 33.5	48.3 $\pm$ 15.6	97.6 $\pm$ 17.7	9.7	–	–	–	–
		Apr	–	–	–	–	–	–	–	–
May		251.5 $\pm$ 33.4	33.2 $\pm$ 8.2	80.6 $\pm$ 18.1	7.9	284.5 $\pm$ 24.1	35.4 $\pm$ 8.9	93.0 $\pm$ 16.3	9.0	
Jun		270.5 $\pm$ 40.1	9.1 $\pm$ 5.8	113.5 $\pm$ 26.1	9.0	283.5 $\pm$ 52.0	14.5 $\pm$ 8.2	92.1 $\pm$ 18.8	8.6	
Jul		276.6 $\pm$ 38.2	23.1 $\pm$ 5.4	100.2 $\pm$ 35.5	8.9	293.2 $\pm$ 22.5	18.2 $\pm$ 4.1	112.1 $\pm$ 15.1	9.5	
Aug		265.4 $\pm$ 31.5	39.7 $\pm$ 12.8*	66.3 $\pm$ 12.2	7.8	349.2 $\pm$ 37.5	15.3 $\pm$ 2.3*	68.6 $\pm$ 12.2	9.0	
Sep		–	–	–	–	–	–	–	–	
Oct		298.7 $\pm$ 36.0	50.2 $\pm$ 13.4*	69.6 $\pm$ 13.4	8.7	393.7 $\pm$ 29.7	18.3 $\pm$ 4.1*	95.6 $\pm$ 14.1	10.8	
Nov		243.0 $\pm$ 76.7	50.7 $\pm$ 19.7*	62.3 $\pm$ 11.5	7.4	248.6 $\pm$ 62.7	21.7 $\pm$ 4.6*	82.5 $\pm$ 10.0	7.8	
Dec		–	–	–	–	–	–	–	–	
2012	Jan	–	–	–	–	–	–	–	–	
	Feb	297.1 $\pm$ 34.1	28.3 $\pm$ 23.1	59.4 $\pm$ 7.7	7.9*	475.4 $\pm$ 38.4	25.0 $\pm$ 13.0	90.9 $\pm$ 9.6	12.2*	
	Mar	–	–	–	–	–	–	–	–	
	Apr	235.1 $\pm$ 37.9	34.2 $\pm$ 12.6	118.1 $\pm$ 20.5	9.0	229.2 $\pm$ 37.7	35.2 $\pm$ 10.5	88.6 $\pm$ 15.2	7.9	

The onset of the gametogenic cycle with the proliferation of gonias seems to associate with the rise of SST and chlorophyll in late winter/early spring for both populations and the development of gametes intensified quickly until the attainment of the ripe stage late spring. These results were generally consistent with the previous findings by Chryssanthakopoulou and Kaspiris (2005), Shafee and Daoudi (1991) and Vilela (1950) in other populations of this species, although, as previously mentioned, these authors reported another onset of gametogenesis in July/August.

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). The positive correlation between these two parameters 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 the CI exhibited significant differences between populations, especially when clams were in the inactive stage (Period of sexual rest), between September and October 2010 and August and November 2011. Although, in the general no significant correlations between CI and chlorophyll were observed in both areas, this fact seems to be associated with the greater food availability in the Ria de Aveiro comparatively with the Ria Formosa lagoon. Despite the fact that a positive correlation between CI and SST was observed in Ria de Aveiro, the CI did not reflect the reproductive cycle of this population, since no significant relationship was found between these parameters. In 2010, the CI of this population followed the SST increase and generally trended upwards until September. After that, the CI decreased, coinciding with the end of the spawning period and SST decrease. In 2011, Ria de Aveiro population showed two peaks in CI that accompanying the SST trend; the first one in May when the majority of the clams were ripe and another one in October, however, in this last one the rest stage was the more frequent stage observed. In contrast, no significant positive correlation was found between CI and SST in the Ria Formosa Lagoon population, however, in this population, the CI was positively correlated with the GI. In 2010, CI remained high when the majority of clams were in advanced gametogenesis and ripe stages, decreasing sharply with partial spawning and consequent rest period. In 2011, Ria Formosa

Lagoon population only showed one peak of CI in May/June which corresponded to the maximum GI value of that year achieved with the contribution of the ripe stage of most of the clams. The reduction of the Ria Formosa Lagoon CI coincided with the rest period of clams.

In previous studies it has been reported that the CI is highly influenced by the energy storage and exploitation strategy of bivalve species (Delgado and Pérez-Camacho, 2005; Joaquim et al., 2008, 2011). Clams from Ria de Aveiro seem to have the ability to recover quickly the reserves after spawning when the SST and food availability (chlorophyll) are still high. These reserves seem to be harnessed to maintain their physiological state during winter. The same was not verified in the Ria Formosa Lagoon population. Clams lost their reserves with the intensification of the spawning event and were only able to recover them, slowly during winter, and more rapidly with the next SST increase. This fact is probably associated to the low levels of chlorophyll observed in Ria Formosa Lagoon, especially in the Summer/Autumn months. The major energetic effort suffered by clams during spawning leads then to their debility which might be at the origin of episodes of severe mortalities in the Ria Formosa Lagoon population after the reproductive period. Several factors can contribute to this difference in the energy storage and exploitation strategy between the two studied populations. da Costa et al. (2012) reports that another environmental variable that affects the energetic strategy of bivalves is immersion time. In our study, the subtidal clams from Ria de Aveiro showed higher CI values than the intertidal clams of Ria Formosa Lagoon during the rest period.

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). The relative amounts of proteins (Ria de Aveiro – 128 to 532  $\mu\text{g mg}^{-1}$  AFDW; Ria Formosa Lagoon – 142 to 520  $\mu\text{g mg}^{-1}$  AFDW), glycogen (Ria de Aveiro – 9 to 54  $\mu\text{g mg}^{-1}$  AFDW; Ria Formosa Lagoon – 7 to 45  $\mu\text{g mg}^{-1}$  AFDW) measured in *R. decussatus* were lower, in term of the proportions, to those previously described in the literature for this

species, however the amount of total lipids (Ria de Aveiro – 35 to 118  $\mu\text{g mg}^{-1}$  AFDW; Ria Formosa Lagoon – 27 to 112  $\mu\text{g mg}^{-1}$  AFDW) was higher (e.g. Anibal et al., 2011; Ojea et al., 2004).

Several authors have suggested that, in bivalves, somatic proteins are used as an energy reserve in situations of nutritional stress and energy imbalance or during gonadal maturation (e.g. Gabbott and Bayne, 1973; Liu et al., 2008). Moreover, it has also been suggested that some species use proteins as a source of energy maintenance when carbohydrate reserves have already been depleted (Albentosa et al., 2007; da Costa et al., 2012; Joaquim et al., 2011). In this study, in 2010 the sudden decrease in protein content in both populations after spawning, when glycogen reserves were also depleted, suggests that proteins were indeed used for maintenance of the species; however, the same was not true for 2011 where no regular seasonal trend was observed. In this study, no correlation was found between proteins and glycogen and GI and significant differences were found between both populations protein content.

Glycogen is the main energy reserve in adult bivalves. In *R. decussatus* it can be an energy source for growth and at the same time stored in specific cells as an energetic reserve for the gametogenesis and gonadal development (Rodríguez et al., 1993). In this study, differences were observed between the two populations concerning glycogen content, although the rapid gonadal development and spawning process forced a striking consumption of the glycogen in both populations, after that, higher and lower values were recorded for Ria de Aveiro and Ria Formosa Lagoon, respectively. During the rest period the glycogen content of both populations also showed opposite trends. Some bivalve species store glycogen when food is abundant and gametogenesis takes place when there is low food availability, thus allowing the first spawning release when seawater temperature increases (conservative strategy), and there are others species in which the gamete production occurs from spring onwards, coupled with the first phytoplanktonic blooms (opportunistic strategy) (da Costa et al., 2012). Ojea et al. (2004) and Urrutia et al. (1999) in studies with Spanish populations (Galician and Basque Country) have considered *R. decussatus* as a conservative species however Anibal et al. (2011) concluded that *R. decussatus* from the Ria Formosa Lagoon exhibited an intermediate strategy. Our results corroborate this last thesis. In fact, although glycogen was positively correlated with GI, which is typical from an opportunistic species, the Ria Formosa Lagoon population stored this reserve during the winter and after that both stored and recently assimilated glycogen content was used for gametogenesis. In Ria de Aveiro population, despite the fact that clams have used the after spawning stored glycogen reserves during autumn and winter, which is typical from a conservative species, gametogenesis was not started and no significant correlations were found between glycogen content and GI. The onset of gamete production was only verified in early spring, associated with the first phytoplanktonic bloom, which is typical of an opportunistic strategy. These differences in the reproductive strategy between geographically distinct populations of the same species were also reported by Cerviño-Otero (2011) and da Costa et al. (2012). As previously mentioned the two populations also differed in the amount of glycogen stored, especially when clams were inactive in terms of reproduction. In fact, reserves were stored as glycogen soon after the spawning period in Ria de Aveiro population which is coincident with the phytoplanktonic bloom observed in autumn, however the same was not verified in the Ria Formosa Lagoon population. Consequently, in rest stage, the reserves of glycogen were considerably higher in Ria de Aveiro clams than in Ria Formosa Lagoon one's, corroborating the CI results. A significant positive correlation between glycogen content and CI was found in Ria Formosa Lagoon population.

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 with glycogen, due to the conversion of glycogen to lipids, biosynthesized during the formation of gametes (Gabbott, 1975). In the present study, total lipids increased with the onset of

gametogenesis. After that and during the reproductive period, an erratic variation of total lipids occurred in both populations that could be related with the successive gamete production and release, typical of a partial spawning species. Total lipids also peaked immediately after spawning. These results suggest that, more than a consequence of gametogenesis, the total lipids content also reflects the energy accumulation process and its consumption during bivalve somatic development, as has been previously reported by other authors (Albentosa et al., 2007; Joaquim et al., 2008, 2011).

The negative correlation found between proteins and total lipids in clams from Ria de Aveiro population reinforce the idea that proteins can be used as an energy reserve in stress situations. Moreover, in our study, protein was the constituent that most contributed to the total energy of *R. decussatus*.

In conclusion, the results of this study show that *R. decussatus* has a partial spawning period, during which occurs successive and simultaneous production of gametes and spawning. Moreover, it was also demonstrated that this species can adopt different energy storage depending on the geographic origin. Clams of both populations show a high reproductive effort that almost depletes its energy reserves; however, while the Ria de Aveiro population retrieves them immediately after spawning, the same is not verified in clams from the Ria Formosa Lagoon which leads to their consequent debilitation, probably due to the different levels of food availability in the two lagoons.

Both populations presented however viable broodstock for intensive hatchery production of juveniles and the extended spawning period of both *R. decussatus* populations has interesting implications for the implementation of profitable aquaculture. Moreover, the high gonadal regeneration capacity presented by this species coupled with its high gonadal development rate would provide larvae during most of the year without the need of performing extensive and expensive broodstock conditioning.

We believe that the results of our study can be useful to *R. decussatus* hatchery production programs management. These programs should take into consideration that: the Ria de Aveiro population provides the most suitable broodstock for hatchery production due to their reproductive strategy; the extended natural spawning period implies that manipulation of broodstock in terms of conditioning, which should be initiated in early winter, would be relatively easy; the 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. Moreover the global information obtained in this study on the gametogenic cycle and consequent energy storage will also allow the determination of the optimal reproductive time for artificial spawning induction for aquaculture production of this species.

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## Appendix 2

# A Microarray-Based Analysis of Gametogenesis in Two Portuguese Populations of the European Clam *Ruditapes decussatus*

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## Abstract

The European clam, *Ruditapes decussatus* is a species with a high commercial importance in Portugal and other Southern European countries. Its production is almost exclusively based on natural recruitment, which is subject to high annual fluctuations. Increased knowledge of the natural reproductive cycle of *R. decussatus* and its molecular mechanisms would be particularly important in providing new highly valuable genomic information for better understanding the regulation of reproduction in this economically important aquaculture species. In this study, the transcriptomic bases of *R. decussatus* reproduction have been analysed using a custom oligonucleotide microarray representing 51,678 assembled contigs. Microarray analyses were performed in four gonadal maturation stages from two different Portuguese wild populations, characterized by different responses to spawning induction when used as progenitors in hatchery. A comparison between the two populations elucidated a specific pathway involved in the recognition signals and binding between the oocyte and components of the sperm plasma membrane. We suggest that this pathway can explain part of the differences in terms of spawning induction success between the two populations. In addition, sexes and reproductive stages were compared and a correlation between mRNA levels and gonadal area was investigated. The lists of differentially expressed genes revealed that sex explains most of the variance in gonadal gene expression. Additionally, genes like *Foxl2*, *vitellogenin*, *condensing 2*, *mitotic apparatus protein p62*, *Cep57*, *sperm associated antigens 6, 16 and 17*, *motile sperm domain containing protein 2*, *sperm surface protein Sp17*, *sperm flagellar proteins 1 and 2* and *dpy-30*, were identified as being correlated with the gonad area and therefore supposedly with the number and/or the size of the gametes produced.

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## Introduction

The European clam, *Ruditapes decussatus* (Linnaeus, 1758) is a bivalve mollusc of the family Veneridae, native to the European Atlantic and Mediterranean coastal waters. The European clam, despite a relatively low European production (3200 tons/year) [1], is considered a high value seafood product and one of the most important bivalve species economically in Southern European countries like Italy, Spain and Portugal [2].

The culture of *R. decussatus* is clearly limited by the availability of seed. Its production is almost exclusively based on natural recruitment, which is subject to high annual fluctuations due to pollution and other environmental factors. To address this situation, artificial spawning and larval rearing programs were developed to provide an alternative source of seed [2], but still need improvement to gain robustness of seed production.

*R. decussatus* is a gonochoric molluscan species (possessing separate sexes) that reproduces annually [3]. Generally, the gonad regresses at the end of the annual reproductive cycle, which is the

end of summer in temperate regions, and regenerates at the beginning of the following one (the end of winter). During the initial stage of gametogenesis, period of sexual rest (I), gonadal follicles are absent and connective and muscular tissue occupies the entire zone from the digestive gland to the foot. There is no evidence of gonadal development and determination of sex is not possible. During the second stage of gametogenesis (II) follicles and gonadal acini begin to appear in females and males, respectively. They increase in size, and appear filled with oocytes in the growth phase in the females and with immature gametes (spermatogonia and spermatocytes) in the males. During advanced gametogenesis (III) the follicles occupy a large part of the visceral mass and it's possible to observe the first signs of partial emission of gametes when it occurs. The maturation period (IV) corresponds to the maturity of the majority of gametes. Throughout this period partial spawning may occur, and it concludes with the total emission of gametes [4].

Currently, the knowledge about the molecular mechanisms of reproduction in marine bivalves is very limited, but is expected to

increase rapidly, due to the advent of high throughput genomic approaches [5]. Here, a large and exhaustive approach is proposed for investigating the molecular bases of variability in reproductive success and, more generally, the reproductive mechanisms in *R. decussatus*. Some specific studies have recently explored this topic. The gonadal transcriptome was described and lists of genes of interest for reproductive stage and sex were established for the alternative and irregular protandrous hermaphrodite, the Pacific oyster, *Crassostrea gigas* [5] and for the functional hermaphrodite scallop *Nodipecten submodosus* [6]. Most importantly, cross-referencing these lists of genes allowed the identification of potential markers of early sex differentiation and new highly valuable information on genes specifically expressed by mature spermatozooids and mature oocytes. Additionally, dedicated studies have been made in *Argopecten purpuratus*, *C. gigas* and *Mytilus galloprovincialis* emphasizing specific reproductive processes [7,8,9,10,11].

In *R. decussatus*, apart from a few specific gene expression analyses (see [12]) and a first version of DNA microarray designed to unravel host-parasite interactions [13], very little information is available on genome-wide expression profiling for physiological, environmental or aquaculture questions.

For the present work, cDNA libraries of oocytes, larval stages and different gonadal maturation stages were sequenced on the Illumina platform, and a custom oligonucleotide microarray representing 51,678 assembled contigs was designed and used to characterize the transcriptomic bases of reproduction in *R. decussatus*. Microarray analyses were performed in four gonadal maturation stages of two Portuguese wild populations, Ria Formosa in Southern Portugal (37°01'N; 07°49'W) and Ria de Aveiro in Western coast of Portugal (40°42'N; 08°40'W). These populations were already characterized by different responses to spawning induction, which is of great interest to study in a context of improvement of aquaculture production [2,14].

The present study provides new highly valuable genomic information for the understanding of reproduction of this species and emphasizes some candidate genes as possible starting points for further functional studies, for instance on spawning and gamete quality with the aim of elucidating how and to what extent environmental factors affect relevant reproductive-gene expression.

## Materials and Methods

### Ethics statement

The European clam is not considered as an endangered or protected species in any Portuguese or international species catalog, including the CITES list ([www.cites.org](http://www.cites.org)). The European clams from Ria de Aveiro (40°42'N; 08°40'W) and Ria Formosa (37°01'N; 07°49'W) were produced and captured with the permission of DGRM (Direção-Geral de Recursos Naturais, Segurança e Serviços Marítimos), APA (Agência Portuguesa do Ambiente) and PNRF (Parque Natural da Ria Formosa - National park defined by the Ramsar Convention).

### Animal sampling for RNA-sequencing

Clams were collected in Ria de Aveiro (40°42'N; 08°40'W), and conditioned in a common garden setting to accelerate their gonad development from October 2011 to January 2012 in the experimental bivalve hatchery of Portuguese Institute of Sea and Atmosphere (IPMA) in Tavira, Portugal. Food regimes consisted of different algal mixtures containing *Isochrysis galbana* (clone T-ISO) and *Skeletonema costatum* (Ria Formosa autochthon clone). Microalgae were cultured in 80 L bags with f/2 medium [15], in a temperature-controlled room at 20±2°C under continuous

illumination (9900 lux) and aeration. Gonad samples were collected and immediately dissected: a transversal section of the gonadal area was fixed for histological examination and the rest of the gonads were frozen immediately in liquid nitrogen. Later, the gonads were crushed into a fine powder at -196 °C with a Dangoumau mill and stored in liquid nitrogen until RNA extraction. For the histological analysis, a 3-mm cross section of the visceral mass of each sample was excised using a microtome cutter in front of the pericardic region and immediately fixed in 10 to 20 ml modified Davidson's solution [16] at 4°C for 48 h. After, the modified Davidson's solution was discarded and 10 to 20 ml 70% ethanol was added and kept at 4°C.

Sections were dehydrated in ascending ethanol solutions, cleared with a xylene substitute named Microclearing (Diapath, Italy), and embedded in paraffin wax. Sections of 5 µm were cut, mounted on glass slides and stained with Harry's hematoxylin-Eosin Y [17]. Slides were then examined under a light microscope and sex and stages were determined according to the 4 stages previously described (see Introduction). Percentage areas of gonadic tubules, connective tissue and digestive gland were then determined on each histological section. Slides were scanned with a digital scanner (hp scanjet 7400 c) and the images saved in \*.TIFF format. Tissue areas were then measured using image analysis software (Imaq Vision Builder, National Instruments Corp.). Gonad area percentage was estimated as pixel number, from gonad / pixel number on total sections, as described in [18]. 35 individuals corresponding to five gonad samples of each reproductive stage and sex were chosen for RNA-sequencing.

### Animal sampling for microarray analysis

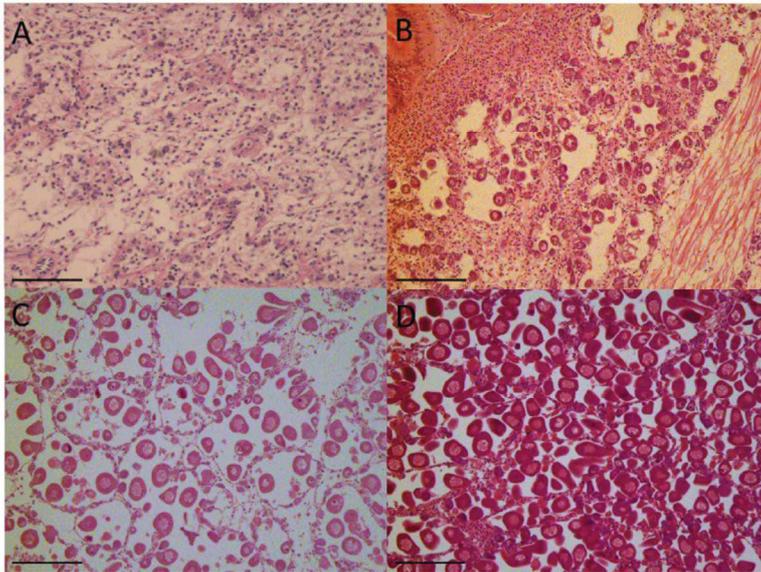
Twenty clams from two wild populations - Ria Formosa (South of Portugal) and Ria de Aveiro (Northern of Portugal) - were sampled monthly between July 2011 and July 2012. At each sampling date the gonads of the collected clams were immediately dissected for RNA extraction and a transversal section of the gonadal area fixed for histological analysis, as described above. Gene expression analysis was done on 64 individual gonads representative of all the reproductive stages and sexes from both populations, chosen on the basis of histological data (Figure 1; Figure 2; Table 1).

### RNA extraction

For RNA-sequencing and microarray analysis, total RNA was isolated using Extract-all (Eurobio) procedure. RNA quality and integrity were analysed on the Agilent bioanalyzer using RNA nano-chips and Agilent RNA 6000 nanoreagents (Agilent Technologies, Waldbronn, Germany). RNA concentrations were measured at 260 nm using a ND-1000 spectrophotometer (Nanodrop Technologies) using the conversion factor 1 OD = 40 mg/mL total RNA. Samples were stored at -80°C until further use.

### cDNA library construction and sequencing

Sequencing of gonadal maturation stages was performed through a multiplexing strategy on the Illumina platform HiSeq2000. A total of 7 cDNA tagged libraries from gonads of 5 individuals, of each developmental stage (sexual rest stage I and stages II/III/IV in both sexes), were prepared on a 7-plex paired-end sequencing lane (2×100 bp). cDNA libraries were constructed, amplified and sequenced at BGI Tech (Shenzhen, China). All the Illumina reads were analyzed with FastQC software in order to assess the sequence quality. Illumina reads have been deposited in GenBank (SRA) with the accession numbers SRR974930,



**Figure 1. Photomicrographs showing stages in the development of *Ruditapes decussatus* female gonad.** A. Sexual rest. B. Initiation of gametogenesis; C. Advanced gametogenesis; D. Maturation. Scale bar: 200  $\mu$ m.  
doi:10.1371/journal.pone.0092202.g001

SRR974931, SRR974932, SRR97493, SRR974934,  
SRR974935, SRR974936.

#### Assembly and transcriptome annotation

To obtain a wider *R. decussatus* transcriptome representation, Roche 454 and Illumina reads have been added to the Illumina reads produced by a 7-plex paired-end sequencing lane (Table S1). Specifically, additional Illumina reads have been obtained through the sequencing of stripped and spawned oocytes, larvae at 48 hours post-fertilization, and larvae at 17, 21 and 30 days post-fertilization cDNA libraries (Pauletto et al. unpublished data; personal communication). In addition, reads obtained by the sequencing by 454 Roche of hemocytes, oocytes and larval stages cDNA libraries have been also considered for the assembly (Novoa et al., unpublished data; personal communication). These libraries have been produced within the European project REPROSEED and will be public with the complete transcriptome of *R. decussatus* (Pauletto et al, Novoa et al. unpublished data). All these data were merged also with publicly available Sanger sequences and nearly 500,000 reads obtained with 454 sequencing of larvae, gonads and several adult tissues (MGE011 and cDN18; SRA058431; [13]). A summary of the origin of *R. decussatus* sequences and assembly information are shown in Table S1.

Assembly was performed separately for each library by using MIRA3 or CLC Genomic Workbench for 454 and Illumina reads, respectively. A total of 990,111 contigs were then merged to reduce the redundancy by using CAP3, resulting in a total of 503,705 contigs (unpublished data). 454 and Illumina reads have been assembled by CLC Genomic Workbench, MIRA 3 and CAP3 with default parameters.

A preliminary annotation was obtained for 141,001 contigs through Blast similarity searches conducted against several protein databases (e-value 1 E-5, see Table S2).

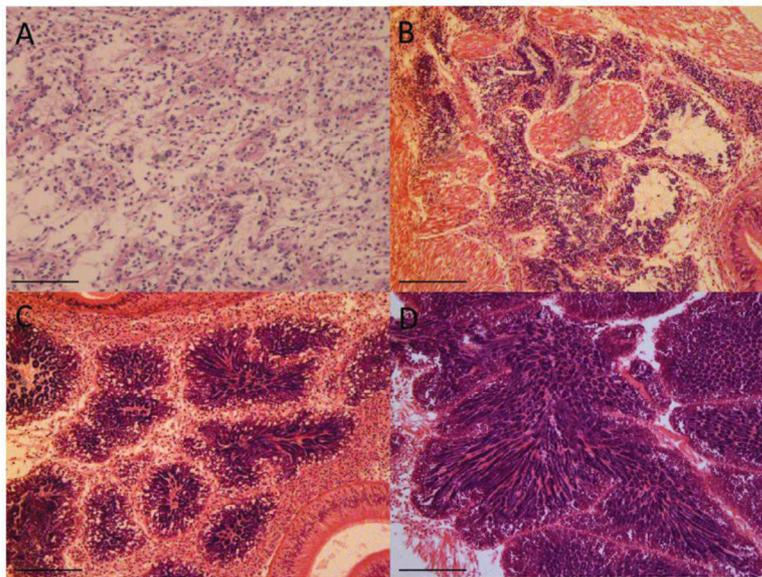
#### Microarray design

All databases used for the annotation step (see Table S2) were considered to reduce the redundancy in annotated contigs. A total of 44,333 contigs found non-redundant (with a unique annotation) in at least one reference database have been considered for microarray design. Putative sense-strand orientation was inferred from the matching protein-coding gene in reference data bases. For 915 contigs that showed ambiguous orientation two probes with opposite orientations (sense and antisense) were designed. For the remaining 43,418 contigs with putatively unambiguous orientation a single (sense) probe was designed.

Since the microarray format could accommodate approximately 60,000 probes, non-annotated contigs that showed the highest expression in gonads based on RNA-seq data (see below) were included in the microarray design. In total 7,376 non-annotated contigs were added, and for each of them, two probes with opposite orientation (sense and antisense) were designed. Probe design was carried out using the Agilent eArray interface (<https://earray.chem.agilent.com/earray/>), which applies proprietary prediction algorithms to design 60-mer probes. A total of 59,951 out of 60,000 probes were successfully obtained, representing 51,709 different *R. decussatus* contigs. The sequences of the 51,709 contigs representing in *R. decussatus* DNA microarray platform have been provided in Table S3. The percentage of annotated transcripts represented on the microarray was 85.7%. In Table S4 has been reported the annotation of each probe represented in DNA microarray platform. Probe sequences and other details on the microarray platform can be found in the GEO database (<http://www.ncbi.nlm.nih.gov/geo/>) under accession number GPL17766.

#### Labeling and microarray hybridization

Sample labeling and hybridization were performed according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol with the Low Input Quick Amp Labeling kit. Briefly, for each sample 100 ng of total RNA was linearly



**Figure 2. Photomicrographs showing stages in the development of *Ruditapes decussatus* male gonad.** A. Sexual rest. B. Initiation of gametogenesis; C. Advanced gametogenesis; D. Maturation. Scale bar: 100  $\mu$ m in A, B, C; 200  $\mu$ m in D. doi:10.1371/journal.pone.0092202.g002

amplified and labeled with Cy3-dCTP. A mixture of 10 different viral poly-adenylated RNAs (Agilent Spike-In Mix) was added to each RNA sample before amplification and labeling to monitor microarray analysis work-flow. Labeled cRNA was purified with Qiagen RNeasy Mini Kit, and sample concentration and specific activity (pmol Cy3/ $\mu$ g cRNA) were measured in a NanoDrop<sup>®</sup> ND-1000 spectrophotometer. A total of 600 ng of labeled cRNA was prepared for fragmentation by adding 5  $\mu$ l 10X Blocking Agent and 1  $\mu$ l of 25X Fragmentation Buffer, heated at 60°C for 30 min, and finally diluted by addition with 25  $\mu$ l 2X GE Hybridization buffer. A volume of 40  $\mu$ l of hybridization solution was then dispensed in the gasket slide and added to the microarray slide (each slide containing eight arrays). Slides were incubated for 17 h at 65°C in an Agilent hybridization oven, subsequently removed from the hybridization chamber, quickly submerged in GE Wash Buffer 1 to disassemble the slides and then washed in GE Wash Buffer 1 for approximately 1 minute followed by one additional wash in pre-warmed (37°C) GE Wash Buffer 2.

**Table 1. Number and sex of the 64 individuals of each gonad developmental stage from the two studied populations used in the microarray analysis.**

Gonad developmental stage	Sex	Ria Formosa	Ria de Aveiro
I		5	5
II	F	5	5
	M	5	5
III	F	5	5
	M	3	7
IV	F	5	3
	M	3	3

doi:10.1371/journal.pone.0092202.t001

#### Data acquisition, correction and normalization

Hybridized slides were scanned at 2  $\mu$ m resolution using an Agilent G2565BA DNA microarray scanner. Default settings were modified to scan the same slide twice at two different sensitivity levels (XDR Hi 100% and XDR Lo 10%). The two linked images generated were analyzed together and data was extracted and background subtracted using the standard procedures contained in the Agilent Feature Extraction (FE) Software version 10.7.3.1. The software returns a series of spot quality measures in order to evaluate the quality and the reliability of spot intensity estimates. All control features (positive, negative, etc.), except for Spike-in (Spike-in Viral RNAs), were excluded from subsequent analyses.

Raw gene expression data of all 64 analyzed individual gonads (Table 1) were deposited in the GEO database under accession number GSE51150.

Spike-in control intensities were used to identify the best normalization procedure for each dataset. After normalization, spike intensities are expected to be uniform across the experiments of a given dataset. Normalization procedures were performed using R statistical software. Quantile normalization always outperformed cyclic loess and quantile-normalized data were used in all subsequent analyses. Normalized data were deposited in GEO archive under accession number GSE51150.

#### Data analysis

Differences in percentage gonad area were analysed using one-way analysis of variance after angular transformation. Comparisons of sex and gametogenic stage distributions between lines were made using Chi-square tests.

A principal component analysis (PCA) using the TMeV 4.5.1 (TIGR MULTIEXPERIMENT VIEWER) [19] was applied to assess the distribution of the studied groups. Statistical tests implemented in the program Significance Analysis of Microarray (SAM) were used to identify differentially expressed probes between the two clam populations. The same approach was used

to identify differentially expressed probes in both populations between males and females. A SAM quantitative correlation analysis was also performed to identify genes with a correlation between mRNA levels and gonad area, after angular transformation. A Pavlidis template matching (PTM) analysis was implemented using the TMeV 4.5.1 in order to verify whether the expression of specific genes increased during gonad development. A one-way ANOVA parametric test was used to identify the probes whose expression changed during gonad development in the two populations, using a p-value cut-off of 0.01 and an adjusted Benjamini & Hochberg correction using R software v2.15.2. Hierarchical clustering was performed using TMeV on statistically significant transcripts, to group experimental samples together based on similarity of the overall experimental expression profiling.

A more systematic and functional interpretation for significant gene lists was obtained using an enrichment analysis with the Database for Annotation, Visualisation, and Integrated Discovery (DAVID) software (Huang et al., 2009). “KEGG Pathway”, “Biological process” (BP), “Molecular function” (MF) and “Cellular component” (CC) analyses were carried out by setting the gene count equal to 2 and the ease equal to 0.1. Because the DAVID database contains functional annotation data for a limited number of species, it was necessary to link *R. decussatus* transcripts with sequence identifiers that could be recognized in DAVID. This process was accomplished using dedicated Blast searches performed with Blastx against zebrafish Ensembl proteins. Finally, *Danio rerio* Ensembl Gene IDs were obtained from the corresponding Ensembl protein entries using the BIOMART data mining tool (<http://www.ensembl.org/biomart/martview/>). *D. rerio* IDs corresponding to differentially transcribed clam genes as well as all of the transcripts that were presented on the array were then used to define a “gene list” and a “background” in DAVID, respectively.

## Results

### Prevalent Gene Expression Patterns

A Principal Component Analysis (PCA) was applied to the entire gene expression database (59,951 probes) for the 64 clams (Figure 3). We observed a clear clustering of the different sexes and gametogenic stages determined by histological methods. Along the first axis, PC1, which explained 35% of the variation, male and female individuals were discriminated. Gonad developmental stages were organized along the second axis, PC2 (15%). A significant divergence in expression patterns between male and female gonads was observed in stages III and IV (PC2). At these later stages, males and females predictably possess the most distinctive expression profiles, while at stages I and II this separation is not as clear along the second axis.

### Two-unpaired class Significance Analysis of Microarray (SAM) between sexes

In order to compare gene expression profiles between sexes in *R. decussatus*, independently from stage and population of origin, a two-unpaired class Significance Analysis of Microarray (SAM) test was carried out on normalized data (FDR = 5%; FC > 1.5). A list of 16,996 significant probes, corresponding to 11,641 unique transcripts, was obtained (Table S5).

Hierarchical clustering using Pearson’s correlation for the set of 16,996 differentially expressed probes identified three main clusters: Stage I, Females and Males (Figure 4). However, there are some exceptions to this hierarchical clustering. Indeed, a stage I individual appeared on the same branch as females.

A total of 7,145 differentially expressed probes were up-regulated in the gonads of males with a FC ranging from 1.5 to 255, while a total of 9,751 differentially expressed probes were up-regulated in the females gonads with a FC ranging from 1.5 to 374.

A putative annotation with zebrafish Ensembl Gene IDs was obtained for 6,506 probes that were differentially expressed between the two sexes. These annotated transcripts were used to define a gene list for functional annotation with DAVID. Enrichment analysis showed 9 KEGG, 95 BP terms, 22 CC terms, and 45 MF terms to be significantly over-represented (Table S6).

“Cell cycle” (dre04110), “oocyte meiosis” (04114) and “Progesterone-mediated oocyte maturation” (dre04914) were the most represented in the enriched KEGG pathway terms, with 49, 45 and 38 genes respectively. Cyclin B1 (*CCNB1*) and cyclin B2 (*CCNB2*); Cyclin-Dependent Kinase 2 (CDK2); Mitotic arrest deficient-like 1 (Mad21) and several genes of the Calmodulin family (*CaM*) were found among the differentially expressed genes.

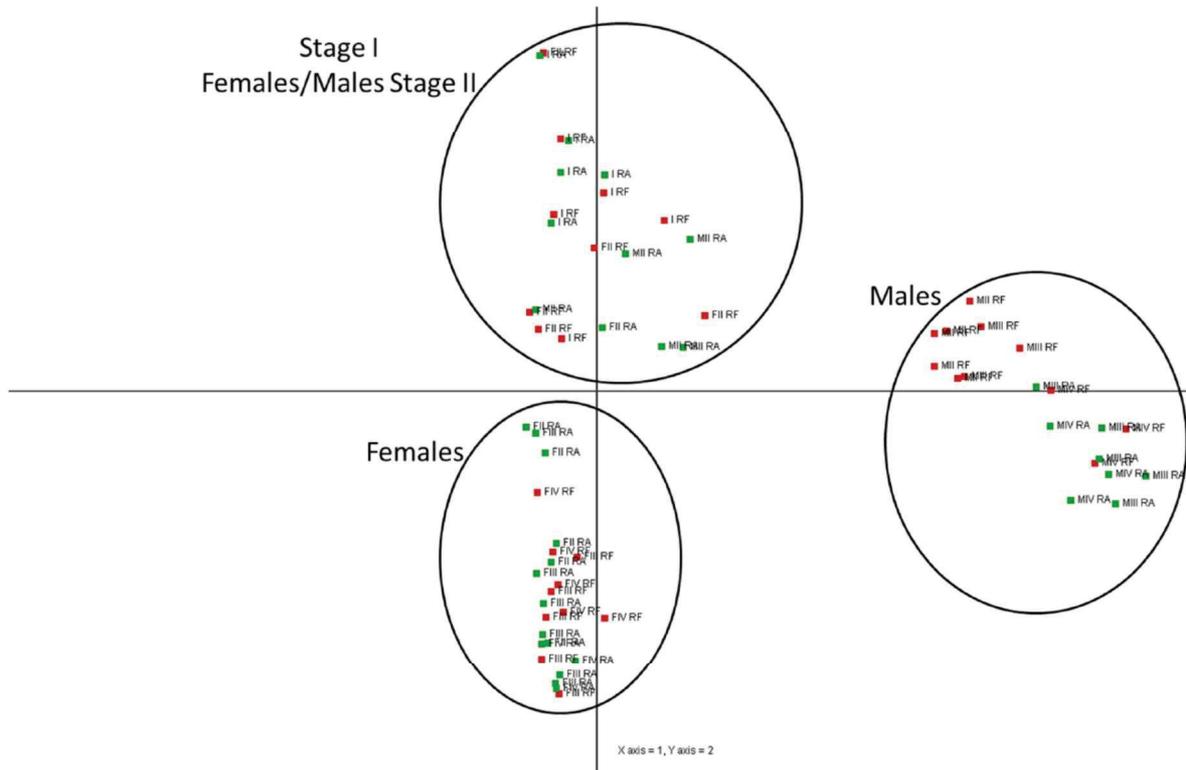
The same analysis was performed in each reproductive stage. The number of differentially expressed probes increased as the reproductive stages developed. At stage II a list of 5,059 significant probes, corresponding to 3,237 unique transcripts was obtained. At stage III a list of 18,714 significant probes, corresponding to 12,870 unique transcripts was obtained. Finally, the stage IV represented the highest number of differentially expressed probes with a list of 20,797 probes, corresponding to 14,260 unique transcripts. At this last stage, “oocyte meiosis” (04114) was once again the most represented term for enriched KEGG pathways, with 49 genes. Genes like Speriolin and testis-specific serine/threonine-protein kinase (*TSSK-5*), were present with a high FC value of 332 and 249, respectively, in the list of differentially expressed probes.

### Identification of Stage-specific Expressed Genes

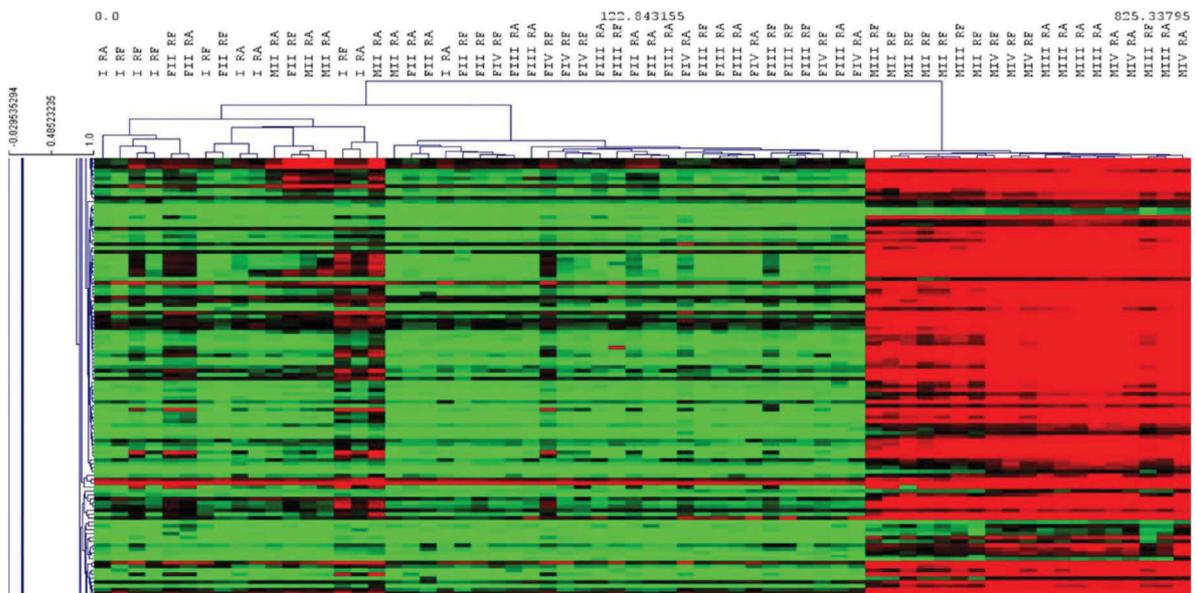
In order to identify those transcripts whose expression changed during gonad development in the two populations, a one-way ANOVA (p < 0.01, adjusted Benjamini & Hochberg correction) was performed. This analysis identified 13,218 probes that exhibited a significant change in expression over the reproductive cycle of male and female gonads (Table S7). A putative annotation with *Danio rerio* Ensembl Gene IDs was obtained for 6,451 probes that were differentially expressed between gametogenic stages. Enrichment analysis showed 9 KEGG, 20 BP terms, 12 CC terms, and 40 MF terms to be significantly over-represented (Table S8).

“Focal adhesion” (dre04510) and “Regulation of actin cytoskeleton” (dre04810) were the most represented within enriched KEGG pathway terms, with 74 and 62 genes respectively. “Microtubule-based process” (GO:0007017) was one of the enriched biological processes with the highest representation.

In addition, we decided to emphasize the expression progression of certain well-known reproduction-related genes in the animal kingdom, in order to verify whether their expression increased during gonad development. Vitellogenin (*VTG*), a female specific glycoprotein, was identified in our female samples with an increase in expression along the gametogenic cycle, from stage I to stage IV, with the higher values achieved in stage IV (maturation period). It was also observed that the females from Ria Formosa presented higher expression values of *VTG*. Another female specific gene, *Condensin-2*, was also over-expressed in females during gonad development, with the same trend as *VTG*. In terms of male specific genes, the motile sperm domain containing protein 2 (*MOSPD2*) and the synaptonemal complex protein 2 (*SCP-2*)



**Figure 3. Principal Component Analysis.** PCA applied to all 59,951 transcripts of the 64 individual clam gonads sampled from Ria de Aveiro (RA) and Ria Formosa (RF). I= Stage I (sexual rest); FII= Female stage II (gametogenesis); FIII= Female stage III (advanced gametogenesis); FIV= Female stage IV (maturation); MII= Male stage II (gametogenesis); MIII= Male stage III (advanced gametogenesis); MIV= Male stage IV (maturation). doi:10.1371/journal.pone.0092202.g003



**Figure 4. Heat map of sex specific genes.** Hierarchical clustering obtained using Pearson's correlation considering the set of 16,996 differentially expressed probes between sexes of the two studied populations of *Ruditapes decussatus*. doi:10.1371/journal.pone.0092202.g004

were identified, showing an increase in expression along the spermatogenesis, from stage I to stage IV.

#### Identification of genes with a correlation between mRNA levels and gonad area

Quantitative correlations between microarray data and values of gonad area of stages II, III and IV from males and females, separately, after angular transformation, were evaluated via a SAM quantitative correlation analysis (FDR = 5%). Females showed a total of 10,415 probes significantly (direct or inverse) correlated with the gonad areas (Table S9). Of these probes, 7,226 were annotated by similarity and associated with a known protein. We noticed the presence of positively correlated genes coding for Forkhead box protein L2 (*Foxl2*), *VTG*, *condensing 2*, Mitotic apparatus protein *p62* and *Cep57*. In relation to male gonads, a total of 18,730 probes showed a significant (direct or inverse) correlation with gonad area (Table S10). A putative annotation was obtained for 12,843 probes. Genes expressed included *dpp-30*, sperm associated antigens 6 (*SPAG6*), 16 (*SPAG16*) and 17 (*SPAG17*), *MOSPD2*, sperm surface protein *Sp17* and sperm flagellar proteins 1 and 2. Additionally, functional analysis of significant transcripts showed that different KEGG pathway terms, such as “Cell cycle” (dre04110), “oocyte meiosis” (04114) and “Regulation of actin cytoskeleton” (dre04810), were influenced by gonad area as well.

#### Comparison between populations using two-unpaired class Significance Analysis of Microarray (SAM)

In order to compare gene expression between the two sampled populations of *R. decussatus*, considering all stages and sexes, a two-unpaired class Significance Analysis of Microarray (SAM) test was carried out on normalized data, enforcing a False Discovery Rate (FDR) of 5% and a Fold Change threshold of 1.5. A list of 2,900 significant probes, corresponding to 2,228 unique transcripts, was obtained.

A putative annotation with *Danio rerio* Ensembl Gene IDs was obtained for 1,777 probes out of the 2,228 differentially expressed transcripts between the two populations (Table S11). Enrichment analysis revealed a total of 2 KEGG pathways, 23 BP terms, 15 CC terms, and 25 MF terms significantly over-represented among the differentially expressed probes (Table S12). “N-Glycan biosynthesis” (dre00510) term was the most represented in the enriched KEGG pathway terms, with 9 genes.

Hierarchical clustering, using Pearson’s correlation, was performed considering the set of 2,900 differentially expressed probes. As reported in Figure 5, with some exceptions, three main clusters were identified: Ria Formosa, Ria de Aveiro and Female Stage III/IV from Ria de Aveiro.

In order to support the results obtained by hierarchical clustering, several comparisons between populations in each reproductive stage and sex were performed. The comparison between stage IV females from both populations was the one that presented the highest number of differentially expressed probes. For this analysis, a two-unpaired class Significance Analysis of Microarray (SAM) test was carried out on normalized data (FDR = 5%; FC > 1.5). A list of 6,765 significant probes, corresponding to 5,133 unique transcripts, was obtained (Table S13). A putative annotation with *Danio rerio* Ensembl Gene IDs was obtained for 3,229 probes. An enrichment functional annotation analysis was performed with DAVID, revealing 11 KEGG pathways, 88 BP terms, 29 CC terms, and 51 MF terms significantly over-represented (Table S14). “Cell cycle” (dre04110), “Spliceosome” (dre03040), “Ubiquitin mediated

proteolysis” (dre04120) and “Oocyte meiosis” (dre04114) terms were the most represented in the enriched KEGG pathway terms, with 45, 32, 30 and 28 genes respectively. Among the genes differentially expressed between females stage IV of the two populations, the great majority were common to “Oocyte meiosis” (dre04114) and “Cell cycle” (dre04110) pathways, like the S-phase kinase-associated protein 1 (*skp1*) and the *Mad211*. Other important differentially expressed genes were also identified, such as the *cdc16*, *cdc20*, *cdc27*. Genes like *CCNB1*, cyclin E2 (*CCNE2*), and *cdk2* were also present in both pathways.

Another comparison that revealed an important number of differentially expressed probes, at the population level, was between stage II males. A list of 5,801 significant probes, corresponding to 3,588 unique transcripts was obtained (Table S15). A putative annotation with *Danio rerio* Ensembl Gene IDs was obtained for 3,181 probes. An enrichment functional annotation analysis has been performed by DAVID, revealing 14 KEGG pathways, 56 BP terms, 23 CC terms, and 50 MF terms significantly over-represented (Table S16). “Insulin signaling pathway” (dre04910) was the most represented in the enriched KEGG pathway terms. Important differentially expressed genes were found, such as, the Mitogen-Activated Protein Kinase 1 (*MAPK1*). However, for the remaining comparisons performed, no additional significant results were observed between populations.

#### Discussion

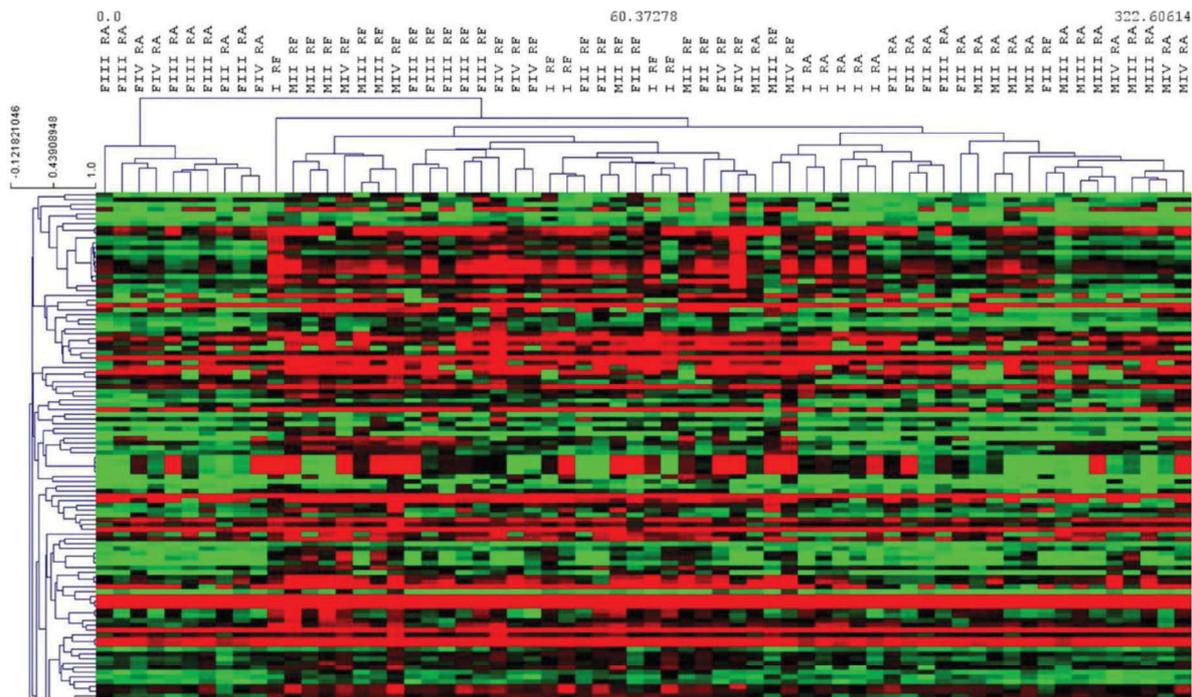
Recent expression profiling studies using microarrays have been successfully employed in order to better understand the cellular and molecular events of reproductive tissue development and unravel some molecular mechanisms involved in reproductive phenotypes (sex differentiation, gametogenesis; e.g. in oyster [5]).

To better understand the different reproductive behaviors observed between northern and southern Portuguese populations, gonads of clams collected in Ria Formosa (Southern Portugal) and Ria Aveiro (Western coast of Portugal) were analyzed (Figure 6).

#### Sex explains most of the variance in gonad gene expression

Although the preliminary analysis using PCA (Figure 3) did not discriminate populations, a clear clustering of the different sexes and gametogenic stages was observed. The main sex effect on gonadic gene expression was also reported for an alternative and irregular protandrous hermaphrodite marine bivalve, the Pacific oyster [5]. The differences between males and females increased over time, following the continuous gametogenesis from stage II to stage IV gonads. Considering gene expression pattern, there is no evidence of sex determination at stage I based on the PCA results, showing that, with this analysis, clam sex is undetermined at stage I.

In the hierarchical clustering performed on the probes differentially expressed between sexes (Figure 4), a stage I individual was detected clustered on the same branch as females. Although the sex of this clam could not be determined histologically, we suggest that this individual had already initiated sex differentiation, being possibly in a later stage I, and could therefore be classified as female according to its gene expression pattern. Indeed, through Pavlidis template matching (PTM) analysis by TMcv, we observed that this specific sample over expressed *VTG*, a female specific glycoprotein identified as being necessary for building up the oocyte in oysters [5], among other species. Oysters, at an undetermined sex stage, were found to express male- or female-specific genes, suggesting that sex differentiation already took place at the first stage of gametogen-



**Figure 5. Heat map of population specific genes.** Hierarchical clustering obtained using Pearson’s correlation considering the set of 2,900 differentially expressed probes between the two studied populations of *Ruditapes decussatus*. doi:10.1371/journal.pone.0092202.g005

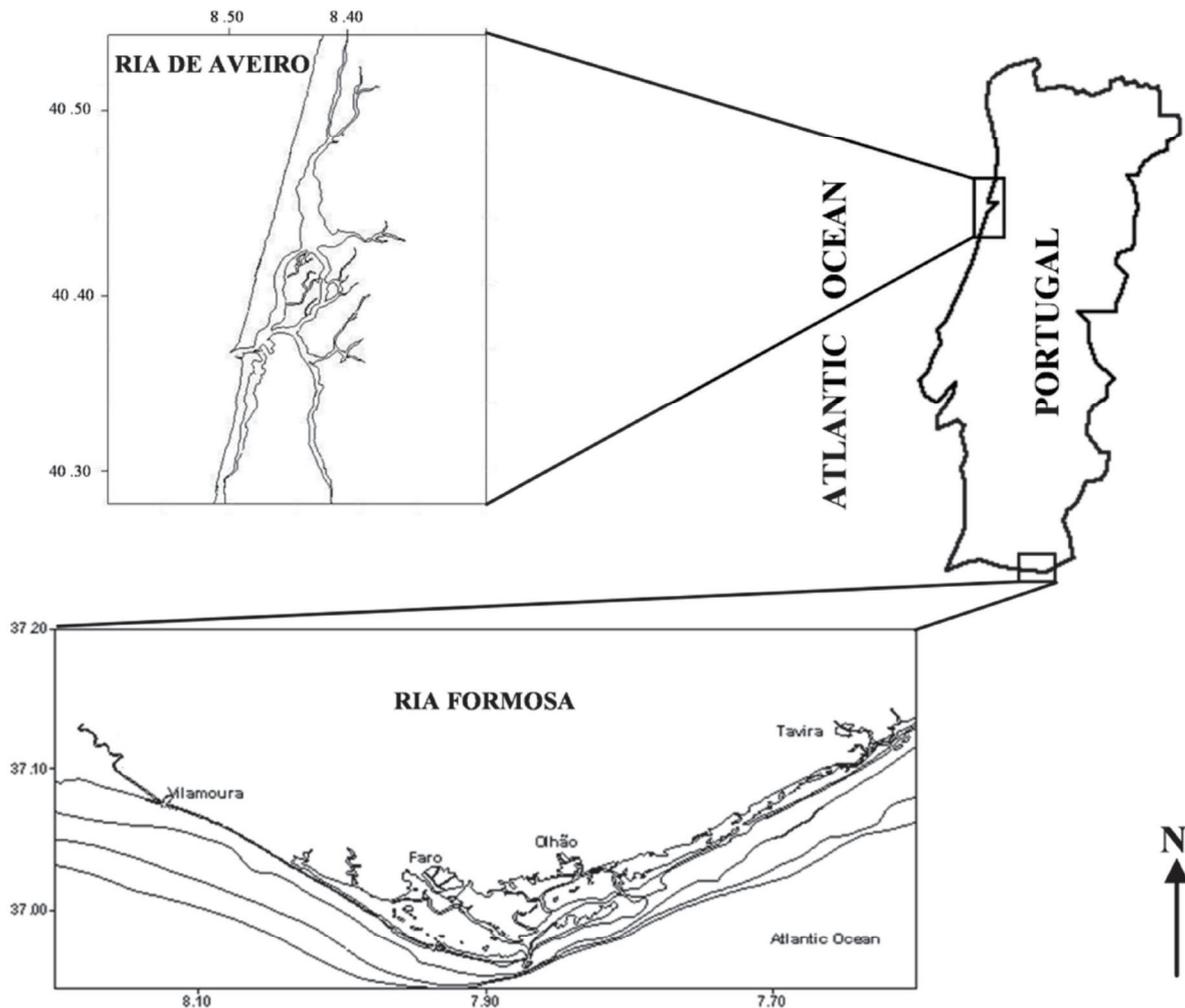
esis, even if it could not be visualized histologically [5]. As a result, in some cases of individuals in later stage I, gene expression patterns could indicate sex determination without histological information.

Following the strong sex effect on gene expression reported above, with 16,996 probes differentially expressed between sexes, 9 KEGG pathways were revealed. Among these pathways, “oocyte meiosis” (dre04114) and “Progesterone-mediated oocyte maturation” (dre04914) were the most represented with 45 and 38 genes, respectively, as well as “Cell cycle” (dre04110) with 49 genes. These pathways are characteristic of females, certainly related to the higher transcription rate in female germ cells compared to male germ cells. Indeed, oocytes provide most of the information for the development of zygotes [20]. Among the genes involved, we found the *Mad21l*, a component of the spindle-assembly checkpoint that prevents the onset of anaphase until all chromosomes are properly aligned at the metaphase plate, and the *CaM* family, which belongs to one of the two main groups of calcium-binding proteins. Calmodulins are supposed to be very important in the oogenesis of *R. decussatus*, since  $Ca^{2+}$  increases are recognized as essential for the oocytes to be released from the metaphase I arrest [21][22][23][24]. The oocytes of the European clam (classified as class II, due to their meiotic maturation distinct from other bivalve species) are held in the ovaries at prophase of the first division of meiosis and their development reinitiates during spawning, by hormonal stimulation or other stimuli. A second barrier to development occurs at metaphase I, prior to extrusion of the first polar body, until fertilization releases this metaphase arrest, allowing further stages of maturation to take place [25]. Furthermore, cyclins, found differentially expressed (*CCNB1*, *CCNB2* and *CDK2*), also trigger metaphase/anaphase

transition and further progress through meiosis by disappearing, due to protein synthesis inhibition [25]. In order to better understand the differences between sexes in each reproductive stage, the same analysis was performed in each one. The number of differentially expressed probes increased as the reproductive stages developed, with stage IV presenting the highest number of differentially expressed probes, confirming the results from PCA analysis. Speriolin, a novel centrosomal protein present in the connecting piece region of mouse and human sperm [26], was present in the list of differentially expressed probes between males and females at stage IV, with a FC value of 332, as well as *TSSK-5* with a FC value of 249. mRNA expression of these genes is highly testis-specific, with a very low level detected in other tissues of the marine bivalve *Argopecten purpuratus* [7].

**Differentiation of gonadal developmental stages**

From the 13,218 probes that exhibited a significant change in expression over the reproductive cycle of male and female gonads, 9 KEGG pathways appeared notably represented. “Focal adhesion” (dre04510) and “Regulation of actin cytoskeleton” (dre04810), were the most represented, with 74 and 62 genes, respectively. In sea star oocytes, the cortical actin cytoskeleton was noticed to play critical roles in modulating  $Ca^{2+}$  release during meiotic maturation, as well as in regulating cortical granule exocytosis [27]. Additionally, “microtubule-based process” (GO:0007017), one of the enriched biological processes with the highest representation, participates in several important events in spermatogenesis, including nuclear elongation, cytoplasmic redistribution and reduction and development of the flagellum [28]. Within the genes involved in this pathway, we found the dynein light chain 2 (*dynll2a* and *dynll2b*), several kinesin family members



**Figure 6. Ria de Aveiro and Ria Formosa Lagoon location.** Ria Formosa (37°01'N; 07°49'W) in Southern Portugal and Ria Aveiro (40°42'N; 08°40'W) in Western coast of Portugal. doi:10.1371/journal.pone.0092202.g006

(*KIF11*, *KIF17*, and *KIF7*). These genes were also found highly expressed in male gonads in *C. gigas* and may be involved in flagella and cilia structure, locomotion and control [5].

*VTG*, a female specific glycoprotein previously described and *Condensin-2*, expressed in oocytes and involved in maintaining the rigidity of chromosomes in prophase were identified in our female samples with increase in expression as oogenesis progressed. In terms of male specific genes, we identified the *MOSPD2* and the *SCP-2*, involved in the meiotic phase of spermatogenesis, also with an increase in expression during progression of spermatogenesis. All these genes also increased in expression along the gametogenic cycle in male and female gonads of *C. gigas* [5]. *scp2* and *scp3*, genes involved in synaptonemal complex formation, were recently found to be highly expressed in the maturing testis of the scallop *Nodiplecten subnodosus* [6]. This synaptonemal complex is a meiosis-specific structure essential for chromosome pairing since disruption of the localization of *scp2* and *scp3* during early recombination resulting in aneuploidy [29]. Our results indicate that synaptonemal

mal complex formation, and therefore meiosis I, was occurring from early to late testis maturation. This results is similar to what was found in *N. subnodosus*, for which the authors concluded that the expression pattern of *scp2* and *scp3* might serve as future markers for the meiotic stage and may prove helpful in differentiating primary and secondary spermatocytes, which is not morphologically possible [6].

A quantitative correlation between microarray data and values of gonad area considered as a proxy of the reproductive effort (e.g. in oyster [30]), were evaluated separately for females and males. The clam gonad undergoes continuous development until it becomes fully mature. At that moment, the gonads or gonadal tissue form a significant portion of the soft parts of the animal. Gonads that will carry the gametes to the body chamber develop, enlarge and are readily observed in the gonad. For this reason, in males as in females, the gonadal area increases with the evolution of the reproductive stages and is considered as a proxy of the reproductive effort [31]. Reproductive effort and the

underlying mechanisms are of great interest for many marine bivalve species that have a very high fecundity, a characteristic of the “r” demographic strategy. Indeed, gametogenesis has a major impact on several physiological functions, generating phenotypic and genetic trade-offs with growth and survival (as established in oysters [32]), meaning that reproductive effort improves at the expense of survival. We identified 7,226 annotated probes correlated with female gonad area and therefore, potentially, with the number and/or the size of the gametes produced. These probes included *Foxl2*, an evolutionarily conserved female-specific transcription factor, which is conserved from the sponge *Suberites domuncula* to humans [33]; *VTC*; *condensing 2*, expressed in oocytes and involved in maintaining the rigidity of chromosomes in prophase; Mitotic apparatus protein *p62* that binds to condensed chromosomes at prophase of meiosis I; and *Cep57*, a centrosomal protein required for microtubule attachment to centrosomes. These female-specific genes, which were positively correlated with gonad areas, were also identified as potentially involved in oogenesis in *C. gigas* [5]. In males, genes expressed included the *SPAG6*, *SPAG16* and *SPAG17*, *MOSPD2*, sperm surface protein *Sp17*, sperm flagellar proteins 1 and 2 and *dpy-30*, all involved in spermatogenesis. *dpy-30* is involved in male development and described as an attractive new candidate gene for the regulation of sex differentiation in oysters [5]. This gene was also identified and characterized as an essential component of the dosage compensation machinery for sex determination in *Caenorhabditis elegans* [34].

#### Differential gene expression between populations

In *R. decussatus*, as in other bivalves, the period of spawning in natural populations differs with geographic location and, once reproductive maturity is reached, it may be triggered by several environmental factors including temperature, chemical and physical stimuli, water currents or a combination of these and other factors [14]. The presence of gametes in the water provides an additional stimulus that generates a spawning response in broodstock, due to the presence of hormones (pheromones) that will act as recognition signals between gametes [35]. Indeed, a surface glycoprotein was identified as the mate recognition signal in the caridean shrimp *Palaemonetes pugio* where glucosamine was considered a pheromone functioning in mate recognition [36]. Although it had a low statistical significance value for enrichment in DAVID ( $P = 0.09$ ), “N-Glycan biosynthesis” (dre00510) was found among the enriched KEGG pathways with the highest number of gene entries. N-glycans have an important role acting as recognition signals and binding between the extracellular coat of the egg and components of the sperm plasma membrane, generally termed “primary binding” [37]. Taking into account that “N-Glycan biosynthesis” (dre00510) pathway was down-regulated in the population of Ria Formosa, we propose that, in some way, this recognition process can explain some of the differences in spawning induction success observed between the two Portuguese populations. Indeed, it was previously demonstrated that environment can strongly affect reproduction of *R. decussatus*, notably in terms of fecundity level [14]. Additionally, among the genes involved in N-Glycan biosynthesis, we found fucosyltransferase (*FucT*) differentially expressed with a fold change of 2.23. This enzyme is implicated in species-specific binding of sperm to eggs in mammals [38][39][40]. In addition, ADAMs family genes (A Disintegrin and A Metalloprotease domain) as well as several integrin ligands appeared differentially expressed between the two populations. Several studies have indicated that these molecules are involved in cell adhesion and sperm-egg interaction (e.g. [41][42][43]), supporting the hypothesis that

“primary binding” could be responsible for the main differences in the reproductive behavior of the two populations.

Recent histological examinations of these two populations showed significant differences in the dynamics of gametogenesis [14]: the maturation process begins earlier in the northern population probably due to contrasted environmental factors mainly temperature, which is already characterized as the main source of variation in the timing of gametogenesis (e.g. [18]). Sea surface temperature (SST) was relatively lower compared to Ria Formosa lagoon, though with higher chlorophyll values [14]. Nonetheless, similar values of oocyte diameter and gonadic area were reported, suggesting similar investment in reproduction in the two populations. In terms of gene expression, hierarchical clustering using Pearson’s correlation, identified three main clusters: Ria Formosa, Ria de Aveiro and Female Stage III and IV from Ria de Aveiro (Figure 5), suggesting that the biggest differences originate from the females in the later stages of gametogenesis, and may be due to a difference in gamete maturity that may be implicated in the differences in response to spawning induction. These differences can originate from environmental differences and/or some genetically based variation between the two populations. Among all the comparisons made between populations for each reproductive stage and sex, the one performed with stage IV females presented the highest number of differentially expressed probes (5,133 probes; Table S13). In this comparison, “Cell cycle” (dre04110), “Spliceosome” (dre03040), “Ubiquitin mediated proteolysis” (dre04120) and “Oocyte meiosis” (dre04114) terms were the most represented in the enriched KEGG pathway terms with 45, 32, 30 and 28 genes, respectively. Due to their specific environments, these populations might adopt different reproductive strategies: the Ria de Aveiro population retrieves its energy reserves immediately after spawning, however, the same is not verified in clams from Ria Formosa Lagoon, with their consequent decline. Considering these facts, it is likely that, at some point, the process of oogenesis diverged between females of the two populations. Among the genes differentially expressed, the *SKP1* and the *Mad21* previously described, were present. The *SKP1* is an essential component of the SCF (SKP1-CUL1-F-box protein) ubiquitin ligase complex, which mediates the ubiquitination of proteins involved in cell cycle progression, signal transduction and transcription. Other important differentially expressed genes were found, such as *cdc16*, *cdc20*, *cdc27*, which are components of the anaphase promoting complex/cyclosome (APC/C), a cell cycle-regulated E3 ubiquitin ligase controlling progression through mitosis and the G1 phase of the cell cycle. In addition, genes like *CCNB1* and *CCNE2* were present and are essential in controlling the progression of cells through the cell cycle, by activating *Cdk* enzymes. Cyclin-encoding transcripts represent maternal mRNAs, known to be stocked in oocytes during oogenesis and maternally transmitted to embryos before the start of embryonic transcription [44].

A total of 3,588 differentially expressed probes were also obtained in the comparison made between males of stage II. “Insulin signaling pathway” (dre04910) was the most represented in the enriched KEGG pathway terms. In addition to its function in somatic cells, it was demonstrated that insulin signaling plays an essential role in cell proliferation and growth during male *Drosophila* gametogenesis where sperm production is regulated by hormonal control via insulin-like peptides [45]. Moreover, the level of some mRNA in insulin pathway was also identified as correlated with gamete quality in fishes [46][47]. Additionally, the *MAPK1* was found differentially expressed. Studies have shown that male reproductive function is modulated via the *MAPK* cascade. The *MAPK* cascade is involved in numerous male

reproductive processes, including spermatogenesis, sperm maturation and activation, capacitation and acrosome reaction, before fertilization of the oocyte [48]. Considering that no additional significant results were observed for the remaining comparisons performed, we suggest that it is in the later stages of oogenesis (stage IV) and in the earlier stages of spermatogenesis (stage II), that the major differences between the two studied populations occur.

## Conclusions

In this study, we identified several differentially expressed probes, which led us to a specific pathway involved in the recognition signals and binding between the oocyte and components of the sperm plasma membrane. We believe that these results can be useful to *R. decussatus* hatchery production program management. Furthermore, as a possible starting point for further research devoted to reproduction and spawning improvement of this species, we identified genes specifically expressed in either males or females that showed increasing expression over the course of gametogenesis.

The designed custom oligonucleotide microarray can be useful for future studies into the molecular mechanisms involved in the European clam differentiation and development. Additionally, we produced lists of relevant pathways and candidate genes that helped to improve our understanding of gametogenesis and that are essential in addressing physiological, environmental or aquaculture questions concerning the European clam *R. decussatus*.

## Supporting Information

**Table S1 Summary of the origin of *Ruditapes decussatus* sequences and assembly information.**  
(XLSX)

**Table S2 List of protein database used for contigs annotation obtained through Blast similarity searches.** Numbers of annotated contigs for each database are also reported.  
(XLSX)

**Table S3 Sequences of the 51,709 different contigs of *R. decussatus* representing in the DNA microarray platform.**  
(ZIP)

**Table S4 List and annotation of each probe represented in the *R. decussatus* DNA microarray platform.**  
(XLSX)

**Table S5 List of significant probes identified by SAM analysis (FDR = 5%; FC > 1.5) by comparing sexes in *Ruditapes decussatus*.**  
(XLSX)

**Table S6 Enrichment analysis showing KEGG pathways, BP terms, CC terms and MF terms significantly represented on differentially expressed probes between sexes in *Ruditapes decussatus*.**  
(XLSX)

**Table S7 List of significant probes identified by One-way ANOVA ( $p < 0.01$ , adjusted Benjamini & Hochberg correction) in order to identify the transcripts whose expression changed over the reproductive cycle of male and female gonads.**  
(XLSX)

**Table S8 Enrichment analysis showing KEGG pathways, BP terms, CC terms and MF terms significantly represented on differentially expressed probes between gametogenic stages in *Ruditapes decussatus*.**  
(XLSX)

**Table S9 List of significant probes identified by SAM quantitative correlation analysis (FDR = 5%) between microarray data and values of female gonad area of stages II, III and IV after angular transformation in *Ruditapes decussatus*.**  
(XLSX)

**Table S10 List of significant probes identified by SAM quantitative correlation analysis (FDR = 5%) between microarray data and values of male gonad area of stages II, III and IV after angular transformation in *Ruditapes decussatus*.**  
(XLSX)

**Table S11 List of significant probes identified by SAM analysis (FDR = 5%; FC > 1.5) by comparing the two populations of *Ruditapes decussatus*.**  
(XLSX)

**Table S12 Enrichment analysis showing KEGG pathways, BP terms, CC terms and MF terms significantly represented on differentially expressed probes between populations of *Ruditapes decussatus*.**  
(XLSX)

**Table S13 List of significant probes identified by SAM analysis (FDR = 5%; FC > 1.5) by comparing stage IV females from both populations of *Ruditapes decussatus*.**  
(XLSX)

**Table S14 Enrichment analysis showing KEGG pathways, BP terms, CC terms and MF terms significantly represented on differentially expressed probes between stage IV females from both populations of *Ruditapes decussatus*.**  
(XLSX)

**Table S15 List of significant probes identified by SAM analysis (FDR = 5%; FC > 1.5) by comparing stage II males from both populations of *Ruditapes decussatus*.**  
(XLSX)

**Table S16 Enrichment analysis showing KEGG pathways, BP terms, CC terms and MF terms significantly represented on differentially expressed probes between stage II males from both populations of *Ruditapes decussatus*.**  
(XLSX)

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## Author Contributions

Conceived and designed the experiments: JTS MM LB AL AH. Performed the experiments: JTS MM DM SJ AMM VQ AH. Analyzed the data: JTS MM LB MP DM SJ AL AH. Contributed reagents/materials/analysis tools: MM LB AL DM SJ AH. Wrote the paper: JTS.

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## Appendix 3

### “Stripped and spawned oocytes in the European clam *Venerupis decussata*: a microarray-based study”

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*Unpublished data*

## Background

The clam *Venerupis decussata* is a native European bivalve species and, even if its global aquaculture production is still relatively low in Europe (4.137 tons in 2011; FAO), it has a high economic value. *V. decussata* production is economically important in many Mediterranean countries, mainly Portugal, Italy and Spain but, due to the difficulties in broodstock conditioning and larval rearing (Hamida et al. 2004), the culture of this species relies mainly on natural recruitment of seed and it is therefore limited by its availability. Among the major hurdles reported in hatchery production of this species, spawning control and gamete quality are the most important issues. Notably, spawning success in the European clam is not predictable, with frequent failures to induce spawning. Furthermore, variability of egg quality, defined as the potential of oocytes to produce a viable progeny (Kjorsvik et al., 1990) is still too variable to ensure a reliable source of seed. A further factor that strongly limits the development of *V. decussata* hatcheries is the impossibility to obtain fertile gametes by gonadal stripping. This practice is widely used to collect oocytes, before natural egg emission, in some bivalve species (e. g. in oyster), whose eggs can be fertilized. Meiotic progression in germ cells is not regulated in the same manner

across molluscan species. While full-grown oocytes of all bivalves are blocked in ovaries at prophase I stage, some crucial differences are observed in spawned eggs. In bivalves such as *Spisula* or *Barnea* spawned oocytes are arrested at prophase I and fertilization occurs at this stage leading to meiosis reinitiation (Dubé and Guerrier, 1982; Longo, 1983; Colas and Dubé, 1998). In contrast, bivalves such as *Venerupis* and *Crassostrea* (Osanai and Kuraishi, 1988) exit from prophase I and undergo germinal vesicle breakdown (GVBD) after spawning and then are further blocked at the first metaphase (metaphase I). The release from metaphase I is naturally triggered by fertilization or can be artificially induced, however in both cases, it seems that an increase in intracellular  $[Ca^{2+}]$  has a pivotal role in meiosis reinitiation (Abdelmajid et al., 1993; Guerrier et al. 1993; Moreau et al., 1996). Although both *Venerupis* and *Crassostrea* oocytes encounter two blockages during meiosis I, their meiotic progression is not regulated in the same way. Naturally spawned oyster oocytes, like in *Venerupis*, are blocked at metaphase I and wait for fertilization to reenter meiosis. However, isolated oocytes from ovaries (stripped) are still at prophase I, yet they can be fertilized at this stage (Osanai, 1985). On the opposite, *Venerupis* oocytes cannot be fertilized at prophase I for reasons that remain unknown. To date, the mechanisms controlling oocytes maturation in *V. decussata* has been scarcely studied (Hamida et al., 2004). Conversely, in other bivalves meiosis in female gametes was extensively analyzed and a few major factors regulating oocyte maturation processes were identified. Notably, it was demonstrated that serotonin (5-HT), thought to be the natural inducer of oocyte maturation in bivalves (Deguchi and Osanai, 1995), triggers in vitro the germinal vesicle breakdown (GVBD) when added to *Spisula*, *Barnea*, *Venerupis philippinarum* or *Crassostrea* isolated prophase I oocytes (Osanai, 1985; Osanai and Kuraishi, 1988; Hirai et al., 1988; Brassart et al., 1988; Dubé, 1988; Krantic et al., 1991; Guerrier et al., 1993). Moreover, it has been suggested that in *V. philippinarum*, the mechanisms by which 5-HT promotes GVBD involve an increase in intracellular  $[Ca^{2+}]$ , which is thought to be mediated in turn by inositol 1,4,5-trisphosphate receptors (IP3r) and specific 5-HT receptors (Guerrier et al., 1996; Gobet et al., 1994; Guerrier et al., 1993). Despite these studies pointed out a few interesting factors driving meiosis progression in bivalves, little is known on cell signaling during gamete maturation in these species. Up to date, only few gene expression and proteomic studies have been carried out in bivalve oocytes (e.g. Ni et al, 2012; Corporeau et al.

2012) and a comprehensive picture of the molecular processes characterizing their maturation is still lacking. Remarkably, when talking about gene expression and mRNA in oocytes, an interesting point should be taken into account: at prophase I immature oocytes show a prominent nucleus (the germinal vesicle), which contains de-condensed chromatin (Tosti, 2006), thus oocytes at this stage are transcriptionally active until meiosis resumption, when transcription is generally thought to cease (Heikinheimo and Gibbons, 1998). However, translation of the stored mRNAs pool continues throughout the final stages of meiosis (Wassarman, 1996) to synthesize proteins that are crucial for supporting not only the oocyte maturation (meiotic maturation) but also the phase prior to zygote-embryonic genome activation (Song and Wessel, 2005, Dheilly et al., 2012; Eichenlaub-Ritter and Peschke, 2002). Beside gamete maturation, several factors are still limiting hatchery production of *V. decussata*. The absence of established methods for larval rearing, a scarce knowledge on the best hatching practices to improve metamorphosis synchronization and settlement are only a few additional issues about European clam seed production, which is an activity far from being completely technically controlled. In this context, the improvement of knowledge on broodstock management and gamete maturation processes seems to be a key step to improve seed production of this emerging bivalve species.

In the present study, combined Next Generation Sequencing (NGS) technologies were employed in order to improve transcriptomic information on *V. decussata*, thus providing a great number of transcripts for further “omic” studies in this species. A microarray platform (8x60,000) for gene expression analysis was then constructed and a dataset of 15 oocytes samples were analyzed. Expression profiles characterizing stripped oocytes and spawned oocytes with different competence levels were evaluated in order to shed light on the molecular processes leading to female gametes maturation and competence.

## Methods

### ***Venerupis decussata* transcriptome: biological samples, assembly and annotation**

Clam tissues, larvae, oocytes and hemocytes were collected in collaboration with the Instituto Português do Mar e da Atmosfera (IPMA) and the Instituto de Investigaciones Marinas (IIM). All samples were stored in RNAlater at -80°C until RNA purification. RNA was isolated with RNeasy Mini Kit (Qiagen), following the manufacturer instructions and a DNase treatment (Qiagen) was carried out. Concentration and purity of RNAs were measured using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). The RNA quality was assessed through the Bioanalyzer 2100 instrument (Agilent).

For the RNA sequencing experiments, 13 non normalized libraries (oocytes, larval stages and gonadal stages) were prepared by using Truseq RNA sample prep Kit (Illumina) following the manufacturer's instruction and sequencing was carried out with Illumina Hi-Seq 2000 by running two multiplexed lanes 2x100bp paired ends (BGI Tech, Shenzhen, China). All the Illumina reads were analyzed with FastQC software in order to assess the sequences quality.

Two additional DSN normalized 454 libraries were synthesized, one starting from a pool of oocytes/larval stages/adults tissues/hemocytes and a second one by pooling different hemocytes sampled at different conditions. The two libraries were sequenced with a Roche 454 GS FLX sequencer using the Titanium chemistry (Genomic unit, CCI-T-UB, Barcelona, Spain) and a sequences quality report was obtained through the 454 Software Release 2.6. In order to build a transcriptome scaffold, a mixed strategy was preferred. Different assembler softwares were employed according to the sequences' origin (454 or Illumina). The 454-sequences were assembled through MIRA3 (default parameters), the reads from each of the Illumina libraries were separately assembled by using CLC Genomic Workbench 5 (default parameters). Finally, to reduce the redundancy, all the obtained contigs plus a set of additional sequences already published in public repositories were merged through CAP3 (default parameters). The contigs not merged in CAP3 were joined to all the meta-contigs, thus obtaining a final transcriptome scaffold.

A functional annotation of the assembled transcriptome was attained through

blastx similarity searches conducted against UniProtKB/SwissProt database and several protein databases available on Ensembl Genome Browser (release 68): *Homo sapiens*, *Danio rerio*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Ciona intestinalis*, *Strongylocentrus purpuratus*, *Gasterosteus aculeatus*, *Daphnia pulex* and *Nematostella vectensis*. In addition, to improve the number of annotated contigs, blastx searches against two molluscs databases were attempted: oysterDB (*Crassostrea gigas*) and *Lottia gigantea* V1.0. Alignments with an E-value of at most  $1 \times 10^{-5}$  were considered significant and only the best hit per each contig was taken into account. Finally, Blast2GO software (Conesa et al., 2005) was used to assign Gene Ontology terms (Ashburner et al., 2000) to all annotated contigs. Default values in Blast2GO were used to perform the analysis and ontology level 2 was selected to construct the level pie chart. To avoid redundant results, when multiple contigs were annotated with the same UniProtKB/SwissProt accession, only the longest one was selected for the Gene Ontology analysis.

### Microarray platform design

All databases used for the annotation step were considered to reduce the redundancy in annotated contigs. In total 44,333 contigs, found non-redundant in at least one reference database, have been considered for microarray design. Of these, 915 contigs showed ambiguous orientation considering the homologs gene in reference databases and for each of them, two probes with opposite orientations (sense and antisense) were designed. For the remaining 43,418 contigs with known orientation, one probe was designed. To fill the microarray platform, non-annotated gonads contigs were employed. Basing on the sum of gonads stage reads mapped against the assembled reference transcriptome the 7,376 most highly expressed contigs were considered and for each of them, two probes with opposite orientations (sense and antisense) were designed. Probe design was carried out using the Agilent eArray interface (<https://earray.chem.agilent.com/earray/>), which applies proprietary prediction algorithms to design 60 mer oligoprobes. A total of 59,951 out of 60,000 probes were successfully obtained, representing 51,709 putative *V. decussata* contigs. The percentage of annotated transcripts represented in the microarray was 85.7%. Probe sequences and other details on the microarray

platform can be found in the GEO database (<http://www.ncbi.nlm.nih.gov/geo/>) under accession number GPL17766.

### **Biological samples for the microarray analyses**

Clams were sampled in Ria de Aveiro (Western coast of Portugal) and conditioned in common garden from May 2013 to June 2013 (one month) in the experimental bivalve hatchery of the National Institute of Biological Resources (IPMA) in Tavira, Portugal, to accelerate their gonad development under common rearing facilities. Food regimes consisted of different algal mixtures containing 1/3 *Isochrysis galbana* (clone T-ISO), 1/3 *Skeletonema costatum* (Ria Formosa autochthones clone) and 1/3 *Chaetoceros calcitrans*. Released oocytes from several females were obtained by a thermal stimulation consisting on the exposition to alternate cycles of 29°C (1 hour) and 5°C (30 minutes) (Joaquim et al., 2008). As each female begun to spawn it was removed from the spawning tank and transferred to an individual spawning beaker with filtered seawater at the same temperature (Joaquim et al., 2008). Once the spawning was completed, the obtained oocytes were gently washed into a clean glass. The rest of each female spawning was mixed with a sperm suspension (from 7 males) during gentle agitation, aiming to obtain around 10 spermatozooids by oocyte in a microscopic view (Cesari and Pellizzato, 1990; Joaquim et al., 2008). Moreover, to evaluate the quality of the collected oocytes, the D-larval rate (ratio between the number of free swimming larvae at 48h post fertilization and the number of starting eggs) of each eggs batch was registered. In addition, gonads from five females were dissected and oocytes were collected through a practice known as “gametes stripping”. As the name indicates, this procedure involves removal of gamete from gonad tissue. Briefly, fully ripe gonads were slashed repeatedly with a scalpel and washed with filtered seawater to harvest the gametes. Finally, sex determination and oocytes appearance were achieved through microscopy examination. About 20,000 oocytes for each spawning/stripping were collected and filtered in a 40 µm sieve. The oocytes were transferred into an eppendorf tube and, after a short spin, the seawater was removed. To remove the salts, the pellet of oocytes was re-suspended with a solution of ammonium formate 3% which was immediately removed after a short spin. Then the oocytes were included in 1,5 ml of Extract all solution (Eurobio) and preserved in liquid nitrogen

until the RNA isolation. RNA was purified by following the manufacturer instructions and a DNase treatment was carried out through RTS DNase Kit (MO-BIO). Samples concentration was measured in a NanoDrop® ND-1000 spectrophotometer and the RNA quality was assessed through the Bioanalyzer 2100 instrument (Agilent).

### **Labeling and microarray hybridization**

Sample labeling and hybridization were performed according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol with the Low Input Quick Amp Labeling kit. Briefly, for each sample 100 ng of total RNA were linearly amplified and labeled with Cy3-dCTP. A mixture of 10 different viral poly-adenylated RNAs (Agilent Spike-In Mix) was added to each RNA sample before amplification and labeling, to monitor microarray analysis workflow. Labeled cRNA was purified with the RNeasy Mini Kit (Qiagen) and samples concentration and specific activity (pmol Cy3/ $\mu$ g cRNA) were measured in a NanoDrop® ND-1000 spectrophotometer. A total of 600 ng of labeled cRNA was prepared for fragmentation adding 5  $\mu$ l 10X Blocking Agent and 1  $\mu$ l of 25X Fragmentation Buffer, heated at 60°C for 30 min, and finally diluted by addition with 25  $\mu$ l 2X GE Hybridization buffer. A volume of 40  $\mu$ l of hybridization solution was then dispensed in the gasket slide and assembled to the microarray slide (each slide containing eight arrays). Slides were incubated for 17 h at 65°C in an Agilent hybridization oven, subsequently removed from the hybridization chamber, quickly submerged in GE Wash Buffer 1 to disassembly the slides and then washed in GE Wash Buffer 1 for approximately 1 minute followed by one additional wash in pre-warmed (37°C) GE Wash Buffer 2.

### **Data acquisition, correction and normalization**

Hybridized slides were scanned at 2  $\mu$ m resolution using an Agilent G2565BA DNA microarray scanner. Each slide was scanned two times at two different sensitivity levels: XDR Hi 100% and XDR Lo 10%. The two generated images were analyzed together, data were extracted and background subtracted using the standard procedures provided in the Agilent Feature Extraction (FE) Software version 10.7.3.1. To evaluate goodness and reliability of spot intensity estimates the software returns a series of spot quality measures. All control features (positive, negative,

etc.), except for Spike-in (Spike-in Viral RNAs), were excluded from subsequent analyses. The fluorescence values were normalized by performing quantile normalization in R statistical software. To improve the reliability of the analysis, only the probes for which the reported fluorescence intensity was greater than 10 in at least 5 samples were taken into consideration. A log base 2 transformation was applied to all the expression values and finally, the parametric Combat algorithm was implemented in R in order to adjust for the known between-experiments batch effects (Johnson et al., 2007). A total of 31,862 probes were used in all the subsequent analyses.

## Data analysis

The analysis was carried out by dividing the oocytes samples in three experimental groups, each characterized by a different quality in terms of developmental competency. The three groups were the following: unfertile stripped oocytes, spawned oocytes with low hatching rate (LHR, 5%-21% of D-larval rate), spawned oocytes with medium hatching rate (MHR, 40%-47% of D-larval rate). Samples used in the analyses and the relative D-larval rates are reported in Table 1. A principal component analysis (PCA), using the TMeV 4.5.1 (TIGR MULTIEXPERIMENT VIEWER) (Saeed et al., 2003) was applied, to assess the distribution of the studied groups. Hierarchical clustering was performed using TMeV on the whole dataset, to group experimental samples based on similarity of the overall expression profiling. Statistical tests implemented in the program Significance Analysis of Microarray (SAM) were used to identify differentially expressed probes between the stripped oocytes and the two groups of spawned oocytes with different hatching rates. A one-way ANOVA parametric test was used to identify the probes whose expression changed between the three tested groups, using a p-value cut-off of 0.05 and a minimum fold change (FC) of 1.5. A more systematic, functional interpretation for significant gene was then obtained using an enrichment analysis from the Database for Annotation, Visualization, and Integrated Discovery (DAVID) software (Huang et al., 2009). “KEGG Pathway”, “Biological process”, “Molecular function” and “Cellular component” annotation were carried out by setting the gene count equal to 3 and the ease equal to 0.1. Because DAVID database contains functional annotation data for a limited number of species, it was necessary to link

the *V. decussata* transcripts with sequence identifiers that could be recognized in DAVID. This process was accomplished using UniProtKB/SwissProt feature identifiers corresponding to each probe. These identifiers were used to define a “gene list” and a “background” in the bioinformatic tool DAVID, corresponding to differentially transcribed clam genes and to all the transcripts that were represented on the array, respectively.

Table 1. Microarray samples dataset. The oocytes samples used for the microarray analysis were divided in three experimental groups: stripped oocytes, low hatching rate oocytes and medium hatching rate oocytes. The D-larval rates obtained after the fertilization of each spawning were reported in round brackets.

Stripped oocytes	Low hatching rate oocytes (LHR)	Medium hatching rate oocytes (MHR)
P1	X14 (12%)	X3 (44%)
P2	X30 (21%)	X5 (41%)
P3	X37 (14%)	X9 (40%)
P4	X44 (11%)	X27 (47%)
P5	X59 (5%)	X32 (46%)

## Results

### Sequencing results

NGS technologies, RNA sequencing and Roche-454, were applied to sequence several cDNA libraries originating from oocytes, hemocytes, larval stages and adult tissues of *V. decussata*. Sequences across the entire length of the mRNA transcripts expressed in the target tissues were attained and two different assembly algorithms were used to obtain a whole scaffold transcriptome. In order to eliminate the redundant sequences, all the contigs produced by CLC bio (850,812) and MIRA3 (98,180) were submitted to the CAP3 software, obtaining a final scaffold of 503,705 sequences. Sequencing results and the de novo assembly’s statistics are summarized in Table 2, while the size distribution of the transcripts after contigs construction is resumed in Figure 1.

Table 2. Statistics. Number of reads obtained from the Illumina (A) and 454 (B) sequencing. Total number of contigs inputted to CAP3 software and statistics of the final scaffold (C).

<b>A. ILLUMINA Sequencing</b>	
Sample	<b>Total reads</b>
<b>Oocytes (stripped)</b>	65 981 907
<b>Oocytes (released)</b>	32 722 892
<b>D-larvae (48 hpf)</b>	36 465 121
<b>Larvae ready to settle (17 dpf)</b>	51 967 552
<b>Larvae settled (21 dpf)</b>	47 752 218
<b>1 month old seed (30 dpf)</b>	45 800 062
<b>Gonads Stage I</b>	41 797 128
<b>Gonads Stage II ♀</b>	49 187 996
<b>Gonads Stage II ♂</b>	47 737 182
<b>Gonads Stage III ♀</b>	44 891 188
<b>Gonads Stage III ♂</b>	52 204 184
<b>Gonads Stage IV ♀</b>	54 690 184
<b>Gonads Stage IV ♂</b>	52 009 646
<b>B. 454 - Sequencing</b>	
Sample	<b>Total reads</b>
<b>Hemocytes naive and in vivo/vitro stimulated</b>	457 559
<b>Larval stages/Oocytes/Hemocytes/Adult tissues</b>	471 192
<b>C. Assembly's statistics</b>	
<b>CAP3 inputated contigs</b>	990 111
<b>CAP3 metacontigs</b>	69740
<b>CAP3 singletons</b>	433 965
<b>complete scaffold</b>	503 705
<b>total nt</b>	408 246 936
<b>mean lenght</b>	810
<b>max lenght</b>	29 774
<b>min lenght</b>	40

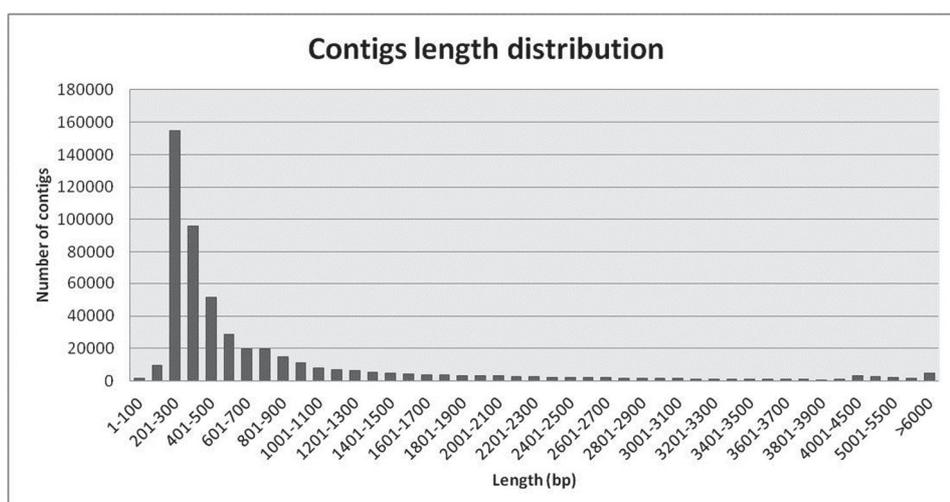


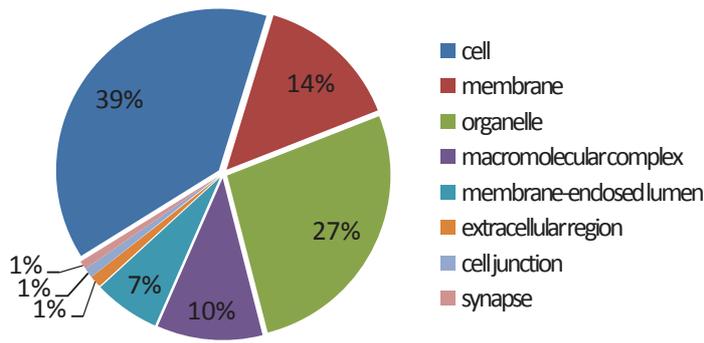
Figure 1. Contigs length distribution. Histogram reporting the number of contigs (y axis) with a specific length range (x axis)

## Whole transcriptome analysis

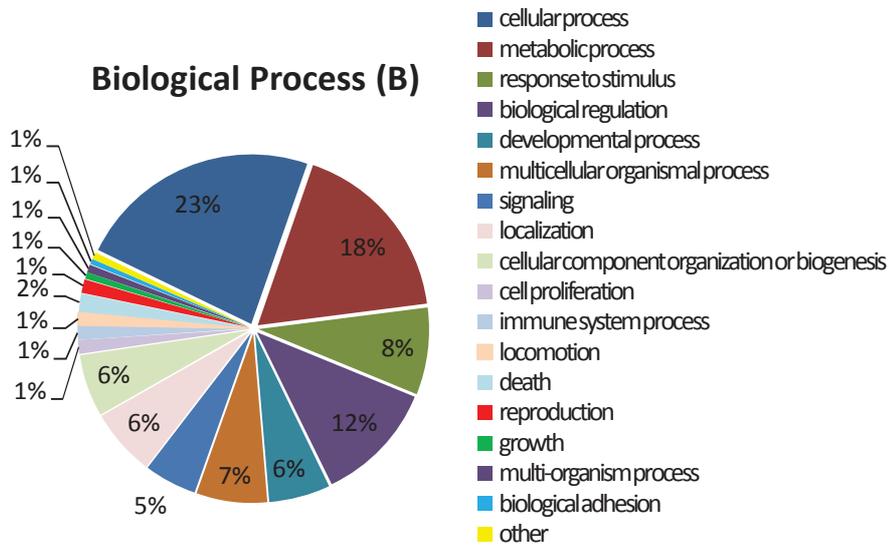
Putative identities of assembled contigs and meta-contigs were obtained by running Blastx similarity searches on several protein databases (see methods). Giving the lack of a reference clam genome as well as the presence of specific sequences features such as high density repetitive regions and increased levels of polymorphism, this approach provided a significant match for 141,728 contigs, leading to a 27% of annotated transcripts.

In order to characterize the annotated transcripts in the de novo assembly, Gene ontology (GO) terms were extracted. Cellular Component (GO CC), Molecular Function (GO MF) and Biological Process (GO BP) terms at ontology level 2 are summarized in pie charts (Figure 2). The GO analysis showed that 39% of the GO CC terms were included in the “cell” class and 27% in the “organelle” class. Concerning the GO BP, “cellular process” (23%), “metabolic process” (18%) and “biological regulation” (12%), were the most represented classes. Also the terms “biological regulation”, “response to stimulus” and “multicellular organismal process” were reported. Finally, within the GO MF, the two most represented groups were “binding” (49%) and “catalytic activity” (33%).

### Cellular Component (A)



### Biological Process (B)



### Molecular Function (C)

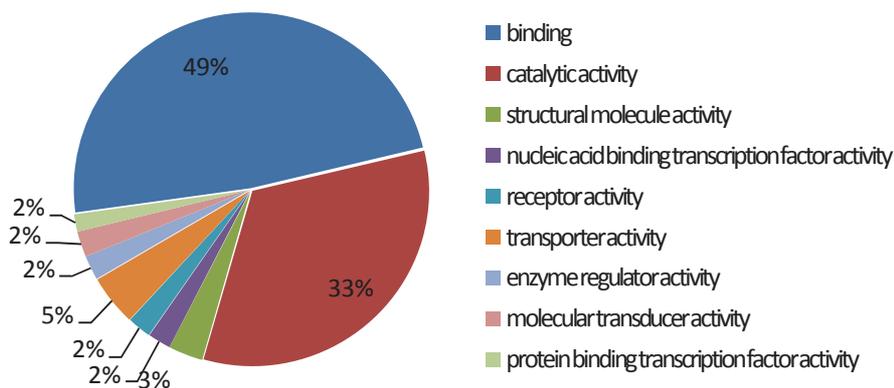


Figure 2. Summary of predicted gene product function and location using level 2 gene ontology terms.

Gene ontology sorted into the three main categories: Cellular Component (A), Biological Process (B) and Molecular Function (C). The GO subcategories are indicated in the legend and the percentage relative to the total number of extracted terms is indicated in the pie chart.

### Clustering analysis

A Principal Component Analysis (PCA) was applied to the selected gene expression dataset (31,862 probes, see Methods) of the 15 oocytes samples (Figure 3). A clear clustering of the three different groups of samples was observed. Stripped eggs, LHR eggs and MHR eggs were clearly separated along the x axis, which explained 29% of the variation. The expression profiles were also separated along the y axis (11% of the variation), but in this case stripped oocytes and MHR oocytes didn't showed a marked divergence in expression patterns, while the separation of LHF eggs was remarkable. Notably, the expression patterns of the 5 stripped oocytes appeared to be similar, while spawned eggs of the two groups seemed less homogeneous.

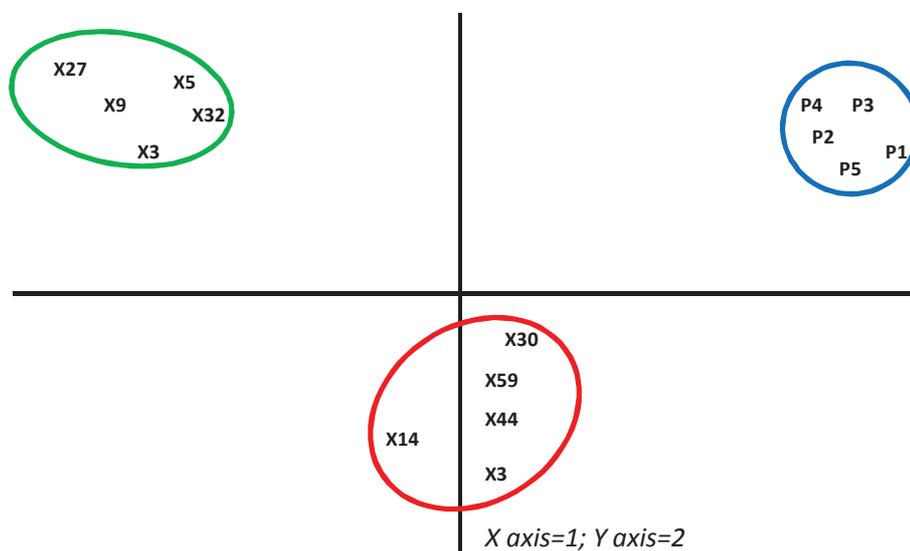


Figure 3. Principal Component Analysis. Samples belonging to the same experimental group are identified by a coloured circle: green for MHR (medium hatching rate) oocytes, red for LHR (low hatching rate) oocytes and blue for stripped oocytes.

Hierarchical clustering of the whole set of probes, using Pearson's correlation, is reported in Figure 4. The analysis identified two main clusters: stripped oocytes

and spawned oocytes. Within the spawned oocytes cluster, samples were further divided in relation to their mean D-larval rate, leading to a definitive separation of the three experimental groups.

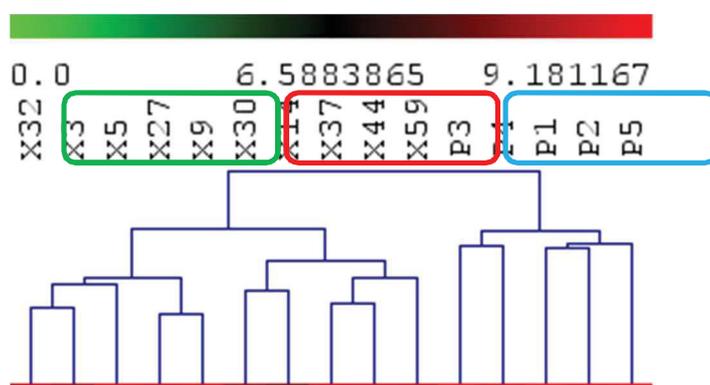


Figure 4. Hierarchical clustering analysis. Stripped oocytes are identified by the “P” letter, while spawned oocytes are labelled with the “X” letter. Red and green rectangles define LHR (low hatching rate) oocytes and MHR (medium hatching rate) oocytes, respectively.

### Comparison between stripped and spawned oocytes

In order to compare gene expression profiles between *V. decussata* oocytes, a two-class unpaired Significance Analysis of Microarray (SAM) test was carried out on normalized/log transformed (base 2) data (FDR<1.5%; FC>1.5). The number of significant probes obtained from the two comparisons is summarized in Table 3.

Table 3. Differential expression analysis. Number of significant probes (FDR<1.5%, FC>1.5) determined through a two-class unpaired SAM (Significance Analysis of Microarray). Unique transcripts refer to the annotation against UniProtKB/SwissProt database. LHR: low hatching rate oocytes; MHR: medium hatching rate oocytes; DEGs: differentially expressed genes.

	<b>ANALYSIS 1</b>	<b>ANALYSIS 2</b>
	<b><i>Stripped oocytes</i></b> <b>VS</b> <b><i>LHR oocytes</i></b>	<b><i>Stripped oocytes</i></b> <b>VS</b> <b><i>MHR oocytes</i></b>
<b>Up-regulated transcripts</b>	105	4,166
<b>Down-regulated transcripts</b>	372	4,594
<b>Total DEGs</b> <b>(unique transcripts)</b>	477 (217)	8,760 (4,772)

A comparison between the significant probes in the two analyses allowed us to identify a set of 439 transcripts which were differentially expressed between stripped

and spawned oocytes, independently of the D-larval rate. A putative UniProtKB/SwissProt accession IDs was obtained for 235 probes corresponding to 198 unique genes (reported in Appendix A1) that were differentially expressed between the two oocytes conditions. Among the differentially expressed genes (DEGs), important transcripts encoding regulators of sex steroids synthesis and activity, such as a progestin and adipoQ receptor family member 3 (PAQR3) and steroid 17-alpha-hydroxylase/17,20 lyase (CYP450-C17), were expressed at a higher level in the stripped oocytes. Gene expression of enzymes involved in the metabolism of ceramide, a signal sphingolipid thought to influence the oocytes maturation and quality, showed significant variations: sphingomyelin phosphodiesterase and neutral ceramidase B were expressed at a higher level in the stripped oocytes, while a putative sphingomyelinase transcript was more abundant in spawned oocytes.

Interestingly, also a putative vitellogenin (Vg), the major egg yolk protein precursor, was more abundant in released oocytes.

Moreover two transcripts encoding proteins suggested to be involved in regulating the maternal mRNAs transcription were differentially expressed. Putative translin-associated protein X and a oocyte zinc finger protein XICOF28 were reported to be more and less abundant in ovarian oocytes, respectively.

Also the regulation of intracellular calcium levels seemed to highly differentiate the ovarian from the spawned oocytes: putative homologs of *C. gigas* calcium-activated chloride channel regulator 4 (two probes), regucalcin and calmodulin were more abundant in stripped oocytes, while sodium/calcium exchanger 3 had a higher expression level in spawned oocytes.

All putative annotated DEGs were used to define a gene list for functional annotation with DAVID. Enrichment analysis showed 7 CC terms, 16 BP terms, 9 MF terms and 1 KEGG to be significantly over-represented (Appendix A2). The only significant KEGG pathway was “ribosome” (dme03010), represented by 3 more abundant transcripts in the stripped oocytes: 60S ribosomal protein L3, 60S acidic ribosomal protein P1 and 40S ribosomal protein S9. “Mitotic cell cycle” (GO:0000278), “translation” (GO:0006412), “WNT receptor signaling pathway” (GO:0016055) and “dephosphorylation” (GO:0016311) were the most represented in the enriched BP terms. Among the DEGs belonging to the biological processes above, 5 M-phase inducer phosphatases (MPIP), a MPIP-like protein, frizzled 8, four-

jointed protein and protein WNT-4 were found. With regard to the CC terms, one of the most represented ones was “ribonucleoprotein complex” (GO:0030529), which in oocytes is involved in the storing and compartmentalization of inactive mRNA incompletely polyadenylated, thus rendering them inactive until the resumption of meiosis (de Moor and Richter, 1997; Mendez et al., 2000). Significant MF terms concerned activities which are classically involved in the cell cycle regulation such as peptidase (GO:0008233) and phosphatase (GO:0016791) activities, and the action of molecules which contribute to the structural integrity (GO:0005198).

### **Correlation between gene expression and hatching rate**

In order to identify the probes whose expression changed in relation to different oocytes competence, a SAM quantitative correlation analysis was carried out on normalized/log-transformed data (FDR<5%). A total of 930 differentially expressed probes were detected, 453 of them were more abundant in oocytes with the highest D-larval rate and 477 in LHR oocytes. A putative annotation in the UniProtKB/SwissProt database was obtained for 587 probes that were differentially regulated in relation to the oocytes competence. The protein names corresponding to the 555 unique UniProtKB/SwissProt accession IDs, out of the 587 matches, were reported in Appendix A3. Genes encoding proteins putatively involved in the oocytes and embryo development were detected. The whole set of annotated transcripts were used to define a gene list for functional annotation with DAVID. Enrichment analysis showed 31 BP terms, 17 CC terms, 18 MF terms and 9 KEGG to be significantly over-represented (Appendix A4). Notably, with 19 genes the BP term “biological adhesion” (GO:0022610) was the most represented. A further BP term significantly enriched was “male gamete generation” (GO:0048232) represented by putative homologs positively correlated with the D-larval rate such as diaphanous and sperm-associated antigen 6 (Spag6). An additional enriched KEGG was “negative regulation of cell cycle” (GO:0045786) represented, among other, by a cyclin-dependent kinase 20 (two probes positively correlated with the oocytes quality).

As concern the CC terms, in addition to the “extracellular region” (GO:0005576), enriched also in the comparison between stripped and spawned oocytes, the cellular compartment showing a consistent enrichment was those

sustaining the vesicular trafficking. Genes annotated with CC terms like “Golgi-associated vesicle membrane” (GO:0030660), “early endosome” (GO:0005769) and “vesicle coat” (GO:0030120) were listed among the genes whose expression changed in relation to the oocytes competence on larval development. Interestingly, the 136 genes belonging to the enriched MF term “ion binding” (GO:0043167) reflected the important role of charge regulation in oocytes development, most notably of calcium (GO:0005509). Furthermore, the importance of calcium was confirmed by the oocytes quality-correlated mRNA expression of sodium/calcium exchanger members 2 and 3, calcineurin (higher expression in MHR oocytes) and IP3r, resulting in the enriched KEGG pathway “calcium signaling pathway” (rno04020). IP3r and calcineurin, together with adenylate cyclase led also to the over-representation of the KEGG pathway “oocyte meiosis” (rno04114). Moreover, within the Panther database, the most represented enriched pathways (p-value<0.01) was “WNT signaling pathway” (P00057), a molecular pathway that also characterized part of the differences between stripped and spawned oocytes. Notably the most interesting probes belonging to this pathway and positively correlated with the oocytes quality were those putatively encoding the WNT proteins receptors frizzled-4 and frizzled-10, and the calcium-dependent cell adhesion molecules cadherins type 4, 7 and 23.

### **ANOVA one way analysis**

To investigate the set of probes whose expression level increased or decreased in relation to the increment of oocytes competence (stripped oocytes=null; LHR oocytes=low; MHR oocytes=medium), an ANOVA one way analysis was carried out on all samples from the three experimental groups. The analysis allowed to obtain a list of 14,118 probes with a significant change in expression between at least two groups (pval<0.5). A total of 568 probes for which the transcriptional variations were positively correlated to the oocytes competence were detected by selecting probes with a maximum expression value in MHR oocytes, intermediate in LHR oocytes and minimum in stripped oocytes. The same approach was adopted to find a list of 1,505 probes inversely correlated to the competence level (maximum expression value in stripped oocytes, intermediate in MHR oocytes and minimum in LHR oocytes). Finally, to provide a more comprehensive functional interpretation of

the genes putatively involved in the biological mechanisms regulating the acquisition of oocytes competence, an enrichment analysis was carried out. Out of 2,073 probes, positively or negatively correlated to the oocytes competence, 1,342 annotated transcripts were used to define a gene list in DAVID. Results reported the over-representation of 3 KEGG, 48 BP terms, 16 CC terms and 31 MF terms (Appendix A5). Among KEGG pathways the higher fold enrichment was shown for “ribosome” (dme03010 and hsa03010), a term found significantly enriched also in the comparison between stripped and spawned oocytes, attesting the important involvement of the ribosomal activities during the oocytes development. Looking at the BP terms, “response to corticosteroid stimulus” (GO:0031960), spindle organization (GO:0007051 and GO:0000022), “steroid metabolic process” (GO:0008202) and sperm-egg recognition (GO:0035036 and GO:0007339) were significantly over-represented. Furthermore, enriched general mechanisms such as “translation” (GO:0006412), adhesion (GO:0007155 and GO:0022610) and oxidation reduction (GO:0055114) were identified.

## Discussion

The gene expression analysis and the evaluation of DEGs between oocytes at different maturation stage and quality pointed out some interesting results and provided a first overview on the molecular mechanisms responsible for stripped oocytes infertility and oocytes competence. Among the 198 annotated DEGs between stripped and spawned oocytes, several transcripts were implicated in the regulation of the cell cycle. Notably *V. decussata* stripped ovarian oocytes have already replicated their DNA and are blocked at the germinal vesicle stage during late prophase of the first meiotic division. Conversely in spawned oocytes meiosis reinitiates and during metaphase I occurs a second block which is only released upon fertilization or artificial activation (Lippai et al. 1995). Several proteins are involved in the regulation of prophase I arrest occurring in ovarian oocytes, the most crucial factor being the maturation promoting factor (MPF) (Jones, 2004). MPF, a key G2/M phase regulator in eukaryotic cells consisting of cdc2 kinase (known also as cdk1) and cyclin B (Nurse, 1990), is induced during the meiosis resumption and its activity is regulated by the phosphorylation of cdc2 kinase. Cdc2 kinase is activated when M-phase inducer phosphatases (cdc25) dephosphorylate threonin-14 and

tyrosine-15 sites (Millar et al., 1991; Strausfeld et al., 1991; Trunnell et al., 2011), leading, in turn, to MPF activation and meiosis resumption. In this study, six probes coding for *cdc25* or *cdc25*-like proteins were reported to be more abundant in released oocytes, suggesting a prominent role of these phosphatases in the resumption of the meiotic cell cycle progression in *V. decussata*. The controlling function of *cdc25* phosphatases in the meiosis I progression has been proposed in a wide range of species (e.g. Kim et al., 2010 in *Caenorhabditis elegans*; Alphey et al., 1992 in *Drosophila*; Kishimoto, 2011 in startfish; Oh et al. 2010 in mouse; Gaffrè et al., 2011 in *Xenopus*). In mouse, both *cdc25A* and *cdc25B* were demonstrated to be critical for meiotic maturation and metaphase I spindle formation of oocytes (Solc et al., 2008). In mice *cdc25B*<sup>-/-</sup> knockout female are sterile because their oocytes cannot exit developmental arrest at meiosis prophase 1 (Lincoln et al., 2002), whereas *cdc25A*<sup>-/-</sup> mice exhibit early embryonic lethality (Ray et al., 2007), indicating that they are required for the control of oocyte meiotic cell cycle and embryonic mitotic cell cycle, respectively. Interestingly, among the six phosphatases differentially expressed between stripped and spawned oocytes, three followed a trend of expression correlated to the D-larval rate and their expression values were lower in stripped oocytes, intermediate in LHR oocytes and higher in MHR oocytes. Unfortunately in the European clam these proteins have not been studied yet and the number of *cdc25* isoforms is still unknown, but these findings allowed to hypothesize that in *V. decussata* more than one *cdc25* family members exist and that they may function as distinct but related cell cycle regulators. Moreover, despite a role in meiosis I resumption, it seems that, at least for a few putative members, also the transcript abundance is fundamental for the maturation processes leading to a correct embryo development.

A crucial role in the molecular processes which differentiate stripped and spawned oocytes was predicted also for a few members of the WNT signaling pathway (GO:0016055), a powerful signaling pathways that play crucial roles in the animal life by controlling the genetic programs of embryonic development and adult homeostasis (Grigoryan et al., 2008). Recently, in mammals WNT pathway signaling has been implicated in ovarian development, oogenesis, and early development. Multiple WNT signaling pathway genes are expressed in mouse oocytes and pre-implantation stage embryos, as revealed by microarray analyses (Wang et al., 2004; Zeng et al., 2004), and this has led to the hypothesis that WNTs

may function in early cell fate determination events (Wang et al., 2004). Other studies, however, indicate that WNT signaling pathways are likely not functional in the early embryo (Kemler et al., 2008), raising the possibility that expression of WNT signaling genes in oocytes and early embryos are most likely related to functions in oogenesis (e.g., oocyte growth or maturation) (Zheng et al., 2006). In the present study the expression levels of three probes putatively encoding a WNT proteins receptor, frizzled-8, were more abundant in released oocytes. This data might suggest that frizzled-8 expression supports the WNT signaling pathway activation by favouring the recognition of WNT proteins. Moreover, our results reported that a putative ortholog of *C. gigas* WNT4 was less abundant in the female gametes extracted from mature gonads, in comparison with the released oocytes, and its mRNA expression tended to increase with the eggs quality. These findings suggest that not only WNT4 transcript could be implied in the *V. decussata* oocytes development but it could also be an important transcript whose abundance affects the oocyte quality, like it has been proposed in mouse. Notably, mice null for WNT4 exhibit sex reversal and a reduced number of oocytes in new-born ovaries (Jeays-Ward et al., 2004). In the same species, ovaries of WNT4-mutant females were characterized by a scarce amount of oocytes and these were in the process of degenerating (Vainio et al., 1999) and the 80% of WNT4 deficient germs cells failed to enter meiosis (Naillat et al., 2010). Finally a putative four jointed protein (Fj) encoding transcript was significantly (FDR<1.5%) more expressed in spawned oocytes than in stripped ones, and its expression, similarly to what reported for WNT4, has the highest level in MHR oocytes. The role of Fj has been poorly investigated in both vertebrates and invertebrates. However, few studies focusing on this gene have been performed in *D. melanogaster*, where it has been demonstrated that this protein directly interacts with WNT4 and they act synergistically to induce planar polarity during early development (Lim et al., 2005; Bosveld et al., 2012). Considering the lack of functional information concerning Fj, it's difficult to establish a specific role of this gene in *V. decussata*, nevertheless it can be suggested that its expression in oocytes maturation is probably linked to the oocytes competence on fertilization and larval development.

Interestingly, the pivotal role suggested for the WNT signaling pathway was confirmed by the expression patterns evaluated in LHR and MHR oocytes, in which the mRNA abundance of a few putative frizzled receptors and cadherins was

correlated to the developmental competence level. Notably, in vertebrates, a cross-talk between WNT-mediated signals and cadherins have been reported to have a major role in morphogenesis and tissue formation (Marie and Haÿ, 2013). Taken together these results allowed us to hypothesize that also in *V. decussata* oocytes, the amount of mRNAs encoding proteins involved in the WNT signaling could be extremely important to permit the complete oocytes maturation and fertilization.

Another interesting finding was the significant change in expression of a putative Vg transcript. Vgs are the major precursor of the egg-yolk proteins, vitellins (Vn), which are stored in developing oocytes and are required for oocyte growth and maturation (Kanungo et al., 1990; LaFleur et al., 2005). In addition, yolk storage proteins are traditionally regarded as the energy reserve for nourishment of the developing embryos (Zhang et al., 2011; Li et al., 1998a, 1998b). Vgs have been shown to be present in almost all species of oviparous animals ranging from nematodes to vertebrates, and extensive sequence conservation is observed among these groups (Chen et al., 1997). To date, full length sequence characterization and gene expression levels of Vg have mainly focused on insects, crustaceans and fish (LaFleur et al., 1995, Mouchel et al., 1996; Okuno et al., 2002; Tsutsui et al., 2000). As regards molluscs, only a few studies were reported and Vg transcriptional levels were mainly evaluated in the gonadal tissue (Osada et al., 2003, 2004; Matsumoto et al., 1997, 2003, 2008; Zheng et al. 2012). In *Patinopecten yessoensis* and *C. gigas*, it has been demonstrated that the synthesis of Vg mRNA occurs in auxiliary cells inside the ovary (hetero-synthetic synthesis pathway), and is controlled by 17beta-estradiol (E2) and vitellogenesis promoting factor (VPF) via estrogen receptor (ER) (Osada et al., 2003, 2004; Matsumoto et al., 1997, 2003). Conversely, studies performed in *Mytilus edulis* and *C. gigas* suggested that yolk proteins are synthesized in developing oocytes through an auto-synthetic pathway (Pipe, 1987; Suzuki et al, 1992). However, there has not been direct evidence on Vg synthesis, and it is still unclear whether the synthesis of a major yolk protein occurs through an auto- or hetero-synthetic pathway in bivalve molluscs. The fact that in the present study Vg mRNA was detected in the oocytes, let us thinking that in *V. decussata* Vg is synthesized through an auto-synthetic pathway. Despite that, we cannot exclude that in *V. decussata* a hetero-synthetic synthesis occurs, thus further investigations are needed. In addition, a higher level of Vg transcript was reported in the spawned oocytes. Despite the importance of Vg during oocytes growth/maturation and

embryonic development has been demonstrated mainly at a protein level, this result might suggest that also Vg mRNA levels play a pivotal role, probably by providing a reserve of transcripts ready to be translated into functional protein. Thus it can be hypothesized that highest levels of stocked Vg mRNA in released oocytes provide a resource to support maturation and following embryo development. However, since the site of Vg synthesis in molluscs is not yet clarified and major regulations seem to occur at a protein level, gene expression results reported here cannot be sufficient to propose general conclusions regarding the involvement of Vg in *V. decussata* released oocytes.

Nonetheless, the presence of Vg mRNA stocks in bivalves was demonstrated also by Osada and colleagues (2004), who observed high level of Vg mRNA in *P. yessoensis* gonads through the spawning stage. A possible explanation they proposed, hypothesized also in *Xenopus* liver cell (Brock and Shapiro, 1983), is that Vg mRNA might be stabilized and remained un-translated, resulting in the retained high amount of Vg mRNA. The mechanism supposed to be involved in the retention of Vg mRNA requires the activity of RNA-binding proteins, which are known to function as translational repressors in the cytoplasm of several eukaryotic cells (Wilkinson and Shyu, 2001; Wickens et al., 2002). This mechanism is supposed to be crucial in oocytes since, during oogenesis, maternal mRNAs are synthesised and stored in a translationally dormant form and are activated either upon re-entry into the meiotic divisions or after fertilisation (Song and Wessel, 2005, Dheilly et al., 2012). In *Xenopus* oocyte maturation and early embryogenesis, translin, a RNA-binding protein, was demonstrated to play a major role to repress maternal mRNA translation (Castro et al., 2000). Notably in this study, a translin-associated protein X, supposed to be involved in the nuclear transport of translin in mice (Aoky et al., 1997; Cho et al., 2004), was expressed in all the oocytes condition and particularly abundant in stripped oocytes. The relevance of mRNA translation in oocytes was demonstrated also by the significant variations reported for a putative homolog of the *Xenopus* oocyte zinc finger protein XICOF28 whose mRNA amount was more abundant in spawned oocytes. The *Xenopus* zinc finger protein XICOF28 belongs to the family of C2H2 zinc finger proteins, which are known to function as RNA-binding molecules (Hall, 2005). Noteworthy, it has been recently reported that proteins belonging to this family are required for regulation of maternally supplied mRNAs during oogenesis, oocyte to embryo transition, and early embryogenesis in both

vertebrates and invertebrates (Yamamoto et al., 2013; Kaymak and Ryder, 2013). Consistent with these evidences, it can be hypothesized that C2H2 zinc finger proteins take part to the mechanisms which regulate mRNAs silencing in *V. decussata* oocytes.

A further interesting result concerns the differential expression of several transcripts involved in calcium ( $\text{Ca}^{2+}$ ) signaling. There has been a long-standing debate as to whether  $\text{Ca}^{2+}$  signals are required for oocyte meiosis and numerous conflicting studies argue that the relationship between  $\text{Ca}^{2+}$  and oocyte maturation is complex (Sun and Machaca, 2004, Tosti et al., 2006). The role that external calcium, through voltage-gated channels, might play in the induction of GVBD was first reported in molluscs that are both fertilized at the PI stage (Allen, 1953; Dubè, 1988; Deguchi and Osanai, 1994), or undergo the second arrest in MI (Dubé and Guerrier Guerrier, 1982; Cuomo et al., 2005). It was soon recognized that also the intracellular calcium increase plays a crucial role in almost all species studied independently from their peculiar meiotic arrest (Deguchi and Osanai, 1994; Juneja et al., 1994; Guerrier et al., 1993). In particular, the interplay between external and internal calcium currents is evident in *Venerupis*, where a serotonin-induced surge of intracellular calcium was shown to trigger maturation even in the absence of external calcium (Guerrier et al., 1993). In the present study, the differences in the amount of mRNA encoding putative calcium-activated chloride channel regulator 4 confirmed that the regulation of intracellular  $\text{Ca}^{2+}$  plays an important role in *V. decussata* oocytes maturation. Likewise in the present work, a putative sodium/calcium exchanger 3 was reported to be more abundant in the released oocytes and homologs of *C. gigas* regucalcin and calmodulin showed higher expression levels in ovarian oocytes. Calmodulin and regucalcin are calcium-binding proteins supposed to contribute to the meiosis regulation (Wasserman and Smith, 1981; van der Voet et al, 2009) and their mRNA variations suggest an involvement in maintaining the calcium homeostasis in immature oocytes. Interestingly, the regucalcin mRNA was expressed at lowest level in the MHR oocytes, thus indicating that low levels of this transcript could be related to higher quality eggs. Despite little is known about the molecular regulation of intracellular  $\text{Ca}^{2+}$  occurring during oocytes maturation in bivalves, these preliminary results pointed out a few important genes possibly involved in such a complex mechanism.

In the present study, changes in mRNA levels were reported also for putative PAQR3 and CYP450-C17, which were both more abundant in ovarian oocytes. Progesterin and adipoQ receptor family is a group of G protein-coupled receptors including membrane progesterone receptors (mPRs) that mediate a variety of rapid cell surface-initiated progesterone actions in the reproductive system involving activation of intracellular signaling pathways. Cytochrome P450-C17 is an enzyme involved in the synthesis of E2 during steroidogenesis. Despite in invertebrates there have been conflicting lines of evidence concerning the existence of enzymes necessary to synthesize vertebrate steroids and related nuclear receptors (Scott, 2013), several studies suggested a role of E2 and progesterone in gonadal development, oocytes maturation and spawning in several bivalve species (Li et al., 1998a; Matsumoto et al., 1997; Osada et al., 2003, 2004; Reis-Henriques and Coimbra, 1990; Varaksina and Varaksin, 1991; Varaksina et al. 1992; Wang and Croll, 2004). Consistent with these studies, we demonstrated that, during *V. decussata* oocytes maturation, significant variations of transcripts involved in the sex steroids synthesis and activity occurred. In particular the highest PAQR3 and CYP450-C17 mRNAs levels in ovarian oocytes may be associated to an higher E2 synthesis and progesterone activity in comparison with released oocytes. Accordingly, evidences indicate that sex steroids have a pivotal role in the pre-spawning stage since they have stimulatory effects on gamete release in *P. yessoensis* and *Placopecten magellanicus* (Osada et al., 1992; Wang and Croll, 2003; Wang and Croll, 2007).

Another metabolic process possibly implied in the oocytes maturation events in *V. decussata* was the regulation of ceramide levels. The enzymes controlling the metabolism of ceramide in oocytes have been poorly studied in molluscs and only recently sequences of genes associated with ceramide metabolism and signalling have been investigated in the Pacific oyster (Timmins-Schiffman and Roberts, 2012). Conversely, in vertebrates quite a few studies have been focused on the role of ceramide in oocytes and two main hypotheses have been suggested (Coll et al., 2007). First, it has been proposed that the generation of ceramide is a part of the signal transduction pathway activated in response to progesterone and that the increase in ceramide is likely to be functionally important in the resumption of the meiotic cycle (Strum et al., 1995; Morrill and Kostellow, 1998; Buschiazzo et al. 2011). Second, it has been recently demonstrated that ceramide induces the default

apoptosis process in oocytes and it has a central role in the age-related decrease of eggs quality (Perez et al., 2005; Miao et al., 2005). In the present study, at least three enzymes involved in the ceramide metabolism were found differentially expressed between stripped and spawned oocytes: a ceramide synthase, less abundant in ovarian oocytes, a ceramidase and a sphingomyelinase, both less abundant in released oocytes. In addition, the expression of the sphingomyelinase and a sphingosine kinase were higher in stripped oocytes and lower in MHR oocytes, suggesting that a low amount of mRNAs encoding this enzyme could be somehow linked to higher eggs quality. Since no data are available on the normal ceramide homeostasis in bivalve oocytes, a comprehensive interpretation of the reported mRNA fluctuations is particularly challenging. Thus we can only legitimately conclude that, also in *V. decussata*, the ceramide metabolism most likely plays an important role in oocytes maturation and competence development.

A second aim of the present study was the identification of molecular mechanisms possibly involved in the development of oocytes competence once fertilization occurred. Since a high variability occurs in the spawned oocytes quality, we hypothesized that differences among the mRNA abundance of some transcripts could affect the oocyte maturation and the acquisition of developmental competence. Notably, mature oocytes of marine invertebrates contain a set of messenger RNAs which are not translated to a significant extent until after fertilization, when they provide most of the metabolic resources and information required for the early development of zygotes (Watson, 2007).

The analysis of the expression profiles of LHR and MHR oocytes allowed us to identify a total of 930 probes whose fluorescence intensity was positively or negatively correlated to the eggs quality, measured by the D-larval rate following oocytes fertilization. A functional annotation of those probes was carried out and it pointed out some interesting results.

Important variations occur in the abundance of transcripts encoding proteins regulating the oocyte meiosis, such as adenylate cyclases (ADCY), IP3r-1, IP3r-2 and the catalytic subunit alpha isoform of protein phosphatase 2B. ADCYs are membrane-associated enzymes that catalyse the formation of the secondary messenger cyclic adenosine monophosphate (cAMP) which is thought to have a pivotal role in the meiotic maturation in several species (Richard, 2007). In fact, accumulated evidence indicates that the release from prophase I arrest is

accompanied by a decrease in oocyte intracellular levels of cAMP, which is most probably a major prophase-I-arresting factor in amphibian and mammalian oocytes. Despite some exceptional cases seem to exist, such as the oocytes of the bivalve *Spisula solidissima*, in which the onset of meiosis reinitiation is associated with an increase in cAMP levels (Yi et al., 2002), it is generally accepted that an almost universal decrease in oocyte cAMP is sufficient to trigger the release of oocytes from prophase I arrest or at least positively affects it. In the present study the mRNA transcripts encoding ADCY type 5 and 6 were more abundant in oocytes with a lower developmental competence. Thus, despite cAMP levels are regulated also by other factors, such as progesterone and mPRs, more likely ADCYs have a key role in cAMP balancing and meiotic progression in *V. decussata*.

Moreover, the variations in the mRNA levels reported for IP3 receptors could provide interesting information concerning the bivalve oocytes maturation. IP3r are membrane glycoprotein complexes acting as Ca<sup>2+</sup> channels and are activated by inositol trisphosphate (IP3), which has been strongly implicated in the conversion of external stimuli to intracellular Ca<sup>2+</sup> signals (Yoshida and Imai, 1997). IP3r have been shown to have a predominant role also in oocytes, in both the formation and propagation of Ca<sup>2+</sup> waves at fertilization (Kume et al., 1993) and it has been demonstrated that intracellular injections of IP-3 triggered GVBD in *Spisula* (Bloom et al., 1988) and *Venerupis* (Guerrier et al., 1996). In the present work, putative orthologs of the mammalian IP3r-1 and IP3r-2 were differentially expressed in relation to the oocytes competence. In particular the IP3r-1 was more abundant in the MHR oocytes, while IP3r-2 expression decreased as the oocytes quality increased. The opposite trend of expression reported for the two IP3r types allows us to think that in the European clam oocytes, at least two IP3r are expressed and that not only the presence of this receptor is crucial in determining a complete oocytes maturation and fertilization, but most probably also the isoform identity. While the isoform-specific cellular function of these channel proteins have not been investigated in molluscs, conversely in mammals IP3r isoforms, their subcellular distribution and peculiar functions have been studied (e.g. Vanlingen et al., 2000; Vermassen et al., 2004). Thus these preliminary results suggest the importance of IP3r messengers during the oocytes maturation phases occurring after the spawning in *V. decussata*.

A further interesting gene differentially expressed in released oocytes and implied in the meiotic maturation was the one coding for the catalytic subunit alpha

isoform of a protein phosphatase 2B, known also as calcineurin. This enzyme is a calcium/calmodulin-dependent phosphatase and plays a major role in the transduction of calcium signals in a variety of cell types from fungi to vertebrates (Stewart et al., 1982 and Aramburu et al., 2000). The significant role of calcineurin in meiotic maturation has been demonstrated in invertebrates (Takeo et al., 2006; Takeo et al., 2010) and in lower vertebrates (Nishiyama et al., 2007; Mochida and Hunt, 2007), while there is very limited information about calcineurin expression and distribution in mammalian oocytes. In pig oocytes, specific inhibitors of calcineurin affect the in vitro maturation of both growing pig oocytes with partial meiotic competence and fully grown pig oocytes with full meiotic competence (Petr et al., 2013; Tumova et al., 2013). These calcineurin inhibitors affected the exit of oocytes from the metaphase I of meiotic maturation. However, the role of calcineurin in the regulation of oogenesis and meiosis of gametes of mammalian females remain unclear. In *Drosophila* it has been demonstrated that oocytes lacking family members of positive regulators of calcineurin (RCANs) are arrested in anaphase of meiosis I (Takeo et al., 2006; Takeo et al., 2010). Consistent with these evidences, we found that calcineurin mRNA was more abundant in high quality oocytes, paving an important function of this enzyme also in *V. decussata* meiotic maturation.

Beside Ca<sup>2+</sup> regulation, also cyclin-dependent protein kinases (cdks) have a crucial role during the oocytes maturation, since they regulate cell cycle transitions (Morgan, 1995; 1997). The importance of cdc2 kinase (cdk1) for the meiosis progression has been previously discussed. Interestingly, variations in the mRNA expression of a putative cyclin-dependent kinase 20 (cdk20 or p42) pointed out an additional mark differentiating oocytes with different developmental competence. In fact, the amount of cdk20 RNA messenger showed a positive correlation with the oocytes quality, suggesting an involvement of this kinase in the mechanisms promoting a correct gametes maturation and fertilization. The role of cdk20 has been recently investigated in mammals and it has been demonstrated that it is essential for the phosphorylation and consequent activation of cdk2 (Liu et al., 2004). Cdk2 is thought to be essential in the mammalian cell cycle, by driving cells through the G1/S transition (Heichman and Roberts, 1994), and in the *Drosophila* early embryogenesis as well (Knoblich et al, 1994). However, several lines of evidence indicated that cdk2 was essential in meiosis, but not in mitosis in mice (Ortega et al., 2003). Consistent with this hypothesis, cdk2 knockout mice grew normally, but both male and female

mice were sterile due to meiotic defects (Berthet et al., 2003). Cdk2 activity is thought to be essential also in porcine oocytes, in particular for the first to second meiosis transition (Sugiura et al., 2005). Furthermore a recent study in the giant prawn *Macrobrachium rosenbergii* suggested that cdk2 kinase might have an essential role during the meiotic maturation also in invertebrates (Chen et al., 2013). Despite the role of cdk20 is not fully resolved, not even in vertebrates, these studies allow us to propose that its abundance in MHR clam oocytes could be implicated in a major activation of cdk2, thus being one of the several factors which contribute to their higher developmental competence.

The acquisition of oocytes competence in *V. decussata* seemed to be regulated also by other important factors. One of them was the diaphanous protein, a formin required for cytokinesis in both mitosis and meiosis. It has been demonstrated in *Drosophila* that it has a role in actin cytoskeleton organization and is essential for many, if not all, actin-mediated events involving membrane invagination (Castrillon and Wasserman, 1994). Moreover a role as a mediator between signaling molecules and actin organizers at specific phases of the cell cycle (e.g. contractile ring) has been proposed (Afshar et al., 2000). The importance of diaphanous during the meiotic maturation has been demonstrated by Castrillon and Wasserman (1994) that reported that mutated alleles of this gene affected spermatogenesis and oogenesis. Later, Bione and colleagues (1998) proposed that the homolog of *Drosophila* diaphanous in human is one of the genes responsible for premature ovarian failure and that it affects the cell divisions that lead to ovarian follicle formation. In molluscs this transcripts has never been reported but, since its mRNA abundance was significantly correlated to *V. decussata* oocytes quality, we can suppose that this protein may have an important role in cytokinesis and other actin-mediated morphogenetic processes that are required in early steps of development.

A further transcript showing the same expression pattern as diaphanous was Spag6, a protein, mostly expressed in testis, thought to be important for structural integrity of the central apparatus in the sperm tail and for flagellar motility in mouse (Sapiro et al., 2000). In the same species, this protein has been later implicated in mouse infertility (Sapiro et al., 2000) since mice lacking Spag6 produced sperm with marked motility defects, morphological abnormalities, disruption of flagellar structures, including loss of the central pair of microtubules and disorganization of the outer dense fibers. It's intriguing that a similar transcript has been detected in *V.*

*decussata* oocytes with the highest quality. We can suppose that most probably this transcript encodes a protein with functions similar to Spag6 in male gametes and thus is implied in the gamete structural integrity.

Finally, in the present study, a pivotal role of the WNT signaling pathway in the oocyte maturation process was suggested. In fact, the abundance of few transcripts corresponding to putative frizzled receptors and cadherins was correlated to the developmental competence. Notably, in vertebrates, a cross-talk between WNT-mediated signals and cadherins have been reported to have a major role in morphogenesis and tissue formation (Marie and Hay, 2013). Thus, these results allowed us to hypothesize that also in *V. decussata* released oocytes, the amount of stocked mRNAs encoding proteins involved in the WNT signaling could be extremely important to permit the complete oocytes maturation and fertilization.

## Conclusion

The gene expression analysis performed in this study allowed to identify a few important mechanism which could have an key role in the process of bivalve oocyte development. Differences in the mRNA expression of some important genes have been detected and the enrichment analysis helped in the interpretation of the DEGs lists, providing a more comprehensive picture of the key processes affecting *V. decussata* oocytes maturation and competence acquisition. Noteworthy, the transcripts which seemed to play a major role in the female gametes maturation and competence acquisition were those encoding proteins involved in the cell cycle progression, calcium regulation and WNT signalling.

Despite the analyses performed within this study provided first interesting findings, however more detailed investigations are still required and alternative molecular approaches should be implemented. For example, to deeply characterize specific events leading to the completion of meiosis and to elucidate the activity of target transcripts, reverse genetic experiments should be exploited.

Notwithstanding the fact that *V. decussata* reproduction is still far from being completely controlled in hatchery, as far as we know, this study represents the most effective attempt to improve the knowledge of oocytes maturation processes in this species.

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## Dissemination

### Publications:

de Sousa JT, Milan M, Bargelloni L, Pauletto M, Matias D, Joaquim J, Matias AM, Quillien V, Leitão A, Huvet A (2014). A microarray-based analysis of gametogenesis in two Portuguese populations of the European clam *Ruditapes decussatus*. PlosOne.

de Sousa JT, Milan M, Pauletto M, Bargelloni L, Joaquim S, Matias D, Matias AM, Quillien V, Leitão A, Huvet A. (2014). A microarray-based analysis of oocytes quality of the European clam *Ruditapes decussatus*. Submitted to Marine Biotechnology.

### Communications:

Teixeira de Sousa J., Huvet A., Matias D., Joaquim S., Leitão A. Histological study of reproductive characteristics of two Portuguese populations of the European clam *Ruditapes decussatus*. Physiomar 2012, 4-8 September 2012, Santiago de Compostella, Spain. Oral presentation

Teixeira de Sousa J. "Ma thèse en 180 secondes" - "La Palourde Européenne - Une espèce, deux populations". Doctoriales Bretagne 2013, December 9-13, Rennes, France. Oral presentation

Teixeira de Sousa J., Milan M., Bargelloni L., Pauletto M., Matias D., Joaquim S., Matias A.M., Quillien V., Leitão A., Huvet A. A microarrays-based analysis of gametogenesis in two Portuguese populations of the European clam *Ruditapes decussatus*. Genomics in Aquaculture 2013, September 4-6, Bodø, Norway. Oral presentation



## Abstract

The European clam, *Ruditapes decussatus* is considered a high value seafood product in Southern Europe. Its production is almost based on natural recruitment, which is subject to annual fluctuations. In Portugal, two of the main production areas of this species are Ria de Aveiro and Ria Formosa Lagoons. These populations were characterized by different responses to spawning induction, which is of great interest in a context of improvement of aquaculture. The purpose of this thesis was to improve the cellular and genomic knowledge on the reproduction of *R. decussatus* with major applications: broodstock conditioning and gamete quality.

The reproductive cycle of the two populations was histologically characterized by comparing the gonadal development, gonadal area and oocyte diameter. With the exception of the dynamics of gonadal development, which may originate in the environment, no differences concerning gametogenesis were found. cDNA libraries of oocytes, larvae and gonads were sequenced on Illumina platform, to enrich resources in reproductive Expressed Sequence Tags, and a custom oligoarray representing 51,678 assembled contigs was then designed. To characterize the transcriptomic bases of reproduction, microarray analyses were performed in four gonadal maturation stages of the two populations. Differentially expressed probes and the “N-Glycan biosynthesis” pathway, potentially involved in sperm-egg interaction, were identified, suggesting that gamete recognition can explain part of the differences in terms of spawning induction success between populations. Moreover, microarray analyses were also performed in oocytes, with the objective of identifying potential markers of oocyte quality in this species. Genes coding for chaperone proteins demonstrated to be important markers of oocyte quality, with some of them being maternal mRNAs essential for early development.

The present study provides new highly valuable genomic information for the understanding of reproduction of *R. decussatus* and emphasizes some candidate genes like protein disulfide isomerase (PDI), Calmodulin family and caspase 8 as possible starting points for further functional studies.

**Keywords:** *Ruditapes decussatus*, marine bivalve, reproduction, gene expression, oligoarray, aquaculture

## Résumé

La palourde grise européenne, *Ruditapes decussatus* est un coquillage d'importance socio-économique en Europe du Sud. Sa production est basée sur le recrutement naturel, qui est sujet à de fortes fluctuations annuelles. Au Portugal, les principales zones de production de cette espèce sont les lagunes de Ria de Aveiro et Ria Formosa. Ces populations présentent des réponses différentes à l'induction de la ponte, résultat d'intérêt dans un contexte d'amélioration de la production aquacole. L'objectif de cette thèse était d'améliorer les connaissances sur la reproduction de *R. decussatus* en utilisant des approches génomiques et cellulaires avec pour principales applications : le conditionnement de géniteurs et la qualité des gamètes.

Le cycle de reproduction de ces populations a été caractérisé par histologie pour le développement gonadique, l'aire gonadique et le diamètre de l'ovocyte. À l'exception de la dynamique de la gamétogénèse, aucune différence significative n'a été identifiée entre populations. Grâce à un effort de séquençage (Illumina) de banque d'ADNc de différents tissus/stades pour enrichir en transcrits liés à la reproduction, une puce à ADN (oligoarray) représentant 51 678 contigs a été produite et utilisée afin de caractériser les bases transcriptomiques de la reproduction de *R. decussatus*. Des gènes différentiellement exprimés et la voie "N-Glycan biosynthesis", impliquées dans l'interaction spermatozoïde – ovocyte, ont été soulignés, ce qui suggère que la reconnaissance entre gamètes puisse expliquer en partie les différences de succès d'induction de ponte entre ces populations. De plus, le transcriptome d'ovocytes collectés chez 15 femelles a été analysé par puce à ADN avec pour objectif d'identifier des potentiels marqueurs de la qualité des ovocytes. Des gènes codant pour des protéines chaperonnes, dont certains caractérisés comme des ARNm maternels essentiels pour le développement précoce, sont apparus différentiellement exprimés entre ovocytes de bonne et mauvaise qualité établie sur le taux de larves D obtenu. La présente étude fournit de nouvelles informations génomiques précieuses pour la compréhension de la reproduction de cette espèce et liste des gènes candidats codant la protéine disulfuride isomerase (PDI), des calmodulines et la caspase 8 comme points de départ possibles pour des études fonctionnelles. Leur implication dans les phases importantes de la reproduction comme l'interaction spermatozoïde-ovocyte, la protection de l'ovocyte, la régulation du calcium et l'apoptose ont font des marqueurs potentiels de la qualité ovocytaire de cette espèce.

**Mots-clefs :** *Ruditapes decussatus*, bivalve marin, reproduction, expression génique, puce à ADN, aquaculture