U.PORTO

**DOUTORAMENTO EM CIÊNCIA ANIMAL**
GENÉTICA E MELHORAMENTO ANIMAL

# Search for new biomarkers of resistance to *Perkinsus olsenii* parasite in *Ruditapes decussatus* clams

João Luís Correia Estêvão

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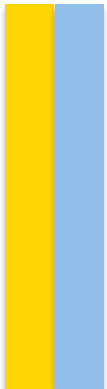
**2025**



INSTITUTO DE **CIÊNCIAS BIOMÉDICAS ABEL SALAZAR**

U.PORTO
INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

PhD THESIS

ANIMAL SCIENCE DOCTORAL PROGRAMME

SPECIALIZATION IN GENETICS AND ANIMAL BREEDING

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PhD Thesis of Doctorate in Animal Sciences, Specialization in Genetics and Animal Breeding, submitted to the Institute of Biomedical Sciences Abel Salazar of the University of Porto

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This Thesis includes one scientific paper published in an international peer-review journal originating from part of the results obtained in the experimental work, referenced to as:

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And an article submitted to Evolutionary Applications journal, that at this moment is in the second round of revisions:

Inés M. Sambade[§], João Estêvão[§], Marina Pampín, Andreia Cruz, Eric Guévélou,Andrés Blanco,Francisco Câmara, Jessica Gómez-Garrido, Fernando Cruz, Luca Bargelloni, Stefano Carboni, Tyler Alioto, Benjamin Costas, Sergio Fernández-Boo[*], Paulino Martínez[*]. Signatures of selection for resistance / tolerance to *Perkinsus olseni* in grooved carpet shell clam (*Ruditapes decussatus*) using a population genomics approach. Evolutionary Applications (under review).

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## Summary

*Ruditapes decussatus* is a native clam species from Europe and North Africa basin which is facing several problems at this time. The introduction of the invasive species *Ruditapes philippinarum* from Asia originated the breakdown of *R. decussatus* production in several countries due to competition for the ecological space. Also, the introduction of this new species brings a new pathological problem, the Alveolata parasite *Perkinsus olsenii*. This parasite, described in Europe in 1989 after detection of high mortalities in clams from Algarve (Portugal), is now the major pathological problem of the clam species. Therefore, the aim of this PhD is to understand the mechanisms of infection of the parasite, as well as to identify molecular markers of resistance by new generation sequencing techniques. For that, clams from several heavily affected beds of Europe by the parasite were collected and diagnosed to identify the resistant and susceptible individuals. In these clams, a proteomic and a genomic approach was conducted in order to identify patterns of protein expression in haemolymph and also by DNA single nucleotide polymorphisms to try to identify molecular markers of resistance. Finally, seed from three different clam species produced in Portugal were challenged against *P. olsenii*. The interaction between parasite and host at short- and long-term post infection was analyzed via transcriptomics and also the differences on prevalence and susceptibility to disease were addressed.

This project will be the basis for the selection of new resistant families of clams. The supply of the seed to producers all along Europe could increase the production and promote the local economy of coastal regions by enhancing their incomes.

## Resumo

*Ruditapes decussatus* é uma espécie de ameijoa nativa da Europa e Norte de África que enfrenta vários problemas atualmente. A introdução da espécie invasiva *Ruditapes philippinarum* da Ásia originou a quebra de produção de *R. decussatus* em vários países devido à competição por espaço entre estas espécies. Para além disso, a introdução desta nova espécie originou um novo problema patológico, trazendo consigo o parasita Alveolata denominado *Perkinsus olsenii*. Este parasita, descrito pela primeira vez na Europa em 1989 após a deteção de altas mortalidades de ameijoas no Algarve (Portugal), é agora o maior problema patológico desta espécie. Portanto, o objetivo deste doutoramento é a compreensão dos mecanismos de infeção do parasita, bem como a identificação de marcadores moleculares de resistência através do uso de técnicas de sequenciação massiva. Para tal, ameijoas de várias áreas altamente infetadas pelo parasita ao largo da Europa foram colhidas e diagnosticadas de modo a identificar os indivíduos resistentes e suscetíveis. Uma aproximação proteómica e outra genómica foram realizadas a estas ameijoas de modo a identificar padrões de expressão proteica na hemolinfa e inclusive através de polimorfismos nucleotídeos simples no ADN a fim de identificar marcadores moleculares de resistência. Finalmente, semente de três diferentes espécies produzidas em Portugal foi submetida ao *P. olsenii.* A interação entre parasita e hospedeiro a curto e longo prazo após infeção foi analisada via análise transcriptómica e também as diferenças na prevalência e suscetibilidade à doença foram abordadas.

Este projeto pretende ser a base para a seleção de novas famílias de ameijoas resistentes. O fornecimento de semente proveniente destas famílias a produtores ao largo da Europa poderá incrementar a produção e promover a economia local de regiões costeiras.

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# Chapter 1

# General introduction

# Chapter 1 - General introduction

## 1.1. Clam production

Bivalve production is on the rise, with its production increasing every year (Figure 1). In the last ten years it increased almost 4 million tonnes, being China the top producer, followed by Chile and Japan. The bivalve production in 2021 reached 18.18 million tonnes. The three most produced bivalves are oyster, clams, and scallops, in which Manila clam leads as the most produced clam worldwide (FAO, 2024).

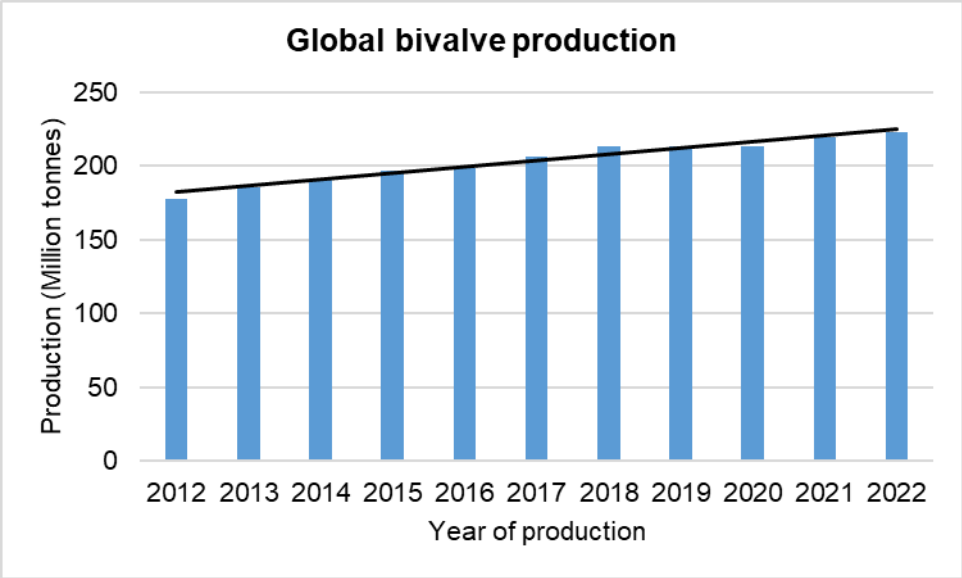


**Figure 1.** World bivalve production during the last 10 years. Data from FAO 2024.

In Europe, the top 5 most produced clams are *Ruditapes philippinarum*, *Spisula solida, Chamelea gallina*, *Ruditapes decussatus*, and *Callista chione* (Figure 2). Portugal is the most productive country of *R. decussatus* in Europe with a mean production of 3,201.12 tonnes (between 2012 and 2022, Figure 3) (FAO, 2024). Despite not produced as highly as *R. decussatus*, the other two *Veneridae* family clams (*R. philippinarum* and *V. corrugata*) also have importance for Portugal economy (Coelho, Carvalho, Goulding, Chainho, & Guerreiro, 2021; Joaquim et al., 2016). *R. philippinarum* is considered as an allochthonous species in Portugal and, therefore, its cultivation is not allowed. Only the clam extraction is permitted. This bivalve was first introduced in France in the 1970s (Flassch & Leborgne, 1994). It is known for its high adaptability to different coastal environments, competing with native European clams, such as the other two veneridae species (Marin, Moschino, Deppieri, & Lucchetta, 2003).

Clam production, nowadays, faces major challenges of inconsistent seed supply and drastic annual fluctuations of seed recruitment in wild beds (Matias, Joaquim, Leitão, & Massapina, 2009). It has also been affected by anthropogenic factors and climate change (e. g. temperature increase, low salinity, ocean acidification) and disease outbreaks (e. g. bacteria, viruses and protozoa) (Carella, Feist, Bignell, & De Vico, 2015; Velez, Figueira, Soares, & Freitas, 2016).

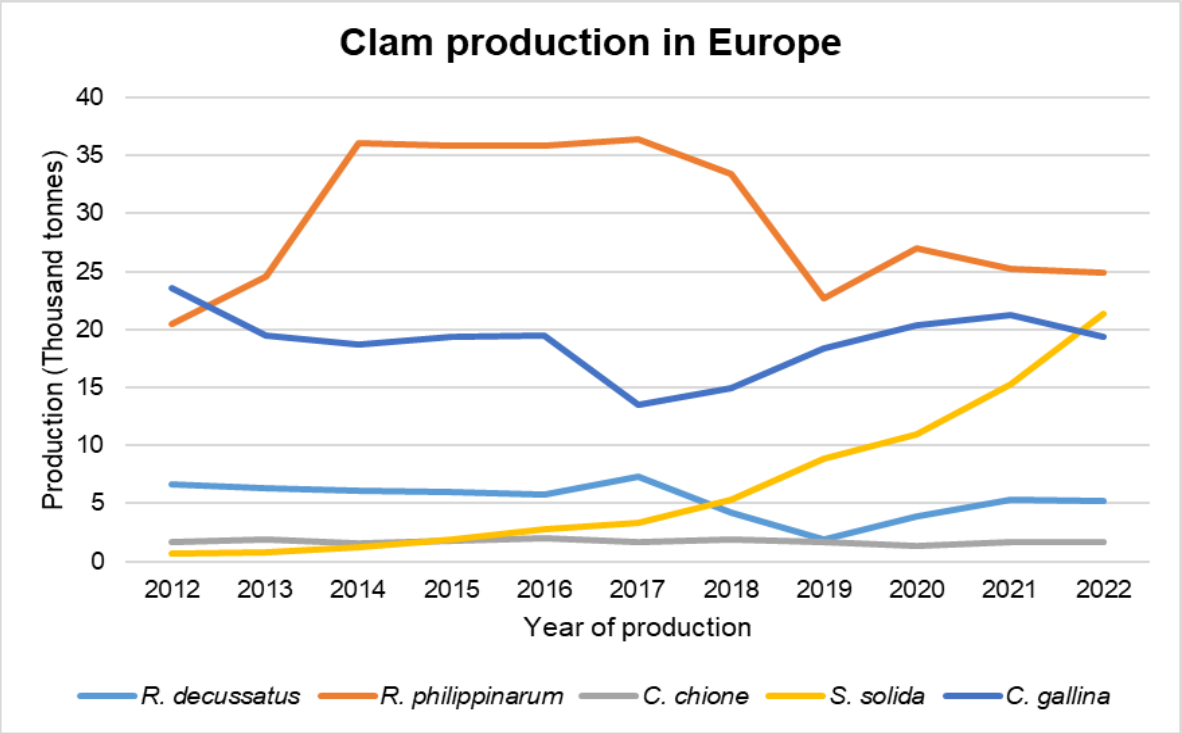


**Figure 2.** Total production of the main five species of clams in Europe during the last 10 years. Data collected from FAO 2024.

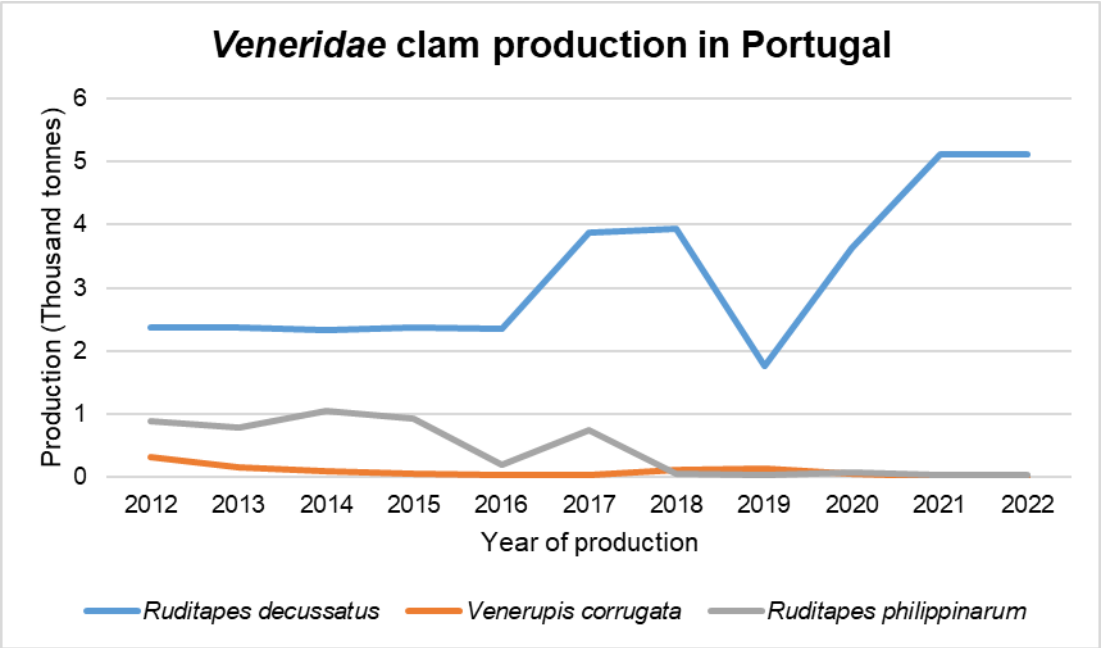


**Figure 3.** *Veneridae* clam production in Portugal during the last 10 years. Data from FAO 2024.

Stocks of *V. corrugata* have decreased in recent years due to overfishing and recruitment failure (Joaquim et al., 2016). Harvesting and aquaculture production of *R. decussatus* have being limited due to low recruitment, high mortality rates caused by *Perkinsus spp.*, and high cost of spat production in hatcheries (Fernández-Reiriz, Labarta, Albentosa, & Pérez-Camacho, 1999; Matias, 2013; Matias et al., 2009).

*R. philippinarum* is the most produced clam species in Europe due to its simplicity of production coupled with fast growth rates and high market demands (Robert et al., 2013). All the three *Veneridae* species are produced in commercial hatcheries in Europe being, however, the volume of the exotic clam seed produced at much higher rate than the autochthonous ones. Therefore, a major scientific literature for Manila clam is reported.

Selective breeding is an alternative to produce improved clam varieties with faster growth and biotic and abiotic stress resistance. Due to its ease in the manipulation of the biological cycle, the high levels of genetic variability and high fecundity, bivalves are strong candidates for selective breeding programs (Gosling, 2003). Rapid gains in growth and disease resistance has been demonstrated with the application of selective breeding programs, in which growth rate can be improved by 10% per generation and disease resistance by 15% per generation (Hollenbeck & Johnston, 2018).

Clam production is, therefore, essential to support jobs and local companies that rely on these sources, leading ultimately to sustainable local development.

### 1.2. *Perkinsus* parasite – Historical perspective

Parasite species belonging to the genus *Perkinsus* were first described in 1946 as the main cause of mass mortalities in the eastern oyster *Crassostrea virginica* in the Gulf coast of the United States. First, it was denominated as *Dermocystidium marinum* (Mackin, Owen, & Collier, 1950; Ray & Chandler, 1955). After changing the species designation to *Perkinsus marinus* and describing it for the first time, *Perkinsus*-like organisms have been found and described in various bivalve species around the world (oysters, abalones, clams, scallops, pearl oysters, cockles, mussels, etc.) from temperate to tropical zones (Figure 4). The following species to be described was *Perkinsus olsenii*, a parasite affecting the abalone *Haliotis ruber* in the South of Australia (Lester & Davis, 1981). Since then, *P. olsenii* have been reported in several mollusc species ranging different geographical zones, being detected also in the Manila clam *Ruditapes philippinarum* (Choi & Park, 1997; Kang et al., 2015; Park, Choi, & Choi, 1999) and later in different areas of the Atlantic and Mediterranean coasts in *Ruditapes decussatus*, *R. philippinarum*, *Venerupis pullastra* and *Paphia aurea* (Azevedo, 1989; Canestri-Trotti, Baccarani, Paesanti, & Turolla, 2000; Casas, Villalba, & Reece, 2002; Cigarría, Rodríguez, & Fernández, 1997; Figueras Huerta, Robledo, & Novoa, 1996; Goggin, 1992; Navas, Castillo, Vera, & Ruiz-Rico, 1992; Ordás, Gómez-León, & Figueras, 2001; Sagistrà, Durfort, & Azevedo, 1996). Following this scenario, *Perkinsus*-like parasites have been reported in various bivalve species from several European countries, including France, Italy and Spain (Ruano, Batista, & Arcangeli, 2015; Villalba, Reece, Camino Ordás, Casas, & Figueras, 2004). Azevedo (1989) described the species *Perkinsus atlanticus* in the grooved carpet shell clam (*R. decussatus*) in 1989, after mass mortalities of this species in Southern Portugal. Later, *P. olsenii* and *P. atlanticus* were found to be conspecific upon high DNA sequence similarity in both internal transcribed spacer (ITS) and non-transcribed spacer (NTS) regions of the ribosomal RNA gene cluster, taking precedence the name *P. olsenii* (Murrell, Kleeman, Barker, & Lester, 2002).

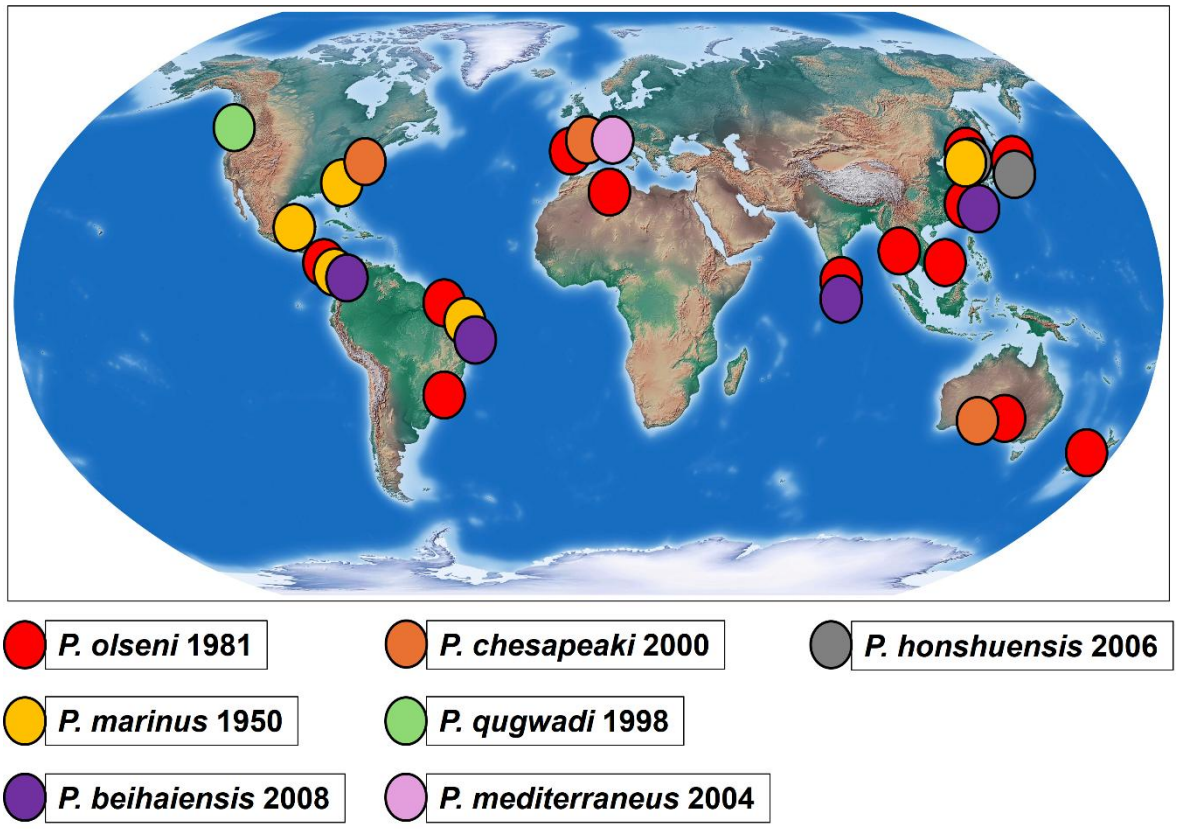


**Figure 4.** *Perkinsus* parasite species distribution worldwide.

Other *Perkinsus* species have been identified meanwhile. *Perkinsus qugwadi* was initially found in 1998 as the causative agent of high mortalities in cultured Japanese scallops *Patinopecten yessoensis* in British Columbia, Canada (Bower, Blackbourn, Meyer, & Nishimura, 1992). *Perkinsus chesapeaki* (Mclaughlin, Tall, Shaheen, Elsayed, & Faisal, 2000) and *Perkinsus andrewsi* (Coss, Robledo, Ruiz, & Vasta, 2001) were both found in the Chesapeake Bay but later it was demonstrated that *P. andrewsi* was a sympatric species of *P. chesapeaki* (Burreson, Reece, & Dungan, 2005). Later, *P. mediterraneus* (Casas et al., 2004; Ramilo, Carrasco, Kimberly S. Reece, et al., 2015) *and P. chesapeaki* were found infecting different bivalve species in Europe (Ruano et al., 2015). Lastly, *Perkinsus beihaiensis* (Moss, Xiao, Dungan, & Reece, 2008) and *Perkinsus honshuensis* (Dungan & Reece, 2006) were found in China and Japan, respectively, also affecting different bivalve species.

Today, seven species of the genus *Perkinsus* are recognized and two of them (*P. marinus* and *P. olsenii*) are included in the list of notifiable diseases of the OIE (World Organization for Animal Health).

### 1.3. *Perkinsus olsenii* as *Ruditapes decussatus* parasite – Causes and consequences

The grooved carpet shell clam (*R. decussatus*) is a bivalve species distributed along the coastal and estuarine areas of Europe and Northern Africa. This species is known to be of a high economic and social importance, especially in Portugal, Spain and Italy (Ruano et al., 2015). Portugal heads the list of top producers in Europe (FAO, 2024), being the major production originated from Ria Formosa in southern Portugal. Aquaculture production has been performed upon cultivation in intertidal parks using juveniles (10–20 mm) from natural recruitment and clams are cultured until commercial size (i.e., more than 35 mm). Since the 1980s, annual production of this species suffered a decline caused by massive mortalities in intertidal areas of Formosa and Alvor lagoons of southern Portugal, in wild clam beds, but especially in culture parks. These mortalities were associated to synergetic factors that contributed to these fluctuations, such as biotic (*P. olsenii* infection) and abiotic (environmental factors) stress and poor management methods (namely high density of clams in culture parks) (Azevedo, 1989; Ruano et al., 2015).

It has been hypothesized that *P. olsenii* species were introduced through the transfer of *R. philippinarum* from Asia (Cigarría et al., 1997; Vilas et al., 2011). The Manila clam was first introduced in France in 1972 by a commercial hatchery from US (Goulletquer, 1997). After that, it was introduced in other European countries, such as England and Spain in 1980, and Italy in 1983 and in some cases from unknown origin (Cordero, Delgado, Liu, Ruesink, & Saavedra, 2017). Since then, this species was spread along the Atlantic coast and has almost replaced *R. decussatus* in some areas. *P. olsenii* is known to have a high infection prevalence in *R. philippinarum* in Asia (Choi & Park, 2010). Moreover, this parasite has been found infecting both *Veneridae* clam species in Spain and Italy (Elandaloussi et al., 2009; Pretto et al., 2014; Ramilo et al., 2015; J. A. F. Robledo et al., 2000; Ros & Canzonier, 1985; Villalba et al., 2004, 2005). In some places of France, in 1990, it was already pointed out a *Perkinsus spp*. infection prevalence of 75 % and 80 % in *R. decussatus* and *R. philippinarum*, respectively (Goggin, 1992). The high infection prevalence of *Perkinsus* in Europe creates biotic stress in the host up to a point that can lead to death directly or indirectly under unfavourable conditions (salinity and temperature changes - abiotic stress). This makes *P. olsenii* a threat for clam culture and production.

### 1.4. *Perkinsus* infection conditions

As mentioned above for *R. decussatus*, different synergetic factors may contribute for infection prevalence of *P. olsenii*. This may be the case of adverse environmental factors that cause physiological stress, which can compromise the defence mechanisms and increase the severity of diseases such as perkinsosis. Water temperature and salinity, presence of specific pollutants and the composition of sediment, where the clams live in, are abiotic factors that have been taken into consideration (de Montaudouin et al., 2010). For instance, it has been shown that the optimal range of temperature and salinity for sporulation of *P. olsenii* is between 24 and 28 ºC and between 25 and 35 ‰, respectively (Auzoux-Bordenave, Vigario, Ruano, Domart-Coulon, & Doumenc, 1996), and that it is inhibited at temperatures below 10 ºC, slows at salinities between 10 and 15 ‰ and it is not possible at salinities below 6 ‰ (Ahn & Kim, 2001; Azevedo et al., 1990; Ruano et al., 2015). Moreover, the organic matter content of the sediment where clams live in plays a crucial role in its survival. If high levels of organic carbon are present, as well as high nitrate and ammonia concentrations, anoxic conditions may happen which can reduce and compromise their respiratory capacity when coupled with lesions in the gills caused by parasite infection, making them less resistant to low oxygen levels (Binias et al., 2014). Lastly, the size/age of the host suggests a relationship with higher mortality rates caused by perkinsosis. In individuals with size larger than 34 mm, a higher mortality rate in *R. decussatus* infected with *P. olsenii* has been observed (Villalba, Casas, López, & Carballal, 2005). This might be explained by the need of higher filtration capacity in larger individuals and that older animals have potentially spent more time in contact with the parasite (Ruano et al., 2015).

### 1.5. *Perkinsus* life cycle

Parasite cells of *Perkinsus spp.* are usually present in the connective tissue in different organs, especially in gill and digestive gland, of the affected individuals. Therefore, it was assumed that infection was initiated with the entrance of biflagellated zoospores through the gills (Villalba et al., 2004). However, zoospores can infect all exposed epithelia, showing a preference for the digestive tract and in particular the epithelium of the oesophagus (Ruano et al., 2015). On the other side, Allam et al., (2013) revealed that *P. marinus* can infect the Eastern oyster by accumulation of infective stages of the parasite in the mantle. Trophozoites appear on the affected tissues of the live host as a spherical cell with a large vacuole and a peripheral nucleus. These cells

encompass vegetative proliferation by successive bipartitioning which by cell wall rupture will release daughter cells. The daughter cells later enlarge forming mature trophozoites. The parasite cells continue to be infective after host death developing then into hypnospores (resistant phase) and after release to the sea water and under optimal conditions then developing into pre-zoosporangia which can produce thousands of free and motile invasive biflagellated cells, the zoospores (Figure 5) (Auzoux-Bordenave et al., 1996; Azevedo et al., 1990; Villalba et al., 2004). Moreover, parasite transmission can still occur without the death of the host through dispersion of trophozoites into water or through infected faeces (Bushek, Ford, & Chintala, 2002).

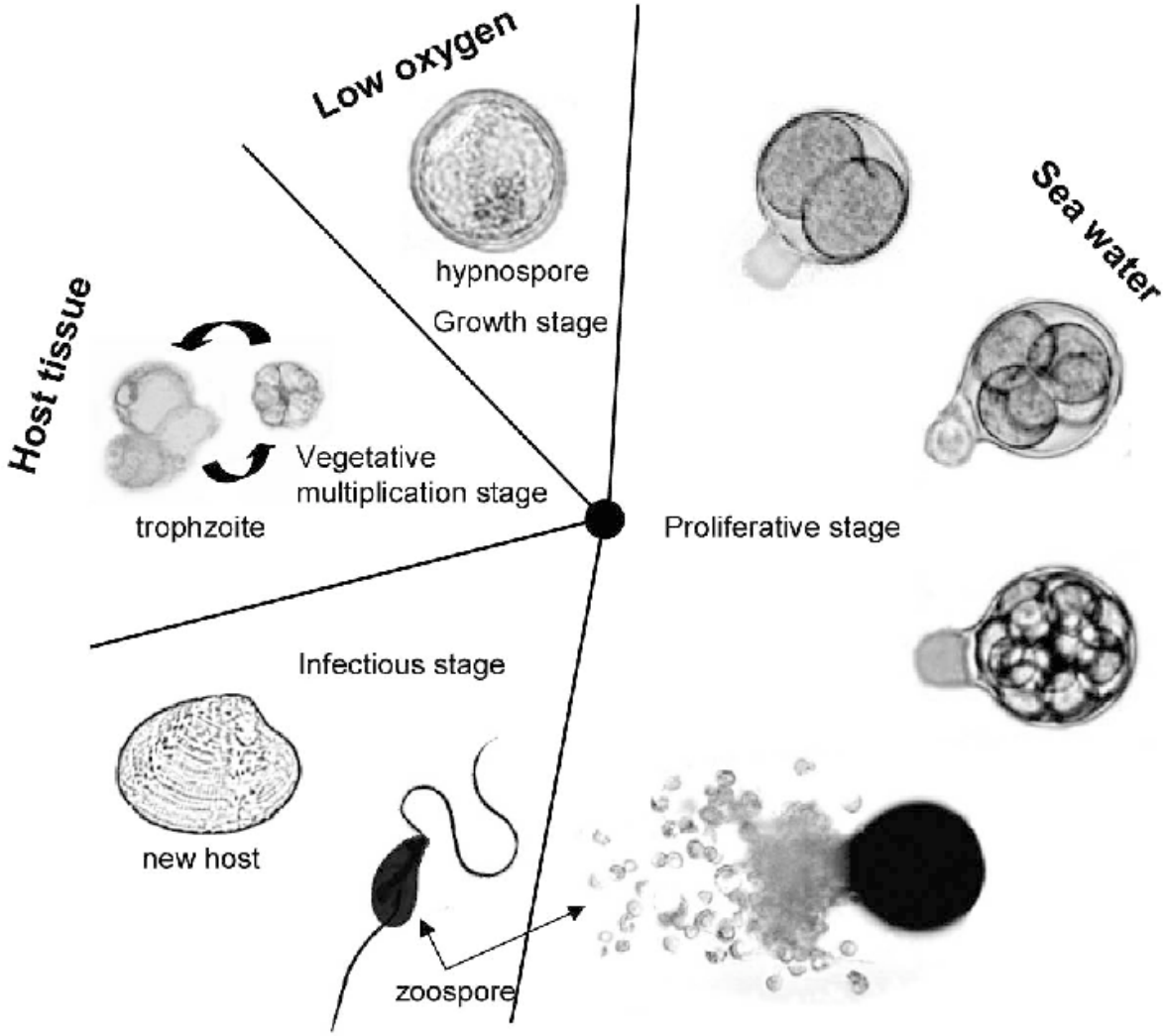


**Figure 5.** *Perkinsus* life cycle (Choi et al., 2010).

### 1.6. *Perkinsus* effects on clams

Infection by *Perkinsus* parasite can be detected by different manifestations. The first one is the increased number of gaping and poorly buried clams in the sediment in culture parks or in natural beds located in intertidal areas. Also, several gross lesions can be detected, being the most significant and destructive one the presence of milky white nodules (1–2 mm diameter, Figure 6) in gill lamellae, that can also appear in the mantle and foot (Park & Choi, 2001). This parasite can affect large areas of the gill epithelium and occasionally more than 60% of the whole respiratory surface, which might compromise the functionality of this organ. Other gross lesions are pale coloration of the digestive gland, although these are not specific to perkinsosis (Ruano et al., 2015). Effects of parasite infection on the host are energy imbalances that may slow growth and result in poor condition index of market size individuals. Moreover, these imbalances may originate deficiencies in energy reserves necessary for successful gametogenesis (Casas & Villalba, 2012; Villalba et al., 2004). These negative effects can result in lower recruitment and scarcity of seed for grow out purposes. In *R. decussatus*, high infection by *P. olsenii* causes inhibition of gametogenesis, reduction of the number of clams with ripe gonad and a significant decrease of storage tissue and gametes, whereas haemocytic infiltration of the gonad increases. In addition, it affects sexual maturation, the viability of the gametes and fecundity (Fernández-Boo et al., 2023; K. Il Park et al., 2006). In this clam species, clearance rate tends to diminish, giving place to an increase in oxygen consumption. Parasite energy consumption in market-sized clams with heavy infections may exceed the energy available for growth (Casas, 2002; Casas & Villalba, 2012; Leite et al., 2004; Villalba et al., 2011), especially under conditions of high temperature and low food availability, such as during the summer season. In short, infection originates such a host weakening that it becomes highly difficult for affected individuals to overcome any other adverse conditions (Villalba et al., 2004, 2005).

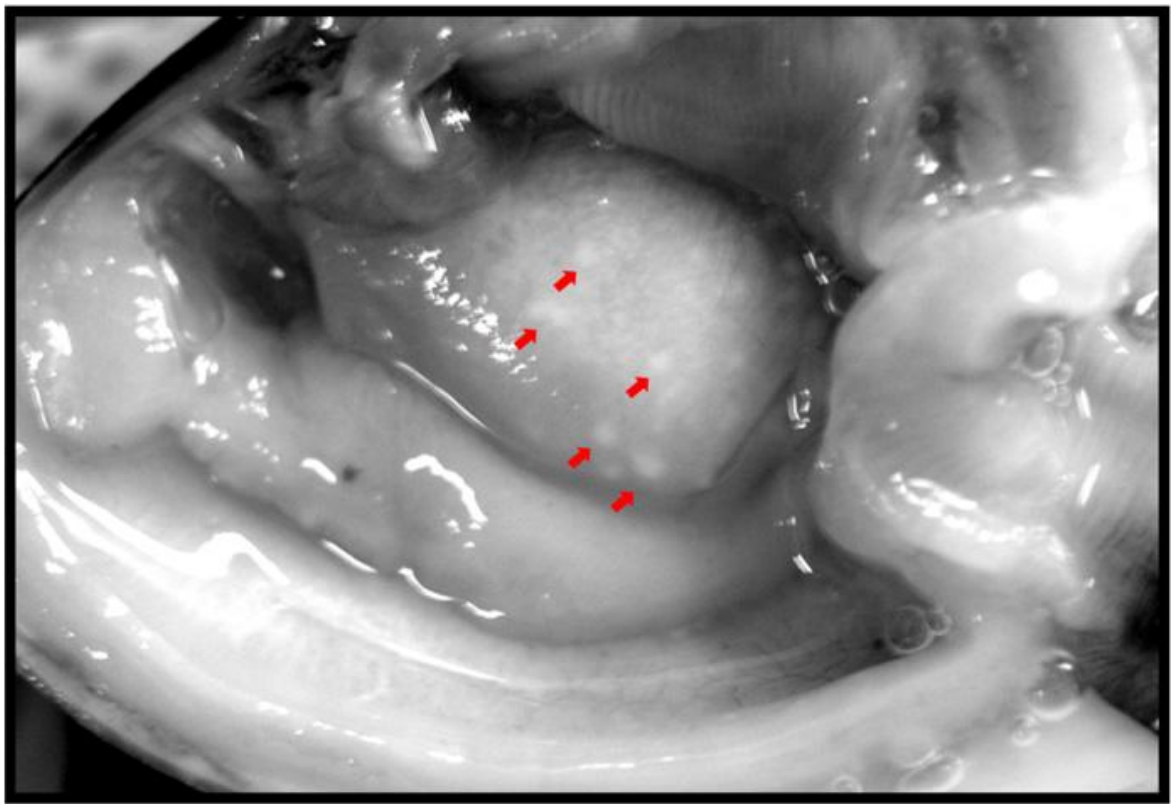

**Figure 6.** Macroscopic observation of milky white nodules (red arrows) on the surface of *R. philippinarum* body (Donaghy, Lambert, Choi, & Soudant, 2009).

After an infection by *Perkinsus* spp., an acute reaction occurs. The primary defensive mechanisms start manifesting in bivalves as an intense cellular reaction, namely by a strong haemocyte infiltration, hemocytosis, involving mostly granulocytes, in different tissues and sometimes associated with parasitic forms during the trophozoite phase (Ruano et al., 2015). As already mentioned, large granuloma-like formations surrounded by haemocytes can be observed in connective tissue at the base of the branchial filaments, occupying all the filament and also part of the lamella (Chagot, Comps, Boulo, Ruano, & Grizel, 1987). At a cellular scale, these lesions give poorly defined contours with a necrotic centre where abundant haemocytes, parasitic forms and cells of the supporting tissue are in a process of autolysis. Also, isolated trophozoite parasite forms in continuous schizogony, both free and intra-haemocytic, and abundant degenerative cellular forms located on the marginal areas on the lesion can be observed. Lastly, these typical lesions include severe disruptions of the cellular organization, autolysis and abundant necrotic material, both inside the injury and in close proximity to it (Azevedo, 1989; Chagot et al., 1987). Another cystic formation can be observed in other tissues, such as in the mantle, labial palps, and vesicular connective tissue or in muscular tissue of the foot. Inside these, multicellular parasite stages are surrounded by haemocytes, and fibrocystic layers are organized in an encapsulation process. Infiltered haemocytes

initiate a mechanism of cellular defence in which synergic effects of lysosomal enzymes and reactive oxygen species (ROS) inactivate and kill the parasite cells by lysis (Soudant, Chu, & Volety, 2013). This encapsulation process can evolve later to a chronic lesion formed by an organized capsule which either progress to the destruction of the disease agent or sequesters latent forms that can be reactivated under more favourable conditions for the pathogen, reaching again high parasitic loads due to its great proliferative capacity (Allam & Raftos, 2015).

### 1.7. Bivalve defence system

The high economic importance of mollusc has been giving space for attention to infectious diseases that affects the production. Consequently, there has been a growth interest in the study of bivalve immunity and in the exploration of mechanisms to fight and resist infectious agents (Bachère et al., 2004; Chu, 1988; L. Song, Wang, Qiu, & Zhang, 2010). This growth of interest follows 3 correlated objectives: i) Understand the bases of bivalve immunity; ii) Create data-based information to produce disease-resistant families of cultured bivalves; iii) Undercover new bioactive compounds for biotechnological applications. The actual literature shows progress in the identification and description of the functional components of the bivalve immune system. However, this information is still fragmentated and based on trial studies making it uneasy to distinguish between immune responses in terms of infection and in a particular defence-related factor as an indirect, general, stress response (Allam & Raftos, 2015).

Any factor that induces a quantifiable pathological effect linked to functional damage of cells tissues and/or organs of an organism is defined as a disease agent. Cause and effects of a disease may be specific and nonspecific, in which specific corresponds to a single cause that originates a single effect and nonspecific to the same effect originated by multiple causes. Moreover, several causes usually cooperate to induce diseases, being some of them necessary (essential to cause the disease) and others predisposing (opening the way for the action of the necessary cause). These causes can be both intrinsic (mutations at gene, chromosome, and genome levels) and extrinsic (physical and chemical insults and pathogens) to the organism (Carella et al., 2015). Environmental stressors, contaminants and physiological factors may also compromise the natural defences of the host. For instance, severe disease-associated mortalities may occur when environmental conditions aid parasite replication (Chu, Soudant, & Lund, 2003). This is the case of *Perkinsus* outbreaks where parasite spatial distribution

clearly depends on climate warming (Cook, Folli, Klinck, Ford, & Miller, 1998). This pathogen is a good example of the complex interactions between host immunocompetence, parasite resilience and the environment (Hofmann & Ford, 2012; Z. Wang et al., 2012).

### 1.7.1. Bivalve defence system: General organization

The defensive system of bivalves is divided by different layers of physical and biological barriers. The first outer barrier is physical and comprises the shell that supports and protects the soft tissue from biological and physico-chemical insults. The next physical barrier is constituted by the skin and the respective mucosal layer that covers it and which entraps intruders facilitating their elimination via ciliary activity. The inner barrier is biological and guaranteed by the effectors of the innate immunity (Allam & Raftos, 2015; Wang et al., 2013; T. Zhang et al., 2014).

Mucosal secretions covering bivalve pallial organs are thought to be the first line of defence in these animals extending the defensive role of mucus beyond that of a simple physical barrier. These secretions are known to contain a wide range of antimicrobial factors, like hydrolytic enzymes (lysozyme and peptidases) and agglutinins (e. g. lectins) (Allam & Espinosa, 2015; Allam & Paillard, 1998; Espinosa, Perrigault, Ward, Shumway, & Allam, 2009; Jing, Espinosa, Perrigault, & Allam, 2011). Also the mucus layer could be highly specific of each species being related within the major pathogens than each species faces (Fernández-Boo et al., 2020).

Haemolymph is a circulatory fluid in the clam tissues that plays several important roles in bivalve physiology, such as internal defence, gas exchange, osmoregulation, nutrient distribution, waste elimination and hydrostatic pressure for structural support of organs (Gosling, 2015). It contains haemocytes and different humoral factors that are secreted by haemocytes and circulate in the plasma. Haemolymph is colourless due to lack of respiratory pigments in most bivalves, showing some species presence of haemoglobin pigments (Bao et al., 2016; Klein, 2017; Terwilliger, Terwilliger, & Schabtach, 1978; Weber & Vinogradov, 2001; Yager, Terwilliger, Terwilliger, Schabtach, & van Holde, 1982).

The internal defence system of bivalves rely mostly on their innate immune response, composed of cellular and humoral components (Allam & Raftos, 2015; L. Song, Wang, Qiu, & Zhang, 2010). Haemocytes are the cells present in the haemolymph vessels and sinuses as well as throughout soft tissues. Together with plasma factors, these cells

comprehend the internal defence of the organism which can be activated by the detection of pathogens via humoral and haemocyte-bound recognition factors. These factors trigger the production of cytokines that mediate the recruitment of additional haemocytes, activation of phagocytosis and the production and/or release of a wide range of antimicrobial compounds. Despite invertebrates do not contain an adaptive immune memory system, they exhibit different recognition factors such as thioester-containing proteins (Rodrigues, Brayner, Alves, Dixit, & Barillas-Mury, 2010), C-type lectins (Wang et al., 2013) or the Down syndrome cell adhesion molecule, Dscam (Ng, Chiang, Yeh, & Wang, 2014), in which some have been associated with mucosal surfaces in interactions with microbes. An innate immune memory has been demonstrated in several invertebrate species that lack acquired immune systems. This innate immune memory, called immune priming, may involve both humoral and cellular arms of invertebrate immune system, being phagocytosis of haemocytes linked to the specific recognition of antigens in primed hosts. Nonetheless, the priming effect and its impact on immune modulation still requires further clarification (W. Yang et al., 2021).

Haemocytes are not only effectors of the internal defence, but they also participate in other functions and are present in outer layers. In addition to immune protection, they participate in biomineralization and shell deposition (Fisher, 2004; Mount, Wheeler, Paradkar, & Snider, 2004; Wilbur, 1964) and can be associated with mucosal secretions covering pallial surfaces (Allam & Raftos, 2015; Takatsuki, 1934). Due to its multi-function, these cells can also move bi-directionally via trans-epithelial migration (Allam & Raftos, 2015).

### 1.7.2. Bivalve defence system: Cellular components

As justified above, haemocytes represent the backbone of the bivalve immune system (Anderson & Good, 1976; L. Song et al., 2010), are multi-potent and contribute to several biological functions (Fisher, 1986). Functions performed by these cells are phagocytosis and encapsulation of pathogens, wound healing, food digestion and transport of nutrients, reproduction, excretion, shell formation, and production and secretion of humoral factors (Allam & Raftos, 2015). It has been suggested that haemocytes are produced from differentiation of connective tissue cells, however there is still a big gap of information regarding their ontology in molluscs (Pila et al., 2016; Smolowitz, Miosky, & Reinisch, 1989). Produced haemocytes are classified according to morphological appearance and ‘‘granularity’’ of their cytoplasm. These can be divided into various sub-types of granular haemocytes, according to their granular and vacuolar

contents. Generally, they are divided into two broad categories: Granulocytes and agranulocytes. Granulocytes are 10-20 mm in size and contain cytoplasmic granules, while agranulocytes lack cytoplasmic granules and are 4-6 mm in size (Gosling, 2015; de la Ballina, Maresca, Cao, & Villalba, 2022). Granulocytes can be classified as distinct basophilic, acidophilic, and mixed forms in Giemsa/May–Grünwald stained preparations. Also, other sub-types of granulocytes have been identified in different mollusc species, including clams (Chang, Tseng, & Chou, 2005; Kuchel, Raftos, Birch, & Vella, 2010; de la Ballina et al., 2022; López, Carballal, Azevedo, & Villalba, 1997; Pipe, Farley, & Coles, 1997). Agranulocytes are sub-divided into three sub-classes: Blast-like cells, basophilic macrophage-like cells and hyalinocytes (Figure 7) (Hine, 1999; de la Ballina et al., 2022). Granulocytes are phagocytic cells and perform roles of phagocytosis and encapsulation of pathogens and other particles, such as algae or cellular debris (Gosling, 2015). In contrast, agranulocytes do not participate in defensive responses, appearing to assist in wound healing aggregation processes (Aladaileh, Nair, Birch, & Raftos, 2007; Gosling, 2015).

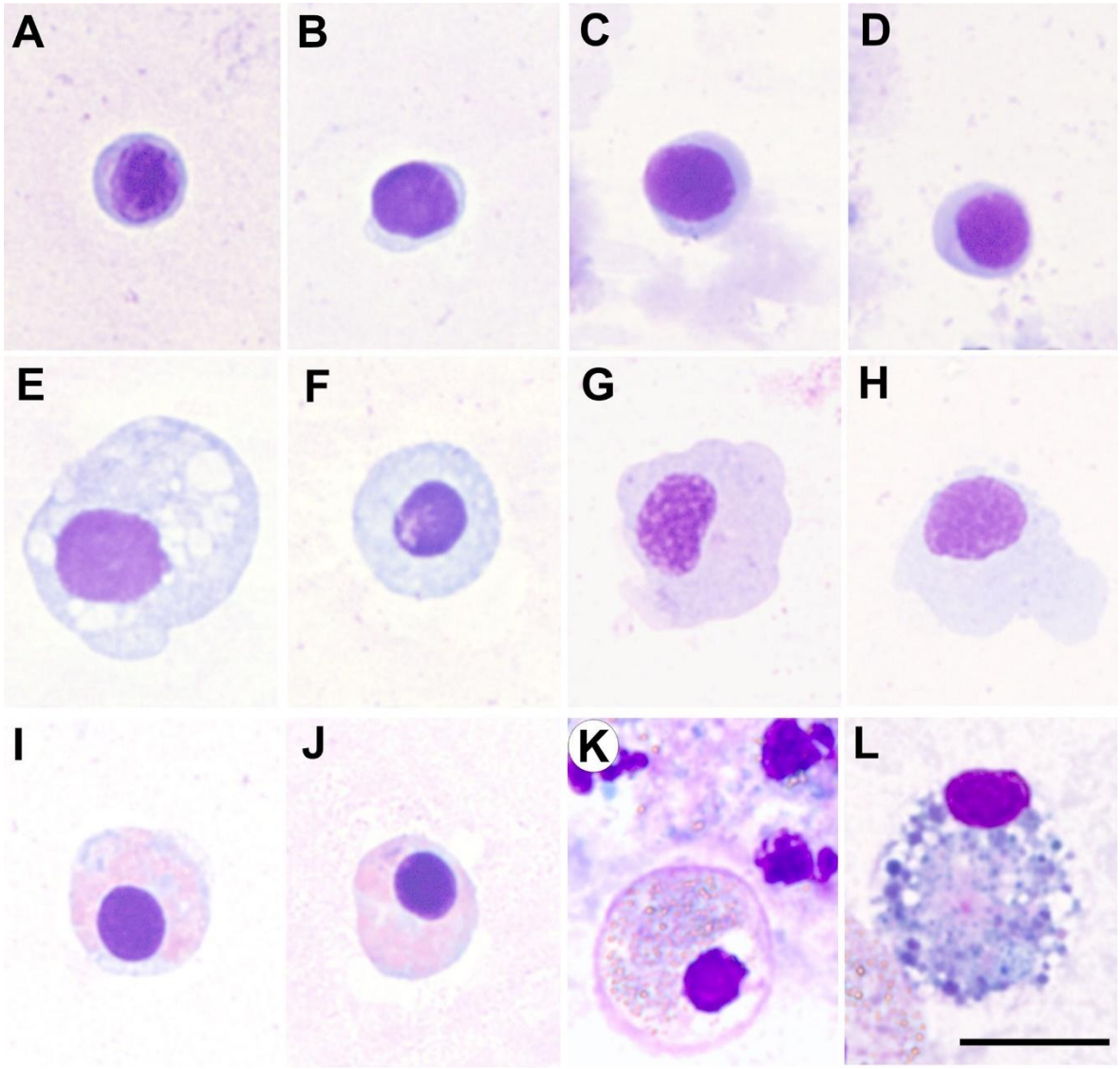


**Figure 7.** Different types of bivalve haemocytes from several bivalve species. (A-D) Blast-like cells (*Ruditapes decussatus*, *Ruditapes philippinarum*, *Aequipecten opercularis* and *Mimachlamys varia*, respectively). (E-H) Hyalinocytes (*R. decussatus*, *R. philippinarum*, *A. opercularis* and *M. varia*, respectively). (I-K) Eosinophilic granulocytes (*R. decussatus*, *R. philippinarum* and *Ostrea edulis*, respectively). (L) Basophilic granulocyte of *O. edulis* (de la Ballina et al., 2022).

As mentioned above, chemotaxis corresponds to recruitment of haemocytes through movement towards pathogen products activated by humoral factors (Cheng & Howland, 1979; Howland & Cheng, 1982). Afterwards, attachment of haemocytes to the particles recognized as non-self and phagocytosis happens. Phagocytosis can be performed by different types of haemocytes, being the granulocytes the most phagocytic (Kuchel et al., 2010). Shortly after engulfment, and as mentioned by Allam & Raftos, (2015), membrane bound granules within the haemocyte migrate to the surface of the phagosome and fuse with the phagosome membrane resulting in the deposition of granular contents onto the surface of the ingested material, followed by a rapid decrease

in the pH of the phagosomes. This intracellular defence mechanism normally neutralizes the ingested pathogen throughout the use of a diverse chemical machinery, such as the production of reactive oxygen species (ROS), nitric oxide, as well as release of a wide range of antimicrobial factors and hydrolytic enzymes packed in lysosomes (Anderson, 1994; Anderson, Paynter, & Burreson, 2016; Buggé, Hégaret, Wikfors, & Allam, 2007; Gourdon, Guérin, Torreilles, & Roch, 2001; Kuchel et al., 2010; Pipe, 1992; L. Song et al., 2010). If this process fails or when cells are too large to be engulfed, a high number of haemocytes are recruited, the intruder surrounded and encapsulated, and a release of cytotoxic products for extracellular killing happens (Dove, Bowser, & Cerrato, 2004; Loker, Bayne, Buckley, & Kruse, 1982). A granuloma might surge if haemocytes accumulate in thin layers around the pathogen.

Despite phagocytic activity of haemocytes possess a high importance for the innate immune response, the various activities of epithelial cells lining mucosal surfaces aids as well to microbial homeostasis at these pathogen portals of entry. Epithelial cells produce and secrete a broad range of bioactive compounds that are secreted to mucus. Moreover, practically all mucosal epithelia of bivalves are capable of endocytosing biotic and abiotic particles and colloids, including epithelial cells lining the external and internal surfaces of the mantle (Bevelander & Nakahara, 1966; Mclean, 1980; Nakahara & Bevelander, 1967), the gill (George, Pirie, & Coombs, 1976; Johnson & Le Pennec, 1995), the foot (Grenon & Walker, 1982; Ryder & Bowen, 1977), or epithelia lining different sections of the digestive gland and gut (Yonge, 1926, 1928, 1935). Phagocytic activity at these tissues has a dual function of nutrition/defence through the increase of food uptake and digestion and by keeping pathogens in check and limiting infections. Phagocytosed particles are exposed to toxic reactive oxygen species (ROS) produced by the host cells during the respiratory burst associated with phagocytosis, finally being neutralized by antimicrobial compounds and hydrolytic enzymes present in phagosomes/phagolysosomes. However, pathogens that are able to inhibit or resist intracellular killing and digestion can initiate infection in epithelial cells (Fryer & Lannan, 1994).

Other mechanisms involved in homeostasis of innate immune system are autophagy and apoptosis. Macroautophagy is used to substitute the term autophagy due to acting as a bulk process that captures large portions of cytosol proteins or sequesters organelles like mitochondria or peroxisomes (Deretic & Levine, 2009), which then fuse with lysosomes. This mechanism is also involved in other key processes, including microorganism elimination, adaptation to starvation, and cell death (Mizushima, 2005). Autophagy is activated by multiple environmental stimuli including changes in salinity,

hypoxia, malnutrition, and toxic substances, and is often associated with changes of the lysosomal membranes, especially in the cells of the digestive gland (Chabicovsky, Klepal, & Dallinger, 2004; Moore, Viarengo, Donkin, & Hawkins, 2007). It comprises three main steps (Deretic, 2006): i) the initiation step, where phagophores are formed by the association of membranes thought to come from the endoplasmic reticulum, trans-Golgi, and late endosomes (Glick, Barth, & Macleod, 2010); ii) the elongation step, where phagophore's double-membrane is extended and encloses cytoplasmic components to form an autophagosome; iii) the fusion between the autophagosome and a lysosome, leading to the degradation of the enclosed cytoplasmic components by lysosomal enzymes. Degradation products then return to the cytoplasm, where they can be reused to build new molecules or in metabolism (Mizushima, 2007). These different steps are regulated by various proteins that are encoded by autophagy-related genes. Picot et al., (2019) demonstrated that autophagy occurs in hemocytes of *C. gigas* and can be modulated by molecules known to modulate autophagy in other organisms such as carbamazepine or $NH_4Cl$ (Moreau et al., 2015). Autophagy occurs in two of the three lines of defence activation in response to oxidative damage of environmental origin (Moore, 2008), including autophagy of organelles and/or proteins damaged by oxidative stress and autophagy with cell death when oxidative damage is not further mitigated (Moore, 2008; Moore, Allen, & Somerfield, 2006). Finally, two categories of autophagic vacuoles are commonly described (Eskelinen, 2004, 2008; Eskelinen & Kovács, 2011; Ylä-Anttila, Vihinen, Jokitalo, & Eskelinen, 2009): i) the intermediate/early autophagic vacuoles or autophagosomes, and ii) the degradative/late autophagic vacuoles, also corresponding to autolysosomes or amphisomes. Both types were associated to be present in the haemocytes of *C. gigas* (Picot et al., 2019).

Apoptosis, a type of programmed cell death that produces changes in cell morphology and in biochemical intracellular processes without inflammatory reactions (Elmore, 2007; Norbury & Hickson, 2001), is a fundamental process essential for an organism's development and homeostasis in the immune system of both vertebrates and invertebrates. It constitutes an important immune response that can be triggered by a variety of stimuli, including cytokines, hormones, toxic insults, viruses, and protozoan parasites (Kiss, 2010; Sokolova, 2009; Terahara & Takahashi, 2008). Components belonging to apoptosis pathways conserved throughout evolution, although several differences between lower animals and vertebrates have been described (Metzstein, Stanfield, & Horvitz, 1998; Tittel & Steller, 2000). Caspases are molecules involved in the transduction of the death signal and are responsible for many of the biochemical and morphological changes associated with apoptosis (Hale et al., 1996). It is of current

knowledge that caspases are activated through two major apoptotic pathways: i) the extrinsic or death receptor pathway (Bridgham, Wilder, Hollocher, & Johnson, 2003) and ii) the intrinsic or mitochondrial pathway (Estévez-Calvar, Romero, Figueras, & Novoa, 2013; Zhang, Li, & Zhang, 2011b), being evidence for a third alternative one: the perforin/granzyme pathway (Trapani & Smyth, 2002). Moreover, lectin-like molecules are capable of activating cell death by interaction with proteins involved in the control of apoptosis. Apoptotic cell death caused by hormonal responses to environmental stress normally result in a depletion of haemocyte populations, leading to immunosuppression and susceptibility to several microbial pathogens (Raftos, Kuchel, Aladaileh, & Butt, 2014). The programmed cell death mechanism of molluscs seems to be similar in complexity to that from vertebrates but also has unique features possibly related to their recurrent exposure to environmental changes, pollutants, pathogens, and to the sedentary nature of some stages in the life cycle of bivalves and gastropods (Coles, Farley, & Pipe, 1995; Gagnaire, Thomas-Guyon, Burgeot, & Renault, 2006; LeGrand, 1997; Naimo, 1995; Sokolova, Evans, & Hughes, 2004). Other molecular mechanisms are involved in the modulation of apoptosis in bivalves, such as those induced by oxidative damage from thermal stress and heavy metals. These include the activation of p38-MAPKs and Rho, which usually follows the activation of caspase-3 and alteration in the levels of Hsp70. Lastly, the intrinsic pathway of apoptosis can be variously activated by xenobiotics of distinct nature through the overproduction of ROS, as well as by heavy metals that induce genotoxic damage in the cells of the digestive gland (Kiss, 2010).

### 1.7.3. Bivalve defence system: Interaction between immune cells and infectious agents

Chemoattraction of haemocytes to infectious agents is common in mollusc species but not generic, as some pathogens do not induce any chemotactic response (Howland et al., 1982), while others induce a chemokinetic (increase in random, non-directional movement) response (Alvarez, Friedl, & Roman, 1995; Schneeweiß & Renwrantz, 1993). In addition, haemocytes can as well move in a directional movement to an infection site (focal inflammation), being a common and non-systematic cellular response in bivalve molluscs. However, it is still unclear whether haemocytes primarily migrate to products released by infectious agents, intact and damaged haemocytes, and other host cells already on site (via release of cytokines and other cellular products) or perform both movements (Allam & Raftos, 2015). This combination originates a dynamic and sometimes temporary change in the number of circulatory haemocytes and a general

increase in haemocyte infiltration in affected tissues (Maloy, Ford, Karney, & Boettcher, 2007). General infiltration or systemic response of haemocytes is more usual in systemic infections like those caused by, for instance, *Perkinsus spp.* (Villalba et al., 2004). Therefore, focal and systemic responses of haemocytes might result in fluctuations in total and differential haemocyte counts in the circulatory system as a combined result of movement of haemocytes from and to various compartments, and also by haematopoiesis, degranulation, or lysis and degradation of haemocytes in animals heavily infected with some pathogens such as intra-haemocytic parasites (e.g. *Bonamia ostreae*, *Haplosporidium nelsoni* or *Perkinsus spp.*) (Cochennec-Laureau et al., 2003; Ford, Kanaley, et al., 1993; Villalba et al., 2004).

Haemocytes secrete a wide range of antimicrobial peptides (AMP) and other cytotoxic substances into the haemolymph which together with other non-specific humoral defence factors (e.g. lectins, lysosomal enzymes) form the humoral components of bivalve innate immunity (Gerdol et al., 2018; Zannella et al., 2017). There are two interaction pathways throughout which haemocytes interact with pathogenic agents: i) directly via membrane bound receptors, ii) indirectly via dissolved recognition factors present in the surrounding environment (plasma, extrapallial fluid, mucus).

Lectins are carbohydrate-binding proteins that have been identified for host-pathogen interactions in different taxa (Vasta, Ahmed, Tasumi, Odom, & Saito, 2007). These proteins were also found in the surfaces of haemocytes and are thought to aid for host resistance to infection. Toll-like receptors (TLR) are another group of membrane-bound molecules known to serve as pathogen recognition receptors (PRR). These molecular receptors are underdeveloped in invertebrates. Instead, there were found proteins containing the same domains and functions as these receptors. TLRs are not well characterized in invertebrates, in comparison to superior organisms. They are just identified as a broad protein spectrum that possess common characteristics (Takeda & Akira, 2005; W. Wang et al., 2018; Y. Zhang et al., 2013). Moreover, TLRs are capable of selective recognition and to initiate responses against a broad spectrum of different and complex microbe-associated molecular patterns (MAMP) (Arancibia et al., 2007). They have been identified in various molluscan species, are highly expressed in circulatory haemocytes, and are widely distributed in the tissues (Perrigault, Tanguy, & Allam, 2009; Philipp et al., 2012; Tanguy, Guo, & Ford, 2004; Toubiana et al., 2013). Nevertheless, it has been reported that they are also highly expressed in pallial organs, especially in the mantle, which are in direct contact with external environment (Perrigault et al., 2009; Zhang, Li, & Zhang, 2011a). TLRs, together with superoxide dismutase-like molecule, complement C1q homologue, two inhibitors of NF-kB and a suppressor of

cytokine signalling have been shown to be differentially expressed in *C. gigas* responding to mortality caused by virus and bacterial infections (Fleury & Huvet, 2012). The process by which recognition factors circulating in the haemolymph recognise and bind to the pathogens, facilitating phagocytosis by haemocytes, is called opsonization. There are already several factors identified in this process as well as the opsonic effect of whole bivalve plasma on the phagocytic activity of haemocytes (Canesi, Gallo, Gavioli, & Pruzzo, 2002). For instance, Bulgakov et al. (2004) reported the presence in Manila clams (*R. philippinarum*) of a lectin that binds the hypnospores of *P. olsenii*. Also, a broad range of lectins, galectins and other proteins with microbe-binding site recognition have also been identified in this clam species (Kim et al., 2008).

Haemocyte phagocytic activity towards a pathogen is determined by the dissolved and/or membrane surface bound factors. They phagocytose and digest a broad range of opportunistic microbial pathogens (Canesi et al., 2002), however, some obligate parasites have developed ways to control neutralization by host immune cells. This avoidance of phagocytosis by haemocytes is mediated by cell surface components and chemical modification of these epitopes can lead to phagocytosis (S. E. Ford, Ashton-Alcox, & Kanaley, 1993). Despite this avoidance indicates an efficient defence strategy for some pathogens, others have developed alternative means allowing them to survive phagocytosis and pursue development inside phagocytes. This resistance to digestion is thought to be frequently linked to the pathogen ability to inhibit lysosome-phagosome fusion as shown in the case of *Bonamia sp.* interactions with oyster haemocytes (Hine & Wesney, 1994). Parasite survival inside the haemocytes of the host hence become protected from the various antimicrobial agents present in the plasma (Balquet & Poder, 1985; Chagot et al., 1992; Engelsma, Culloty, Lynch, Arzul, & Carnegie, 2014; Hine et al., 1994). Another strategy to parasite survival is the regulation of host reactive oxygen species (ROS) production by the fabrication of antioxidant chemicals such as superoxide dismutase (SOD) (Ahmed, Schott, Gauthier, & Vasta, 2003; Schott, Pecher, Okafor, & Vasta, 2003; Schott & Vasta, 2003). When haemocytes are unable to eliminate the pathogen via phagocytosis or other means, apoptosis of these cells happens. However, inhibition of apoptosis can still happen through the regulation of ROS production, which are involved in the initiation of caspase-independent apoptosis (Bröker, Kruyt, & Giaccone, 2005; Fleury, Mignotte, & Vayssière, 2002; Kroemer & Martin, 2005). This immune cell hijacking capacity from parasites allows them to survive and spread along host tissues, being an important aspect of pathogen survival on the first stages of infection at mucosal surfaces. The odds of parasite survival increase with the occurrence of local and systemics movements of haemocytes as these can serve as vehicles for

adapted pathogens and result in the use of transepithelial migration of haemocytes as a means to increase infection (Allam & Parvez, 2007). This is the case of *Perkinsus marinus*, which can modulate and suppress apoptosis, helping the parasite survive inside the host (Hughes, Foster, Grewal, & Sokolova, 2010; Soudant, Chu, & Volety, 2013; Sunila & LaBanca, 2003). Finally, *Perkinsus marinus* has been found to survive phagocytosis (La Peyre, F. E. Chu, & Vogelbein, 1995; La Peyre, Yarnall, & Faisal, 1996).

Other described innate defence process in invertebrates is melanisation of pathogens (Cerenius, Jiravanichpaisal, Liu, & Soderhall, 2010). Haemocytes can induce the formation of melanotic nodules that limit the spread of infectious microorganisms or damaged tissues. The phenoloxidase pathway is responsible for the regulation of melanin biosynthesis, may represent a key intracellular killing system in various bivalve species, including *R. decussatus* (Aladaileh et al., 2007; Kuchel et al., 2010; Muñoz, Meseguer, & Esteban, 2006). Therefore, melanisation is not only a normal process of shell formation on molluscs but also an important mechanism involved in wound healing and encapsulation of non-self-entities. The melanisation process has been found in all pallial epithelia of molluscs, in particular the external epithelium of the mantle, being a major trait of several microbial infections affecting this organ. Either on the extrapallial fluid or in the haemocytes contained within or in the mantle, high quantities of melanin are produced to wall off the pathogens, leading to considerable rearrangements of shell matrix deposition (Ford & Borrero, 2001; Paillard, 2004). Beyond this, tyrosinases have been identified as key enzymes in the melanogenic pathway that catalyses the production of melanin (Allam et al., 2014). Melanised pathogens can follow a biomineralization mechanism which embeds them in new calcified shell layers. This process is denominated nacrezation and occurs during pearl formation (Addadi & Weiner, 1997; Bevelander & Nakahara, 1969; Stunkard & Uzmann, 1958). Perculin is one of the biomineralization-related proteins that are responsible for nucleating the growth of calcium carbonate crystals in molluscs (Blank et al., 2003; Weiss, Kaufmann, Mann, & Fritz, 2000) and it is thought to play a dual role as an organic support for biomineralization and as a potential defence molecule against pathogenic microorganisms (Wang, Lee, & Lee, 2008).

### 1.7.4. Bivalve defence system: Immune recognition factors

Pattern recognition receptors (PRRs) are usually associated with host defence response against infection. These correspond C-type lectins, fibrinogen-related proteins (FREPs), complement homologues, Toll-like receptors (TLR), scavenger receptors (SRCR), lipopolysaccharide and b-1,3-glucan-binding proteins (LGBP), and peptidoglycan-recognition proteins (PGRPs). PRRs genes are highly expressed in the digestive glands of bivalves, suggesting that their filter feeding life history may have biased pattern recognition toward the digestive system as a first line of defence. Also, these molecules appear to be up regulated after challenge with pathogens, pointing towards functions related to the immune response (Allam et al., 2014; de Lorgeril, Zenagui, Rosa, Piquemal, & Bachère, 2011; McDowell et al., 2014; Rebeca Moreira et al., 2012; Venier et al., 2011; L. Zhang, Li, Zhu, Zhang, & Guo, 2014). For instance, C-type lectins were found to be commonly expressed in *R. philippinarum* infected with *Perkinsus* spp. parasites (Kang et al., 2006).

Lectins are a group of humoral PRRs that are largely characterized in bivalves. They are identified by their capacity of agglutination, as happens in *C. gigas* against *P. marinus* infection (La Peyre, F.-L. E. Chu, & Meyers, 1995; Romestand, Corbier, & Roch, 2002). Several immunologically active lectins appear to be expressed in bivalves. In *R. philippinarum*, seven different lectins were identified after *Perkinsus olsenii* infection, in which different subsets of these lectins were expressed against this parasite in comparison to *Vibrio tapetis* infection (Kang et al., 2006). Another lectin was induced in *C. farreri* by MAMPs lipopolysaccharide (LPS) and b-glucan, but not by peptidoglycan (PGN) (H. Zhang et al., 2010). This suggests that bivalve lectins have different degrees of selectivity in their target specificities. Some lectins are associated with specific immunity and only responds in the presence of specific cues. This is the case of C-type lectins associated to mucosal immunity that only responds to the presence of pathogens at the water-tissue interface (Jing et al., 2011).

Galectins are β-galactoside binding proteins well characterized as extracellular bivalve lectins. They perform different roles in development and immune responses, comprehending in bivalves defensive PRRs (Kim et al., 2008; X. Song et al., 2010; Tasumi & Vasta, 2007; Yamaura et al., 2008). The activity of these proteins seems to be subverted by some pathogens to facilitate infection (Tasumi et al., 2007; Vasta, 2012). For instance, *P. marinus* seems to mimic the surface carbohydrates to which the *C. virginica* galectin is specific, facilitating phagocytosis and infection of oyster haemocytes. Also, the gene expression of this *C. virginica* galectin is significantly down regulated

during *P. marinus* infection, indicating that the host may be attempting to control parasite uptake and proliferation by decreasing the expression of its co-opted receptor (Wang et al., 2010).

Peptidoglycan-recognition proteins (PGRPs) are PRRs that have been identified and characterized in oysters and scallops (Itoh & Takahashi, 2008, 2009; Ni et al., 2007). These proteins appear to incorporate goose-type lysozyme and defensin-like domains, and additional domains with functions broader than MAMP recognition. These complexes confer bacteriolytic activity and peptidoglycan binding capacity being, however, not induced by LPS (Itoh et al., 2009; Ni et al., 2007).

Thioester bearing proteins (TEPs) are produced by a gene family homologous to mammalian complement components and the protease inhibitor, α2-macroglobulin (Nonaka, 2011). They have been characterized in *C. farreri* and *R. decussatus* (M Prado-Alvarez, Rotllant, Gestal, Novoa, & Figueras, 2009; Zhang et al., 2007, 2009). In *C. farreri*, it is expressed under microbial challenge, pinpointing for its role in host defence. Homologues of other components of the vertebrate complement cascade have also been pointed to be involved in bivalve immune responses. These have been primarily identified as C1q domain-containing proteins (C1qDC), a highly diverse group of molecules displaying important functions in pathogen recognition and in tailored responses to different pathogens (Gerdol et al., 2011; Gestal, Pallavicini, Venier, Novoa, & Figueras, 2010; Li, Yu, Zhao, Su, & Li, 2011; L. Wang et al., 2012). These proteins were found to be differentially expressed in the grooved carpet shell infected with *P. olsenii* (Garcia et al., 2022; Leite et al., 2013) and in *R. philippinarum* infected with *V. tapetis* (Allam et al., 2014). In short, different bivalve C1qDC are able to bind distinct bacteria and yeast and improve their phagocytosis by haemocytes.

Lipopolysaccharide and β-glucan binding proteins have been characterized in oysters, clams, and scallops (Itoh, Kamitaka, et al., 2010; Liu et al., 2014; J. Su et al., 2004; D. Zhang et al., 2010). Their expression is regulated by bacterial challenge and LPS (D. Zhang et al., 2010). These proteins may also mediate both haemocyte-related functions via integrin and phenoloxidase activation (Itoh, Kamitaka, et al., 2010).

Fibrinogen-related proteins (FREPs) are lectin-like proteins that incorporate fibrinogen and immunoglobulin superfamily related domains (Adema, Hertel, Miller, & Loker, 1997; Hanington & Zhang, 2010; Zhang & Loker, 2004; Zhang, Zeng, & Loker, 2008). In *Biomphalaria glabrata*, FREPs may function as an opsonin and favour the encapsulation of parasites (Hanington et al., 2010). Also, they appear to be significantly

up regulated in haemocytes exposed to a range of different MAMPs, suggesting that FREPs may be involved in pathogen recognition.

### 1.7.5. Bivalve defence system: Antimicrobial peptides and proteins, hydrolytic enzymes and protease inhibitors

Antimicrobial peptides (AMP) are small (less than 10 kDa) cationic peptides that, in molluscs, have been mostly characterized in mussels and oysters, comprehending these defensin-like peptides. Defensins represent the most widespread group of AMP among bivalves and also a potent animal defence against a wide range of bacteria and fungi (Lehrer & Ganz, 2002), being some present in epithelial cells associated with mucosal tissues (Allam et al., 2014; Gonzalez et al., 2007; Gueguen et al., 2006; Rosa et al., 2011; Schmitt, Gueguen, Desmarais, Bachère, & de Lorgeril, 2010). Other antimicrobial proteins include histones, lysozymes and larger molecules such as the bactericidal/permeability-increasing protein (BPI) (Dorrington, Villamil, & Gómez-chiarri, 2011; Seo, Lee, Nam, & Park, 2013; Xue et al., 2010; Yang et al., 2019). Nonetheless, the repertoire of antimicrobial compounds is larger than previously reported maintaining limited the information of their effective role in fighting infections (further discussed). A substantial number of studies deduce a role for these compounds in host defence based on changes in the levels of corresponding transcripts after experimental challenge. Moreover, the low number of bacterial diseases affecting adult bivalves indicates that these compounds may be efficient in neutralizing pathogens (Allam & Raftos, 2015). Several AMPs has been observed to keep their expression unchanged following microbial challenge indicating that they are constitutively expressed giving a primary layer of defence against waterborne pathogens (Gueguen et al., 2006). In parallel, other AMPs appear to be up regulated in response to natural or experimental infections, despite some have no direct effect on the primary etiologic agent of these infections (Adhya et al., 2012; Allam et al., 2014; Perrigault et al., 2009; Rosa et al., 2011; Zhao et al., 2007). This is the case of the histone protein H4 that was present in enriched libraries from *C. virginica* infected with *P. marinus* but it had no activity against the parasite (Dorrington, Villamil, & Gómez-chiarri, 2011). Therefore, the main function of AMPs may be the maintenance of microbial homeostasis and supply of a wide-range protection against opportunistic bacterial infections.

The bivalve defence system comprises more than just antimicrobial peptides. Integrative components, such as enzymes (e.g., lysozyme, proteases) that degrade

specific substrates of pathogen cell walls (bacterial peptidoglycan) represent additional arsenal of bivalve immune system. Usually, this defence involves proteins rather than AMP, which is the case for *P. marinus* immune response in mussel (Anderson & Beaven, 2001). For instance, bactericidal/permeability-increasing proteins display high selective cytotoxicity towards Gram-negative bacteria (Boman, 1995) and represent an important family of antimicrobial proteins. Other antimicrobial compounds that display a wide activity spectrum are members of the lysozyme families (N-acetylmuramide glycanhydrolase). Different lysozymes demonstrated antimicrobial activity in distinct tissues, such as mantle and its secretions, digestive gland, plasma and other mucosal tissues (Allam et al., 2014, 1998; Allam, Paillard, Howard, & Pennec, 2000; Allam, Paillard, & Auffret, 2000; Chu & La Peyre, 1993; Itoh, Okada, Takahashi, & Osada, 2010; Itoh & Takahashi, 2007; Xue et al., 2007, 2010). They have shown also to increase antibacterial activity of AMP and to have a hypothetical dual role in immunity and digestion (Haug, Stensvåg, Olsen, Sandsdalen, & Styrvold, 2004; McHenery, Allen, & Birkbeck, 1986; McHenery, Birkbeck, & Allen, 1979). Nonetheless, studies have shown that lysozyme levels in bivalve plasma may respond to environmental cues rather than, or in parallel to, pathogen exposure (Chu & La Peyre, 1989; Paillard, Allam, & Oubella, 2004). Proteases are other proteins present in the mucus and mantle tissues of bivalves that change their profile in response to infection (acid protease, zinc metalloprotease, serine protease, cathepsin L protease) (Allam et al., 2014; Brun, Ross, & Boghen, 2000). Beyond proteases, protease inhibitors are also present in bivalve tissues and secretions. These include serine and cysteine protease and metalloprotease inhibitors and, despite its role in bivalve defence remains unclear, it is suggested that they may provide protection against proteases produced by invading microorganisms (McDowell et al., 2014; Rebeca Moreira et al., 2012; Nikapitiya, McDowell, Sohn, & Gómez-Chiarri, 2013; La Peyre, Xue, Itoh, Li, & Cooper, 2010; Xue et al., 2006; Yu et al., 2011). This may happen through the inactivation of pathogen proteases, limiting their proliferation and growth rates or indirectly facilitating their neutralization via host immune proteins. They may also protect the host from the deleterious effect of endogenous protease activities over-represented in response to pathogen exposure (Phillips et al., 2004). This is the case of *C. virginica* serine protease inhibitors that represent an integrative component of defence against *P. marinus,* where it has been shown that a mutation in the promoter region of these protease inhibitors is correlated with resistance to the parasite (Yu et al., 2011). Lastly, the regulation of protease inhibitors in bivalves in response to microbial challenge is not systemic, highlighting a differential response to infection across different tissues. For instance, infected clams with brown ring disease present serine protease

inhibitors transcripts with up regulation in the extrapallial fluid and mantle and down regulation in the haemolymph (Allam et al., 2014).

## 1.8. New generation biomarkers search tools

### 1.8.1. Proteomics

Proteomics is the study of the whole protein content of any sample including cells, tissues, biofluids or organisms. The proteome changes over time, between cell types and in response to external stimuli (Fliser et al., 2007). Hereupon, proteomics is a snapshot of the protein environment at a given time (Graves & Haystead, 2002), providing information about the expression level of relevant genes and post-translational modifications (Rodrigues, Silva, Dias, & Jessen, 2012). Different gel-based and gel-free proteomics techniques are used in aquaculture (Rodrigues et al., 2012), being liquid chromatography coupled with mass spectrometry (LC-MS) the most currently used. This tool allows the analysis of thousands of peptides in a single sample, making it highly effective in protein quantification and post-translational modification analysis (Rodrigues, Campos, Kuruvilla, Schrama, & Cristobal, 2017). High-throughput proteomics is a powerful tool that has become widely used in bivalve research to identify different responses to stress conditions, and that could be complementary to transcriptomic analysis as not always the expression of a gene led to a differential concentration of a protein, because protein expression is also influenced by epigenetic, post-transcriptional and post-translational modifications (Gómez-Chiarri, Guo, Tanguy, He, & Proestou, 2015).

Proteomics have been employed to analyse the response of bivalves to different stressors including pathogens (Corporeau, Tamayo, Pernet, Quéré, & Madec, 2014; Ji et al., 2013; Vaibhav, Thompson, Raftos, & Haynes, 2017) and environmental stress (Campos, Tedesco, Vasconcelos, & Cristobal, 2012; Tomanek, 2014). These analyses have been also performed on plasma proteins which allowed to uncover their roles in immune responses, heavy metal transport, antioxidation, wound repair and shell mineralization (Itoh et al., 2011; Koutsogiannaki & Kaloyianni, 2010; Xue et al., 2012), suggesting the usefulness of haemolymph for proteomics. Several studies of haemolymph´s proteome on different species in response to stress and diseases have been performed providing novel insights into the role of haemocytes in bivalve innate immunity (Chen et al., 2011; Franco-Martínez et al., 2018; Jiang et al., 2018; Novoa et al., 2016; Thompson et al., 2012). The aim of these studies were principally the

identification of proteome maps, protein biomarkers, and characterization of specific functions of proteins or immune processes. For instance, the bases of tolerance/resistance to bonamiosis in *O. edulis* and *C. gigas* were addressed using a proteomics approach (Cao et al., 2009; Fernández-Boo et al., 2020). Also, the combination of proteomics with transcriptomics allowed to provide insights into the phagocytic killing of *C. gigas* haemocytes (Jiang et al., 2018), revealing a number of antimicrobial-related biological processes of phagocytes, together with oxidation-reduction and lysosomal proteolysis processes. Development of proteomics to understand the molecular mechanisms underlying immune responses and identification of protein biomarkers for environmental pollution and disease resistance in marine bivalves are important for biomonitoring and stock assessment in aquaculture.

The field of proteomics in aquaculture is facing some challenges, being one the limited information at genomic level (genomes and transcriptomes) of the species which hinders protein identification and proteomic data interpretation and results in a high number of unknown proteins (Rodrigues et al., 2012). Integrative omics analysis, such as the parallel use of proteomics, transcriptomics and metabolomics is a useful tool to help understand the state of an organism or a response to a stress (Li et al., 2021; Tan et al., 2024).

### 1.8.2. Genomic tools

Quick advances in sequencing technology have resulted in a wealth of genomic resources for key species with the potential to significantly improve hatchery-based selective breeding of molluscs (Houston et al., 2020). The development of genomic resources for selected species and the application of these tools toward selective breeding are key processes for the improvement of bivalve aquaculture. Unfortunately, the current state-of-the-art is between the developed genomic resources and their application for selective breeding (Hollenbeck et al., 2018).

The decreasing costs of next-generation DNA sequencing has turned it possible to sequence whole genomes of non-model species in a cost-effective manner. A whole genome allows for mapping of genetic markers to a specific location, providing the opportunity to search for nearby genes or genetic elements to identify causative mutations associated with a particular trait. Mollusc genomes are highly polymorphic whose assembly would be facilitated by using highly inbred individuals with reduced heterozygosity (Wang et al., 2017; Zhang et al., 2012) or the usage of modified assembly techniques to account for excess heterozygosity (Li et al., 2017; Takeuchi et al., 2016).

One important aspect retained from genome sequencing in molluscan species is the observation of lineage-specific expansions in gene families related to immune and stress response (Guo, He, Zhang, Lelong, & Jouaux, 2015; Li et al., 2017; Mun et al., 2017; Takeuchi et al., 2012, 2016; Wang et al., 2017; Zhang et al., 2012), a key evolutionary mark related to survival for sessile organisms living in frequent heterogeneous conditions. An example is the C1q gene in *R. philippinarum*, where it is responsible for pathogen detection and activation of the complement system in the innate immune response (Mun et al., 2017).

The majority of economically important traits in aquaculture production are polygenic, being supported by a number of genomic regions explaining a small proportion of the variance of the trait, named quantitative trait loci (QTL). The mapping of QTLs is performed by the identification of genetic marker alleles that show non-random association with phenotypes and by positioning these loci in the genome (Lynch & Walsh, 1998). Once a QTL is identified, individuals can be selected in breeding programs based on the identified markers that segregate associated with the desirable phenotypes (Lande & Thompson, 1990), resulting in marker-assisted selection (MAS), that could be performed in non-directly quantifiable traits, such as disease resistance (Sonesson, 2005). QTL mapping in molluscs has relied on linkage-based methods , in which one or more families are genotyped and scored for a trait of interest, and the co-segregation of markers and trait phenotypes are observed among offspring and families(Lynch et al., 1998). Genome-wide association studies (GWAS) has been an alternative to identify marker-trait associations that are in linkage disequilibrium with QTL, in which it is performed at the population level rather than at family level (Hirschhorn et al., 2005). This technique has the advantage of providing more precise locations of QTL, despite requiring many more loci than linkage-based QTL. QTL studies have focused mostly on growth but also in other phenotypes, including disease resistance (Bai, Han, Liu, Li, & Li, 2016; Harrang et al., 2015; Jiao et al., 2014; Zhong, Li, Guo, Yu, & Kong, 2014) or environmental stressors such as heat response or low salinity tolerance (Du et al., 2024; Marshall et al., 2021).

Incorporating marker information into breeding programs has led to an increase in the accuracy of predicted breeding values, which then caused a proportional increase in the response to selection (Martinez, 2007). Since selection is based on marker genotypes, MAS is useful for traits that are difficult to measure on breeding candidates (Sonesson, 2007), and especially when the markers are linked to large-effect QTL (Robledo et al., 2017). Genomic selection uses a genome-wide scale genotyping to

improve the genomic relatedness between individuals over the pedigree information (BLUP) to render a more precise estimation of breeding values (GBLUP). The procedure assumes that genetic markers (usually SNPs) are genotyped at sufficient density to estimate genomic relatedness between candidates to selection and to detect putative associations of QTL in linkage disequilibrium with closely linked markers (Meuwissen, Hayes, & Goddard, 2001) and all the genetic variances can be used as a complex trait (Meuwissen, Hayes, & Goddard, 2013). The difference relies in the usefulness of each technique, whereas MAS is suitable for identification of large-effect loci, genomic selection is suited for improvement of traits that are under the control of many loci with small effect (Robledo et al., 2017). Both techniques still need to be applied to breeding programs of molluscs. Reduced-representation sequencing techniques (e.g. genotyping by sequencing or RAD-seq) are an important part of the genomics-based selection in molluscs. These techniques are suitable for large marker genotyping across the genome, enough for precise pedigree reconstruction, estimation of heritabilities, genetic map construction, identification of QTL and estimation of genomic breeding values (GBLUP)(Palaiokostas et al., 2016; Robledo et al., 2017).

Despite the alternative techniques used in genomic approaches for breeding in molluscs, several challenges arise. These include high rates of larval mortality, high incidence of marker segregation distortion, self-fertilization, lack of both high-quality annotated reference genomes and comprehensive studies about the genetic structure of important production traits. Also, the low economic value of individual animals and the high cost of genotyping are other challenges in the implementation of genomic selection. Various ideas have been proposed to reduce the cost of genotyping, such as the construction of target multispecies SNP-chips (Gutierrez et al., 2017), pooling of test individuals' DNA (Sonesson, Meuwissen, & Goddard, 2010), usage of low-density genotyping for within-family genomic selection (Lillehammer, Meuwissen, & Sonesson, 2013), and imputation of genotypes using a combination of low- and high-density genotyping (Tsai et al., 2017). Surpassed these challenges, faster rates of genetic improvement in cultured molluscs can be obtained together with optimized control of inbreeding.

### 1.8.3. Single nucleotide polymorphisms

Single nucleotide polymorphisms (SNPs) are variations at single base pair locations in the genome. They are normally bi-allelic (have two alleles), codominant, and exist in

exons, introns, regulatory regions, and in intergenic regions, being suitable for description of both selectively neutral and adaptive genetic variation. SNPs may be selectively neutral when appear in introns or in intergenic regions. Nonetheless, they may have effects on phenotypes and affect fitness when they appear in coding or regulatory regions, providing, in both cases, information on population structure, demographics and adaptation. SNPs are replacing other types of genetic markers, such as microsatellites, due to their larger density across the genomes and low genotyping cost. There are single locus methods for genotyping targeted SNPs and multiple locus techniques for genotyping SNPs at many locations throughout the genome or within targeted genomic regions (Allendorf et al., 2022). SNPs are the most abundant type of polymorphism in the genome, occurring at every 200-500 bp in many wild populations (Brumfield, Beerli, Nickerson, & Edwards, 2003; Morin, Luikart, Wayne, & the SNP workshop group, 2004). In the case of molluscs, SNPs are even more abundant in the genome, appearing at every 60 bp in coding regions and at every 40 bp in non-coding regions (Sauvage, Bierne, Lapègue, & Boudry, 2007).

Next generation sequencing (NGS) technologies have made affordable the genotyping of thousands of SNPs across the whole genome at low prices. Restriction Site associated DNA sequencing (RAD-seq) methodologies are a good example of the emerging technologies for the rapid development of SNPs to be used in breeding programs or population genomics studies (Robledo et al., 2017). Despite RAD-seq can be performed with or without a reference genome, a more robust genotyping is achieved if a whole genome assembly is available. This technology has aided to construct highly dense genetic maps for QTL identification in mollusk species, such as Chinese scallop (*Chlamys farreri*; Gómez-Chiarri et al., 2015; Jiao et al., 2014) or common cockle (*Cerastoderma edule*); (Hermida et al., 2022), but it has been also used for population genomics studies in mollusk, either to disclose the environmental factors shaping the genome of common cockle (Vera et al., 2022, 2023), or to identify genomic regions and genetic markers associated with resilience to parasites in common cockle (Pampín et al., 2023; Villalba et al., 2023). Furthermore, RAD-seq technologies have been the basis to identify SNPs across the whole genome to be selected for their inclusion in SNP-chips, a more robust genotyping technology, but only developed for species with more genomic resources and usually with higher production importance or ecosystemic value, such as *Crassostrea gigas* and *Ostrea edulis* (Gutierrez et al., 2017). This chip was eventually used to identify a main genomic region associated with resilience to the parasite *Bonamia ostreae* using a population genomics approach (Kamermans et al., 2023; Sambade et al., 2022; Vera et al., 2019).

### 1.8.4. Transcriptomics

Transcriptomics provide a snapshot of the total transcripts present in a cell, reflecting the genes that are actively expressed in that cell, at a given time. As the study of transcriptomes, it is the sum of all coding and non-coding RNA transcripts produced by the genome during development or under specific circumstances (Lowe, Shirley, Bleackley, Dolan, & Shafee, 2017). Transcriptomics uses distinct techniques to measure the expression of genes in different cell populations that are affected by different treatments, diseases, or environmental factors at different time points. These include serial/cap analysis of gene expression (SAGE/CAGE), expressed sequence tag (EST), suppression subtractive hybridization (SSH), microarrays and RNA sequencing (RNA-Seq). The most widely used nowadays is RNA-seq, a tool that takes advantage of deep-sequencing technology combined with computational methods to quantify and map transcriptomes (Lowe et al., 2017; Wang, Gerstein, & Snyder, 2009). RNA-seq takes advantage over the other techniques as it presents a higher throughput, sensitivity, accuracy, long read lengths and no actual need of prior knowledge of the organism's genome (Su et al., 2014).

The identification of genome sequences and entire genomes for several bivalve species has revealed many immune-related genes. This allowed to perform targeted transcriptomics studies to identify gene expression and biochemical pathways in different tissues and organs of bivalves in response to biotic and abiotic stress (Gómez-Chiarri et al., 2015). RNA-seq have been used to characterize the whole transcriptome profile of haemocytes from several species, revealing functionally over-represented immune pathways such as signal transduction, complement cascades, PRRs (e.g., lectin, b-glucan recognition proteins, peptidoglycan recognition proteins, toll-like receptors), apoptosis (e.g., IAP, BAX, BAC-2), antimicrobial molecules (e.g., AMPs, lysozyme, protease and protease inhibitors) and others (Dong, Chen, Lu, Wu, & Qi, 2017; Hasanuzzaman et al., 2017; Rebeca Moreira et al., 2012; Pauletto et al., 2014; Tanguy et al., 2013; L. Zhang et al., 2014). The usage of this tool allowed to uncover many sequences from molecules never described in bivalves (Rebeca Moreira et al., 2012), being also used to investigate specific molecular mechanisms involved in cell cycle which have been found to be highly expressed during the development of the disease (Siah, McKenna, Berthe, Afonso, & Danger, 2013). Transcriptomics have also been used to evaluate the response to environmental stressors in bivalves (Detree, Núñez-Acuña, Roberts, & Gallardo-Escárate, 2016; Ertl, O'Connor, Papanicolaou, Wiegand, & Elizur, 2016; Granger Joly de Boissel et al., 2017), in which cathepsins, heat shock proteins,

peroxiredoxin and superoxide dismutase were found to be expressed in the haemolymph, suggesting their important role in haemocyte functioning and innate immunity.

Transcriptomics have significantly improved our understanding of the role of haemocytes in defence mechanisms. The extensive characterization of immune-related genes allows us to obtain genetic markers associated with pathogen susceptibility, which could be used in breeding selection programs through MAS. Haemocyte transcriptomes offer a valuable resource for posterior studies to clarify the defence mechanisms of bivalves against pathogens and environmental stressors which could provide new strategies for management of diseases in bivalve aquaculture and environmental monitoring (Nguyen & Alfaro, 2020). Despite several bivalve species have their complete genome sequenced, the lack of genomic data from other commercially important species presents a bottleneck for transcriptomics in bivalve aquaculture (Li et al., 2017; Mun et al., 2017). Bivalve genomes are highly polymorphic and present high levels of heterozygosity and complexity, making this a challenge for genome assembling. This then results in a high number of unknown transcripts represented in bivalve transcriptomes. Moreover, the lack of knowledge regarding the function of most genes remains a difficulty in data interpretation (Nguyen et al., 2020). Lastly, the cost of RNA-seq is decreasing and in the future will allow to perform studies at large scale.

### 1.9. Biomarkers of tolerance to *P. olsenii* in *R. decussatus*

Several epizootic and molecular studies have already been performed in an effort to identify biomarkers for tolerance diseases (Canesi et al., 2002; R. Moreira et al., 2012; Olafsen, 1995; Ordás, Ordás, Beloso, & Figueras, 2000). Modulation of the immune response processes, like apoptosis, complement system, response to stress (M. Prado-Alvarez, Gestal, Novoa, & Figueras, 2009) and inflammation (Simão et al., 2020), as well as, metabolism regulation (Fernández-Boo, Villalba, & Cao, 2016), parasite recognition and cell migration (Leite et al., 2013) were pinpointed as occurring on the different tissues of the grooved carpet shell in either natural or trial conditions. In addition, the interaction between host and parasite shows a combined expression of genes related to redox and glucose metabolism, protease activity, apoptosis, and iron metabolism in a closely related species (*R. philippinarum*), indicating a crosstalk event between host and parasite (Hasanuzzaman et al., 2017, 2018, 2020). Also, new studies based on SNPs indicates that tolerance to *Perkinsus olsenii* infection in *R. philippinarum* clams is a trait

with high heritability (>50%) suggesting that tolerant clams could have better tools to counteract the infection (Smits et al., 2020).

## 1.10. Aims of the thesis

The main objective of this work is to search for molecular markers and mechanisms of resistance in *Ruditapes decussatus* to *Perkinsus olsenii* infection, while uncovering and describing the processes of host-pathogen interaction. More specifically, the main objectives are:

1. Search for protein biomarkers of tolarance to *P. olsenii* in *R. decussatus* by LC/MS-MS (**Chapter 2**).
2. SNP analysis and identification of genes related to disease resistance/tolerance in *R. decussatus* (**Chapter 3**).
3. Clam seed performance following *P. olsenii* infection in the three most produced clam species in Portugal: *R. decussatus*, *R. philippinarum* and *V. corrugata* (**Chapter 4**).

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# Chapter 2

# Search for new biomarkers of tolerance to *Perkinsus olsenii* parasite infection in *Ruditapes decussatus* clams

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# Chapter 2 - Search for new biomarkers of tolerance to *Perkinsus olsenii* parasite infection in *Ruditapes decussatus* clams

## 2.1. Introduction

The grooved carpet shell clam (*Ruditapes decussatus*) is a bivalve species distributed along the coastal and estuarine areas of NE Atlantic areas and Mediterranean Sea. This species is known to be of a high economic and social importance, especially in Portugal, Spain and Italy (Ruano et al., 2015). Production of this species suffered a decline caused by massive mortalities which were associated to synergetic factors that contributed to these fluctuations, such as biotic (diseases), abiotic (environmental factors) stress, poor management methods and intensive culture of the introduced species *Ruditapes philippinarum* (Cruz et al., 2020; Ruano et al., 2015). After mass mortalities of this species in Southern Portugal (Azevedo, 1989), the parasite species *Perkinsus olsenii* in *R. decussatus*, being nowadays, one of the major issues for clam production (Villalba et al., 2004).

Different synergetic factors contribute for infection prevalence and intensity of *P. olsenii*. This may be the case of adverse environmental factors that cause physiological stress, which can compromise the defence mechanisms and increase the severity of diseases such as perkinsosis. Water temperature and salinity, presence of specific pollutants and the composition of sediment where the clams live in are abiotic factors that have to be taken into consideration (de Montaudouin et al., 2010). These factors are worsening due to climate change and anthropogenic pollution which can reduce and compromise their respiratory capacity when coupled with lesions in the gills caused by parasite infection, making them less resistant to low oxygen levels (Binias et al., 2014). Lastly, the size/age of the host suggests a relationship with infection prevalence and intensity of perkinsosis. *R. decussatus* clams larger than 34 mm and infected with *P. olsenii* present higher mortality rate than younger ones (Villalba et al., 2005). This might be explained by the higher filtration capacity in larger individuals and that older animals have potentially spent more time in contact with the parasite (Ruano et al., 2015). Nonetheless, *Perkinsus olsenii* is not considered to be deadly for clam species, reaching the plateau of chronic disease. It is the synergistic factors above mentioned coupled to a high infection prevalence that cause the incapacity of metabolic homeostasis by the host, ending up in an inability of organism subsistence (Ruano et al., 2015; Villalba et al., 2004).

Parasite cells of *Perkinsus spp.* are usually present in the connective tissue of different organs, especially in gill and digestive gland (Villalba et al., 2004). In *R. decussatus*, high infection by *P. olsenii* causes inhibition of gametogenesis, reduction of the number of clams with ripe gonad and a significant decrease of storage tissue and gametes whereas haemocytic infiltration of the gonad increases (Casas & Villalba, 2012; K. Il Park et al., 2006). Parasite energy consumption in market-sized clams with heavy infections may exceed the energy available for growth, especially under conditions of high temperature and low food availability, such as during the summer season (Villalba et al., 2004, 2005).

Several epizootic and molecular studies have already been performed in an effort to identify biomarkers for tolerance to the disease as clams are invertebrates with a highly specific innate immune system (Gómez-Chiarri et al., 2015; Soudant et al., 2013). Modulation of the immune response processes, like apoptosis, complement system, response to stress (Prado-Alvarez et al., 2009) and inflammation (Simão et al., 2020), as well as, metabolism regulation (Fernández-Boo et al., 2016), parasite recognition and cell migration (Leite et al., 2013) were pinpointed as occurring on the different tissues of clams in either natural or trial conditions. In addition, the interaction between host and parasite shows a combined expression of genes related to redox and glucose metabolism, protease activity, apoptosis, and iron metabolism in a closely related species (*R. philippinarum*), indicating a crosstalk event between host and parasite (Hasanuzzaman et al., 2018). Also, new studies based on single nucleotide polymorphisms (SNPs) indicates that tolerance to *Perkinsus olsenii* infection in *R. philippinarum* clams is a trait with high heritability (>50%) suggesting that tolerant clams could have better tools to counteract the infection (Morgan Smits et al., 2020).

High-throughput proteomics is a powerful tool that has become widely used in bivalve research to identify different responses to stress conditions, and that could be complementary to transcriptomic analysis as not always the expression of a gene led to a differential concentration of a protein, because protein expression is also influenced by epigenetic, post-transcriptional and post-translational modifications (Gómez-Chiarri et al., 2015). Regarding immune response, proteomic analysis was used to characterize the proteomic signature of two oyster families with different susceptibility to ostreid herpesvirus 1 (Leprêtre et al., 2021), identification of recognition proteins that bind parasites in the hard clam *Mercenaria mercenaria* (Hartman, Pales Espinosa, & Allam, 2018), characterization of the protein profile of oyster species with different susceptibility to diseases (Fernández-Boo et al., 2020), and also identify the expression of protein in clams with different susceptibility to Brown Ring disease (M. Smits et al., 2020); showing

the applicability of the technique to study host/pathogen interactions and to identify possible biomarkers of disease resistance in bivalves.

The present study aims to identify new tolerance biomarkers to *P. olsenii* infection in *R. decussatus*. For that, a characterization of the haemolymph proteomic profiles of naturally non/low-infected and highly-infected individuals by the parasite across several heavy affected areas of Europe using a shotgun proteomics approach was performed. Also, the mechanisms that might be involved in the response against the disease in chronic infections were studied. Through identification of proteins which could confer tolerance to the disease, it was expected to open new ways of study to aid in the production of *R. decussatus* seed with increased resistance to the disease.

## 2.2. Material and methods

### 2.2.1. Clam collection and sampling

One hundred adult *R. decussatus* clams (average shell length 41.74 mm) from 5 different heavy *P. olsenii* affected areas/seabed including Pontevedra (Spain), Algarve (Olhão, Portugal), Naples (Italy), Venice (Italy), and Izmir (Turkey) were collected (Figure 1). Haemolymph was withdrawn using a 1 mL syringe with a 25 G disposable needle through a notch in the shell near the adductor muscle and stored immediately at -80ºC until use. A 10 µl aliquot from the collected haemolymph was used for evaluation of the quality of haemolymph avoiding the use of samples with sperm, eggs or bacteria contamination using light microscopy. Only individuals that presented a clean haemolymph and high quantity of haemocytes were chosen for total protein extraction and digestion. Both hemigills were collected for *P. olsenii* infection level quantification. Briefly, one hemigill was collected for RFTM diagnosis according with Ray (Ray, 1966) using the next infection scale: level: 0 – absence of parasite, 1 – very slight infection, 2 – slight infection, 3 – moderate infection, 4 – intensive infection, 5 – very intensive infection. The other hemigill was placed on ethanol at 70% and stored directly at -20ºC for DNA extraction and *P. olsenii* quantification by quantitative PCR according to Garcia et al. (2022) using *P. olsenii* specific primers designed by Ríos et al. (2020).


**Figure 1.** Populations sampled during the study.

### 2.2.2. Total protein extraction and sequencing

Haemolymph samples from individuals of each population were selected for total protein extraction according to the following criteria: i) Five individuals with absence of parasite (0) or very slight infection (1); ii) Five individuals with intensive infection (4) or very intensive infection (5). A total of 10 individuals per population from the 5 different heavy affected areas were selected for total protein extraction. Since clam individuals from Venice area did not present an infection level lower than moderate infection (level 3), individuals with this infection level were considered for tolerant group. Haemolymph was directly homogenized by disruption of haemocytes cellular membrane using sonication (2 x 4s, 23 kHz, 105 µm). The homogenate was then centrifuged at 16 000 x g, for 20 min at 4ºC, to eliminate the cell debrys. Total protein was posteriorly quantified using the Pierce BCA Protein Assay kit (Thermo Scientific™) and all samples were adjusted to the same protein quantity (56.78 µg) at a final volume of 150 µL, finally 150 µL of 2x SDT buffer (2 %(v/v) SDS, 0.1 M DTT, 100 mM Tris, pH 7.6) was added to all samples for protein denaturation and maintained at -20ºC before trypsin digestion.

Enzymatic digestion of the denatured protein was performed with trypsin/LysC (2 µg) overnight at 37ºC. The resulting peptide concentration was measured by fluorescence and adjusted again to the same concentration. Protein identification and quantitation were performed by nLC-MS/MS according to Osório et al. (2021).

### 2.2.3. Data analysis

The raw data were processed using the Proteome Discoverer v.2.5.0.400 software (Thermo Scientific, Germany) and according to Osório et al. (2021). Protein annotation and identification was performed against the database available at UniProt for Bivalvia with 92,220 entries (May, 2020) and a common contaminant database from MaxQuant (v.1.6.2.6, Max Planck Institute of Biochemistry, Germany) was used to eliminate contamination proteins.

Assessments were performed to identify the total expressed proteins (TP) and differentially expressed proteins (DEP) and evaluate the modulation of the proteome; the similarities, and differences between tolerant and susceptible individuals' protein profiles to determine affected functions and mechanisms by *P. olsenii* infection. Two different approaches were made: i) Highly-infected (susceptible) individuals from all areas versus non/low-infected (tolerant) individuals from all areas (S/T); ii) Highly-infected (susceptible) individuals versus non/low-infected (tolerant) individuals from each affected area. Only the first approach was considered for scrutiny in this study and the applied procedure for TP and DEP detection is next described. Nonetheless, the procedure described below was also employed to the remaining analyses and the observed numbers of TP and DEP discussed in the results and discussion section.

After protein identification, filters for raw data processing were applied to confidently detect TP in the S/T analysis. A protein selection criterion was used on the Proteome Discoverer software to find TP: i) No contaminants; ii) Protein should appear in at least 2/3 of the individuals; iii) Number of unique peptide hits with the protein mass spectrum should be ≥2; iv) Number of peptide spectrum matches with the protein mass spectrum should be ≥4. Beyond this selection, a criterion filter to identify exclusive expressed proteins (EP) was employed in the susceptible and in the tolerant individuals. The selection criterion filter utilized to detect exclusive proteins was as following: i) No protein contaminants; ii) Appearance in only one group (tolerant or susceptible); iii) Protein should appear in at least 2/3 of the individuals. A cut-off to determine DEP was established for protein abundance ratios ≥ 1.50 and ≤0.67.

### 2.2.4. Functional annotation and enrichment analysis

Functional annotation and enrichment analysis were performed on the protein sets obtained from the two applied selection criterion filters to identify DEP and EP from S/T

analysis. Therefore, all obtained proteins were gathered and functionally annotated, and the enrichment analysis performed.

Functional annotation was executed using the Blast2GO software (Götz et al., 2008) through a designed workflow in OmicsBox application (BioBam®, Spain). A Gene Ontology annotation of level 3 was elaborated utilizing a local BLAST against a created local database from Bivalvia available in UniProt, as employed in section 2.3. Annotations from Gene Ontology (GO), Enzyme Codes (EC) and KEGG pathways were obtained and level 3 graphs containing the represented functions for GO terms (biological process, molecular function, and cellular component) in each analysis were prepared.

Functional enrichment analysis was performed using aGOtool (https://bio.tools/agotool), an online Gene Ontology enrichment tool (Schölz et al., 2015). Same Uniprot database was treated as background. As foreground, the protein set lists obtained as mentioned above were applied. Functional enrichment analysis was executed utilizing all available categories of functional associations and results exhibited as over- and under-represented functions. Multiple testing per category was used without a GO basic or a slim subset. The following parameters were considered for the enrichment analysis: *p-value* cut-off of 0.01; a corrected *p-value* (False Discovery Rate) cut-off of 0.05; filter of redundant parent terms and foreground count one; filter top 20 publications. Also, functional association to all available categories was deemed, being these either over or under-represented.

This analysis was not performed on the protein set list obtained from the tolerant individuals' group of the S/T analysis. This was due to the very low number of DEP and EP found in this group (N = 5).

## 2.3. Results and discussion

### 2.3.1. Evaluation of *Perkinsus olsenii* infection intensity by RFTM and qPCR

Infection intensity quantification by RFTM was performed on all collected individuals, while by qPCR was only operated on highly and low/non-infected individuals to accurately select the individual from all populations, after RFTM quantification analysis. Therefore, the results presented and discussed further comprehend only the infection quantification made using RFTM on all individuals from each affected area. From almost all areas it was possible to collect individuals with all different infection levels, except for

Venice population which presented only individuals with infection levels of 3, 4 and 5 (mean infection rate (MIR):4.58) (Figure 2). Regarding the Naples population, a higher frequency (40%) of individuals with a moderate infection (level 3) was observed and a MIR of 2.21 (Figure 2). From Izmir (Türkiye) and Venice areas (Italy), a higher number of highly infected individuals (level 5) could be noted (58% and 69%, respectively). Algarve (Portugal) and Pontevedra (Spain) presented both a low infection level in most of the clams (33% for level 1 and 49% for level 2, MIR:1.75 & 2.41 respectively). Statistically significant parasite load was found when comparing the different areas, being Izmir and Venice the most affected ones, while Algarve, Naples and Pontevedra demonstrated to have a lower/moderate prevalence (Supplementary file 1). The differences in the prevalence of *P. olsenii* between the affected areas could be explained by several factors such as water temperature and salinity, intensive farming, clam density, pollution, culture conditions and hydrodynamics of the basin (Ruano et al., 2015; Villalba et al., 2004). While Venice lagoon is an area with an extremely high density of clams and lower water renewal, favouring the reinfection of the animals, other areas such as Algarve and Pontevedra present a higher water renewal and lower clam density. The absence of individuals non or low infected in Venice populations could be explained due to the high prevalence of *P. olsenii* in the area which even caused some events of massive mortality (Pretto et al., 2014). On the other hand, the high pressure of parasite implies that moderate infected clams from Venice lagoon could be considered as tolerant to the disease because those adult clams (more than 3 years old) living in an environment with high density of animals and high disease prevalence were able to attenuate the parasite proliferation while most of the animals present a massive parasite load (level 5).

Regarding the Mediterranean populations (Venice, Izmir and Naples) a higher prevalence of the parasite than in Atlantic populations was expected because higher water temperatures and salinities favours parasite proliferation (Villalba et al., 2004).

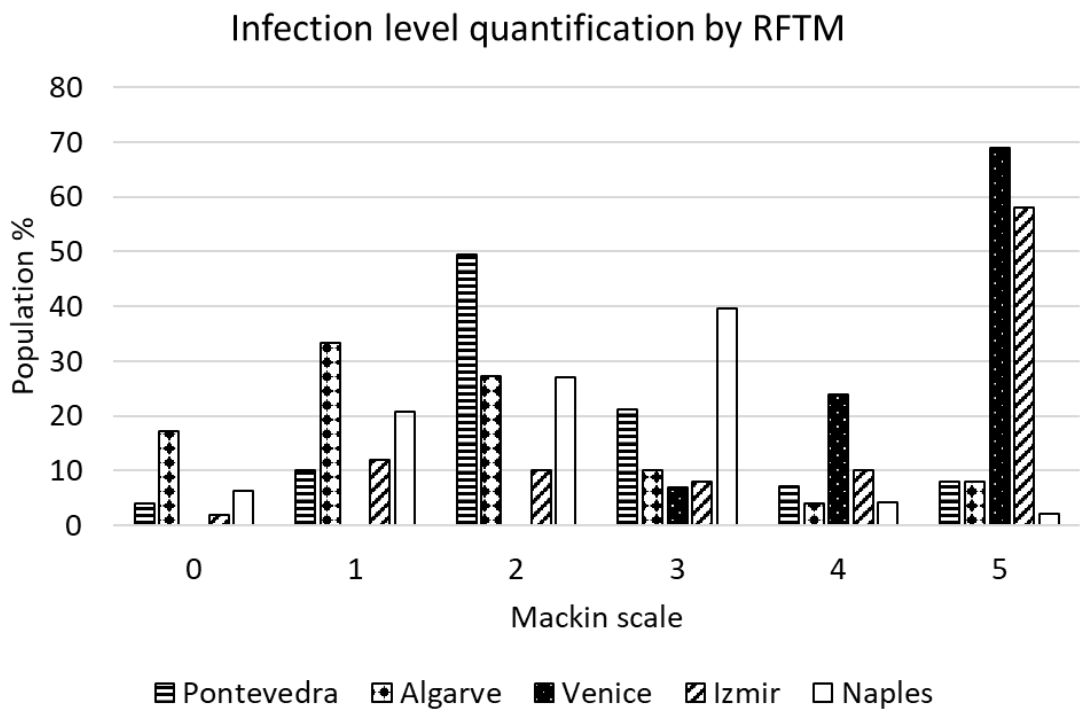


**Figure 2.** Infection level quantification frequencies by RFTM from the individuals collected in the different analysed areas. The numbers represented on the x axis represent the infection ranks attributed by the Mackin scale classification, according to Ray (Ray, 1966), being 0 – absence of parasite, 1 – very slight infection, 2 – slight infection, 3 – moderate infection, 4 – intensive infection, 5 – very intensive infection. The y axis represents the population frequency associated to each infection rank for the analysed areas.

### 2.3.2. Modulation of susceptible (S) vs tolerant (T) clam haemolymph proteome

A total of 4396 different proteins were identified from all individuals. From these, after the application of a filter described in section 2.2.3 a total of 258 TP on S/T were obtained (Table 1, Supplementary datafile 1). Twenty-three DEP in the Susceptible group and 4 DEP in the Tolerant group were detected after applying the respective cut-off. One EP was found in the Tolerant group and 4 EP in the Susceptible group. From these results, it can be stated that 27 possible biomarkers of susceptibility and 5 biomarkers that could confer tolerance to *P. olsenii* infection in *R. decussatus* were identified (Tables 1 & 2).

For the remaining analyses performed, a similar number of TP to the above discussed analysis were also obtained (295.25±71.28 TP per population), with the exception of Pontevedra area, which presented a total number of 692 TP, 2.34 times higher than the average number of the remaining populations (Table 1). This resulted in the identification of 198 DEP on the susceptibility group and 12 in the tolerant group. Also, Naples population, even presenting an average number of TP, presented a

substantial number of EP in the susceptible group and a total of 474 proteins being exclusive or DEP between conditions. In contrast, Algarve revealed to have the lowest number of DEP, displaying in total 71 DEP between treatments.

**Table 1.** Identified expressed protein number after Shotgun proteomic and differential analysis in each comparison.

| Analysis | Total Proteins | Exclusive Proteins | | | DEP[a] (≥1.50 and ≤0.67) | | |
|---|---|---|---|---|---|---|---|
| | | Susceptible | Tolerant | Total | Susceptible | Tolerant | Total |
| **Susceptible vs. Tolerant** | 258 | 4 | 1 | 5 | 23 | 4 | 27 |
| **Algarve (PT)** | 230 | 10 | 9 | 19 | 34 | 37 | 71 |
| **Naples (IT)** | 328 | 255 | 7 | 262 | 181 | 31 | 212 |
| **Pontevedra (SP)** | 692 | 23 | 1 | 24 | 198 | 12 | 210 |
| **Izmir (TU)** | 243 | 10 | 18 | 28 | 57 | 55 | 112 |
| **Venice (IT)** | 380 | 10 | 50 | 60 | 58 | 67 | 125 |

[a]Differentially expressed proteins

A noticeable number of TP present in all individuals from the affected areas could be observed (117, Figure 3), as well as a high number of exclusive TP in the Pontevedra area (298). Moreover, Algarve area demonstrated to have the lowest number of exclusive TP (7). These results indicate that a different amount of molecular processes occur specifically in each affected area and a noticeable quantity of these processes could be found as commonly shared. The fluctuation in the number of TP, DEP and EP between the areas may derivate from abiotic factors mentioned on the section 2.3.1, such as water temperature and salinity, intensive farming, clam density, pollution, culture conditions and hydrodynamics of the basin (Ruano et al., 2015; Villalba et al., 2004). These abiotic factors, if not attenuated, have an impact on the metabolism and the innate immune response of the affected organisms, making them more or less prone to parasite infection (Soudant et al., 2013).

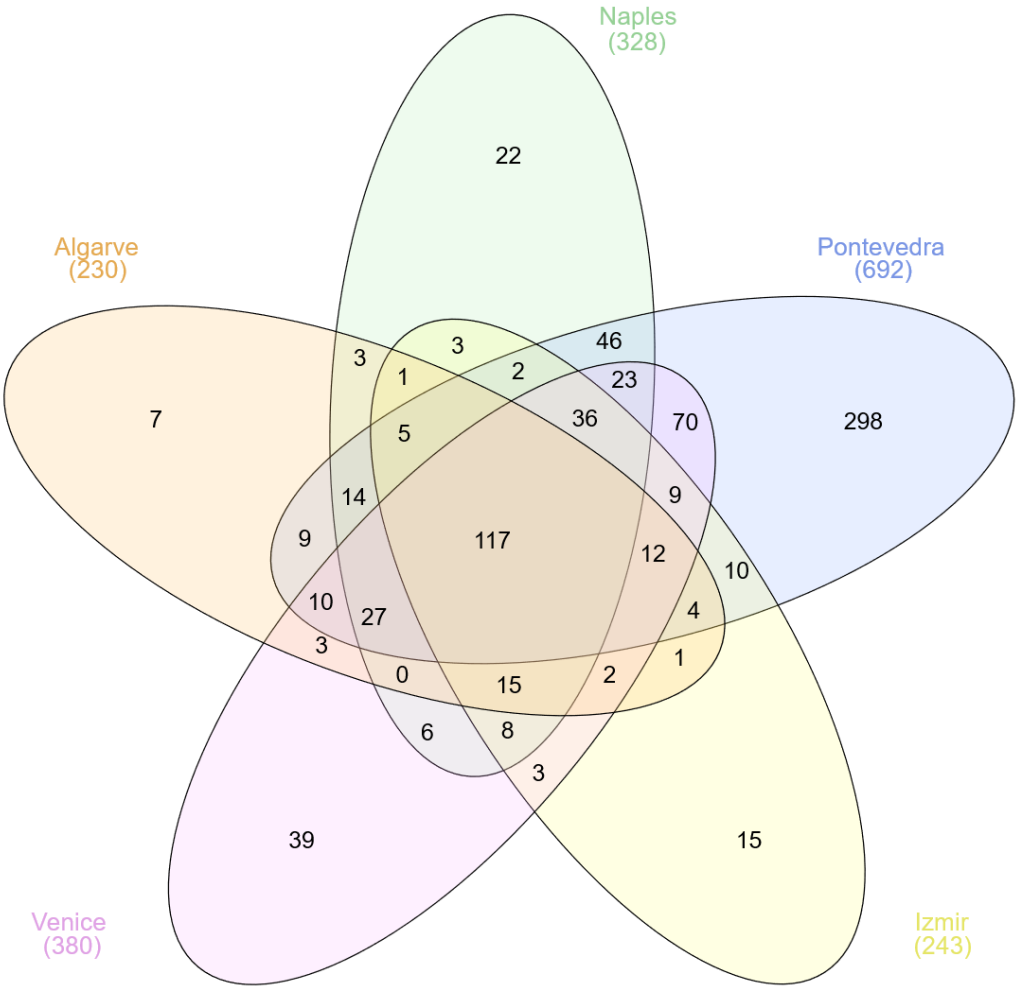


**Figure 3.** Venn diagram of the total expressed proteins in the individuals (both highly infected and low/non-infected) from each affected area.

**Table 2.** Differentially expressed proteins with an abundance ratio (S/T) greater than or equal to 1.50, and below than or equal to 0.67. Functional roles are described as grouped proteins for the susceptibility group and as individual biomarkers for the tolerance group in the results and discussion section of the biomarkers. PSMs: peptide-spectrum matches

| Accession number | Name | Species | Peptides | PSMs | Unique peptides | Abundance ratio S/T | Abundance ratio p-value S/T | Abundance Susceptible | Abundance Tolerant | Functional role discussed |
|---|---|---|---|---|---|---|---|---|---|---|
| **K1QV55** | Ubiquitinyl hydrolase 1 | *Crassostrea gigas* | 1 | 2 | 1 | 100 | 1E-17 | 200 | | ROS production |
| **A0A210PVH9** | Asparagine synthetase [glutamine-hydrolyzing] | *Mizuhopecten yessoensis* | 1 | 2 | 1 | 100 | 1E-17 | 200 | | Energetic stress response |
| **A0A210Q774** | Betaine-homocysteine S-methyltransferase 1 | *Mizuhopecten yessoensis* | 1 | 6 | 1 | 100 | 1E-17 | 200 | | DNA methylation, amminoacid synthesis |
| **A0A210QXB3** | Ubiquitinyl hydrolase 1 | *Mizuhopecten yessoensis* | 1 | 2 | 1 | 100 | 1E-17 | 200 | | ROS production |
| **A0A194AN82** | Putative aminopeptidase W07G4.4 | *Pinctada fucata* | 3 | 204 | 3 | 3.028 | 0.002144833 | 152.6 | 47.4 | ROS production |
| **Q8ITB9** | Ribosomal protein L7 (Fragment) | *Argopecten irradians* | 2 | 107 | 2 | 1.859 | 0.117443406 | 128.3 | 71.7 | Energy restoration and balance |
| **A0A210Q465** | Ras-related protein Rab-1A | *Mizuhopecten yessoensis* | 5 | 208 | 3 | 1.75 | 0.167580242 | 126.2 | 73.8 | ROS production |
| **K1PZ93** | Dihydropyrimidine dehydrogenase [NADP(+)] | *Crassostrea gigas* | 6 | 144 | 6 | 1.718 | 0.185482208 | 129 | 71 | ROS production |

| Accession number | Name | Species | Peptides | PSMs | Unique peptides | Abundance ratio S/T | Abundance ratio p-value S/T | Abundance Susceptible | Abundance Tolerant | Functional role discussed |
|---|---|---|---|---|---|---|---|---|---|---|
| **M5AKT2** | Paramyosin-2 (Fragment) | *Pinctada fucata* | 3 | 102 | 3 | 1.684 | 0.206821194 | 132.6 | 67.4 | Immune response modulation |
| **M5B264** | Paramyosin-1 (Fragment) | *Pinctada fucata* | 3 | 102 | 3 | 1.684 | 0.206821194 | 132.6 | 67.4 | Immune response modulation |
| **K1R150** | Ras-related protein Rab-1A | *Crassostrea gigas* | 6 | 235 | 4 | 1.656 | 0.226098844 | 131.8 | 68.2 | ROS production |
| **Q8ITC5** | 60S ribosomal protein L30 | *Argopecten irradians* | 4 | 93 | 4 | 1.616 | 0.256382439 | 106.1 | 93.9 | Energy restoration and balance |
| **K4HXI8** | Ferritin, isoform H | *Ruditapes philippinarum* | 3 | 103 | 2 | 1.597 | 0.271749348 | 131.3 | 68.7 | Iron regulation, chelation and withholding |
| **J9PJ22** | Arginine kinase | *Hyriopsis schlegelii* | 2 | 107 | 2 | 1.583 | 0.283997895 | 123.2 | 76.8 | Metabolic regulation, immune response |
| **A0A194AM72** | Putative ras-related protein Rab-1A | *Pinctada fucata* | 7 | 240 | 5 | 1.582 | 0.285251664 | 134.8 | 65.2 | ROS production |
| **A0A210QMV4** | 60S ribosomal protein L7 | *Mizuhopecten yessoensis* | 4 | 196 | 3 | 1.578 | 0.288234404 | 124.2 | 75.8 | Energy restoration and balance |
| **A0A2L1TGZ7** | 40S ribosomal protein S3 (Fragment) | *Crassostrea brasiliana* | 7 | 179 | 7 | 1.549 | 0.315704816 | 124 | 76 | Energy restoration and balance |

| Accession number | Name | Species | Peptides | PSMs | Unique peptides | Abundance ratio S/T | Abundance ratio p-value S/T | Abundance Susceptible | Abundance Tolerant | Functional role discussed |
|---|---|---|---|---|---|---|---|---|---|---|
| **K1R6F1** | Proteasome subunit alpha type | *Crassostrea gigas* | 4 | 170 | 4 | 1.549 | 0.315097357 | 126.2 | 73.8 | Parasite cell function and proliferation |
| **A0A210QAJ5** | 40S ribosomal protein S3 | *Mizuhopecten yessoensis* | 7 | 179 | 7 | 1.549 | 0.315704816 | 124 | 76 | Energy restoration and balance |
| **A0A515EJ23** | Cathepsin K | *Meretrix petechialis* | 3 | 410 | 3 | 1.546 | 0.317885769 | 107 | 93 | Parasite cell function and proliferation |
| **K1QJM1** | 60S ribosomal protein L30 | *Crassostrea gigas* | 3 | 89 | 3 | 1.542 | 0.321814599 | 111.7 | 88.3 | Energy restoration and balance |
| **Q70MM6** | Ribosomal protein S3 (Fragment) | *Crassostrea gigas* | 8 | 202 | 8 | 1.52 | 0.344119694 | 119.1 | 80.9 | Energy restoration and balance |
| **K1QEA6** | Phosphoenolpyruvate carboxykinase (GTP) | *Crassostrea gigas* | 5 | 268 | 2 | 1.515 | 0.34903728 | 159 | 41 | Gluconeogenesis, pathogen recognition and clearance |
| **K1RGQ0** | Ras-related C3 botulinum toxin substrate 1 | *Crassostrea gigas* | 3 | 63 | 2 | 1.515 | 0.34976977 | 117.9 | 82.1 | ROS production |
| **Q0KHB7** | Phosphoenolpyruvate carboxykinase (GTP) | *Crassostrea gigas* | 4 | 230 | 2 | 1.515 | 0.34903728 | 159 | 41 | Gluconeogenesis, pathogen recognition and clearance |

| Accession number | Name | Species | Peptides | PSMs | Unique peptides | Abundance ratio S/T | Abundance ratio p-value S/T | Abundance Susceptible | Abundance Tolerant | Functional role discussed |
|---|---|---|---|---|---|---|---|---|---|---|
| **Q70MM5** | 60S ribosomal protein L40 (Fragment) | *Crassostrea gigas* | 3 | 132 | 3 | 1.515 | 0.349013069 | 122.4 | 77.6 | Energy restoration and balance |
| **A0A210R4I2** | Proteasome endopeptidase complex | *Mizuhopecten yessoensis* | 3 | 100 | 3 | 1.504 | 0.360641778 | 131.6 | 68.4 | Parasite cell function and proliferation |
| **J9UEN7** | Myosin essential light chain | *Hyriopsis cumingii* | 6 | 366 | 6 | 0.635 | 0.081099859 | 83.4 | 116.6 | Phagocytosis regulation |
| **A0A210PXL7** | T-complex protein 1 subunit delta | *Mizuhopecten yessoensis* | 4 | 103 | 2 | 0.611 | 0.062360692 | 83.9 | 116.1 | Phagocytosis regulation, cell growth and proliferation, gametogenesis, apoptosis regulation |
| **A0A067XI00** | Ferritin, isoform 4 | *Ruditapes decussatus* | 19 | 1373 | 19 | 0.599 | 0.054326708 | 66.2 | 133.8 | Iron regulation, shell development, organismal growth |
| **K1QRU8** | Myosin heavy chain, striated muscle | *Crassostrea gigas* | 8 | 454 | 5 | 0.539 | 0.024482892 | 77.4 | 122.6 | Phagocytosis regulation |
| **K1R6N5** | Structural maintenance of chromosomes protein 3 | *Crassostrea gigas* | 1 | 1 | 1 | 0.01 | 1E-17 | | 200 | Metabolic regulation |

### 2.3.3. Gene Ontology of modulated clam proteomes

Functional annotation was performed using the Blast2GO software (Götz et al., 2008) to assign GO terms to each protein and allow identification and characterization of the biological processes (BP) occurring in susceptible and tolerant *R. decussatus* individuals to *P. olsenii* infection. In the susceptible individuals, all BPs from DEP were related to cellular and metabolic processes, 21% of the hits (14 hits) were related to organic substance metabolic process, while 19% (13 hits) and 18% (12 hits) were related to primary metabolic process and cellular metabolic process respectively. In tolerant individuals, BPs regarding localization, cellular homeostasis, protein folding and biological regulation could be detected (1 hit for each class) (Figure 4). Also, a functional enrichment analysis was elaborated on the susceptibility markers to ascertain which functions were over-represented and correlate the results with the functional annotation. For this analysis, all identified enriched BPs were assigned to metabolic processes, which goes in accordance with the obtained results in the functional annotation (Figure 5). In this case, processes such as organic substance biosynthetic process and primary metabolic process presented a fold enrichment >3 while other processes such as nitrogen compound, organonitrogen compound, organic substance and macromolecule metabolic process presented a fold enrichment >2. Previous transcriptomic analysis performed on infected *R. philippinarum* by *P. olsenii* parasite suggests a similar response on long-term infected individuals, being metabolism and response to stress the main over-represented functions (Hasanuzzaman et al., 2017). A gene expression study based on Suppression-Subtractive Hybridization (SSH) also demonstrated that genes related to metabolic processes in chronically infected *R. decussatus* clams with *P. olsenii* were modulated (Prado-Alvarez et al., 2009). Leite et al. (2013) used the same clam species and parasite but with a microarray built-in gene expression analysis that suggested a similar scenario, being the genes related to metabolism the most modulated ones in highly infected individuals. Given this and as can be stated and already suggested by Leite et al. (2013), *P. olsenii* infection in *R. decussatus* affect clam survival and growth by altering their metabolic system.


**Figure 4.** Represented GO terms through functional annotation of the DEP in the susceptible and tolerant groups. Circle size represents the number of hits in each GO term while the colour indicates the percentage of representation of the GO term in each group.

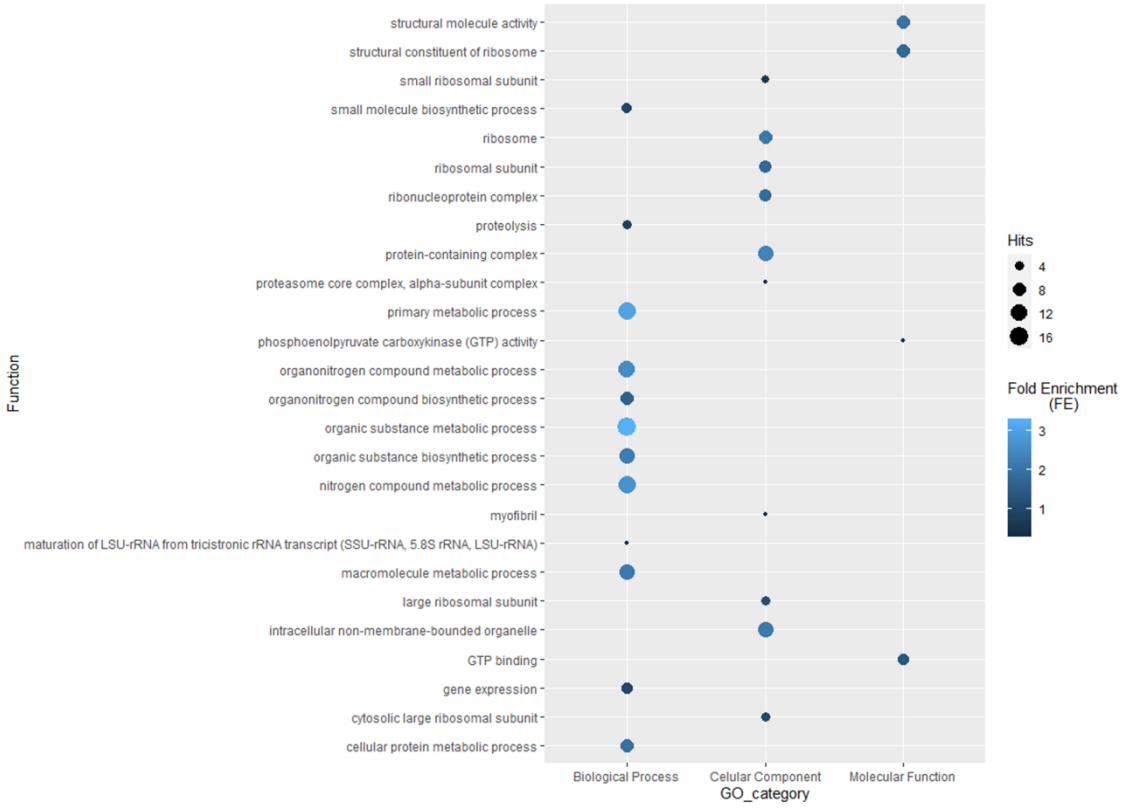


**Figure 5.** Functionally enriched GO terms in the susceptible group. Circle size represents the number of hits in each GO term while the colour shows the fold enrichment (how much it is over-represented) for each respective function.

### 2.3.4. Susceptibility and tolerance biomarkers to *P. olsenii* in *R. decussatus*

Protein function and discussion of the biological role for both susceptibility and tolerance biomarkers will be detailed in this section.

#### 2.3.4.1. Energy balance markers

Several ribosomal proteins appeared to be modulated in the susceptible group, indicating a high demand of energy restoration and balance by the host to not only counteract its survival, growth (Meyer & Manahan, 2010) and maturation (Wang et al., 2012) demands, as well as to actively use its immune defence system to fight the parasite spread and usage of the host resources for survival (Soudant et al., 2013). Also, increased demand for respiratory burst and oxygen uptake by the gills due to agglomerate concentrations of the parasite at this tissue leads to a higher transport by haemocytes and higher energy rates (Hasanuzzaman et al., 2020). Asparagine synthetase was found overexpressed in the group of highly infected individuals, being its expression propelled by a feedback network

within the stress response, which includes an amino acid response and an unfolded protein response (Tsai et al., 2020). This network might be activated under energetic stress caused by the parasite usage of host's energy reserves. At host-parasite interaction level there is usually a modulation of genes related to metabolic processes, since there is a requirement of energy for parasite survival, growth, and reproduction and for host activation of its immune response for infection counteraction. Interconnected pathways of glycolysis, tricarboxylic acid cycle and oxidative phosphorylation are usually up-regulated to activate immune cells in order to synthesize macromolecules and generate antimicrobial burst (Hasanuzzaman et al., 2020). At high infection intensities of *Perkinsus spp.*, a direct sub-lethal impact on the host growth and reproduction happens, leading to interference of the parasite with energy balance of the clams (Leite et al., 2004; Soudant et al., 2013).

In this same group of susceptible individuals, another marker related to metabolic regulation and physiological function was overexpressed. Arginine kinase (AK) is kinase that catalyzes the conversion of adenosine triphosphate to arginine-phosphagen in invertebrates (Arockiaraj et al., 2011). It has also the ability to create metabolic energetic reservoirs and to recycle ATP when it is metabolically needed (López-Zavala et al., 2013). AK could be an effector of the immune response in bivalves by the accumulation of ATP and activation of purinergic signalling pathway, significant enhancement of $Ca^{2+}$ influx, ROS production and lysosome release of haemocytes (Jiang, Jia, Chen, Wang, & Song, 2016). High level of AK in highly infected individuals indicates an enhancement of metabolic modulation by *R. decussatus* towards parasite infection and activation of its immune responses by ATP accumulation and activation of ROS production and lysosomal activity (Jiang et al., 2016). Phosphoenolpyruvate carboxykinase (PEPCK) is another overexpressed protein from the susceptible group involved in the metabolic pathway of gluconeogenesis in organisms, catalysing the formation of phosphoenolpyruvate and oxaloacetate. It was suggested that could also mediate pathogen recognition and clearance through recognition and binding to pathogen's PAMPs in *C. gigas* (Lv et al., 2017).

#### 2.3.4.2. Oxidative stress and DNA damage markers

Betaine-homocysteine S-methyltransferase 1 is a protein involved in the protection against stress and aminoacid synthesis (Sun et al., 2017). The higher energy requirements to spend by the host for its survival, activation of the immune system and metabolic maintenance leads to a higher rate of aminoacid and protein synthesis, resulting in the observed higher abundance of this molecule in the susceptible individuals.

During phagocytosis and encapsulation, lysosomal enzymes act together with ROS production enzymes to destroy the encapsulated parasites (Cheng, 2000). Several markers related to ROS production could be identified as modulated on the susceptible group. In this specific case, proteins like dihydropyrimidine dehydrogenase [NADP(+)], ubiquitinyl hydrolase 1, aminopeptidase, ras-related protein Rab-1A and ras-related C3 botulinum toxin substrate 1 were observed in these individuals to be acting together on the reactive oxygen species (ROS) production. ROS production is a highly efficient mechanism of invertebrates to fight against pathogens and this response was widely documented during *Perkinsus* spp. infection (Soudant et al., 2013).

Proteases could also be identified as overrepresented among the susceptible individuals. Proteasome endopeptidase complex, proteasome subunit alpha type and cathepsin K are serine proteases that can be found as extracellular products. In the case of individuals highly affected by *P. olsenii*, these proteases can be acting for cellular homeostasis maintenance and inhibition of parasite proliferation (Hasanuzzaman et al., 2018).

#### 2.3.4.3. Effects on muscle and cytoskeleton

A differential expression of proteins related to muscle and cytoskeleton composition was identified in the proteomic analysis. Paramyosins were over-represented in susceptible individuals while myosin heavy chain (MHC) and myosin essential light chain (MELC) were over-represented in tolerant individuals. Alteration of the expression of these protein could imply disorganization of the cytoskeleton induced by different stressors (Chan et al., 2021; Rodríguez-Ortega, Grøsvik, Rodríguez-Ariza, Goksøyr, & López-Barea, 2003). Another important fact that must be stated is the cooperation function of myosin and actin in phagocytosis of invertebrate immune defences (Castellano et al., 2001; Liu et al., 2009). The high levels of MELC and MHC in tolerant individuals could indicate an elevated phagocytic regulation capacity (Liu et al., 2009) that could allow for a higher capacity of pathogen elimination of *P. olsenii* by tolerant clam.

Tail-less complex polypeptide 1 (TCP-1) ring complex (TRiC), which is also called Chaperonin-containing TCP-1 (CCT) complex is a highly conserved, hetero-oligomeric complex that ensures proper folding of actin, tubulin, and regulators of mitosis (Valpuesta, Martín-Benito, Gómez-Puertas, Carrascosa, & Willison, 2002). This mechanism has a direct impact on phagocytosis regulation by producing functional actin which was above described to actively participate in parasite encapsulation (Castellano et al., 2001). Therefore, tolerant

individuals with an increase expression of this protein could have a higher phagocytic regulation capacity and can more efficiently remove invading pathogens (Liu et al., 2009). CCT complex is also required for controlling cell and organismal growth by interacting at the intercellular level with the insulin/TOR signalling pathway in *Drosophila* (Kim & Choi, 2019) that also plays an essential role during gametogenesis (Ueishi, Shimizu, & Inoue, 2009). The lower expression of CCT in susceptible animals could be the result of the high parasitic load in the individuals leading to a suppression of gametogenesis in infected animals.

#### 2.3.4.4. Ferritin

Ferritins are conserved proteins responsible for the storage and detoxification of iron (S. C. Andrews, 2010). In molluscs most of the ferritins are cytosolic (Chen et al., 2016; Zhang et al., 2013), but extracellular ones have also been identified (Huan, Liu, Wang, & Liu, 2014; Sun et al., 2014). Their modulation in this phylum has been principally linked to the response to parasite infections (Chen et al., 2016; M. F. Simão et al., 2010; Zhang et al., 2013) specifically by decreasing iron concentrations available to protozoa parasites and bacteria, whose proliferation depends on it (Soudant et al., 2013). This is also the case for *P. olsenii* infection in *R. decussatus* (Elandalloussi et al., 2003; Leite et al., 2004). A fast proliferation of the parasite can only occur by a readily available supply of nutrients. To counteract the parasite pressure, immune related cells developed the capacity to produce ion-binding proteins to reduce the levels of available iron, slowing down parasite growth and proliferation rates (Elandalloussi et al., 2003).

Ferritins could also be part of the response of the clams to infection, while susceptible group is expressing Ferritin H which is a cytosolic protein involved in iron uptake to avoid pathogen spread and proliferation (Kim et al., 2012), the tolerant group has a higher expression of ferritin subunit 4 which is a secreted ferritin more involved in shell formation and growth (M. Simão et al., 2020; Zhang et al., 2003). Ferritin H gene was observed to be up-regulated upon *V. tapetis* induction in *R. philippinarum*, exhibiting a noticeable suppression of bacterial growth (Kim et al., 2012). During a bacterial infection, plasma iron levels are drastically reduced as the pathogen searches iron for growth and proliferation (Ong, Lihui Wang, Yong Zhu, Ho, & Jeak Ling Ding, 2005). Following this, both host and pathogen have developed strategies to compete for iron – the former retains the iron (Ong et al., 2005) while the latter tries to capture it. This subunit was also identified as an important mediator of the antioxidant and protective activities of NF-kB. Accumulation of ROS is suppressed through iron sequestration, inhibiting JNK signaling (Ong et al., 2005).

#### 2.3.4.5. Structural maintenance of chromosomes protein 3

This protein is present only in the tolerant individuals showing to be exclusive to this group. Structural maintenance of chromosomes protein 3 is a component of the multimeric cohesin complex that holds together sister chromatids during mitosis, enabling proper chromosome segregation (Nasmyth & Haering, 2009). The specific role of this protein in infection by parasites is not clear yet and more research needs to be done in order to understand its function.

## 2.4. Conclusion

The protozoan parasite *Perkinsus olsenii* is one of the major issues in *Ruditapes decussatus* culture. Efforts were already made to identify the routes through which the parasite infects the clam and what mechanisms of defence are used to counteract it. The enrichment functional analysis in the protein susceptibility markers detects functions assigned to metabolic processes as the most enriched ones. Also, in this work 32 proteins related to higher susceptibility or tolerance to *P. olsenii* infection in *R. decussatus* were identified. Twenty-seven of them were related to possible markers of susceptibility and 5 of tolerance to *P. olsenii* infection in *R. decussatus*.

In the susceptible group, different ribosomal proteins appeared to be modulated, suggesting a high demand of energy restoration and balance by the host, not only for growth and maturation, but also to actively use its immune defence system to fight the parasite replication and usage of the host resources for survival. An increase of metabolic regulation is also suggested by another marker towards parasite infection and activation of its immune responses by ATP accumulation, activation of ROS production and lysosomal activity. Ferritin was found to be modulated in both susceptible and tolerant groups with modulation of the expression of different isoforms. In the susceptible ones, its expression was found to be related to iron availability regulation, hijacking free iron to inhibit parasite cell growth and proliferation while in tolerant individuals the presence of ferritin seems to be involved in shell formation and growth. Also a dysregulation of the cytoskeleton seems to occur with a different proportion of myosin and paramyosin between groups. Regulation of apoptosis by the T-complex protein 1 identified in the tolerant individuals is an indicator that suppression of host defence mechanism by the parasite can be prevented, avoiding pathogen survival inside the host.

In summary, the identification of new markers of tolerance suggests that an efficient pathogen elimination mechanism coupled to a better metabolic regulation and higher organismal development capacity are signatures present in tolerant individuals.

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## 2.6. Sample credit author statement

João Estêvão: Formal analysis, Investigation, Data curation, Writing – Original Draft, Visualization. Hugo Osório: Software, Data curation, Writing - Review & Editing. Benjamin Costas: Writing - Review & Editing, Supervision. Andreia Cruz: Writing - Review & Editing, Project administration, Funding acquisition. Sergio Fernández-Boo: Conceptualization, Methodology, Writing - Review & Editing, Supervision, Project administration.

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# Chapter 3

# Signatures of selection for resistance / tolerance to *Perkinsus olsenii* in grooved carpet shell clam (*Ruditapes decussatus*) using a population genomics approach

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# Chapter 3 - Signatures of selection for resistance / tolerance to *Perkinsus olsenii* in grooved carpet shell clam (*Ruditapes decussatus*) using a population genomics approach

## 3.1. Introduction

The grooved carpet shell clam (*Ruditapes decussatus*) is a bivalve species distributed throughout the coastal and estuarine areas of Northeast (NE) Atlantic Ocean and Mediterranean Sea (Cordero et al., 2014; Juanes et al., 2012). This species is of high economic and social importance, especially in southern Europe, with Portugal standing as the largest producer (FAO, 2024). However, its production has suffered a decline caused by massive mortalities, attributed to a combination of different biotic (pathogens) and abiotic (heat waves and salinity stress) factors, poor management protocols, and intensive culture of Manila clam (*Ruditapes philippinarum*) (Azevedo, 1989; Ruano et al., 2015). The parasite *P. olsenii* was first reported in Europe in *R. decussatus* in 1989 after massive mortalities in Southern Portugal (Azevedo, 1989), and since then, it has become a major issue for clam culture (Villalba et al., 2004). *P. olsenii* life cycle is based on trophozoites detected on the affected tissues of host, as spherical cells with a large vacuole and a peripheral nucleus. These cells encompass vegetative proliferation by successive bipartitioning that, after cell wall rupture, release daughter cells. The daughter cells later enlarge giving rise to mature trophozoites, which can infect new hosts after release trough pseudo-faeces (Bushek et al., 2002) or after tissue decomposition from death host. In this case, trophozoites develop a resistant state called hypnospore by enlarging and forming a new cell wall. After release to the sea water and under optimal conditions, they develop into pre-zoosporangia which can produce thousands of free and motile invasive biflagellated zoospores (Auzoux-Bordenave et al., 1996; Azevedo et al., 1990; Villalba et al., 2004). All stages of the parasite life cycle are infective. Currently, collectors and farmers of this clam species face significant challenges of seed supply and recruitment from shellfish beds, together with important annual mortalities of adults close to commercialization caused by high prevalence of *P. olsenii* (da Costa et al., 2020).

To counteract mortalities due to environmental factors and pathogenic infections, such as perkinsosis, breeding programs aimed at obtaining improved bivalve strains resilient to biotic and abiotic stress conditions have been launched in different bivalve species, such as oysters, mussels and clams (Hollenbeck & Johnston, 2018; Potts et al., 2021). Genetic diversity is a cornerstone for broodstock foundation to ensure long-term genetic response and appropriate handling of genotype by the specific environment interactions in the areas of production (Gjedrem & Baranski, 2009). A sufficiently large and genetically diverse

broodstock also diminishes the risk of inbreeding depression and unintended selection (Duncan et al., 2013). Therefore, understanding genetic structure and population dynamics of the species throughout its distribution is essential for a sustainable production and for efficient breeding programs. Bivalves usually have a bipartite life cycle characterized by a dispersive, planktonic larval phase and a sedentary adult phase (D'Aloia et al., 2015). While larvae dispersal by marine currents during the planktonic phase contributes to homogenize populations (Hellberg, 2009; Vera et al., 2022), oceanic barriers and fronts determine some differentiation across the whole genome (Hellberg, 2009). Additional differentiation at specific genomic regions might be related to adaptation to diverse environmental factors across the species distribution range (Miller et al., 2019; Wu et al., 2022).

Population genetics studies of *R. decussatus* have been performed to date with a limited number of markers, and low, but significant, population differentiation has been detected throughout the distribution range, especially pronounced between Atlantic and Mediterranean regions (Arias-Pérez et al., 2016; Cordero et al., 2014; Cruz et al., 2020). Three main genetic groups, corresponding to Atlantic, Western Mediterranean and Eastern Mediterranean, were identified, with a main discontinuity separating the Atlantic and Mediterranean groups. However, mtDNA haplotypes placed Atlantic and Western Mediterranean groups closer regarding the Eastern Mediterranean. The most comprehensive study detected genetic differences between infected and non-infected clams at several microsatellite loci, suggesting that resistance to the *P. olsenii* parasite could have a genetic basis (Cruz et al., 2020).

Despite the relevant information achieved, a whole genomic screening would provide more refined data regarding the factors shaping the genome of the species across its distribution. This is essential for disclosing the mechanisms of adaptation, either at local or broader scale. Next generation sequencing (NGS) technologies have made affordable contiguous and consistent genome assemblies, which have been used to call and genotype thousands of single nucleotide polymorphisms (SNP) across the whole genome (Houston et al., 2020; Yáñez et al., 2023; G. H. Yue & Wang, 2017). Restriction site associated DNA sequencing (RAD-Seq) methodologies represent a powerful tool for genomic screening to be applied in breeding programs or population genomics studies (Robledo et al., 2017). RAD-Seq technologies have been employed for population genomics studies in mollusk (Gutierrez et al., 2017; C. Yue et al., 2020), either to disclose the environmental factors shaping the genome (Vera et al., 2022, 2023) or to identify genomic regions and genetic markers associated with resilience to parasites (Hornick & Plough, 2022; Pampín et al., 2023; Sambade et al., 2022; Vera et al., 2019).

In this study, the first chromosome-level genome of *R. decussatus* was assembled and annotated, and it was further used for a preliminary evaluation of the genetic structure of the species using 2bRAD-Seq. The study also focused on the genetic variation associated with resistance / tolerance to perkinsosis through individual evaluation of parasite load on shellfish beds affected by a wide range of perkinsosis incidence. A total of 90 markers and several candidate genes associated with perkinsosis resistance / tolerance were identified representing an invaluable information for controlling this parasitosis.

## 3.2. Materials and methods

### 3.2.1. Whole Genome Sequencing (WGS)

#### 3.2.1.1. Long-read WGS

High molecular weight DNA was obtained with E.Z.N.A.® Mollusc DNA kit (Omega Biotek, USA) from one *R. decussatus* muscle foot from Algarve (Portugal) following manufacturer´s instructions. The sequencing libraries were prepared using the Ligation sequencing kit SQK-LSK109 from Oxford Nanopore Technologies (ONT) and the quality parameters monitored by the MinKNOW platform version 4.1.2 in real time and base-called with Guppy version 4.2.3 (Supplementary Methods).

#### 3.2.1.2. Short-read whole genome sequencing

The short-insert paired-end libraries for the whole genome sequencing (WGS) were prepared using DNA from the same individual used for long-read sequencing with a PCR free protocol using KAPA HyperPrep kit (Roche) with some modifications (Supplementary Methods) and the quality evaluated on an Agilent 2100 Bioanalyzer with the DNA 7500 assay (Agilent) for size and quantified by Kapa Library Quantification Kit for Illumina platforms (Roche).

#### 3.2.1.3. Hi-C sequencing

Five *R. decussatus* males and five *R. decussatus* females were dissected and several organs (mantle, haemocytes, gills, muscle and gonads) were snap frozen in liquid nitrogen, pooled together and maintained at -80ºC until use. The different samples were pulverized

using a mortar and pestle immersed in a liquid nitrogen bath. Hi-C libraries were prepared using the Omni-C kit (Dovetail Genomics), following the manufacturer's protocol (Supplementary Methods). The library was sequenced on NovaSeq 6000 (Illumina, 2 × 151 bp) following the manufacturer's protocol for dual indexing.

### 3.2.2. Genome assembly

Before assembly, long- and short-reads were preprocessed and filtered following a specific pipeline to ensure a minimum length and quality (Supplementary Methods; Figure S1). The Omni-C reads were mapped to the assembly using BWA-MEM and pre-processed using the Dovetail pipeline (https://omni-c.readthedocs.io/en/latest/fastq_to_bam.html). After removal PCR duplicates, they were scaffolded with YaHS30 v1.1 using default parameters and the assembly error corrected by ten rounds of scaffolding. The resulting assembly (fRudec1) was evaluated with BUSCO v 5.4.0 (Simão et al., 2015) using the metazoan_odb10 lineage dataset and Merqury v 1.1 (Rhie et al., 2020) for consensus quality (QV) and k-mer completeness. Finally, to compute the contiguity CNAG's in-house script Nseries.pl was used (https://github.com/cnag-aat/assembly_pipeline/blob/v2.0.0/scripts/Nseries.pl).

### 3.2.3. Genome annotation

#### 3.2.3.1. RNA-Seq

For genome annotation, RNA-Seq was carried out on gill, mantle, foot, haemocytes, and digestive gland using pools of 10 individuals for each tissue. Total RNA extraction was performed using the RNeasy mini kit (Qiagen) with DNase treatment. RNA quantity and quality were evaluated with the Qubit® RNA BR Assay kit (Thermo Fisher Scientific) and the RNA integrity estimated by using RNA 6000 Nano Bioanalyser 2100 Assay (Agilent). Next, equimolar RNA pools of 10 individuals were used for library construction of each tissue after evaluation of individual RNA extractions.

The RNA-Seq libraries were prepared with KAPA Stranded mRNA-Seq Illumina® Platforms Kit (Roche) following the manufacturer´s recommendations (Supplementary Methods). The final library was validated on an Agilent 2100 Bioanalyser with the DNA 7500 assay.

#### 3.2.3.2. Repetitive elements

Repeats present in the *R. decussatus* genome assembly were annotated with RepeatMasker v4-1-5-0 (http://www.repeatmasker.org) using the custom repeat library available for Mollusca, after excluding those repeats that were part of repetitive protein families (performing a BLAST search against UniProt) (Supplementary Methods). Bedtools v2.31.1 (Quinlan & Hall, 2010) was used to produce the final repeat-masked version of the genome.

#### 3.2.3.3. Gene annotation

Gene annotation was done by combining transcript alignments, protein alignments and *ab initio* gene predictions following the CNAG structural genome annotation pipeline (https://github.com/cnag-aat/Annotation_AAT) (Figure S2; Supplementary Methods). RNA-Seq reads obtained from several tissues, either sequenced specifically in this study (gill, mantle, foot, haemocytes, and digestive gland) or from public databases, were aligned against the genome with STAR v-2.7.10a (Dobin et al., 2013) and used to generate transcript models with Stingtie v2.2.1 (Niknafs et al., 2017). The TransDecoder program, which is part of the PASA package, was run on the PASA assemblies to detect coding regions in the transcripts. Additionally, the complete proteomes of *Crassostrea virginica*, *C. gigas*, *Mytilus coruscus*, *M. galloprovincialis*, and *M. edulis* were downloaded from Uniprot in April 2024 and aligned to the genome using Miniprot v0.6 (H. Li, 2023). *Ab initio* gene predictions were performed on the repeat-masked *R. decussatus* assembly with several programs with and without incorporating evidence from the RNA-Seq data. Finally, all the data were combined into consensus CDS models using EvidenceModeler-2.1 (EVM, Haas et al., 2008) and functional annotation was performed on the annotated proteins with Blast2go (Conesa et al., 2005). Additionally, UTRs and alternative splicing forms were annotated via two rounds of PASA annotation updates.

The annotation of non-coding RNAs (ncRNAs) was obtained using the repeat-masked version of the genome assembly and after removing RNA gene families identified with Infernal (Nawrocki & Eddy, 2013) and tRNAscan-SE (Chan & Lowe, 2019) packages. Long non-coding RNAs (lncRNAs) were identified after filtering protein-coding genes to retain read clusters longer than 200bp and not covered more than 80% by a small ncRNA. Due to the lack of conservation between species, no functional annotation of the lncRNAs was performed. The final non-coding annotation contains the lncRNAs and the sncRNAs.

### 3.2.4. Population genomics analysis

#### 3.2.4.1. Sampling and parasite load

Between 2019 and 2022 one hundred clams per bed were collected and analyzed from six shellfish beds with different perkinsosis prevalence : i) three sampling sites from NE Atlantic Ocean from Algarve (ALG; 37°01'11.9"N 7°50'11.3"W), Pontevedra (PO; 42°25'41.0"N 8°41'15.3"W), and Noia (NO; 42°47'31.7"N 8°54'56.6"W) (FAO Major Fishing Area 27s - ATLANTIC, NORTHEAST; Subarea 27.9); and ii) four from the Mediterranean Sea from Sardinia (SAR; 39°50'04.0"N 8°29'02.2"E), Izmir (TU; 38°27'09.6"N 26°59'09.3"E), and two from Venice (VEN1 and VEN2; 45°13'02.6"N 12°13'50.9"E9) (FAO Major fishing Area 37, Mediterranean and Black sea; subareas 37.1.3, 37.3.1 and 37.2.1) (Table 1, Figure 1). The two samples from the Venice lagoon (2019 and 2022) were collected to confirm the results observed in 2019 suggesting an intermediate genetic constitution between Atlantic and Mediterranean regions (see Results).

Both hemigills from each clam were collected for *P. olsenii* load evaluation through histology and quantitative PCR (qPCR). Briefly, one hemigill was used for Ray's fluid thioglycollate medium (RFTM) diagnosis according to Ray (Ray, 1966) using the following infection scale: level: 0 – absence of parasite, 1 – very slight infection, 2 – slight infection, 3 – moderate infection, 4 – heavy infection, 5 – critical infection. The other hemigill was placed on ethanol at 70% and stored directly at -20ºC for DNA extraction used for qPCR parasite load evaluation according to Garcia et al. (2022) with the *P. olsenii* specific primers designed by Ríos et al. (2020). After diagnosis, a total of 213 carpet-shell clams were selected for genotyping trying to include all infection levels from each population (Table 1).

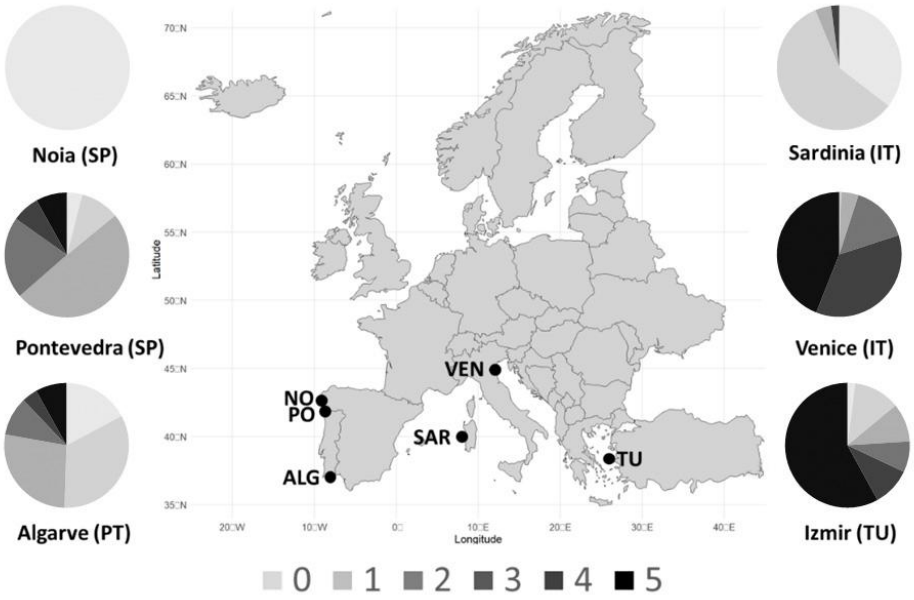


**Figure 1.** Geographical location of *R. decussatus* shellfish beds showing *P. olsenii* prevalence. Levels of infection are shown as pie charts From the RFTM categories in progressive grey scale at the bottom. Prevalence of Venice was calculated as averages of infected clams for each level from both samplings, 2019 and 2022.

**Table 1.** Main features of *R. decussatus* samples. N: Number of genotyped individuals per population. Date: year of sample collection. Prev: prevalence of infection. Perkinsosis status: LTA (Long Term Affected), Naïve (Non-affected).

| Sample code | Location | Country | N | Date | Prev. (%) | Perkins. status | Genotyped individuals from each infection level | | | | | | % of individuals at each infection level | | | | | | Coordinates |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | L0 | L1 | L2 | L3 | L4 | L5 | L0 | L1 | L2 | L3 | L4 | L5 | |
| ALG | Algarve | Portugal | 31 | 2020 | 83 | LTA | 6 | 5 | 5 | 5 | 3 | 7 | 17.2 | 33.3 | 27.3 | 10.1 | 4 | 8.1 | 37°01'11.9"N 7°50'11.3"W |
| NO | Noia | Spain | 31 | 2020 | 0 | Naïve | 31 | 0 | 0 | 0 | 0 | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 42°47'31.7"N 8°54'56.6"W |
| PO | Pontevedra | Spain | 31 | 2020 | 96 | LTA | 4 | 5 | 5 | 9 | 4 | 4 | 4.0 | 10.1 | 49.5 | 21.2 | 7.1 | 8.1 | 42°25'41.0"N 8°41'15.3"W |
| SAR | Sardinia | Italy | 30 | 2022 | 65 | LTA | 14 | 13 | 2 | 0 | 1 | 0 | 35.4 | 58.3 | 4.2 | 0 | 2.1 | 0 | 39°50'04.0"N 8°29'02.2"E |
| TU | Izmir | Turkey | 30 | 2019 | 98 | LTA | 1 | 6 | 5 | 4 | 5 | 9 | 2 | 12 | 10 | 8 | 10 | 58 | 38°27'09.6"N 26°59'09.3"E |
| VEN19 | Venice | Italy | 30 | 2019 | 100 | LTA | 0 | 0 | 0 | 8 | 11 | 11 | 0 | 0 | 0 | 7 | 24 | 69 | 45°13'02.6"N 12°13'50.9"E |
| VEN22 | Venice | Italy | 32 | 2022 | 100 | LTA | 0 | 2 | 10 | 8 | 7 | 3 | 0 | 1 | 9 | 23 | 48 | 19 | 45°13'02.6"N 12°13'50.9"E |

### 3.2.5. RAD-Seq SNP genotyping

Total DNA was extracted from foot muscle (for genotyping) or gill (for qPCR) using the E.Z.N.A.® Mollusc DNA kit (Omega Biotek, USA) following manufacturer instructions. SNP identification and selection, as well as genotyping and validation protocols followed (Maroso et al., 2019). Briefly, AlfI IIb restriction enzyme (RE) was used to construct the 2b-RAD libraries following the protocol by (Manuzzi et al., 2019); samples were evenly pooled for sequencing in Illumina Next-Seq500 including 90 individuals per run at the Genomics Platform of Universidad de Valencia (Spain). Then, home-made scripts were used to cut and remove fastq sequences of unexpected length and to filter out sequences that did not include the Alf1 restriction site in the right position. Individuals with < 250,000 reads were discarded. The assembled *R. decussatus* genome was used as reference to align reads from each individual using Bowtie 1.1.2 (Langmead et al., 2009), allowing a maximum of three mismatches and a unique genome alignment (-v 3 -m 1). STACKS 2.0 (Catchen et al., 2013) was then used to call SNPs and genotype a common set of markers in the whole dataset, applying the marukilow model with default parameters in the gstacks module. This SNP panel was further filtered using STACKS 2.0 and PLINK 1.9 (Purcell et al., 2007) by applying the following criteria: i) minimum allele count (MAC) ≥ 3 in the whole sample; ii) depth > 6 reads iii) genotyped in > 60% individuals in the whole sample; iv) conformance to Hardy–Weinberg equilibrium (HWE; $p > 0.05$) in at least three shellfish beds across the whole collection; and v) selection of the most polymorphic SNP within each RAD-tag.

### 3.2.6. Genetic markers and genomic regions under divergent selection

We applied a method based on haplotype differentiation between population pairs around focal SNPs to detect signals of divergent selection for the two scenarios tested, geographic origin and perkinsosis infection status. Extended haplotype homozygosity (EHH) is a method used to detect selection signatures by measuring the persistence of haplotype homozygosity around a focal variant. Under directional selection, beneficial alleles rapidly increase in frequency, carrying linked haplotypes with them, which results in an extended region of high homozygosity (Gautier et al., 2017). Selection signals can be further investigated using the cross-population EHH (xp-EHH) test, which compares the decay of haplotype homozygosity between two populations to identify loci under selection in one population relative to the other. Alternatively, the Rsb test provides a standardized measure of selection by comparing the integrated EHH scores (iES) between populations.

In our study, we applied the Rsb test, as it is well suited for identifying selection signatures when the genetic structure between populations is not high. Haplotype phasing was performed separately for each chromosome using SHAPEIT5 (Hofmeister et al., 2023) from VCF files. The Rsb test was then applied to all population pairs, and significant genomic windows were identified when the standardized score exceeded 2.33 ($p < 0.01$). Within each significant window, the SNP with the highest signal (focal SNP) was selected for further analysis of genetic diversity and population structure using outlier loci associated with divergent selection.

For the geographic scenario, a total of 15 population pairwise comparisons were performed within the Atlantic Ocean (NO, PO, ALG; 3 comparisons), within the Mediterranean Sea (SAR, VEN, TUR, 3 comparisons), and between Atlantic and Mediterranean regions (9 comparisons). All SNPs for divergent selection detected in the three scenarios were pooled (eliminating duplications) to obtain the total set of SNPs showing significant divergence between any population pair in the total sampling.

For the perkinsosis infection scenario, we compared samples across infection levels using populations with similar infection profile and from the same geographic area, to avoid the geographic differentiation component that could bias the results. To gain statistical power (sample size), we considered three main infection levels: low (L0, L1), moderate (L2, L3) and heavy (L4, L5). This approach assumes a similar selective pressure on the individuals from the populations compared, which it could be expected considering their age (adults) and the previous records of perkinsosis on those populations. Accordingly, for the infection-level approach we used the information of PO and ALG from the Atlantic Ocean and of VEN and TU from the Mediterranean Sea. Common outliers and overlapping genomic windows across the two comparisons were identified and used to detect the most consistent candidate genes through gene mining.

### 3.2.7. Genetic diversity

Genetic diversity per sample was estimated using expected (He) and observed (Ho) heterozygosity, and allelic richness (Ar), computed using the rarefaction method to correct the bias due to sample size. These analyses were performed with the DiveRsity R package v. 1.9 using the ‘basicStats’ function (Keenan et al., 2013). Conformance to HWE was evaluated with an exact test implemented in the R package Genepop 4.7.5 (Rousset, 2008). The sense and magnitude of the deviation from random mating was estimated with $F_{IS}$ (intrapopulation fixation index) with exact tests using Genepop 4.7.5.

### 3.2.8. Genetic structure

Global and pairwise population differentiation ($F_{ST}$; (Weir & Cockerham, 1984)) were estimated using different grouping criteria. Pairwise $F_{ST}$ between populations, as well as global $F_{ST}$ for the whole dataset and for each region and their significance were obtained with the 'Fst' function of the R package Genepop 4.7.5 (Pembleton et al., 2013)

StructureSelector software (Y. Li & Liu, 2018) was used to obtain K estimators and CLUMPAK outputs (Kopelman et al., 2015). Three K estimators were used to identify the most likely number of clusters: the deltaK *ad hoc* estimator (Evanno et al., 2005), Mean LnP(K) (Pritchard et al., 2000) and MedMeaK (Puechmaille, 2016). CLUMPAK output files rendered STRUCTURE bar plots illustrating membership of individuals to inferred genomic clusters. Discriminant analysis of principal components (DAPC), a multivariant method to infer the number of clusters within a group of genetically related individuals, was employed as a complementary approach to disclose the structure in the studied samples. The Adegenet package function 'dapc' in RStudio was used (Jombart & Ahmed, 2011). A principal component analysis (PCA) from the matrix of genotypes was performed and then, a selected number of principal components (PCs) used as input for linear discriminant analysis (LDA). To determine the optimal number of PCs for LDA, a cross-validation process was implemented, and the PCs associated with the lowest Root Mean Square Error (RMSE) retained. Furthermore, DAPCs that preserved at least 90% of the cumulative data variance were subject to evaluation.

### 3.2.9. Gene mining

For each locus, the Rsb score was calculated using the rehh package in R and the candidate genomic regions under divergent selection identified. Genes within those genomic regions were retrieved from the *R. decussatus* genome and further inspected to look for candidate genes related to resistance / tolerance to *P. olsenii* considering their immune function and the previous functional information in response to *P. olsenii* infections in *R. decussatus* (Estêvão et al., 2023) and *R. phillipinarum* (Hasanuzzaman et al., 2018). Gene Ontology (GO) enrichment on the whole gene list within windows was performed with GOfuncR (https://bioconductor.org/packages/release/bioc/html/GOfuncR.html) using gene IDs of the annotated genome taking as reference the whole transcriptome of *R. decussatus*.

#### 3.2.10. Ethics approval and consent to participate

Not applicable.

## 3.3. Results

### 3.3.1. Genome assembly

A total of 159.23 Gb was obtained for long-read ONT sequencing (113x), 2926.26 Gb for 150 bp PE Illumina sequencing (191x) and 542,25 Gb for OmniC sequencing (386x). The genome assembly of *R. decussatus* comprised 1,677 contigs with a contig N50 of 1.868 Mb for a total assembly size of 1,406 Mb (Table 2). This assembly was rather fragmented displaying consensus quality (QV = 39.13) and k-mer (83.9%) completeness. After scaffolding with Hi-C, contigs were assembled in 598 scaffolds, the largest 19 superscaffolds corresponding to the haploid chromosome number of the species (n = 19) and comprising 1,301 Mb (92.5% of the total assembly) (Figures 2 and S3, Table S1). Furthermore, another 90 scaffolds comprising 419 Mb could be placed but not mapped in one of the 19 superscaffolds (95.4% total assembly). The remaining 489 unplaced scaffolds comprised 56 Mb, ranging in length from 1000 bp to 533,292 bp. Genome completeness as assessed by BUSCO using the metazoa_odb10 lineage was 94.3% complete (C:94.5%[S:93.5%,D:1.0%],F:3.7%,M:1.8%,n:954), in the upper range reported for bivalve assemblies in the last five years (Boutet et al., 2022; Gundappa et al., 2022; Peñaloza et al., 2021; Ran et al., 2019).

**Table 2**: Statistics of the chromosome-level genome assembly of *R decussatus.*

| | |
|---|---|
| Contig N50 | 1,867,564 |
| Contig N90 | 378,298 |
| Contig length_max | 9,656,817 |
| Contig len_mean | 838,478 |
| Genome length | 1,406,128,519 |
| No. contigs | 1,677 |
| No. gaps | 316 |
| GC content | 32.34 |
| QV | 39.13 |
| kmer-completeness | 83.9 |
| BUSCO5.2.2 metazoa_odb10 | C:94.8%[S:93.4%,D:1.4%],F:3.5%,M:1.7%,n:954 |

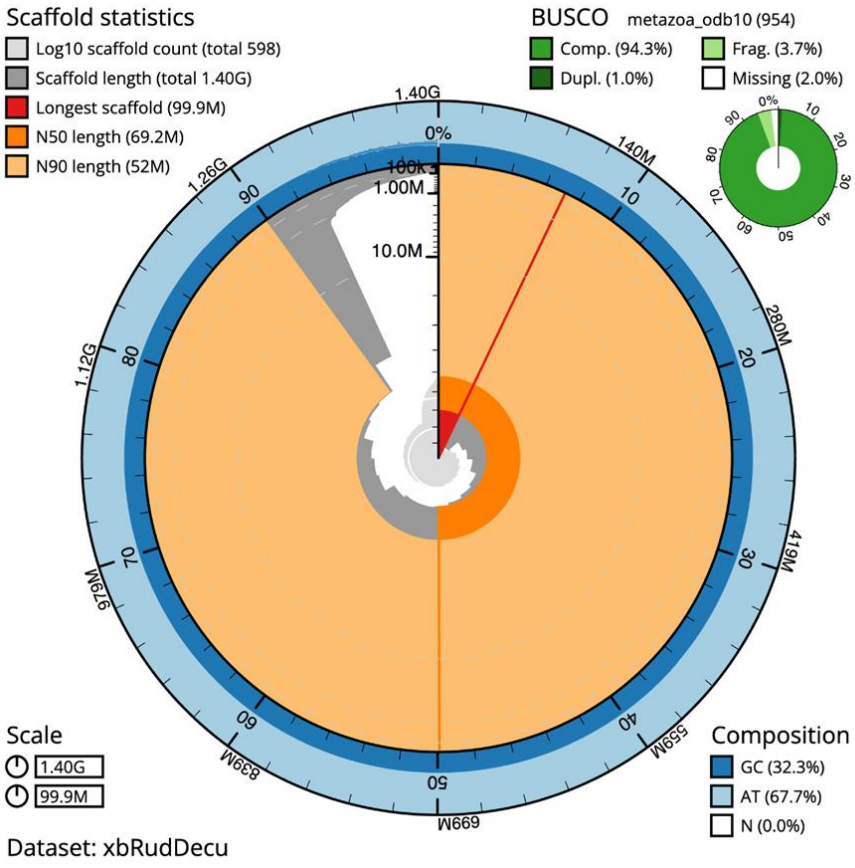


**Figure 2:** Snail plot showing contiguity and quality of *R. decussatus* genome assembly.

### 3.3.2. Genome annotation

In total, 38,276 protein-coding genes that produce 54,530 transcripts (1.42 transcripts per gene) were annotated and encoded for 49,539 unique protein products (Tables 3 and S2). Functional labels to 8.5% of the annotated proteins could be assigned (Table S3). The annotated transcripts contain 8.5 exons on average, with 86% being multi-exonic. In addition, 13,056 non-coding transcripts were annotated, of which 11,425 and 1,631 corresponded to long and short non-coding RNA genes, respectively.

**Table 3**: Annotation statistics of the *R. decussatus* genome.

| | |
|---|---|
| Number of protein-coding genes | 38,276 |
| Median gene length (bp) | 7,087 |
| Number of transcripts | 54,530 |
| Number of exons | 283,949 |
| Median UTR length (bp) | 1,585 |
| Median intron length (bp) | 811 |
| Exons/transcript | 8.49 |
| Transcripts/gene | 1.42 |
| Multi-exonic transcripts | 86% |
| Gene density (gene/Mb) | 27.38 |

### 3.3.3. SNP calling

From a total of 994.5 million reads obtained by 2bRAD-Seq in the 215 individuals studied, 81.3% were retained after quality filtering. On average 46% of reads aligned to unique sites in the *R. decussatus* genome, representing 3.5 M reads per individual (ranging from 90 K to 4.3 M reads), most being discarded due to multiple alignments (34%) and only 1.4% did not aligned to the assembled genome. The gstacks module, using the marukilow model applied to all samples yielded 160,871 RAD loci. After filtering, a total of 13,438 SNPs were retained, which constituted the common catalogue for analyses in the six studied shellfish beds (Figure 1). The main filtering steps were “loci with MAC ≥ 3” (54.5 %),

"genotyped in 60% individuals" (57.4%) and "keeping only one SNP per RAD" (50.4%) (Figure 3).

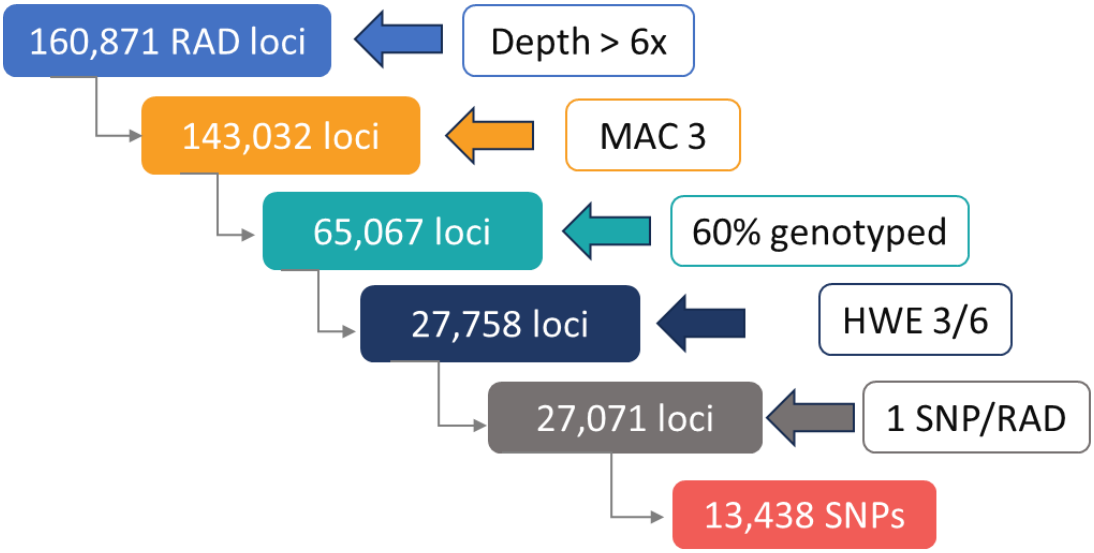


**Figure 3.** Bioinformatic filtering pipeline followed for SNP calling and selection in *R. decussatus*.

### 3.3.4. Outlier identification

Outliers and genomic regions under divergent selection were explored with the Rbs method of EHH software considering the main goals of the study: i) to obtain a preliminary picture of the genetic structure of *R. decussatus* also considering outliers for divergent selection in Atlantic and Mediterranean shellfish beds; ii) to identify candidate genes and markers associated with resistance / tolerance to *P. olsenii* by comparing groups of individuals classified by infection level (low, moderate, heavy) across shellfish beds with similar infection profile. From outlier information, the following SNP datasets were used to analyse genetic diversity and population structure: i) the whole SNP dataset; ii) SNP dataset under divergent selection obtained from the geographic scenarios explored; iii) neutral SNPs, obtained by excluding all outliers (geographical and perkinsosis infection) from the whole SNP dataset. For the analyses, both Venice samples were pooled (60 individuals), after confirming no significant genetic differentiation between them (see below).

The geographical analysis revealed 394 SNPs under divergent selection in the 12 pair-wise comparisons tested (ATL (3), MED (3), ATL vs MED (9)) (Tables S4-S7). Many outliers were specific of each pairwise comparison in all scenarios and the number of outliers detected were higher in the ATL vs MED (176, Table S6) and the ATL (145, Table S4) scenarios, than in the MED scenario (105, Table S5).

When exploring signals of selection for resistance / tolerance to *P. olsenii*, we detected a total of 343 outliers by comparing the level of infection (low: L0+L1; moderate: L2+L3; heavy: L4+L5) using two population pairs showing similar infection profile (ATL scenario: ALG and PO; MED scenario: VEN and TU) (Tables S8-S10); among them, 191 were detected in the ATL scenario (Table S8), 158 in the MED scenario (Table S9), and 90 were shared between both scenarios or lied within the same genomic window showing signals of divergent selection (< 1 Mb distance; 29 genomic windows, Table S10). Genes within these genomic windows were inspected for their putative association with immune response and with previous studies on perkinsosis.

### 3.3.5. Genetic diversity

Average observed (Ho) and expected (He) heterozygosity and allelic richness (Ar) were first estimated with the whole SNP dataset. All estimators showed significantly higher genetic diversity in the Mediterranean than in the Atlantic region (average Ar: 1.797 vs 1.604; He: 0.242 vs 0.173; Mann-Whitney test $p < 0.001$ in all cases) (Table 4A). VEN samples were by far the most diverse samples among all studied (Ar = 1.854; He = 0.233). All sampling locations conformed to HWE ($p > 0.05$). Similar observations were obtained when using the neutral dataset (data not shown).

**Table 4**. Genetic diversity and intrapopulation fixation index ($F_{IS}$) of *R. decussatus* populations with: (A) whole dataset; (B) geographical outliers for divergent selection.

| A) | | | | |
|---|---|---|---|---|
| | Ar | He | Ho | $F_{IS}$ |
| ALG | 1.595 | 0.166 | 0.166 | 0.003 |
| NO | 1.606 | 0.17 | 0.167 | 0.021 |
| PO | 1.610 | 0.168 | 0.169 | 0.002 |
| SAR | 1.711 | 0.195 | 0.200 | -0.008 |
| TU | 1.769 | 0.203 | 0.198 | 0.027 |
| VEN | 1.854 | 0.233 | 0.228 | 0.037 |
| Atlantic | 1.604 | 0.173 | 0.166 | 0.009 |
| Mediterranean | 1.797 | 0.242 | 0.211 | -0.008 |
| B) | | | | |
| | Ar | Ho | He | $F_{IS}$ |
| ALG | 1.660 | 0.156 | 0.157 | 0.012 |
| NO | 1.715 | 0.199 | 0.209 | 0.044 |
| PO | 1.735 | 0.219 | 0.226 | 0.017 |
| SAR | 1.850 | 0.303 | 0.297 | -0.005 |
| TU | 1.885 | 0.264 | 0.276 | 0.038 |
| VEN | 1.967 | 0.346 | 0.365 | 0.051 |
| Atlantic | 1.797 | 0.191 | 0.212 | 0.024 |
| Mediterranean | 1.993 | 0.315 | 0.372 | 0.028 |

In bold values with p <0.05

When using the geographical outlier panel for divergence selection (Table 4B), genetic diversity was significantly higher for all estimators in the Mediterranean than in the Atlantic region as for the whole SNP dataset; but additionally, genetic diversity was significantly higher for the outlier than for the neutral dataset both in the Atlantic (average Ar: 1.797 vs 1.615; He: 0.212 vs 0.170; Mann-Whitney test $p < 0.05$) and the Mediterranean (average Ar: 1.993 vs 1.797; He: 0.372 vs 0.237; Mann-Whitney test $p < 0.05$) regions.

### 3.3.6. Genetic structure

The two samples from Venice lagoon (VE19 and VE22) showed no genetic differentiation between them ($F_{ST} = 0.002$; $p > 0.05$) and accordingly, they were pooled into a single sample of 60 individuals. The global $F_{ST}$ for all samples using the whole SNP dataset was 0.180 ($p < 0.001$). The most pronounced genetic differentiation was detected between Atlantic and Mediterranean samples (average pairwise $F_{ST} = 0.224$; Table 5A). No significant genetic differentiation was detected between NO and PO ($p > 0.05$ after Bonferroni correction), while ALG and TU were the most differentiated populations ($F_{ST}$ =

0.356; p = 0). Higher differentiation was detected within the Mediterranean Sea than within the Atlantic Ocean (average pairwise $F_{ST}$ = 0.169 vs 0.031, respectively). Pairwise $F_{ST}$ comparisons using the neutral SNP panel showed very similar results (data not shown).

A much higher differentiation was found when using the geographic divergent outlier SNPs, although following a similar pattern to that observed with the whole and neutral datasets (Table 5B). No significant differentiation was detected between the two close Spanish populations (NO and PO), but figures increased abruptly in all other cases with $F_{ST}$ > 0.2 in all Atlantic vs Mediterranean comparisons (average $F_{ST}$ = 0.318). TU displayed the highest differentiation with Atlantic populations ($F_{ST}$ > 0.4), but also important with the other Mediterranean populations, especially SAR ($F_{ST}$ = 0.346).

**Table 5.** Pairwise $F_{ST}$ values between populations of *R. decussatus* with (A) whole SNP dataset; (B) geographic divergent selection outliers. Below and above the diagonal $F_{ST}$ and probability values, respectively.

| A) | ALG | NO | PO | SAR | TU | VEN |
|---|---|---|---|---|---|---|
| ALG | - | * | * | * | * | * |
| NO | 0.045 | - | 0.032 | * | * | * |
| PO | 0.048 | **0.001** | - | * | * | * |
| SAR | 0.158 | 0.152 | 0.150 | - | * | * |
| TU | 0.355 | 0.349 | 0.349 | 0.2841 | - | * |
| VEN | 0.173 | 0.168 | 0.166 | 0.085 | 0.137 | - |

| B) | ALG | NO | PO | SAR | TU | VEN |
|---|---|---|---|---|---|---|
| ALG | - | * | * | * | * | * |
| NO | 0.129 | - | 0.293 | * | * | * |
| PO | 0.127 | **0.010** | - | * | * | * |
| SAR | 0.272 | 0.224 | 0.206 | - | * | * |
| TU | 0.485 | 0.434 | 0.416 | 0.346 | - | * |
| VEN | 0.266 | 0.224 | 0.209 | 0.123 | 0.135 | - |

In bold highlighted no significant $F_{ST}$ values after Bonferroni correction (p < 0.003); * p < 0.001

The clustering method of STRUCTURE was run for K values ranging from 1 to 7 (number of samples plus 1; Figure S4) using deltaK and Mean LnP(K) estimators; K = 4 was the optimal number of clusters with the whole and neutral SNP datasets. The results revealed a single cluster comprising the three Atlantic populations, while the three Mediterranean samples, TU, SAR and VEN, constituted separate entities, with SAR

showing a certain Atlantic component and VEN some admixture from different clusters (Figure 4A).

When employing the divergent geographic outlier panel, the results were slightly different depending on the method used, the most likely number of clusters (K) being either 4 or 5 (Figures 4B and 4C; Figure S5). The three Atlantic populations constituted a single cluster for K = 4, while the three Mediterranean populations clearly constituted separated units, more than with the whole SNP dataset. However, a notable differentiation of the southernmost population (ALG) was detected for K = 5 in the Atlantic, and within the Mediterranean Sea, the Venice Lagoon (VE) showed an admixed composition mainly from ALG but also, to a minor extent, from the other clusters.

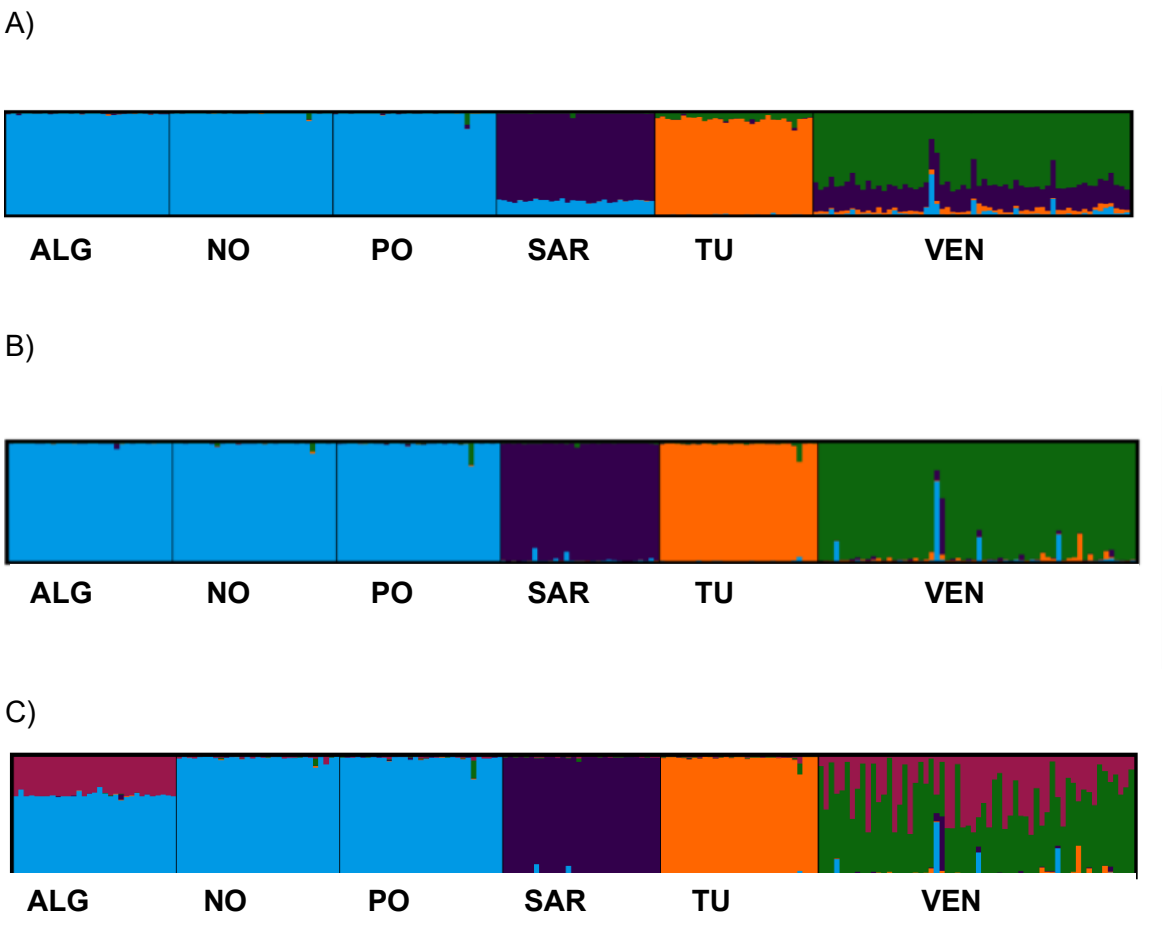


**Figure 4**. STRUCTURE analysis in *R. decussatus* with (A) the whole panel for K=4; (B) and (C) geographical outlier panel for K=4 and K=5, respectively.

The discriminant analyses of principal components (DAPC) complemented and confirmed the results observed with STRUCTURE (Figure S6). Atlantic samples were tightly clustered with the whole SNP dataset, while the Mediterranean were highly differentiated.

Some nuances could be unveiled with the divergent outlier panel, which depicted the ALG slightly differentiated from the northern Atlantic NO and PO; additionally, VE was nearly equidistant from TU and SAR, but also to the Atlantic cluster.

### 3.3.7. Gene mining

A total of 90 outliers related to the *Perkinsus* resistance / tolerance were shared or lied within the same genomic window (< 1 Mb) when comparing the ATL (ALG and PO) and MED (VEN and TU) infection-level approach (Table S10). The 29 genomic windows, where the infection-level outliers were located (43Mb; 3.07% of *R. decussatus* genome) included 439 genes that were inspected to identify candidates related to perkinsosis resistance / tolerance (Table S11). No functional enrichment GO terms were identified in this set of genes using GOfuncR, despite the very important immune-related gene families included, such as complement C1q, E3 ubiquitin-protein ligase, glutathione peroxidase, MAM and LDL-receptor class A, lysozyme, cytochrome P450, lymphocyte antigen, N-acetylglucosamine-1-phosphotransferase, peptidoglycan recognition protein, peroxidase, proteasome, prostaglandin E2 receptor, serine/threonine-protein kinase, toll-like receptor, and tripartite motif-containing protein, among others (Table S11B). The poor functional annotation of *R. decussatus* genome (8.5%; Table S3), a usual feature of mollusc genomes (Liu et al., 2021), likely underlies this outcome. Then, we compared our list of 439 genes with those from the functional studies performed by Estêvão et al., (2023; proteomic) in *R. decussatus* and by Hasanuzzaman et al. (2018; transcriptomic) in the congeneric species *R. philippinarum* in response to *P. olsenii* (Table S12). Among them, we could identify five genes related to iron storage, cytoskeleton organization, proteases and energy balance with identical or similar annotation to the list of 32 proteins differentially expressed when comparing heavily infected vs non-infected clams by Estêvão et al. (2023). Specifically, phosphoenolpyruvate carboxykinase [GTP] and proteasome subunit alpha type were also detected as differentially expressed (DEG) in response to infection in the wild and controlled laboratory conditions by Hasanuzzaman et al. (2018). Furthermore, we could identify 12 genes with identical annotation to the DEGs reported by Hasanuzzaman et al. (2018) (Table S12). Four of them, complement C1q-like protein 4, cytochrome P450 2A6, tyrosine-protein kinase, and ubiquitin-conjugating enzyme E2 U, where considered as crucial in the response of *R. phillipinarum* to *P. olsenii* by these authors. Other 15 DEGs showed very similar annotation or pertained to the same gene family, and among these, caprin-1, low-density lipoprotein receptor-related protein 2, palmitoyltransferase B, and universal stress

protein A. R, were also considered critical by Hasanuzzaman et al. (2018) in the response to *Perkinsus*.

### 3.4. Discussion

The increasing prevalence of *P. olsenii* poses a significant challenge to aquaculture, resulting in extensive mortalities and adverse impacts on clam health, which translates into economic losses and disruption of marine ecosystem balance. Perkinsosis shows variable incidence throughout *R. decussatus* shellfish beds, from the Atlantic Ocean to the Mediterranean Sea, embracing east and west Mediterranean beds. Thus, the first goal of the present study was to obtain information on the genetic diversity and structure of the species across the whole distribution range, applying for the first time SNP markers covering the whole genome at medium density (13,438 SNPs; ~ 1 SNP / 100 kb), taking as reference a new chromosome-level genome here assembled. This enabled to contrast previous information obtained on genetic structure of the species with a low number of mtDNA, isozyme, microsatellite and SNP markers (Arias-Pérez et al., 2016; Cordero et al., 2014; Cruz et al., 2020) and to identify suggestive outlier loci associated with divergent selection across the grooved carpet clam distribution to be thoroughly explored in the future.

#### 3.4.1. Genetic structure

Genetic diversity was significantly higher in Mediterranean than in Atlantic populations, as previously reported (Arias-Pérez et al., 2016; Cordero et al., 2014). Consistent geographic differentiation was detected between Atlantic and Mediterranean populations using the whole or the neutral SNP datasets (average pairwise $F_{ST}$ = 0.224 and 0.193, respectively), in the range reported in other studies using nuclear RFLPs and microsatellites (Arias-Pérez et al., 2016; Saavedra & Cordero, 2024). A much lower differentiation was found between the Atlantic samples (average pairwise $F_{ST}$ = 0.031), which constituted a single cluster with STRUCTURE and DAPC analyses, than between the Mediterranean ones ($F_{ST}$ = 0.169), which essentially represented three differentiated clusters. This observation could be partially related to the different geographic extension surveyed in the Atlantic Ocean and the Mediterranean Sea, but it has also been reported in other aquatic species across a similar distribution range (Maroso et al., 2019; Saavedra & Cordero, 2024). Our data suggest a higher isolation of Mediterranean populations either by a more complex current / front marine pattern or by less efficient larval dispersion mechanisms that could be

operating in an inner sea. Within the Mediterranean Sea, the TU and SAR samples, located in the eastern and western regions, displayed the greatest differentiation ($F_{ST}$ = 0.283), and as previously suggested with mtDNA markers (Arias-Pérez et al., 2016), the SAR population was closer to the Atlantic samples than to the other Mediterranean shellfish beds. All data soundly point towards a mixed origin of the population from Venice lagoon that showed higher genetic diversity and admixed constitution, including a certain component from the Atlantic which agrees anectodical evidence of restocking in that area using clams of various origins (L. Bargelloni, personal communication). Genetic differentiation greatly increased when exploring outlier loci under divergent selection, particularly when comparing Atlantic and Mediterranean samples (average $F_{ST}$ = 0.318), suggesting adaptation to the very different environmental conditions between both body waters. Despite the Atlantic Ocean showed lower differentiation with divergent outliers than the Mediterranean Sea (average $F_{ST}$ = 0.089 vs 0.201), the percentage with respect to the neutral dataset increased much more in the Atlantic Ocean (average $F_{ST}$ = 0.089 vs 0.028) than in the Mediterranean Sea (0.201 vs 0.168), which could suggest a more heterogeneous environment in the Atlantic Ocean. In fact, this is also supported by the higher number of divergent outliers detected within the Atlantic than within the Mediterranean area (156 vs 116). The results, although based on a limited sampling collection, support previous observations, but provide new insights on genetic diversity and structure across the *R. decussatus* distribution range, emphasizing the important differentiation of a species living in highly diverse environmental conditions that should be considered both for broodstock foundation as well as for genetic-environmental interactions in production areas.

### 3.4.2. Genetic and environmental factors underlying perkinsosis

*Perkinsus* infection of clams exhibits variable outcomes depending on environmental conditions, especially temperature and salinity, which have been reported to play an important role in parasite prevalence (Ruano et al., 2015; Villalba et al., 2004). Elevated water temperatures have been linked to increased *Perkinsus* prevalence and variations in salinity levels have been observed to influence the susceptibility of *R. decussatus* to *P. olsenii*, emphasizing the need for a comprehensive examination of these factors (Casas & Villalba, 2012). However, high prevalence of the parasite has been detected both in Atlantic and Mediterranean populations, despite the temperature and salinity differences in both regions (this study; Ruano et al., 2015). Parasite prevalence may be also related to the genetic constitution of populations, and the genetic differentiation observed between them in our study might reflect differences in resistance / tolerance to perkinsosis. Noia estuary

was the only perkinsosis-free area among the populations studied, but this appears to be related to the particular current patterns in NW Spain, since this estuary has proved to be also free of other emergent parasites in bivalves, such as *Marteilia cochillia* in common cockle (*Cerastoderma edule*) (Pampín et al., 2023; Villalba et al., 2023). Within the Mediterranean Sea, the prevalence was much lower in SAR, which could be related to its insular condition or less impact of clam species transference from other areas. Despite the other two Atlantic (PO, ALG) and Mediterranean (VEN, TU) showed nearly 100% prevalence, the average infection level was significantly lower in the Atlantic than in the Mediterranean populations. Indeed, perkinsosis incidence has been associated with temperature and salinity. However, a higher parasite pressure should determine changes in the genetic constitution of populations associated either to resistance or tolerance (or both), provided a significant heritability, and in fact, genetic divergence has been detected for specific genomic regions including immune-related genes in the two scenarios studied (ALG & PO; VEN & TU). Furthermore, although common genomic regions were detected in the two scenarios, specific signals were also detected within each scenario, suggesting an adjusted response depending on parasite diversity, environmental factors or their interaction.

### 3.4.3. Genetic markers and candidate genes for resistance / tolerance to *P. olsenii*

Resistance, tolerance, and resilience are terms featuring host-parasite interactions that have been largely discussed in the literature (Holbrook et al., 2021; Paraskevopoulou et al., 2022; Råberg et al., 2008). While resistance refers to the capacity of the host to avoid the parasite entrance or to eliminate it once inside, tolerance is related to the ability of the host to maintain a certain level of health/fitness and an ability to neutralise the virulence of the parasite. On the other hand, resilience defines the capacity of recovery from a parasite infection. Discrimination of the different components are more affordable on controlled experimental conditions, so in the wild samples of our study we could hardly discriminate between them, especially resilience. Furthermore, it is also possible that individuals with no infection have been recovered from a previous infection, and this cannot be distinguished from resistant individuals to infection. Bearing in mind these considerations, we decided with all cautions to use only the combination of resistance / tolerance to interpret the association of markers with the different infection levels, despite some features could be associated to resistance (or recovery) and tolerance.

Most samples in the present study have been probably in contact with the parasite at any time of their life considering the long infection records of shellfish beds (Ruano et al. 2015) and the age of the individuals collected (adults), resulting in the high prevalence observed (close to 100 % in most cases, excluding NO, naïve). However, as outlined before, perkinsosis pressure probably differs across the shellfish beds studied and accordingly the genetic response underlying the resistance or tolerance to perkinsosis. Thus, despite detecting signals of selection to perkinsosis in the wild is more complex and might be confounded with other environmental factors associated to geography, it offers the opportunity to check broader scenarios to be interpreted for a more comprehensive understanding of this parasitosis. On the other hand, challenge in laboratory conditions enables a much better control of environmental factors, and thus an increasing statistical power to detect QTL associated with resistance / tolerance, but it does not consider the environment variation in the wild, where clam production takes place.

In our study, we could identify a set of 90 SNPs located in 29 genomic windows (43 Mb, 3.07% genome) related to divergent selection for resistance / tolerance to perkinsosis shared in the two infection-level scenarios (low, moderate, heavy), Atlantic (ALG and PO) and Mediterranean (VEN, TU), mapping on the assembled chromosomes (supercaffolds) of *R. decussatus*. The list of 439 genes within those genomic windows enabled to identify candidates related to resistance / tolerance and to compare them with those previously reported using functional approaches in response to *P. olsenii* in *R. decussatus* (Estêvão et al., 2023) and *R. philippinarum* (Hasanuzzaman et al., 2018). It should be noted that the study by Hasanuzzaman et al. (2018) involved the comparison of transcriptomic response between different levels of infection in the wild, a similar experimental scenario to our infection-level comparison. Among the most relevant genes here identified in genomic windows in the two infection-level scenarios inspected, phosphoenolpyruvate carboxykinase [GTP], a key enzyme related to energy balance through gluconeogenesis but also involved in bacteria recognition and elimination (Lv et al., 2017), and several proteasome related genes, involved in cell proliferation and differentiation, replication of protozoan parasites and stress response (Fernández-Boo et al., 2014; Portilho et al., 2019), were also differentially expressed in the two functional studies outlined before. The same occurred with several DEGs detected in the transcriptome response of *R. phillipinarum* to *P. olsenii* in the wild, some of them considered critical in host-parasite interaction by Hasanuzzaman et al. (2018). Among them, complement C1q-like protein 4 and cytochrome P450 2A6, involved in immune and stress response (Lüchmann et al., 2015; Nie et al., 2016; Wang et al., 2024); tyrosine-protein kinase, ubiquitin-conjugating enzyme E2 U, and caprin 1, involved in apotosis and cell proliferation pathways (Leite et al., 2013; Prado-Alvarez et

al., 2009; Romero et al., 2015). On the other hand, low-density lipoprotein receptor-related protein 2 and palmitoyltransferase related to lipid homeostasis, has also been identified as potential biomarkers by Hasanuzzaman et al. (2018). Finally, several genes pertaining to relevant immune and stress related families in molluscs, such as caprin, E3 ubiquitin, ras-related proteins, heat shock proteins, serine/threonine proteins, universal stress proteins and vacuolar protein sorting-associated protein (Cheng et al., 2016; de la Ballina et al., 2021; Guo et al., 2018; Huang et al., 2015; Lou et al., 2020; Ronza et al., 2018; Smits et al., 2020), identified in our study and by Hasanuzzaman et al. (2018), will deserve further investigation to understand the response of grooved carpet shell clam to perkinsosis.

### 3.5. Conclusions

The first chromosome-level genome of *R. decussatus* was assembled and used as reference for a population genomics study throughout the distribution range of the species. We could consistently genotype > 13,000 SNPs using 2b-RADseq for the first genomic screening of *R. decussatus* involving Mediterranean and Atlantic regions. Our data confirmed the main features of the genetic structure previously reported but also made possible identifying a subset of SNPs related to divergent selection putatively associated with environmental factors to be further explored. The comparison of perkinsosis infection across populations with similar infection profile allowed the consistent identification of 90 markers and associated genomic windows related to divergent selection for resistance / tolerance to *P. olsenii*, including candidate genes previously considered as critical in the response of infected clams in functional studies in the wild. This information should be considered for understanding the genetic basis of this parasitosis, which should be helpful to devise strategies for its control and to stablish founder population in hatcheries for an improved seed resistance.

### 3.6. Availability of data and materials

To be uploaded to public repositories upon acceptance.

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# Chapter 4

# Insights on the susceptibility of three commercial clam species to *Perkinsus olsenii* infection and its response to infection

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# Chapter 4 - Insights on the susceptibility of three commercial clam species to *Perkinsus olsenii* infection and its response to infection

## 4.1. Introduction

Bivalve production is reaching new highs in the last 10 years of production, surpassing already 2 billion dollars of global trade value (imports, exports and reexports) (FAO, 2023). In Portugal, the three main commercial clam species are *Ruditapes decussatus*, *Ruditapes philippinarum*, and *Venerupis corrugata*, which production has been affected by biotic and abiotic factors such as disease infection (Ramilo et al., 2016) and salinity and temperature (Carregosa et al., 2014; Macho et al., 2016; Woodin et al., 2020), respectively, which interferes with the production volume due to mass mortalities and also to product quality impairing the condition index and flesh quality. One of the major concerns of the industry has been the infection of these species with the protozoan parasite *Perkinsus olsenii*, known to infect a wide range of mollusc species (Villalba et al., 2004). Individuals affected by this parasite present a disorder in the homeostasis which, in the presence of adverse conditions, may lead to death. The main effects in clams, caused by the infection of this pathogen, are tissue inflammation, followed by growth reduction, reproductive impairment, and low gamete quality, ultimately leading to mass mortalities (Choi & Park, 2010; Fernández-Boo et al., 2023; Villalba et al., 2004).

Perkinsosis was largely studied in *R. philippinarum* but there is limited information in other hosts such as *R. decussatus* and specially in *V. corrugata* (Choi & Park, 2010; Estêvão et al., 2023; Garcia et al., 2022; Hasanuzzaman et al., 2020; Ramilo et al., 2016; Villalba et al., 2004). Also, the information regarding the differential prevalence of the parasite or the susceptibility of each clam species to perkinsosis is missing in available bibliography. There are just a few research articles studying the prevalence of *P. olsenii* in those three clam species, but none of them present all species from the same clam bed, which could be desirable to have a comparison with the same parasite pressure in the different species (Balseiro et al., 2010; Ordás et al., 2001; Ramilo et al., 2016). There are also some histopathological reports from the INTECMAR Institute where information about the disease prevalence of several bivalve species is found since the year 2000 up to date, but information of the three clam species at the same place is not founded (http://www.intecmar.gal/Informacion/Patoloxia/Default.aspx?sm=e). Only previous research performed by Rodríguez et al. (1994) in adults from the same three clam species showed that *Perkinsus atlanticus* (=*olsenii*) is able to infect and multiply in those species. Also, results showed that parasite multiplies faster in *R. philippinarum* than *R. decussatus* and *V. pullastra* (=*V. corrugata*).

In order to shed some light into this matter, an *in vivo* trial was designed with seeds from the three species. The objective of the present study is to answer three questions: I) Is the parasite able to infect all species at the same intensity? II) Is *P. olsenii* more infective in the target species *R. philippinarum*? III) What is the immune response of each species following short and long-time exposure? Here, the Perkinsosis problematic is addressed in three commercial clam species using qPCR for infection quantification and prevalence determination, mortality, and also using transcriptomics to analyse hosts immune responses at short and long-term.

## 4.2. Material and methods

### 4.2.1. Isolation of *Perkinsus olsenii* hypnospores

One hundred and twenty adult individuals from *R. decussatus* collected from Ria Formosa (Portugal). One hemigill was incubated in RFTM for 7 days for *Perkinsus olsenii* diagnosis, while the rest of the body was incubated in RFTM for *P. olsenii* isolation (Casas et al, 2002). Diagnosis was evaluated according to Ray (1954) and animals with an infection level of 4 and 5 were selected to isolate the maximum quantity of parasites. Hypnospore isolation was performed according to Casas et al. (2002). Isolated pre-zoosporangia were incubated for 5 days in sterile seawater with antibiotics to avoid bacteria proliferation. After that, the percentage of developed zoosporangia was calculated in a Malassez chamber under light microscopy.

### 4.2.2. Experimental infection of clam seed by *Perkinsus olsenii*

Clam seed (5-7 mm size) from the three species was produced at Oceano Fresco S.A. facilities (Nazaré, Portugal) from parental individuals of *R. decussatus* from Povoa do Caramiñal, Galicia, Spain (42.61315, -8.928174); *R. philippinarum* from Enseada de Ribeira, Galicia, Spain (42.564005, -8.990126) and *V. corrugata* from Povoa do Caramiñal, Galicia, Spain (42.61315, -8.928174), using the protocol developed by Matias et al. (2016). Briefly, clams were induced to spawn by thermal stimulation, through a rapid increase in temperature from 20 to 22 ºC and to 28 ºC at each interval of 2 h, over a 6-h interval. Fertilization of oocytes from females by nutritional regime was carried out by addition of a mixture of sperm from males. Embryos from each treatment were incubated in triplicate 5-L tanks, with 1-µm filtered and UV-irradiated seawater, maintained at 20 °C, at a density of 100 eggs per ml. After 48 h of incubation, the D-larvae were collected.

For the experimental infection, seeds from the three species were reared in six 25 L tanks (2 control, 2 low infection, 2 high infection) at 18 ± 1 ºC. Each tank contained 2 replicates of each species in a net mesh with 50 clam seed per mesh (Figure 1). A *P. olsenii* solution of isolated pre-zoosporangia containing a percentage of zoosporangia of 18% and approximately 680,000 zoospores/mL was used to infect the reared clam seed in the tanks. From this *P. olsenii* preparation, a total of 4.5 x $10^5$ hypnospores and 3.26 x $10^6$ zoospores were immersed in the low infection (LI) condition tanks, and a total of 4.5 x $10^6$ hypnospores and 3.26 x $10^7$ zoospores were immersed in the high infection (HI) condition tanks. Clam seed were exposed to *P. olsenii* parasites for 48 hours to allow parasite entrance and infection of the individuals; time at which the first sampling was performed, and water was changed for the first time. After this, water was changed every two days. A total of 3 clams per mesh (n = 6 clams per tank, n = 12 per species and condition) were collected and stored individually in 1.5 mL tubes at -20 ºC for diagnosis of *P. olsenii* by qPCR. Two clams per mesh (n = 4 clams per tank, n = 8 per species and condition) were collected for transcriptome analysis and they were placed into 1.5 mL tubes containing 0.5 mL of RNA Later (Sigma, US) (Figure 2). Samples were taken at 48 hours, 1 week, 2 weeks, and 4 weeks after parasite challenge.

**Figure 1.** Net-mesh used in the *in vivo* trial. Each mesh contains 50 clam seed from one species. At each tank 6 net-mesh were placed, two per clam species.

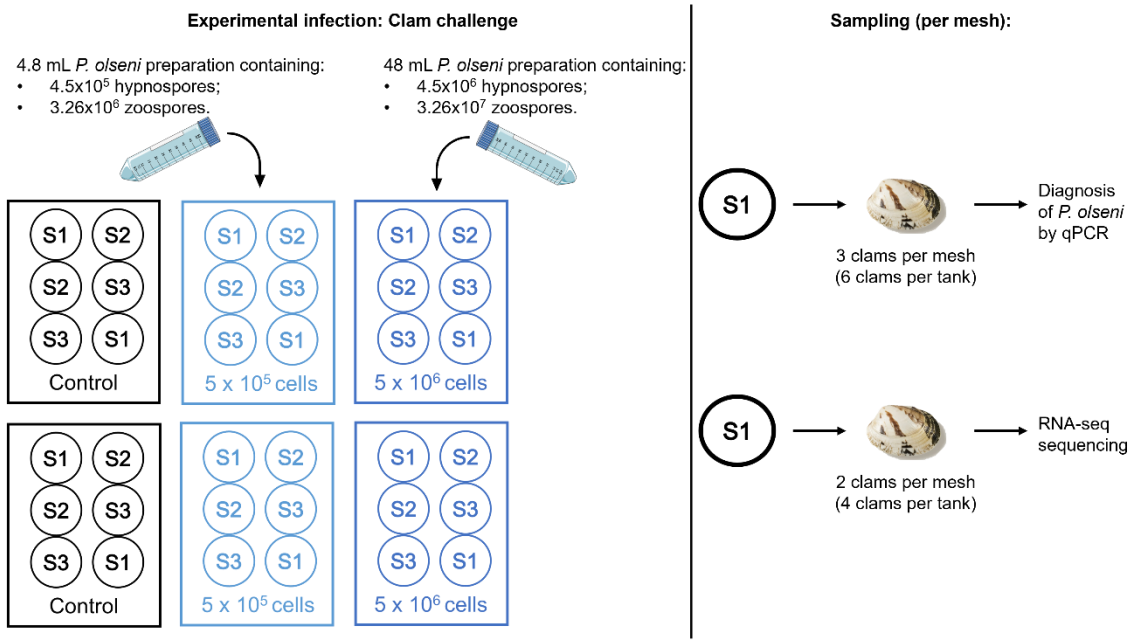


**Figure 2.** Experimental set up of the infection of clam seed by *P. olsenii*. S1: *R. decussatus*; S2: *V. corrugata*; S3: *R. philippinarum*. 5 x $10^5$ and 5 x $10^6$ cells tanks represents low and high infection tanks, respectively.

### 4.2.3. DNA extraction and *Perkinsus olsenii* quantification

DNA was extracted from N = 12 whole clam seed individuals from each species and condition using the E.Z.N.A.® Mollusc DNA kit (Omega Biotek, USA), according to manufacturer's instructions and quantified on a DeNovix DS-11 FX (DeNovix Inc. US). Quantitative PCR was carried out to quantify the *P. olsenii* parasite burden in the individuals using the primers designed by Ríos et al. (2020) with slight modifications (Garcia et al., 2022).

### 4.2.4. RNA extraction and clam seed transcriptomics

RNA was extracted from N = 8 whole clam seed individuals from each species and condition at 48 hours and 4 weeks post challenge. RNA extraction was performed using the E.Z.N.A.® DNA/RNA kit (Omega Biotek, USA), according to manufacturer's instructions and quantified on a DeNovix DS-11 FX (DeNovix Inc. US). Three individuals per condition at each collection time (48 hours and 4 weeks) and each species were selected according to their quality and quantity and sent to Novogene UK Company limitied for mRNA sequencing in a Nova Seq X plus (PE150) (Illumina Inc., US) with 20 million reads pair-end.

The quality of the reads was assessed by using FastQC v.0.12.1 (Andrews, 2010). Quality filtering and removal of residual adaptor sequences were performed on read pairs using TrimGalore (Krueger et al., 2021). Residual Illumina-specific adaptors were eliminated, and reads were trimmed if a sliding window average Phred score over five bases was <20, and only reads where both pairs had a length longer than 75 bp post-filtering were retained. Filtered reads of each species were aligned against their own genome (*Ruditapes decussatus* – Sambade et al. (2025) under revision; *Ruditapes philippinarum* – a new improved version from Mun et al. (2017), unpublished) and assigned to genes based on the latest annotation of their genome using STAR v. 2.7.10b (Dobin et al., 2013). The option --quantMode GeneCounts was used to obtain the number of reads for each gene (gene count data). Also, filtered reads of each species were aligned against *P. olsenii* genome (Bogema et al., 2021) in order to try to identify differential expressed genes in the parasite and describe the crosstalk between clam seed and the pathogen.

Gene count data were used to calculate gene expression and estimate differential expression (DE) using the Bioconductor package DESeq2 v.1.44.0 (Love et al., 2014), in R v.4.4.1 (R Core Team, 2017). Differential gene expression was performed using the standard parameters in DESeq2, with an application of an adjusted p-value cut-off of $p < 0.05$ to the obtained results. Hierarchical clustering and principal component analyses (PCA) were performed to assess the clustering of the samples and identify potential outliers over the general gene expression background. The R packages “pheatmap”, “PCAtools” and “EnhancedVolcano” were used to plot heatmaps, principal component analysis (PCA) and volcano plots, respectively.

Functional enrichment analyses were performed using the R package “clusterProfiler” and a Gene Ontology (GO) annotation database for each species, created by the R package “AnnotationForge”, as reference. Posteriorly, the R package “enrichplot” was used to create the functional enrichment plots. In these analyses, only genes that fall into the following criteria were used: An adjusted p-value cut-off of $p < 0.05$ and a logarithmic Fold Change $> 1$ and $< -1$. Since a low quantity of differentially expressed genes were obtained in each comparison (see below in section 4.3.2.3), very few or absent over- and infra-represented functions were observed under an established p-value cut-off of $p < 0.05$. Therefore, we decided to not establish a p-value cut-off and represent the most significantly over- and infra-represented functions.

#### 4.2.5. Statistical analysis

One-way ANOVA followed by a Tukey post-hoc test was used to compare infection intensity between time post-exposure, conditions and species with a level of significance of $p \leq 0.05$. All tests were performed with SPSS v.29 (IBM, USA) and JASP v.0.17.

## 4.3. Results and discussion

### 4.3.1. Clam seed mortality, prevalence and infection intensity

#### 4.3.1.1. Mortality

Prior the beginning of the trial, a pre-trial was done some months before using *R. decussatus* and *V. corrugata* seeds with a parasite concentration in the water of $10^7$ and $10^6$ hypnospores for high-infection (HI) and low-infection (LI) treatment respectively. Since all animals reached the same level of infection during that trial (data not shown), the concentration of hypnospores was decreased to $10^6$ and $10^5$ in the present trial to be able to detect the differential susceptibility of clams to the parasite. If *P. olsenii* concentration in water is too high (more than $10^6$ cells), clams would be surrounded by too many cells and at the end they become infected. If, as shown in this research study, *P. olsenii* concentration is lower, animals are able to deal with the parasite and they can avoid the infection.

During the 28-day trial, *V. corrugata* presented the highest cumulative mortality, while *R. decussatus* practically presented no mortality. Unfortunately, no mortality data for *R. philippinarum* was obtained due to the fact that some dead clams of this species could be placed in the tanks without notice because their shells were closed at the beginning of the experience with normal appearance, making it impossible to distinguish between the alive and dead individuals. There were no significant differences among treatments ($p < 0.01$). *R. decussatus* control and highly infected groups presented both 0 % cumulative mortality, while low infected group presented 1.7 % cumulative mortality. In *V. corrugata*, control group presented 37.8 % cumulative mortality, and low and high infection groups presented 53.9 % and 44.4 %, respectively (Figure 3). A higher mortality was found in *V. corrugata* in all groups but without statistical differences between control and infected groups. Parasite infection does not seem to be affecting mortality as a higher parasite burden did not correspond to a higher mortality being the mortality considered normal for this species at this stage. Moreover, all three species were maintained at 18 +/-1 ºC, which is close to 20 ºC, the optimal temperature reported for *V. corrugata* juveniles (Albentosa et al., 1994). Beyond that, no mortality has been found in *V. corrugata* when exposed to temperatures up

to 27 ºC, despite being under thermal stress at this temperature (Macho et al., 2016). Therefore, the possible reasonable explanation for high mortality in this species is that the individuals that died were in poor health condition. Waki et al. (2012) performed a similar experiment with juvenile Manila clam (3-15 mm shell length), in which they were challenged with $10^6$ and $10^7$ prezoosporangia. Despite they obtained high mortality rates with $10^7$ prezoosporangia, there was survival rates as high as the control group in juveniles infected with $10^6$ prezoosporangia. Shimokawa et al. (2010) also challenged juvenile Manila clams (3–10 mm shell length) with the same quantity of prezoosporangia as Waki et al. (2012). They did not obtain high levels of mortality in both parasite loads for the first 4 weeks post challenge. This indicates that small clam challenges with $10^6$ prezoosporangia parasite cells are suitable to induce an infection without causing high mortality rates during the first 4 weeks post challenge. Also, the origin of the clams and the parasite as well as the batch can affect the results.

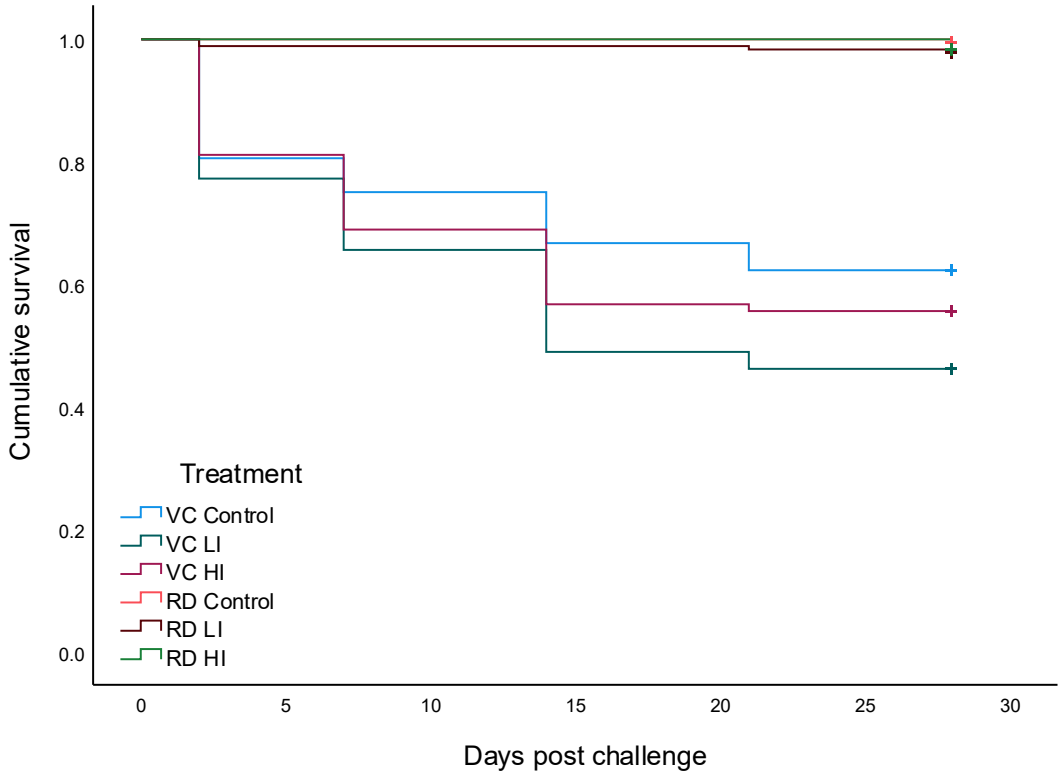


**Figure 3.** Kaplan-Meier cumulative survival curve of *R. decussatus* and *V. corrugata* clam seed during the 28 days post challenge with *P. olsenii*. VC*: Venerupis corrugata*; RD: *Ruditapes decussatus*; LI: Low infected; HI: Highly infected.

#### 4.3.1.2. Prevalence

Regarding prevalence, 100 % of the individuals were found infected in all species at high infection (HI) condition, indicating that all species could be infected by *P. olsenii* at a high parasite cell density. In contrast, in the low infection (LI) condition some differences in the prevalence of the parasite were observed, where *R. decussatus* and *R. philippinarum* presented the highest percentage of infection, and *V. corrugata* presented the lowest prevalence at this condition (Figure 4). Ramilo et al., (2016) surveyed *P. olsenii* prevalence in 4 different clam species (including the 3 species used in this study) along the Galician coast (NW Spain) and verified the highest prevalence in *R. decussatus* and *V. corrugata*, while *R. philippinarum* presented a lower prevalence. Dang et al. (2013) also surveyed the prevalence of *R. decussatus* and *R. philippinarum* in Mundaka Estuary (Spain) and Arcachon Bay (France), respectively. Parasite prevalence in *R. decussatus* ranged between 68% and 100%, while *R. philippinarum* ranged between 77% and 100%. A small survey of the prevalence from the last 10 years (between 2012 and 2022) was performed with the INTECMAR data (http://www.intecmar.gal/Informacion/Patoloxia/Default.aspx?sm=e) using *R. decussatus* and *R. philippinarum* from Praceres bed, Spain (Figure 5). The survey of the prevalence was performed in Praceres because it is the only place were *P. olsenii* prevalence data is available for both clam species. Prevalence in *R. decussatus* ranged between 37% and 100 %, while in *R. philippinarum* ranged between 7 % and 40 %. This survey indicates that *R. philippinarum* appears to be less predisposed to parasite infection than *R. decussatus* confirming our in vivo results. Also, several juvenile Manila clam challenge studies demonstrate that nearly 100 % of individuals were infected with *Perkinsus olsenii* using similar parasite loads (Shimokawa et al., 2010; Waki et al., 2012; Waki & Yoshinaga, 2013, 2018). This indicates that the health condition of individual clams and the condition of prezoosporangial suspensions have influence in the mortality, prevalence, and intensity of infection, resulting in either high or low affected clams. Moreover, since the *P. olsenii* cells were isolated from *R. decussatus* clams, this could justify why *R. decussatus* appeared to have a higher predisposition to the parasite. Nonetheless, an in-depth study of the predisposition of the different clam species to the parasite, using cells derived from different hosts, would help to a better understanding whether there is an effect on predisposition depending on the parasite host species. As above mentioned, a similar experiment in the three *Veneridae* species using an approximate, but smaller, quantity of motile zoospores than our LI condition was performed by Rodriguez et al. (1994). *R. philippinarum* appeared to be the most predisposed, followed by *R. decussatus* and *V. corrugata*. These results are similar to our results of parasite prevalence in the LI condition,

where *V. corrugata* appears to be the less predisposed to *P. olsenii* infection at lower parasite loads.

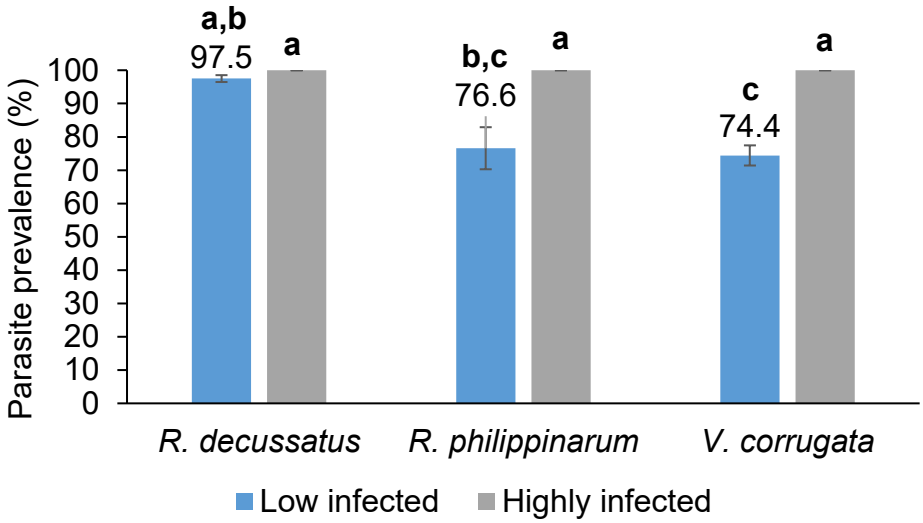


**Figure 4.** Parasite prevalence (%) in each of the studied species. Highlighted values represent mean prevalence (%) in the low infected individuals. Different letters denotes statistical differences among groups ($p<0.05$).

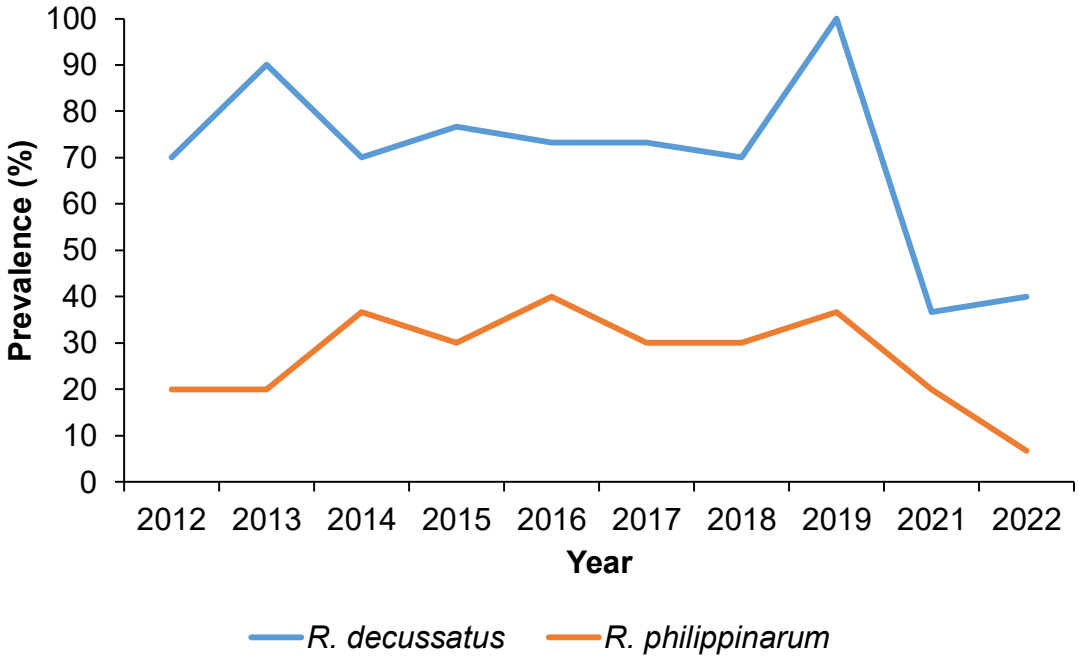


**Figure 5.** *Perkinsus olsenii* prevalence (%) in *R. decussatus* and *R. philippinarum* obtained from INTECMAR data in Praceres, Spain (http://www.intecmar.gal/Informacion/Patoloxia/Default.aspx?sm=e) (2012-2022).

#### 4.3.1.3. Infection intensity

Infection intensity demonstrated the same pattern for both conditions, being *V. corrugata* and *R. philippinarum* the species with the lowest and highest parasite load, respectively. *R. decussatus* appears as the intermediate affected species (Figure 6). Beyond that, we can observe a clustering between the *V. corrugata* high infection condition and the *R. decussatus* and *R. philippinarum* low infection condition, indicating that *V. corrugata* species seems to be less predisposed to *P. olsenii* infection. Hereupon, *R. philippinarum*, when infected, appears to be the most affected species by the parasite in accordance to Rodriguez et al. (1994), which found that *R. philippinarum* presented a higher infection intensity than *R. decussatus* and *V. corrugata*, in a similar infection challenge with *P. olsenii*. Also, the host of *P. olsenii* hypnospores was *R. decussatus* suggesting that host could have no influence on the infection but in our case the origin of the clams could be the explanation.

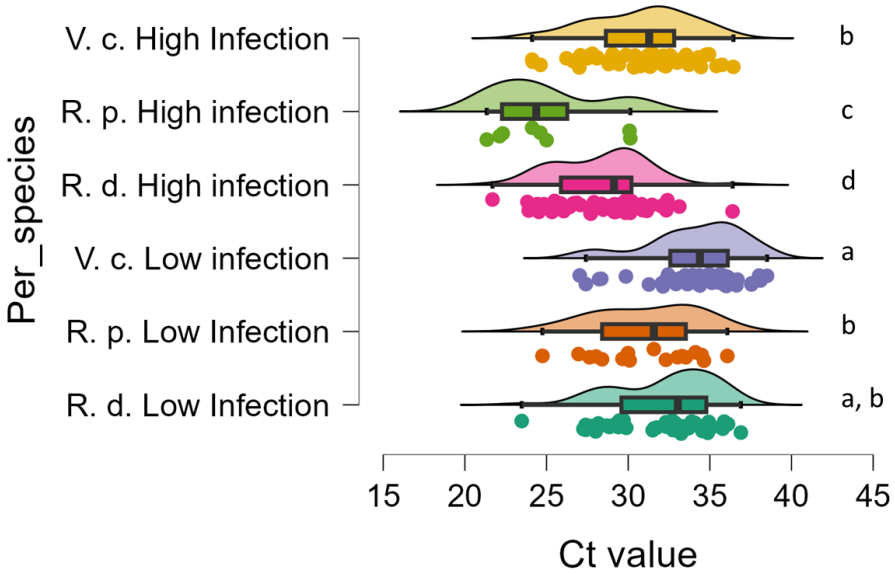


**Figure 6.** Parasite burden (Ct value obtained from qPCR) obtained in each of the studied species and implemented condition (low and high infection). The lower the Ct value, the higher the parasite burden derived from the higher number of parasite DNA copies that were amplified in the qPCR. Different letters denotes statistical differences among groups ($p<0.05$). Species: V. c.(*Venerupis corrugata*); R. p.(*Ruditapes philippinarum*); R. d.(*Ruditapes decussatus*).

The progression of the infection through time presented similar results at HI and LI infection condition for *R. decussatus* and *V. corrugata*. Due to a low number of *R. philippinarum* individuals at HI condition this comparison could not be done in this species. The results obtained for the progression of infection in the LI condition throughout the 4 weeks post challenge in the three species are represented in Figure 7. All three species in the LI condition presented a low to moderate infection level (mean Ct value between 28 and

32) at 48 hours post challenge, according to the results previously obtained in adult *R. decussatus* clams at 48h post-infection by Garcia et al. (2022), in which a low to moderate infection corresponds to a Ct value approximately between 28 and 32, correlating to values on RFTM scale between 2 and 3. At one week post challenge, a significant decrease in the parasite burden was observed in all species being the point with the lowest parasite load. After this time point, the parasite seems to proliferate in the 3 species with a non-significant progression of the infection at 4 weeks post challenge. It seems that a higher burden of the parasite was present at 48 hours post challenge due to the direct contact of the clams with the parasite zoosporangia/zoospores in the water and with several possible zoospores in the clam´s perivisceral cavity and also in gills due to filtration. Since water was changed at 48 hours post challenge and after this point a 48 hour cycle change of water occurred, only the parasite cells that were capable to infect the individuals during the first 48 hours remained on clams' tissues. These parasite cells were then able to spread in the host tissues and increment the infection throughout time, as can be observed between 1 week and 4 weeks post challenge. A similar pattern occurred in previous studies where an increase of parasite cells in the host tissues increases in the first hours post challenge. This is then followed by a non-significant decrease of the parasite burden in the tissues during the first- and second-week post challenge, which afterwards return to increase and progress the infection towards the 4 weeks post challenge (Garcia et al., 2022; Shimokawa et al., 2010; Waki et al., 2012; Waki & Yoshinaga, 2013, 2015, 2018). This indicates that despite the host can counteract at some point an initial infection by *P. olsenii*, this pathogen is able to surpass the immune response by the host and spread throughout its body, progressing the infection. It also seems that a strong immune response of the host is detected during the first week of infection, for after that came back to initial levels (Garcia et al., 2022), that means that once parasite is able to surpass the immune response of the host then the energy cost allocated to keep fighting against infection is too high to keep it because parasite demands a high quantity of energy increasing the metabolism of clams (Estêvão et al., 2023).

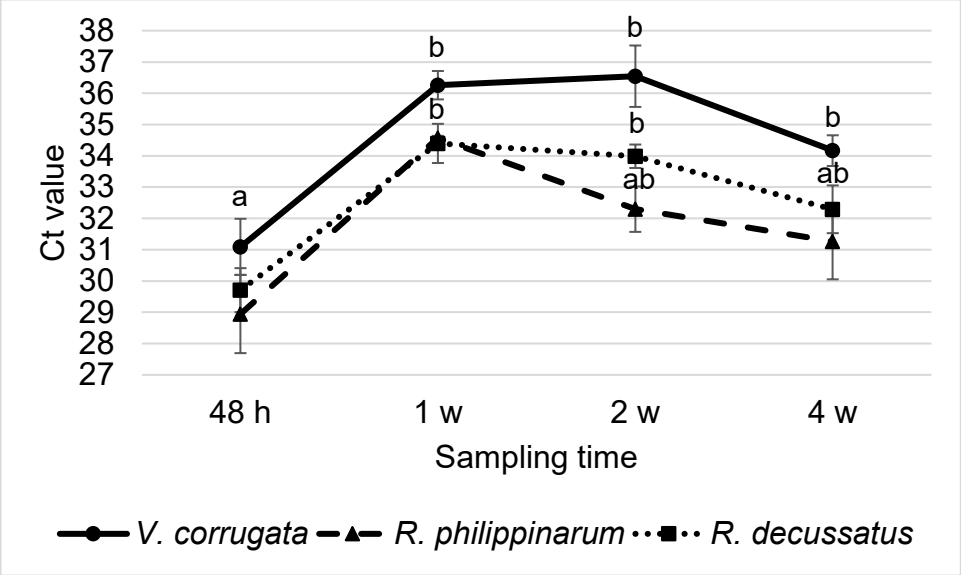


**Figure 7.** Parasite burden (Ct value obtained from qPCR) obtained in each of the studied species for 4 weeks post challenge in the low infection condition. The lower the Ct value, the higher the parasite burden derived from the higher number of parasite DNA copies that were amplified in the qPCR. Different letters denote statistical differences among sampling times ($p<0.05$).

With this experimental infection of clam seed it can be concluded that: i) *Ruditapes philippinarum,* when infected, presents higher levels of infection than the autochthonous species being in that way the possible target species of the parasite; ii) *Ruditapes decussatus* is the most predisposed species, since it presents a high parasite prevalence but with a lower infection level; iii) *Venerupis corrugata* is the species that presents the lowest prevalence and infection levels and, despite being able to be infected by the parasite, it seems to be not much affected by the infection as the other two *Veneridae* clam species indicating that it is the less predisposed and susceptible of the three species to *P. olsenii* infection.

### 4.3.2. Transcriptomic analysis of the immune response

#### 4.3.2.1. RNA sequencing output

A total of ~2,466 million paired-end (PE) reads were generated, for an average of ~41 million reads for each of the 52 samples analysed and 100 % of them were retained after quality filtering.

#### 4.3.2.2. Transcriptomic approach

For the transcriptomic approach, only samples collected from 48 hours and 4 weeks post exposure were used for RNA processing. The criteria were to look onto the initial days of the infection and how the animals respond after a long-time infection. Reads for *R. decussatus* and *R. philippinarum* were mapped against their own genomes. Reads from *V. corrugata* were blasted against both genomes with percentage of alignment lower than 6% on both cases. As the project Tree of Life (https://www.darwintreeoflife.org/) included this species on those 70k species that are planned to be sequenced, we took the decision to wait until release of *V. corrugata* genome before continue with a *de novo* approach. So, hereafter only results from response to infection in *R. decussatus* and *R. philippinarum* will be presented.

Regarding the percentage of alignment of the individuals from each species against their own genome, a good percentage could be observed (Table 1). These results demonstrate the suitability of the genomes to use as reference for the transcriptomic analysis. Nonetheless, very low percentage of alignment was observed between individuals of each species and the genomes of the other *Veneridae* species. This poor alignment percentage might result from the high polymorphism and heterozygosity that is observable on the bivalve genomes (Nguyen & Alfaro, 2020).

Less than 1 % of the reads aligned against *P. olsenii* genome (data not shown) and, therefore, the analysis of the crosstalk between host and parasite was discarded from this study.

**Table 1.** Alignment percentage (%) test of the 3 *Veneridae* species. Rp: *Ruditapes philippinarum*; Vc: *Venerupis corrugata*; Rd: *Ruditapes decussatus*.

| | | Genomes | |
|---|---|---|---|
| **ID** | **Species** | ***R. decussatus*** | ***R. philippinarum*** |
| 11 | Rp | 2.65 | **48.78** |
| 14 | Rp | 3.64 | **51.75** |
| 1 | Rp | 2.97 | **56.69** |
| 18 | Vc | 4.83 | 2 |
| 21 | Vc | 5.04 | 2.04 |
| 33 | Vc | 5.56 | 2.25 |
| 36 | Rd | **82.4** | 2.51 |
| 39 | Rd | **84.23** | 3.24 |
| 42 | Rd | **83.46** | 2.61 |

In *R. decussatus*, the mean number of genes with at least one read were ~25,039 (65 %), being 83 % annotated using its reference genome while in *R. philippinarum*, the mean number of genes with at least one read were ~24,289 (66 %), being 43 % annotated using its reference genome.

#### 4.3.2.3. Differential expressed genes between conditions

The differential gene expression profile between the control and the LI and HI conditions throughout the 48 hours and 4 weeks post exposure to *P. olsenii*, in *R. decussatus* and in *R. philippinarum* were studied. For *R. decussatus*, there were small differences between the control and the LI and HI conditions at both 48 hours and 4 weeks post exposure as shown in the PCAs (Figure 8). Moreover, a high dispersion between individuals in each condition was observed which precluded detection of DEGs. For *R. philippinarum*, the same scenario appeared, where small differences between the control and the LI and HI conditions at both 48 hours and 4 weeks post exposure were found (Figure 9), and even a higher dispersion between individuals in each condition than in *R. decussatus*. We can conclude that clams had not a clear response to the parasite that could be due to the immature immune system at this size. Also if we take a look on the number of genes shared between species using a Venn diagram at 48 hours and 4 weeks post exposure, a

substantial number of shared expressed genes were found at both sampling times in *R. decussatus* (26074 at 48 hours post exposure and 25240 at 4 weeks post exposure; Figure 10). A higher quantity of expressed genes exclusive to the low infected individuals (1057) was found at 48 hours post exposure while at 4 weeks post exposure there was an approximate number of expressed genes exclusive to each group of individuals (between 705 and 846). In *R. philippinarum* there was also a substantial number of shared expressed genes at both sampling times (25899 at 48 hours post exposure and 25055 at 4 weeks post exposure; Figure 11). A higher number of expressed genes exclusive to the highly infected individuals (2237) was found at 48 hours post exposure while a higher number of expressed genes exclusive to the control individuals (1191) was found at 4 weeks post exposure. These results indicates that for *R. decussatus* a higher number of exclusive genes could be observed on the low infected individuals at 48 hours post exposure, which then stabilizes with the other groups of individuals at 4 weeks post exposure. For *R. philippinarum*, a higher number of exclusive genes could be observed in the highly infected individuals at 48 hours post exposure, which then decreases to a lower number suggesting a reduced immune response.

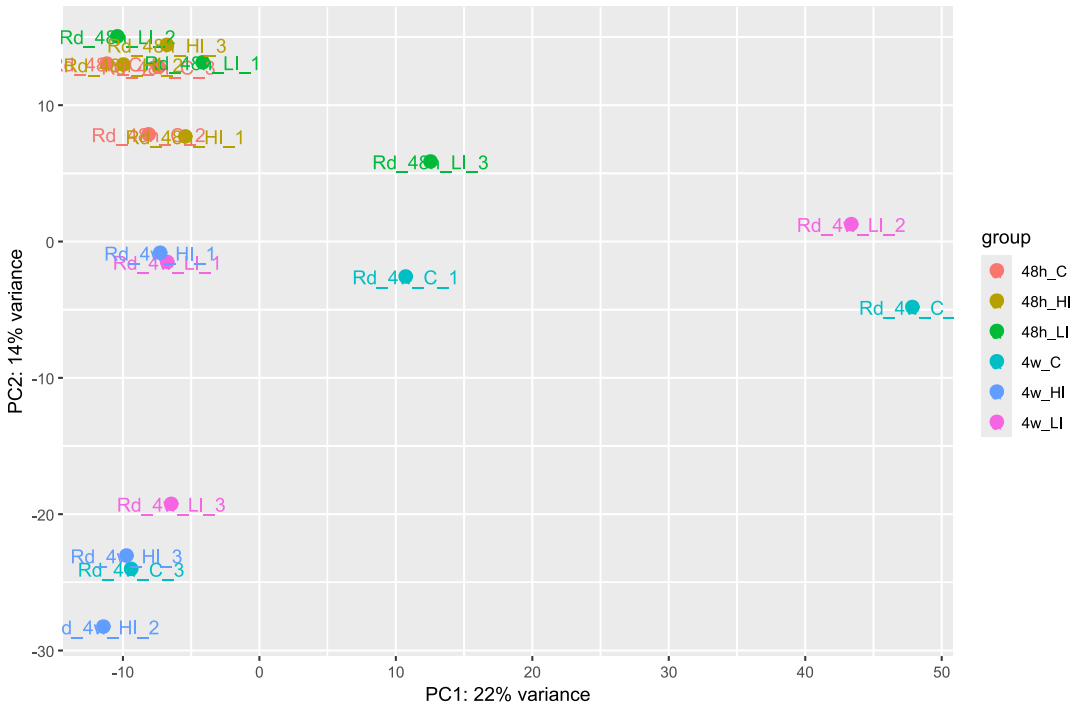


**Figure 8.** Principal Components Analysis (PCA) showing the clustering of the *R. decussatus* (Rd) RNA-Seq samples, with the samples coloured according to their condition (C: control; HI and LI: high and low infection, respectively; h: hours and W: weeks post infection).

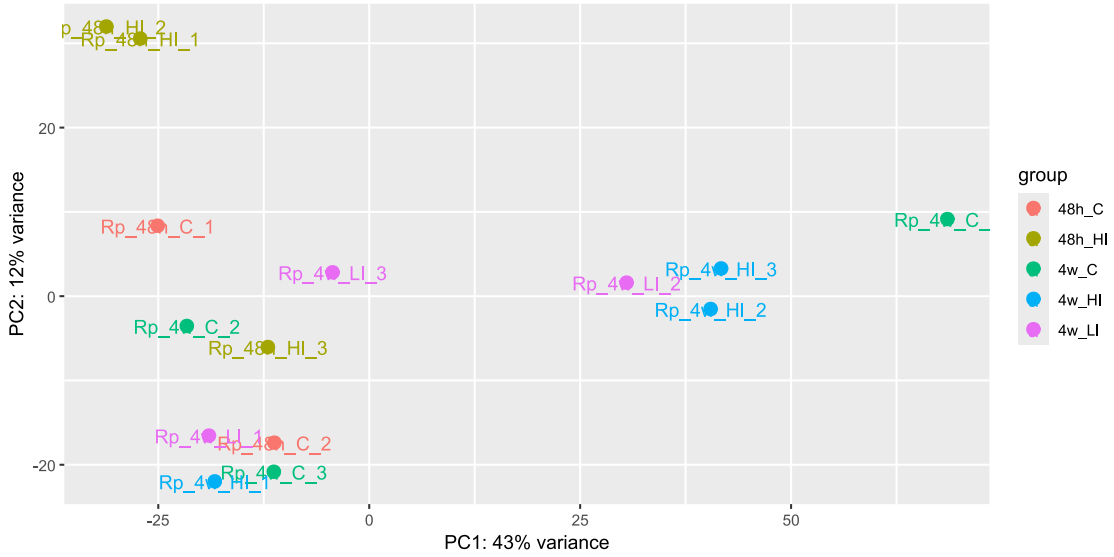


**Figure 9.** Principal Components Analysis (PCA) showing the clustering of the *R. philippinarum* RNA-Seq samples, with the samples coloured according to their condition (C: control; HI and LI: high and low infection, respectively; h: hours and W: weeks post infection).

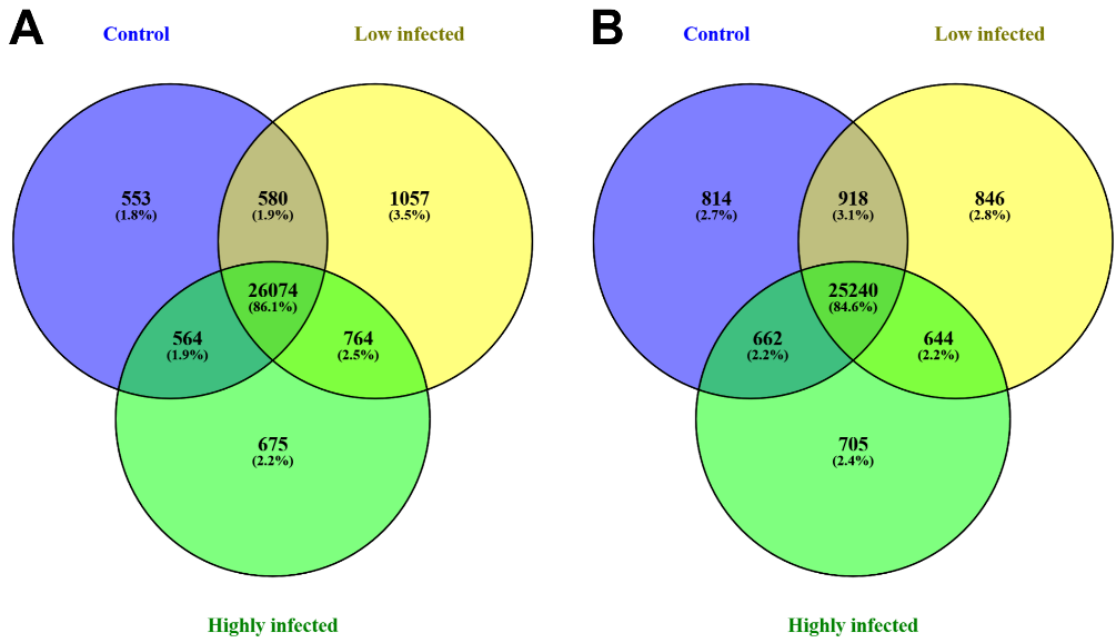


**Figure 10.** Venn diagram of the expressed genes in *R. decussatus* at 48 hours (A) and 4 weeks (B) post exposure, containing at least one read.

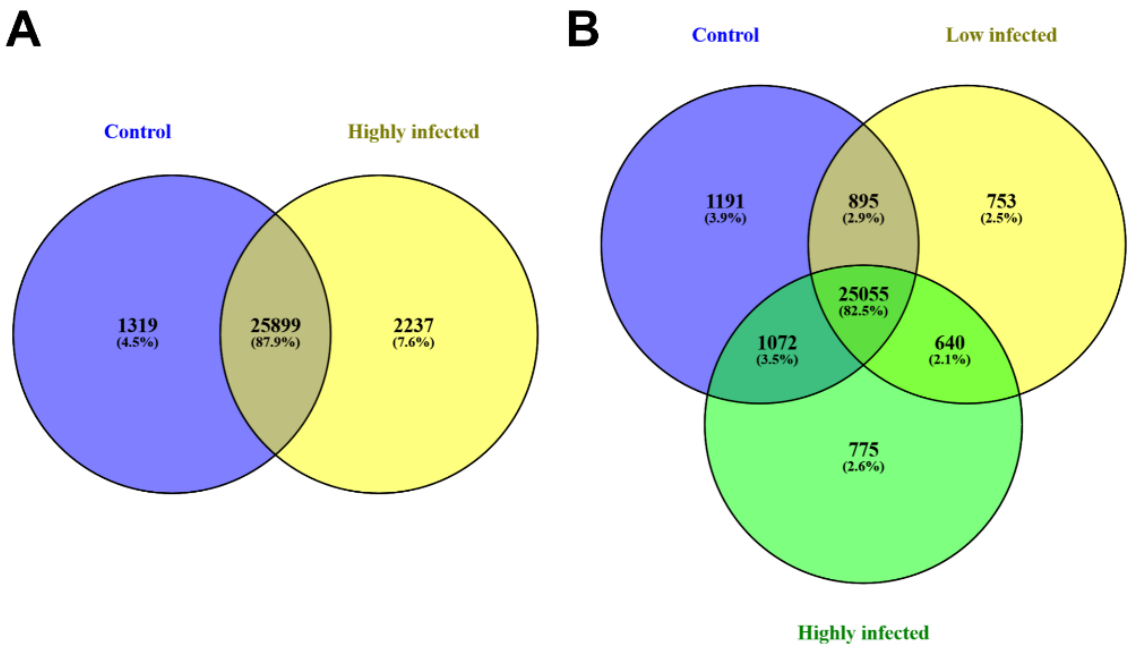


**Figure 11.** Venn diagram of the expressed genes in *R. philippinarum* at 48 hours (A) and 4 weeks (B) post exposure, containing at least one read.

Regarding the differentially expressed genes, different number of genes were identified in each species depending on the condition and time point (Table 2).

**Table 2.** Number of differentially expressed genes with respect to controls in *R. decussatus* and *R. philippinarum* in the low and high infection conditions, at both 48 hours and 4 weeks post exposure. NP: Not present.

| | **LI Treatment** | | | | **HI Treatment** | | | |
|---|---|---|---|---|---|---|---|---|
| | **48 hours** | | **4 weeks** | | **48 hours** | | **4 weeks** | |
| | Up | Down | Up | Down | Up | Down | Up | Down |
| *Ruditapes decussatus* | 30 | 39 | 12 | 22 | 34 | 45 | 17 | 35 |
| *Ruditapes phiippinarum* | NP | NP | 11 | 13 | 23 | 34 | 5 | 8 |

For *R. decussatus* in the low infection condition, 30 and 39 genes were up- and down-regulated at 48 hours post-exposure. At 4 weeks, 12 and 22 genes were up- and down-regulated (supplementary tables 1 & 2). For the same species in the high infection condition, 34 and 45 genes were up- and down-regulated at 48 hours post-exposure. At 4 weeks, 17 and 35 genes were up- and down-regulated (supplementary tables 3 & 4). For *R. philippinarum* in the low infection condition, 11 and 13 genes were up- and down-regulated at 4 weeks post-exposure. No DEGs were identified at 48 hours post exposure because there were not enough individuals at this condition and time point to perform the gene

expression analysis, since some of the individuals collected were already dead at the time of collection without noticing it (supplementary table 5). For the same species in the high infection condition, 23 and 34 genes were up- and down-regulated at 48 hours post-exposure. At 4 weeks, 5 and 8 genes were up- and down-regulated (supplementary tables 6 & 7). A low number of differentially expressed genes were identified in all the analyses. This low number might result from the usage of clam seed in this experiment, since this is a premature stage in the clam development and since the immune system is not completely developed a less complex response is expected (Schelonka & Infante, 1998).

Functional enrichment analyses were performed in each condition, at each sampling point for each species, and the over- and infra-represented functions represented in a treeplot. As described above, a low number of DEGs were obtained in each comparison resulting, therefore, in non-significantly enriched functions in some functional enrichment analyses. Hereupon, we only present and discuss the most significantly over- and infra-represented functions for each comparison.

Regarding *R. decussatus* in the HI condition, at 48 hours post exposure, functions related to cell junction (alcohol C21-steroid junction), metabolism (diol folic acid-containing biosynthetic), immune response (immune biotic other organism), pathogen elimination (melanization modification regulation filopodium), and cell migration and division (actomyosin contractile ring apparatus) were identified as over-represented, despite not significant (Figure 12). In this same time point, functions related to cell signalling (fucosylation nucleotide-sugar Notch transmembrane), immune response (gamma-aminobutyric organic acid anion), reproductive system development (post-embryonic reproduction process structure), regulation of intracellular pH (intracellular acidification pH organization) and epithelial cell migration (ameboidal-type epithelial cell migration) were found as infra-represented, despite not significant (Figure 13). At 4 weeks post exposure, functions regarding cell cycle (deoxyribonucleoside deoxyribonucleotide triphosphate metabolic, nucleoside phosphate catabolic process, purine-containing compound catabolic process) and innate immune response (innate immune response, regulation immune system process) were pinpointed as over-represented (Figure 14). In this same time point, functions regarding cell cycle (cGMP purine ribonucleotide compound, nucleoside phosphate metabolic process, nucleobase-containing small molecule metabolic), metabolism (carbohydrate derivative biosynthetic process) and cell signalling (cell surface receptor pathway) were found as infra-represented, despite some were not significant (Figure 15). These results, in this HI condition, seems to indicate that the clams are initially (at 48 hours post exposure) fighting the high parasite presence and infection in their tissues by spending energy on pathogen elimination and immune response, while trying to maintain

some metabolic homeostasis, in the expense of mechanisms related to organismal development, as well as other immune response mechanisms, such as regulation of the intracellular pH and epithelial cell migration, possibly due to the invasion of the parasite in the tissues and cells of the host and to avoid further proliferation of the parasite cells. Posteriorly, at 4 weeks post exposure, the clams seem to still fight to eliminate the parasite from their tissues, while some modulation of the clams' cellular proliferation seems to be performed by the parasite in order to maintain the proliferation of the invader cells in the host. Moreover, metabolic mechanisms seem to be suppressed in the clams, possibly due to exhaustion of energetic reserves from the host to fight the pathogen.

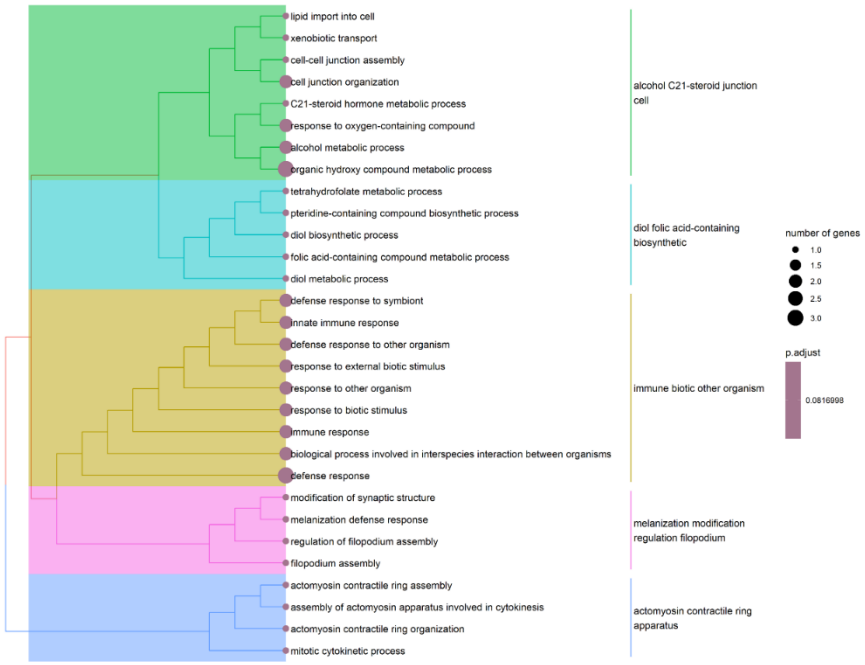


**Figure 12.** Top 30 over-represented functions from the functional enrichment of *R. decussatus* DEGs in the high infection condition, at 48 hours post exposure. Circles size is proportional to the number of the genes and circle colours represent the adjusted p-value of the correspondent enriched term.

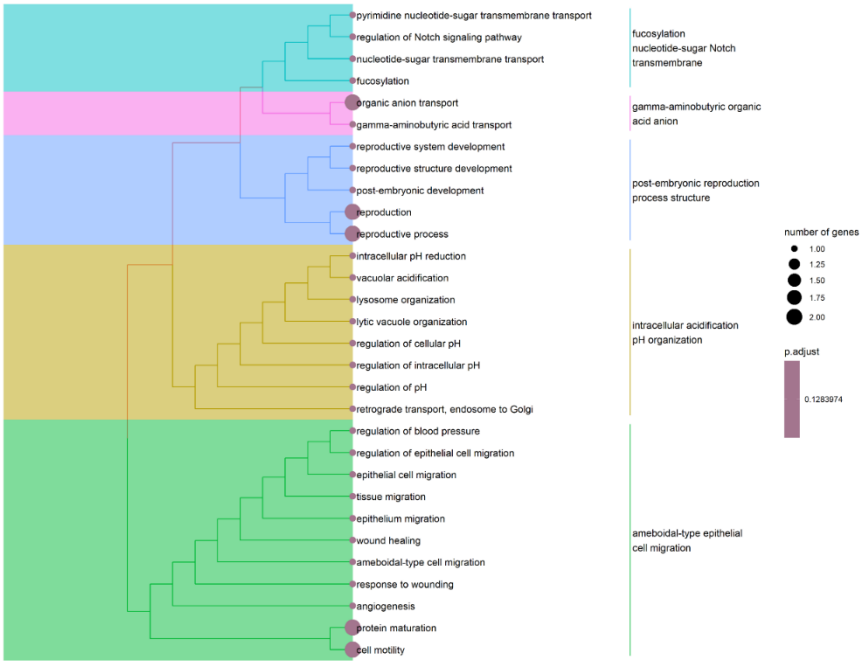


**Figure 13.** Top 30 infra-represented functions from the functional enrichment of *R. decussatus* RNA-Seq samples in the high infection condition, at 48 hours post exposure. Circles size represent the number of the genes and circle colours represent the adjusted p-value of the correspondent enriched term.


**Figure 14.** Top 30 over-represented functions from the functional enrichment of *R. decussatus* RNA-Seq samples in the high infection condition, at 4 weeks post exposure. Circles size represent the number of the genes and circle colours represent the adjusted p-value of the correspondent enriched term.


**Figure 15.** Top 30 infra-represented functions from the functional enrichment of *R. decussatus* RNA-Seq samples in the high infection condition, at 4 weeks post exposure. Circles size represent the number of the genes and circle colours represent the adjusted p-value of the correspondent enriched term.

Regarding *R. decussatus* in the LI condition, at 48 hours post exposure, functions related to the neuroendocrine system (dopamine norepinephrine biogenic amine), osmoregulation (glycine amino-acid betaine choline), cell cycle (cellular modified amino acid, mRNA processing metabolic process) and cytoskeleton organization (regulation microtubule cytoskeleton organization) were identified as over-represented, despite not significant (Figure 16). In this same time point, functions related to cellular transport (L-alpha-amino L-amino amino transmembrane, intracellular monocarboxylic anion protein), immune response (response to abiotic stimulus), organism development (brain central system development) and cell cycle (positive regulation biosynthetic process) were found as infra-represented but without significance in relation to control condition (Figure 17). At 4 weeks post exposure, functions regarding stress response (amino aminoglycan carbohydrate biosynthetic) and metabolism (positive regulation cellular metabolic, positive regulation macromolecule metabolic, positive regulation metabolic process, macromolecule catabolic process) were pinpointed as over-represented, being some of the functions non-significant (Figure 18). In this same time point, functions regarding cell cycle (sulfur alpha-amino aspartate amino, small molecule biosynthetic process), metabolism (carboxylic acid metabolic process, oxoacid metabolic process) and response to extracellular signal (protein-coupled receptor signalling pathway) were found as infra-represented but some of the functions without significance in relation to control condition ($p>0.05$) (Figure 19). These

results, in this LI condition, indicate that the clams are initially (at 48 hours post exposure) trying to maintain physiological homeostasis together with cellular proliferation (probably hemocyte division) triggered by the infection by the parasite. Posteriorly, at 4 weeks post exposure, the clams seem to be responding to the stress caused by the prolonged infection by trying to maintain a metabolic homeostasis. At this condition, it seems that clams are trying to activate functions to counteract the presence of the parasite but the level of parasites is not enough to cause a significant decrease of functions in the individuals.

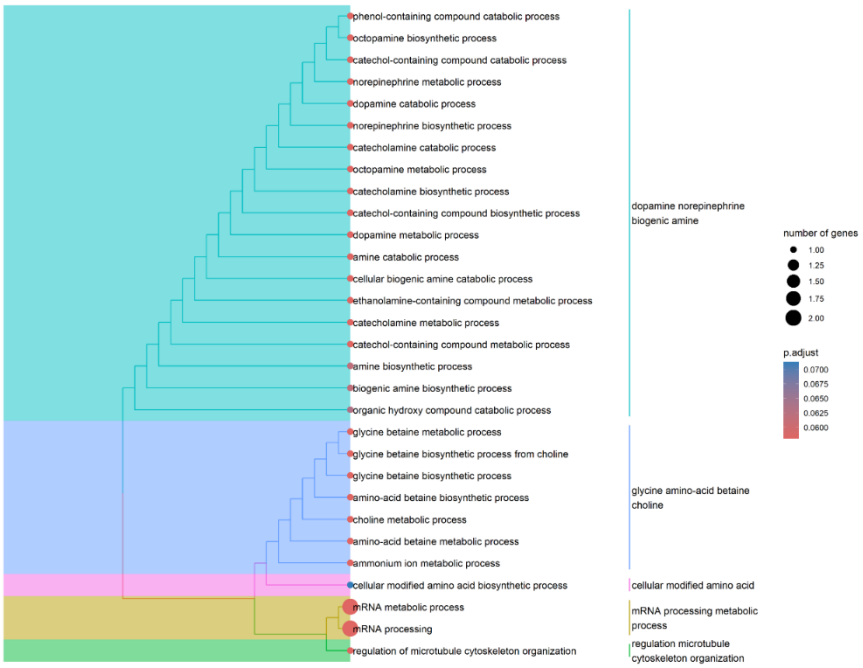


**Figure 16.** Top 30 over-represented functions from the functional enrichment of *R. decussatus* RNA-Seq samples in the low infection condition, at 48 hours post exposure. Circles size represent the number of the genes and circle colours represent the adjusted p-value of the correspondent enriched term.

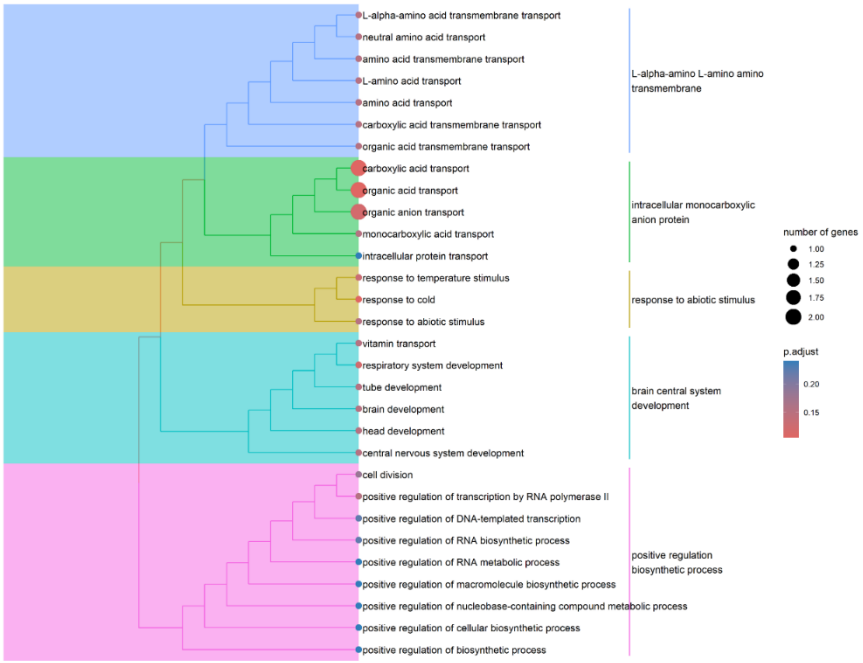


**Figure 17.** Top 30 infra-represented functions from the functional enrichment of *R. decussatus* RNA-Seq samples in the low infection condition, at 48 hours post exposure. Circles size represent the number of the genes and circle colours represent the adjusted p-value of the correspondent enriched term.

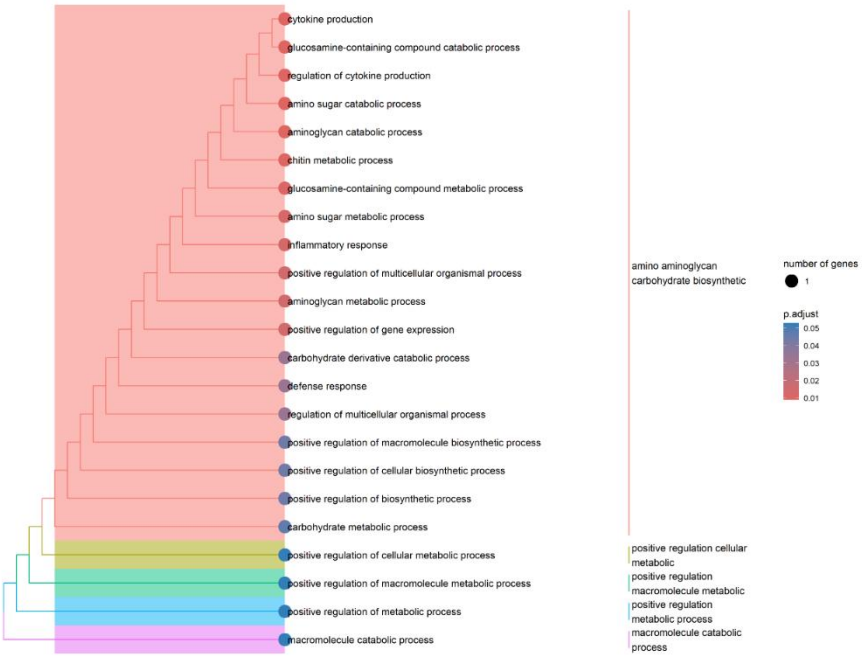


**Figure 18.** Over-represented functions from the functional enrichment of *R. decussatus* RNA-Seq samples in the low infection condition, at 4 weeks post exposure. Circles size represent the number of the genes and circle colours represent the adjusted p-value of the correspondent enriched term.

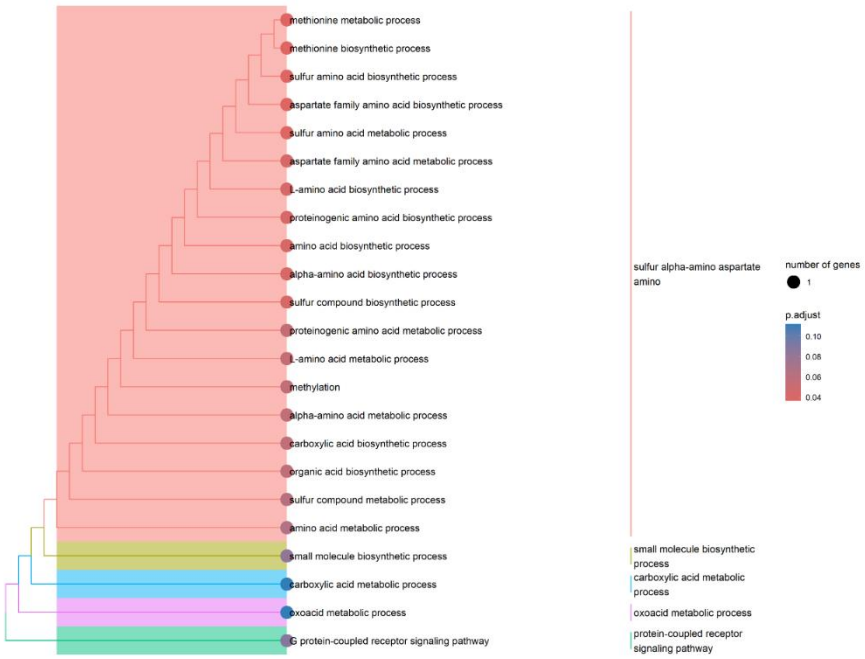


**Figure 19.** Infra-represented functions from the functional enrichment of *R. decussatus* RNA-Seq samples in the low infection condition, at 4 weeks post exposure. Circles size represent the number of the genes and circle colours represent the adjusted p-value of the correspondent enriched term.

In the species *R. philippinarum* in the HI condition, at 48 hours post exposure, just the function related to cell cycle was identified as over-represented (DNA integration, adjusted p-value = 0.016). At this same time point, functions related to cell cycle (double-strand break change replication, chromosome organization) and response to stress (DNA repair, DNA damage response, cellular response to stress) were found as infra-represented, despite some functions were not significantly represented (Figure 20). At 4 weeks post exposure, no functions were found as over-represented due to the low number of up-regulated genes (5 up-regulated genes). In this same time point, functions regarding cell cycle (alpha-amino aspartate family amino), metabolism (oxoacid metabolic process, organic acid metabolic process, cellular catabolic process) and response to extracellular signal (protein-coupled receptor signalling pathway) were found as infra-represented without significance in relation to control condition ($p>0.05$) (Figure 21). These results, in this HI condition, indicate that the clams are initially (at 48 hours post exposure) spending their energetic sources on cellular proliferation, while certain cellular proliferation mechanisms as well as a response to the stress caused by the parasite infection is being suppressed. Later, on 4 weeks post exposure, clams appear to have some mechanisms of cellular proliferation, metabolism and response to extracellular signal suppressed by the prolonged presence of the parasite. In the same *R. philippinarum* species in the LI condition, at 48 hours post exposure, no over- or infra-represented functions were identified due to the lack of differentially expressed

genes (see above). At 4 weeks post exposure, no functions were found as over-represented due to the lack of genes containing any associated GO term. In this same time point, functions regarding cell cycle (rRNA processing metabolic biogenesis, cell cycle), and reproductive development (meiotic sexual cell cycle, reproduction, reproductive process) were found as infra-represented without significance in relation to control condition ($p>0.05$) (Figure 22). These results, despite lacking information regarding of what was being spent as resources by the clams at 4 weeks post exposure, indicate that processes of cellular proliferation and development of reproductive organs were being suppressed by the prolonged presence of the parasite in the clam tissues.


**Figure 20.** Infra-represented functions from the functional enrichment of *R. philippinarum* RNA-Seq samples in the high infection condition, at 48 hours post exposure. Circles size represent the number of the genes and circle colours represent the adjusted p-value of the correspondent enriched term.

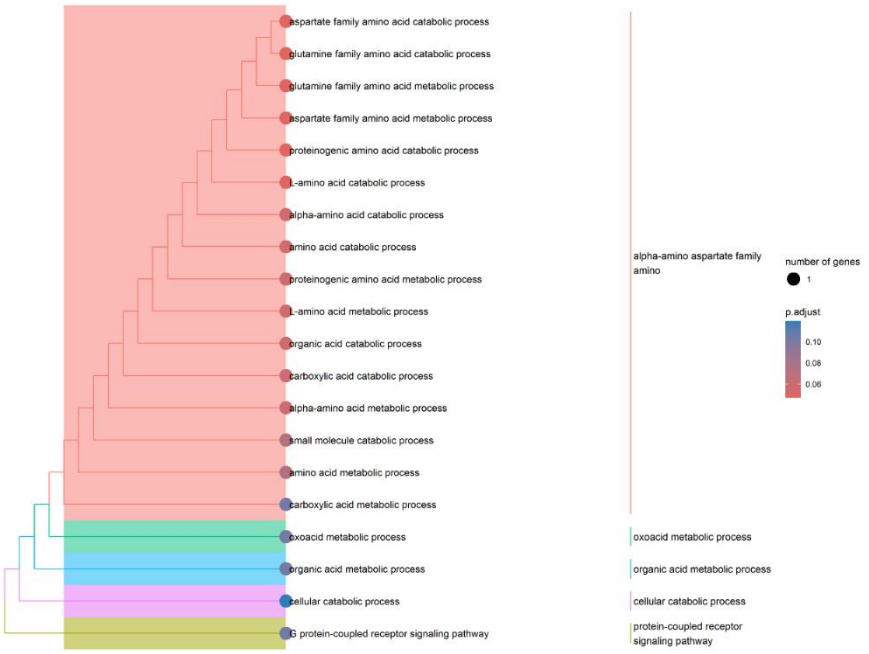


**Figure 21.** Infra-represented functions from the functional enrichment of *R. philippinarum* RNA-Seq samples in the high infection condition, at 4 weeks post exposure. Circles size represent the number of the genes and circle colours represent the adjusted p-value of the correspondent enriched term.

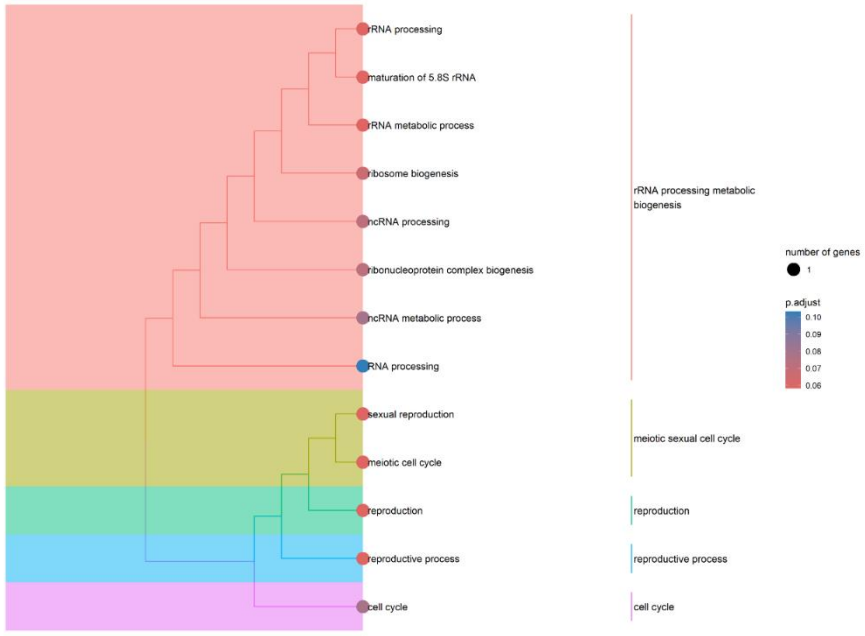


**Figure 22.** Infra-represented functions from the functional enrichment of *R. philippinarum* RNA-Seq samples in the low infection condition, at 4 weeks post exposure. Circles size represent the number of the genes and circle colours represent the adjusted p-value of the correspondent enriched term.

Despite a clear lack of information in *R. philippinarum* responses to parasite infection due to the low number of DEGs, some similarities and differences could be observed when comparing conditions and time points between both Veneridae species. For instance, in the presence of a high quantity of parasite (HI condition), both clams appear to clearly fight the parasite invasion in their tissues by activating their defensive mechanisms on the first stages of the infection. Nonetheless, differences appear between both species in what is suppressed by the parasite at this initial stage. *Ruditapes decussatus* clams appear to have their development immediately compromised by the presence of the parasite, despite both species present mechanisms of defence being modulated by the parasite in order for the invader to proliferate in the host tissues. Later on, when the infection starts to chronically progress at 4 weeks post exposure, both clam species appear to be prejudiced at metabolic and immune response level which indicate a compromise of the organisms by the prolonged presence of the parasite at high quantities. In the presence of a lower quantity of parasite (LI condition), the only possible comparison between the two clam species was at 4 weeks post exposure, where both presented suppression in cellular proliferation. Nonetheless, *R. philippinarum* appeared to have the development of their reproductive system compromised by the parasite presence. Interestingly, and contrary of what was observed at higher infection doses, *R. philippinarum* appears to have the reproductive system development compromised at chronic exposure to the parasite at lower infection doses. It is also remarkable that those genes related to reproductive status or performance identified, could have other functions too, since at this stage, clams are not in reproductive age. The results described here for both clam seed species at chronic infection are similar to what have previously been reported in the adult stages, despite clam seed present a less developed immune response and, therefore, a possible less complex response (Estêvão et al., 2023; Hasanuzzaman et al., 2017, 2018; Leite et al., 2013; Prado-Alvarez et al., 2009). For instance, Estêvão et al. (2023) presents a high representation of the metabolic functions in the long term affected *R. decussatus* by the parasite. Hasanuzzaman et al. (2017) presents metabolism and stress response as the main over-represented functions in *R. philippinarum* chronically infected by *P. olsenii*. Other two studies on *P. olsenii* chronic infection in *R. decussatus* also demonstrated metabolism as the most modulated process (Leite et al., 2013; Prado-Alvarez et al., 2009). Despite there are similarities between our study and the literature, this work presents the first gene study in an infection challenge performed in clam seed. The already existing studies used adult clams collected from the wild and were either challenged or not with the parasite.

#### 4.3.2.4. Gene expression patterns of differentially expressed genes

Gene expression patterns of differentially expressed genes were observed between conditions and time points in the same species and between species at 48 hours post exposure, in the HI condition. For *R. decussatus*, 6 DEGs were identified for their expression pattern between the HI and LI conditions (Table 3). *EMILIN-1-A* gene encodes a multifunctional protein involved in cell migration, proliferation and adhesion, being present in various connective tissues (Choudhary et al., 2021). This gene was down-regulated in both LI and HI conditions at 48 hours post exposure, indicating that it was possibly suppressing cell migration, proliferation and adhesion in *R. decussatus* tissues to avoid parasite proliferation through these cells to other tissues. *Hemicentin-1* is a gene that encodes an extracellular protein with a wide variety of functions required for embryonic development, cell migration and invasion, cleavage furrow maturation, maintenance of cell polarity, age-related macular degeneration and response to environmental stress (Luo et al., 2017). This gene was found up-regulated in the LI condition and down-regulated in the HI condition at 48 hours post exposure, indicating that in the LI condition it might be participating in organismal development of clam seed, as well as in cell migration and invasion to counteract parasite infection. On the other side, in the HI condition this gene and the mentioned functions may be suppressed by the high presence of the parasite. *Pathogenesis-related protein 5* coding gene has been identified to participate in response to various stresses, such as pathogen infection and anti-fungal activity (Schreiber et al., 1997). This gene was strongly up-regulated in both conditions at 48 hours post exposure, indicating that it is activated in response to *P. olsenii* infection, possibly participating in pathogen elimination. *Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1* gene encodes a calcium-binding EGF-like (EGF_CA) domain that is considered to play a key role in growth control (Chen et al., 2022). The up-regulation of this gene in both conditions at 48 hours post exposure indicates that it is actively controlling the growth of *R. decussatus* clam seed that could be due to the inhibition of the clam seed growth caused by the parasite infection. *Tenascin-R* belongs to a group of extracellular matrix molecules that have been considered specific to the phylum Chordata, acting as integrin ligands and participating in the regulation of morphogenic events. In mice it has been pointed out to perform an important role in neurogenesis and the maintenance of perineuronal nets (Adams & Tucker, 2024). It's up-regulation in both conditions at 4 weeks post exposure points towards active neurogenesis in clam seed even with the progression of the infection by *P. olsenii*. *ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase* gene encodes an enzyme that catalyses the cyclization of $NAD^{++}$ to produce cyclic ADP-ribose (cADPR), which is involved in $Ca^{2+}$ mobilization and therefore, $Ca^{2+}$ signalling (Aarhus et

al., 1995). This gene is strongly up-regulated in both conditions at 4 weeks post exposure, indicating that it is activated in response to *P. olsenii* infection. However, its function in molluscs is not yet understood and more research is needed to comprehend the mechanisms in which this gene is participating towards parasite infection. Anyway, in other species the Cyclic ADP-ribose is an important calcium mobilizing metabolite produced by the ADP-rybosyl cyclase. The up-regulation of this gene could be derived to the need of the clam to continue building and maintaining the shell, since the parasite infection could compromise the metabolic functions for the maintaining of the shells (Schuber & Lund, 2004).

**Table 3.** Differentially expressed genes in *R. decussatus* and *R. philippinarum* between conditions and sampling times and between *R. decussatus* and *R. philippinarum* at 48 hours post infection, in the high infection condition. HI: Highly infected; LI: Low infected; Rd: *Ruditapes decussatus*; Rp: *Ruditapes philippinarum*; 48h: 48 hours post exposure; 4w: 4 weeks post exposure.

| | | **Fold change (Rd)** | | |
|---|---|---|---|---|
| **Gene name** | **Time** | **LI** | **HI** | **Function** |
| EMILIN-1-A | 48h | -1.62 | -0.81 | Cell migration, proliferation and adhesion |
| Hemicentin-1 | 48h | 0.53 | -5.41 | Development, cell migration and invasion |
| Pathogenesis-related protein 5 | 48h | 7.89 | 7.14 | Response to pathogen infection, wound healing |
| Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1 | 48h | 4.46 | 2.75 | Growth control |
| Tenascin-R | 4w | 2.22 | 2.45 | Neurogenesis |
| ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase | 4w | 7.35 | 7.37 | Calcium signalling |
| | | **Fold change (Rd HI)** | | |
| **Gene name** | | **48h** | **4w** | **Function** |
| Cartilage intermediate layer protein 1 | | -2.94 | -12.35 | Shell formation |
| Interleukin-6 receptor subunit beta | | 4.35 | 5.01 | Immune response |
| | | **Fold change (Rd LI)** | | |
| **Gene name** | | **48h** | **4w** | **Function** |
| Complement C1q-like protein 4 | | -2.13 | -5.69 | Immune response |
| Thrombospondin-2 | | -2.83 | 2.18 | Angiogenesis, fibroblast adhesion |
| | | **Fold change (Rp)** | | |
| **Gene name** | **Time** | **LI** | **HI** | **Function** |
| Major facilitator superfamily domain-containing protein 4A | 4w | -3.51 | -2.49 | Transportation of saccharides, amino acids and peptides |
| | | **Fold change (48h C vs HI)** | | |
| **Gene name** | | **Rd** | **Rp** | **Function** |
| Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1 | | 2.75 | -8.98 | Growth control |

Also, in *R. decussatus*, two genes were compared for their expression in the HI condition between 48 hours and 4 weeks post exposure (Table 3). *Cartilage intermediate layer protein 1* coding gene has been shown to be a mediator of cardiac extracellular matrix remodelling in humans (van Nieuwenhoven et al., 2017). This gene is known to interact with cartilage matrix proteins which have been known to be involved in shell formation in *C. gigas* (Zhu et al., 2023). The down-regulation of this gene at both 48 hours and 4 weeks post exposure indicates that shell formation mechanisms are being suppressed due to parasite infection, giving space for immune response processes to eliminate the parasite. *Interleukin-6 receptor subunit beta* codes an Interleukin-6 (IL-6) receptor. IL-6 is a cytokine with multifunctional properties, affecting cell development and differentiation, and has important roles in immune response and acute phase responses (Ma et al., 2024). The up-regulation of this gene may be related with the recognition of the parasite and its infection, activating the immune responses of the clams at 48 hours and 4 weeks post exposure, respectively. Another two genes were compared for their expression in the same species and in the LI condition, between the same time points (Table 3). *Complement C1q-like protein 4* codes a protein that has been related to the immune system and is modulated differently according to different types of stress in *R. philippinarum* (Liu et al., 2024; Wang et al., 2018). This gene is down-regulated at both 48 hours and 4 weeks post exposure, indicating that the immune system of the clams is being modulated due to the infection caused by *P. olsenii*. This modulation derives from the fact that C1q proteins belong to the complement system, which is an important immune defence mechanism against invading bacteria (Jiang et al., 2015). Said that, this gene may be down-regulated to give protagonism for other important genes of the immune system to actively fight and eliminate the parasite. *Thrombospondin-2* gene encodes an extracellular matrix glycoprotein that functions as a modulator of cell-matrix interactions. This protein inhibits angiogenesis, but also modulates fibroblast adhesion, bone formation, and haemostasis (Kyriakides et al., 2001). This gene is down-regulated at 48 hours post exposure and up-regulated at 4 weeks post exposure, indicating that in the first hours of contact of the clams with the parasite, there is no modulation of tissue formation, as well as of fibroblast adhesion, while when the infection progresses chronically, there is a modulation of these processes possibly to avoid parasite proliferation through the tissues. This gene may also be involved in shell formation and its down-regulation of the gene at 48 hours post exposure may be related to the acute infection caused by the initial contact with the parasite, while at 4 weeks post exposure its up-regulation may be related to the stabilization of the infection and necessity to invest in mechanisms of shell formation and growth. Interestingly, these two genes only appear regulated in the LI condition and not in the HI condition. This may be due to the effect of the different parasite loads in the host response, since a higher parasite load may result in a

more acute and dysregulated response, while a lower parasite load may result a more organized response by the host, giving energy for the clams to have a better recovery from the infection. Another gene was compared in *R. philippinarum* for its expression pattern between the HI and LI conditions (Table 3). The gene *Major facilitator superfamily domain-containing protein 4A* is a member of the major facilitator superfamily (MFS), being responsible for the transportation of substances such as monosaccharides, polysaccharides, amino acids, and peptides (Yang et al., 2022). This gene is down-regulated in both conditions at 4 weeks post exposure, indicating that transportation of saccharides, amino acids and peptides are being suppressed in order to give place to other mechanisms due to *P. olsenii* infection. Finally, the gene *Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1* was differently expressed between *R. decussatus* and *R. philippinarum* at 48 hours post exposure, in the HI condition (Table 3). The function of this gene is above mentioned in the text and it is up-regulated in *R. decussatus* and down-regulated in *R. philippinarum*. This difference indicates that growth in *R. decussatus* is being actively controlled, while in *R. philippinarum* this mechanism appears to be suppressed. This indicates that both species reacts differently on the beginning of the infection by the parasite when highly infected, suggesting that *R. philippinarum* growth may be more compromised due to *P. olsenii* infection.

The DEGs obtained at 4 weeks post exposure were compared with other studies regarding the response of *R. decussatus* or *R. philippinarum* to *Perkinsus olsenii* infection at long term exposure, to ascertain the presence of commonly present genes. The time point of 4 weeks post exposure was selected for comparison with long term affected clams from the wild because at this time phase of the infection it is presumable that the infection progresses at chronic level. Moreover, the DEGs obtained at both 48 hours and 4 weeks post exposure were compared with the study of Sambade et al. (2025) to verify the presence of any markers of resistance or tolerance to *P. olsenii* in this study. Regarding the 48 hours post exposure, a total of 23 genes were found commonly present in the study of Sambade et al. (2025) (Table 4). These genes were related to functions regarding angiogenesis, fibroblast adhesion, cell adhesion, migration, invasion, organization and interaction, cell cycle, development, response to stress, fibrillogenesis, growth control, immune response, metabolism, mucus adhesion, pigmentation and toxicity. At 4 weeks post exposure, 13 genes were found commonly present in the study of Sambade et al. (2025) and 3 genes were found commonly present in the study of Hasanuzzaman et al. (2018) (Table 4). These genes were related to functions regarding angiogenesis, fibroblast adhesion, calcification, development, digestion, growth control, immune response, metabolism, response to stress, cellular proliferation and wound healing. Despite these studies used adult clams to evaluate

the chronic response to infection caused by *P. olsenii*, a substantial quantity of genes could be found in common, despite using clam seed in this study. This was specially observed at 48 hours post exposure, indicating that the genetic markers of resistance or tolerance to *P. olsenii* identified by Sambade et al. (2025) can be also detected in clam seed at the beginning of the infection, indicating that these genes are extremely important for the development of the infection.

**Table 2.** Differentially expressed genes common with previous studies.

| Species, Time, Condition | Gene description | Fold Change | Function | Common with study | Fold Change in the study |
|---|---|---|---|---|---|
| Rd 48h C vs LI | Thrombospondin-2 | -2.830 | Angiogenesis, fibroblast adhesion | Sambade et al. 2025 | NA |
| Rd 48h C vs HI | Cadherin-related tumor suppressor | 1.042 | Cell adhesion and interaction | Sambade et al. 2025 | NA |
| Rp 48h C vs HI | Hemagglutinin/amebocyte aggregation factor | 7.844 | Cell adhesion and organization | Sambade et al. 2025 | NA |
| Rd 48h C vs LI | ATP-dependent DNA helicase PIF1 | -1.096 | Cell cycle | Sambade et al. 2025 | NA |
| Rd 48h C vs HI | E3 ubiquitin-protein ligase TRIM33 | -3.794 | Development | Sambade et al. 2025 | NA |
| Rd 48h C vs HI | Hemicentin-1 | -5.637 | Embryonic development, cell migration and invasion and response to environmental stress | Sambade et al. 2025 | NA |
| Rd 48h C vs HI | Hemicentin-1 | -5.180 | Embryonic development, cell migration and invasion and response to environmental stress | Sambade et al. 2025 | NA |
| Rd 48h C vs LI | Hemicentin-1 | -2.499 | Embryonic development, cell migration and invasion and response to environmental stress | Sambade et al. 2025 | NA |
| Rd 48h C vs LI | Hemicentin-1 | -2.118 | Embryonic development, cell migration and invasion and response to environmental stress | Sambade et al. 2025 | NA |
| Rd 48h C vs LI | Hemicentin-1 | 6.221 | Embryonic development, cell migration and invasion and response to environmental stress | Sambade et al. 2025 | NA |
| Rd 48h C vs LI | Fibrillin-1 | 1.711 | Fibrillogenesis | Sambade et al. 2025 | NA |
| Rd 48h C vs HI | Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1 | 2.752 | Growth control | Sambade et al. 2025 | NA |
| Rd 48h C vs LI | Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1 | 4.462 | Growth control | Sambade et al. 2025 | NA |
| Rp 48h C vs HI | Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1 | -8.979 | Growth control | Sambade et al. 2025 | NA |
| Rp 48h C vs HI | CD109 antigen | 10.882 | Immune response | Sambade et al. 2025 | NA |
| Rp 48h C vs HI | Cyclic GMP-AMP synthase-like receptor 2 | 4.493 | Immune response | Sambade et al. 2025 | NA |
| Rp 48h C vs HI | Hemicentin-2 | -10.355 | Immune response | Sambade et al. 2025 | NA |
| Rd 48h C vs HI | Matrilin-2 | -1.664 | Immune response | Sambade et al. 2025 | NA |
| Rd 48h C vs HI | Multidrug resistance-associated protein 1 | 1.659 | Metabolism | Sambade et al. 2025 | NA |

| Species, Time, Condition | Gene description | Fold Change | Function | Common with study | Fold Change in the study |
|---|---|---|---|---|---|
| Rd 48h C vs LI | Perlucin-like protein | -1.170 | Mucus adhesion | Sambade et al. 2025 | NA |
| Rd 48h C vs LI | Perlucin-like protein | -0.713 | Mucus adhesion | Sambade et al. 2025 | NA |
| Rp 48h C vs HI | Perlucin-like protein | -7.494 | Mucus adhesion | Sambade et al. 2025 | NA |
| Rp 48h C vs HI | SCO-spondin | -5.704 | Neuronal development | Sambade et al. 2025 | NA |
| Rd 48h C vs LI | E3 ubiquitin-protein ligase HERC2 | 4.578 | Pigmentation | Sambade et al. 2025 | NA |
| Rd 48h C vs HI | Lambda-crystallin homolog | -1.633 | Response to stress | Sambade et al. 2025 | NA |
| Rd 48h C vs HI | ATP-binding cassette sub-family C member 2 | 1.333 | Toxicity | Sambade et al. 2025 | NA |
| Rd 48h C vs LI | Glucose dehydrogenase [FAD, quinone] | 2.336 | Unknown | Sambade et al. 2025 | NA |
| Rp 48h C vs HI | Inositol phosphoceramide mannosyltransferase 3 | -4.356 | Unknown | Sambade et al. 2025 | NA |
| Rp 48h C vs HI | Methyltransferase-like protein 27 | 5.765 | Unknown | Sambade et al. 2025 | NA |
| Rd 48h C vs HI | Sodium- and chloride-dependent glycine transporter 2 | -1.968 | Unknown | Sambade et al. 2025 | NA |
| Rd 48h C vs HI | Stimulated by retinoic acid gene 6 protein-like | 1.825 | Unknown | Sambade et al. 2025 | NA |
| Rd 4w C vs LI | Thrombospondin-2 | 2.176 | Angiogenesis, fibroblast adhesion | Sambade et al. 2025 | NA |
| Rd 4w C vs LI | Ectin | 7.690 | Calcification | Sambade et al. 2025 | NA |
| Rd 4w C vs HI | Transcription intermediary factor 1-alpha | -6.001 | Development and organogenesis | Sambade et al. 2025 | NA |
| Rd 4w C vs LI | FMRFamide receptor | -4.201 | Development of the nervous system | Sambade et al. 2025 | NA |
| Rp 4w C vs HI | FMRFamide receptor | -3.849 | Development of the nervous system | Sambade et al. 2025 | NA |
| Rd 4w C vs LI | Chitotriosidase-1 | 2.758 | Digestion and immune response | Sambade et al. 2025 | NA |
| Rp 4w C vs LI | RNA-binding protein RO60 | 2.300 | Embryogenesis | Sambade et al. 2025 | NA |
| Rd 4w C vs HI | Protocadherin Fat 4 | -5.801 | Growth control | Hasanuzzaman et al. 2018 | 0.753 |

| Species, Time, Condition | Gene description | Fold Change | Function | Common with study | Fold Change in the study |
|---|---|---|---|---|---|
| Rp 4w C vs LI | Protocadherin Fat 4 | 3.360 | Growth control | Hasanuzzaman et al. 2018 | 0.753 |
| Rd 4w C vs HI | CD109 antigen | -10.816 | Immune response | Sambade et al. 2025 | NA |
| Rd 4w C vs HI | CD109 antigen | -8.830 | Immune response | Sambade et al. 2025 | NA |
| Rd 4w C vs HI | Complement C1q-like protein 4 | -7.060 | Immune response | Hasanuzzaman et al. 2018 | 1.002 |
| Rd 4w C vs HI | Complement C1q-like protein 4 | 7.619 | Immune response | Hasanuzzaman et al. 2018 | 1.002 |
| Rd 4w C vs LI | Complement C1q-like protein 4 | -5.692 | Immune response | Hasanuzzaman et al. 2018 | 1.002 |
| Rd 4w C vs LI | IgGFc-binding protein | -1.213 | Immune response | Sambade et al. 2025 | NA |
| Rp 4w C vs LI | Integrin alpha-4 | 2.760 | Immune response | Sambade et al. 2025 | NA |
| Rp 4w C vs LI | Complement C1q tumor necrosis factor-related protein 3 | -6.314 | Immune resposne and metabolism | Sambade et al. 2025 | NA |
| Rd 4w C vs HI | Atrial natriuretic peptide receptor 1 | -7.655 | Response to hypoxia | Sambade et al. 2025 | NA |
| Rd 4w C vs HI | Atrial natriuretic peptide receptor 3 | 7.275 | Response to hypoxia | Sambade et al. 2025 | NA |
| Rd 4w C vs HI | Caprin-2 | -5.314 | Synaptic plasticity, stress response, immune response, and cellular proliferation | Sambade et al. 2025 | NA |
| Rd 4w C vs LI | T-complex protein 1 subunit gamma | -2.558 | Wound healing | Hasanuzzaman et al. 2018 | 1.031 |

### 4.4. Conclusion

This study evaluated the susceptibility of clam seed from *Ruditapes decussatus*, *Ruditapes philippinarum* and *Venerupis corrugata* to *Perkinsus olsenii* infection during a laboratory challenge of 28 days. With this experimental infection of clam seed we can conclude that: i) *Ruditapes philippinarum* is the most susceptible species to *P. olsenii* infection, presenting higher levels of infection than the autochthonous species when infected; ii) *Ruditapes decussatus* is the most predisposed species, demonstrating a high parasite prevalence but a lower infection level; iii) *Venerupis corrugata* presents the lowest prevalence and infection levels and, despite being able to be infected by the parasite, it seems to be not much affected by the infection; iv) the infection curve of *P. olsenii* appears to be similar on the three species, progressing as a chronic infection by the end of the 4 weeks post exposure; v) the transcriptomic analysis adds new insights into the mechanisms involved in the early and chronic stages of *P. olsenii* infection in *R. decussatus* and *R. philippinarum*; vi) a series of candidate genes related to the response to *P. olsenii* infection could be identified involving processes related to cellular proliferation, response to stress, growth, immune response, angiogenesis, neurogenesis, metabolism, pigmentation, calcification, digestion and wound healing. This work provides a new comparison on the susceptibility between the three *Veneridae* clam species, highlighting susceptibility and predisposition to parasite infection. Finally, it provides and confirms genomic markers of response to *P. olsenii* infection, constituting a transcriptomic reference applicable to future studies of genomic selection in clam seed.

### 4.5. References

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# Chapter 5

# General discussion

## Chapter 5 – General discussion

The infection caused by the parasite *Perkinsus olsenii* is responsible for high mortalities in different clam species around the world, being one of them the European native species *Ruditapes decussatus*. The main objective of this thesis was the search for new biomarkers of resistance to *P. olsenii* in *R. decussatus* in an attempt to increase the knowledge on this clam species with a clear decrease production trend but with high market demand and consumer interest. The work described in this dissertation provides information regarding new and reported biomarkers in this species, as well as new information related to infection and biomarkers in other *Veneridae* clam species. Moreover, it gives insight of parasite prevalence and infection level in *R. decussatus* populations from different affected clam beds around Europe, together with genetic variability and differentiation between these populations and their respective areas. Finally, a reference genome of *R. decussatus* plus transcriptomic and proteomic resources obtained from the interaction between the host and the parasite are provided. This work provides bases and sources of information for the host clam response to *P. olsenii* infection, being useful for future genomic studies of clam selection for aquaculture and population restoration purposes.

Regarding the parasite prevalence and infection levels of populations used in this study, results represent a snapshot of the parasite epizootics in a time point regarding the presence of *P. olsenii* in the analysed areas. This, additionally, made feasible the selection of appropriate samples for the identification of the resistance or tolerance biomarkers. Therefore, the information obtained regarding these clam beds is only preliminary and need a more in-depth study along time. Nonetheless, the data obtained indicates a high presence of the parasite in all the affected areas, being the prevalence equal or greater than 65 %. *Perkinsus* infection of clams exhibits variable infection intensities depending on environmental conditions, especially temperature and salinity, which have been reported to play an important role in parasite prevalence (Ruano et al., 2015; Villalba et al., 2004). However, high prevalence of the parasite has been detected in Atlantic and Mediterranean populations, despite the temperature and salinity differences in both areas (Estêvão et al. 2023; Ruano et al., 2015).

The genomic and proteomic approaches in this dissertation allowed to understand not only the populations' genetic structure and diversity, but also proteomic composition of each population in response to *P. olsenii* infection, and also in relation to the environment, although this approach was not fully covered in the thesis since it was not the principal aim of the study. Also, the complete dataset of environmental parameters of each bed at collection time should be taking into consideration as the protein expression could be highly

variable with small changes in temperature, salinity, pH or other paramentes as well as with their gonadal status and sex, and these data was not taken since our unique variable was *P. olsenii* infection level. Moreover, a genomic screening in the identified genomic markers allowed to uncover mechanisms of tolerance or resistance that are common to all the analysed populations, providing basic information of the chronic response in adult clams to *P. olsenii* infection. Also, more biomarkers were provided for the construction of a novel SNP-Chip with 15k SNP under the European projects IGNITION (GA101084651; https://ignition-project.eu/) and ShellFishBoost (SBEP0009_2023; https://bluepartnership.eu/projects/boosting-resilience-european-shellfish-production-against-climate-change-related.

Finally, an experimental infection of clam seed from the three *Veneridae* species provided additional information on the susceptibility and response to the parasite. This last work allowed to understand which species juveniles are more affected and which are more predisposed, together with the information regarding how the infection progresses along 28 days. Insights on the progression of the infection allowed to correlate with the transcriptomics data subsequently analysed, where a higher number of mechanisms of response to parasite infection in the juvenile clams were observed on the first 48 hours of infection, in comparison to four weeks post infection when the infection starts progressing to a chronic infection. This contrast may possibly derive from the establishment of the chronic infection by *P. olsenii*, where clams may mostly spend their resources in maintaining metabolic homeostasis together with pathogen elimination, comparing with the first contact with the parasite where they may spend most of their resources fighting the parasite entrance in their tissues in expense of growth and reproductive differentiation.

Regarding the biomarkers identified in this dissertation, 27 were proteins related to susceptibility and 5 were proteins related to tolerance to *P. olsenii* infection in the proteomic study. The susceptibility biomarkers included different ribosomal proteins, suggesting a high demand of energy restoration and balance by the host, not only for growth and maturation, but also to actively use its immune defence system to fight the parasite replication and usage of the host resources for survival. Ferritin was also identified in the susceptibility markers, being related to iron availability regulation, hijacking free iron to inhibit parasite cell growth and proliferation. In the tolerance biomarkers, a different ferritin was identified that appears to be involved in shell formation and growth, coupled with another biomarker involved in regulation of apoptosis, indicating that suppression of host defence mechanism by the parasite can be prevented, avoiding pathogen survival inside the host.

In the genomic study, 90 SNPs linked to 439 candidate genes associated with resistance / tolerance were identified. From these candidate genes, some were associated with the proteomic study of Estêvão et al., (2023) and the transcriptomic study of Hasanuzzaman et al. (2018). Among these, five genes related to iron storage, cytoskeleton organization, proteases and energy balance with identical or similar annotation to the list of 32 proteins differentially expressed when comparing heavily infected vs non-infected clams by Estêvão et al. (2023). Phosphoenolpyruvate carboxykinase [GTP] was identified in these three studies, indicating that it is a strong biomarker of resistance / tolerance to *P. olsenii*. This biomarker is related to energy balance through gluconeogenesis but also involved in bacteria recognition and elimination (Lv et al., 2017) . Four other relevant biomarkers were found in common with the study of Hasanuzzaman et al. (2018), related to response to perkinsosis in Manila clam (complement C1q-like protein 4, cytochrome P450 2A6, tyrosine-protein kinase, and ubiquitin-conjugating enzyme E2 U). Other several genes pertaining to relevant immune and stress related families in mollusc were also identified in common with the same study.

From the transcriptomic study, a total of 328 differentially expressed genes could be identified on two clam species, conditions and sampling points. A panoply of candidate genes was analysed for their expression pattern in each or both *R. decussatus* and *R. philippinarum* species. These were related to diverse processes of response to *P. olsenii* infection, such as cellular proliferation (*EMILIN-1-A, Hemicentin-1*), response to stress (*Pathogenesis-related protein 5*), growth (*Hemicentin-1, Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1*), immune response (*Interleukin-6 receptor subunit beta, Complement C1q-like protein 4*), angiogenesis (*Thrombospondin-2*), and neurogenesis (*Tenascin-R*). From the differentially expressed genes identified in this study, 39 were common with the genomic study from this dissertation and with the study of Hasanuzzaman et al. (2018). These genes included important processes of response to *P. olsenii* infection, reinforcing their role as resistance / tolerance markers, as 23 of them already identified at 48 hours post exposure to the parasite. The most prominent processes identified in these genes included angiogenesis (*Thrombospondin-2*), cellular proliferation (*Hemicentin-1*), growth (*Hemicentin-1, Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1*), immune response (*Hemicentin-2, Complement C1q-like protein 4*), metabolism (*Multidrug resistance-associated protein 1*), pigmentation (*E3 ubiquitin-protein ligase HERC2*), response to stress (*Caprin-2*), calcification (*Ectin*), digestion (*Chitotriosidase-1*) and wound healing (*T-complex protein 1 subunit gamma*).

The biomarkers obtained in this dissertation compose a solid foundational basis for evaluating the presence of resistance or tolerance to *P. olsenii* in individual clams, being

them either adults or juveniles. Hereupon, future studies or field experiments of marker-assisted selection breeding programs will certainly benefit from this data to produce more resistant *R. decussatus* seed and to continue improving the knowledge on the species.

## 5.1. Final remarks

- The results of prevalence derived from this study represent a picture of the parasite epizootics in a single time point with a high presence of the parasite in all the affected areas (prevalence ≥ 65%).
- The genetic structure, diversity, and proteomic composition of the populations were covered, as well as mechanisms of tolerance / resistance common to all the populations in chronic infection by *P. olsenii*.
- The experimental infection of three *Veneridae* species clam seed provided additional information on the susceptibility and response to parasite, where a higher number of mechanisms of response to parasite infection in juvenile clams were found at 48 hours in comparison to 4 weeks post infection, possibly due to the establishment of a chronic infection by *P. olsenii*.
- 27 proteins related to susceptibility and 5 related to tolerance to *P. olsenii* infection were identified in the proteomic study.
- 90 SNPs including 439 candidate genes associated with resistance/ tolerance to *P. olsenii* infection were identified in the genomic study.
- 328 differentially expressed genes could be identified in *R. decussatus* and *R. philippinarum* after exposition to the parasite and evaluation of infection progression.
- A solid foundational basis of biomarkers was obtained with implications in future studies of marker-assisted selection breeding programs in *Ruditapes decussatus*.

## 5.2. Future perspectives

This work provided a foundational basis of biomarkers that were obtained mostly in wild conditions, except of the clam seed experiment, where the infection took place in a controlled condition. Therefore, improvements can be done in order to more accurately detect markers of resistance or tolerance to *Perkinsus olsenii* infection in *Ruditapes decussatus*. A common garden experimental infection of several families (from parents coming from a naïve or an infected bed by the parasite) will allow for a more precise genotype and genomic screening of genes interfering with the tolerance or resistance to the parasite. Moreover, these experiments will allow to distinguish between events of tolerance, resistance or resilience, as it will be possible to monitor the progression of the infection over time on the same group of individuals, despite a high labour may be required. Another approach that deserves consideration is to test if trained immunity on seed or juveniles could be a possible measure to counteract infection or diminish the damage of the parasite.

A breeding program using individuals from Venice population could be the best approach since this population presents the higher genetic diversity of the all populations studied, making it a good candidate for searching signatures of resistance or tolerance to *P. olsenii*. Finally, this thesis contributes as an information source to aid for stakeholders to make informed decisions on the clam production in the European coast, enhancing this branch of the aquaculture sector as well as the economic sector of community coastal areas that are affected by this disease.

### 5.3. References

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# Appendix I

# Search for new biomarkers of tolerance to *Perkinsus olsenii* parasite infection in *Ruditapes decussatus* clams

**Table S1.** Total expressed proteins from the analysis Susceptible versus Tolerant. For the complete version of this table, visit the link: https://ars.els-cdn.com/content/image/1-s2.0-S1050464823000529-mmc1.xlsx

| Accession | Description | Abundance Ratio: (Infected) / (Non_infected) | Abundance Ratio P-Value: (Infected) / (Non_infected) |
|---|---|---|---|
| A0A210Q774 | Betaine--homocysteine S-methyltransferase 1 | 100 | 1E-17 |
| A0A210PVH9 | Asparagine synthetase [glutamine-hydrolyzing] | 100 | 1E-17 |
| K1QV55 | Ubiquitinyl hydrolase 1 | 100 | 1E-17 |
| A0A210QXB3 | Ubiquitinyl hydrolase 1 | 100 | 1E-17 |
| A0A194AN82 | Putative aminopeptidase W07G4.4 | 3.028 | 0.002144833 |
| Q8ITB9 | Ribosomal protein L7 (Fragment) | 1.859 | 0.117443406 |
| A0A210Q465 | Ras-related protein Rab-1A | 1.75 | 0.167580242 |
| K1PZ93 | Dihydropyrimidine dehydrogenase [NADP(+)] | 1.718 | 0.185482208 |
| M5AKT2 | Paramyosin-2 (Fragment) | 1.684 | 0.206821194 |
| M5B264 | Paramyosin-1 (Fragment) | 1.684 | 0.206821194 |
| K1R150 | Ras-related protein Rab-1A | 1.656 | 0.226098844 |
| Q8ITC5 | 60S ribosomal protein L30 | 1.616 | 0.256382439 |
| K4HXI8 | Ferritin | 1.597 | 0.271749348 |
| J9PJ22 | Arginine kinase | 1.583 | 0.283997895 |
| A0A194AM72 | Putative ras-related protein Rab-1A | 1.582 | 0.285251664 |
| A0A210QMV4 | 60S ribosomal protein L7 | 1.578 | 0.288234404 |
| K1R6F1 | Proteasome subunit alpha type | 1.549 | 0.315097357 |
| A0A210QAJ5 | 40S ribosomal protein S3 | 1.549 | 0.315704816 |
| A0A2L1TGZ7 | 40S ribosomal protein S3 (Fragment) | 1.549 | 0.315704816 |
| A0A515EJ23 | Cathepsin K | 1.546 | 0.317885769 |
| K1QJM1 | 60S ribosomal protein L30 | 1.542 | 0.321814599 |
| Q70MM6 | Ribosomal protein S3 (Fragment) | 1.52 | 0.344119694 |
| K1QEA6 | Phosphoenolpyruvate carboxykinase (GTP) | 1.515 | 0.34903728 |
| Q0KHB7 | Phosphoenolpyruvate carboxykinase (GTP) | 1.515 | 0.34903728 |
| Q70MM5 | 60S ribosomal protein L40 (Fragment) | 1.515 | 0.349013069 |

| Accession | Description | Abundance Ratio: (Infected) / (Non_infected) | Abundance Ratio P-Value: (Infected) / (Non_infected) |
|---|---|---|---|
| K1RGQ0 | Ras-related C3 botulinum toxin substrate 1 | 1.515 | 0.34976977 |
| A0A210R4I2 | Proteasome endopeptidase complex | 1.504 | 0.360641778 |
| A0A0N9HES5 | Ras-related C3 botulinum toxin substrate 1 | 1.498 | 0.367342508 |
| A0A0N9HJP7 | Rho GTPase | 1.498 | 0.367342508 |
| K1QWC3 | 40S ribosomal protein S3 | 1.49 | 0.375985509 |
| A0A210PQH0 | Ras-related protein Rab-11A | 1.489 | 0.377380455 |
| K1QX44 | Ras-related protein Rab-11B | 1.489 | 0.377380455 |
| A0A194AM89 | Proteasome subunit alpha type | 1.486 | 0.380375276 |
| K1R008 | Proteasome subunit alpha type | 1.486 | 0.380375276 |
| K1PD48 | Beta-ureidopropionase | 1.486 | 0.380437533 |
| K1QCB0 | 40S ribosomal protein S5 | 1.468 | 0.402033843 |
| Q4H451 | Ribosomal protein S5 | 1.468 | 0.402033843 |
| A0A210PU23 | 40S ribosomal protein S5 | 1.468 | 0.402033843 |
| Q70ML8 | Ribosomal protein S5 (Fragment) | 1.468 | 0.402033843 |
| A0A194AJD6 | Putative paramyosin-3 | 1.467 | 0.402744578 |
| B1AAP1 | Galectin | 1.438 | 0.437770628 |
| A0A210QN06 | Proteasome subunit alpha type | 1.433 | 0.444085792 |
| A0A210QAC9 | Filamin-A | 1.42 | 0.461501747 |
| C8CBM2 | Glutathione peroxidase A | 1.416 | 0.467215424 |
| K1QFW9 | Tr-type G domain-containing protein | 1.408 | 0.477700363 |
| A0A159WJ17 | Heat shock protein 60 | 1.405 | 0.481616248 |
| A0A0K0YAZ1 | Filamin-like protein-3 (Fragment) | 1.403 | 0.485037851 |
| A0A210QPM8 | 1,5-anhydro-D-fructose reductase | 1.401 | 0.487403297 |
| K1QHI5 | Pyruvate carboxylase | 1.392 | 0.499381814 |
| K1QS07 | Proteasome subunit beta type-3 | 1.391 | 0.50052143 |
| K1PPK1 | Actin-related protein 2/3 complex subunit 4 | 1.387 | 0.507262675 |
| L0BTB2 | Heat shock protein 90 | 1.378 | 0.520073484 |

| Accession | Description | Abundance Ratio: (Infected) / (Non_infected) | Abundance Ratio P-Value: (Infected) / (Non_infected) |
|---|---|---|---|
| A0A194AJE0 | Putative filamin-A-like protein isoform X7 | 1.375 | 0.523439041 |
| J9Z5K1 | Elongation factor 1 alpha (Fragment) | 1.374 | 0.525287897 |
| A0A067XIK1 | Ferritin | 1.366 | 0.537860759 |
| J9Q7E3 | Ubiquitin | 1.361 | 0.544137393 |
| G9FRU1 | Polyubiquitin | 1.361 | 0.544137393 |
| K1RA77 | Ubiquitin | 1.361 | 0.544137393 |
| J7FBH7 | 40S ribosomal protein S27a | 1.361 | 0.544137393 |
| Q75W47 | Polyubiquitin | 1.361 | 0.544137393 |
| A0A077H3L5 | 40S ribosomal protein S27a (Fragment) | 1.361 | 0.544137393 |
| J7K156 | Polyubiquitin (Fragment) | 1.361 | 0.544137393 |
| H1ADR7 | GTP-binding nuclear protein | 1.357 | 0.550815736 |
| A0A210PRC6 | Proteasome subunit alpha type | 1.356 | 0.55177634 |
| K1RM80 | Citrate synthase | 1.352 | 0.558767771 |
| K1PFH4 | Talin-2 | 1.347 | 0.566421155 |
| A0A1P8SD54 | Ubiquitin | 1.338 | 0.580264627 |
| K1PD36 | 60S ribosomal protein L40 | 1.334 | 0.586341213 |
| D2XEB0 | 60S ribosomal protein L40 | 1.334 | 0.586341213 |
| A0A210QBW4 | Elongation factor 2 | 1.333 | 0.588407858 |
| C4N894 | Complement factor B-like protein | 1.331 | 0.591821756 |
| A0A210Q890 | Major vault protein | 1.331 | 0.591572342 |
| A0A5P8PEG2 | Histone H4 | 1.329 | 0.594866459 |
| Q6WV74 | Histone H4 | 1.329 | 0.594866459 |
| B3FEB2 | Histone H4 | 1.329 | 0.594866459 |
| A0A5P8PEI5 | Histone H4 | 1.329 | 0.594866459 |
| A0A5P8PFI1 | Histone H4 | 1.329 | 0.594866459 |
| B3FEA7 | Histone H4 | 1.329 | 0.594866459 |
| A0A6C0N4Q3 | 60S ribosomal protein L40 | 1.322 | 0.606583366 |

| Accession | Description | Abundance Ratio: (Infected) / (Non_infected) | Abundance Ratio P-Value: (Infected) / (Non_infected) |
|---|---|---|---|
| Q4A487 | Histone H4 (Fragment) | 1.32 | 0.608584456 |
| D2DLE9 | Histone H4 | 1.32 | 0.608584456 |
| Q4A488 | Histone H4 (Fragment) | 1.32 | 0.608584456 |
| A0A210PZN8 | Inosine-5'-monophosphate dehydrogenase | 1.317 | 0.614224517 |
| K1PCA0 | Septin-7 | 1.308 | 0.628203663 |
| K1R5V4 | GTP-binding nuclear protein | 1.308 | 0.629355167 |
| A0A140H125 | GTP-binding nuclear protein | 1.302 | 0.638686559 |
| K1RNB5 | Propionyl-CoA carboxylase beta chain, mitochondrial | 1.295 | 0.651277265 |
| H6B8P0 | Troponin C | 1.287 | 0.665639685 |
| K1QA50 | V-type proton ATPase subunit H | 1.286 | 0.667414375 |
| K1QIR8 | 78 kDa glucose-regulated protein | 1.283 | 0.671829527 |
| Q75W49 | 78kDa glucose regulated protein | 1.283 | 0.671829527 |
| Q5Y1E4 | Tubulin beta chain (Fragment) | 1.268 | 0.699041118 |
| S4S355 | Histone H4 | 1.265 | 0.703062981 |
| A8DQX1 | Histone H4 (Fragment) | 1.265 | 0.703062981 |
| C3VUU2 | Arginine kinase | 1.262 | 0.709997949 |
| K1PR25 | Regulator of differentiation 1 | 1.262 | 0.708982405 |
| B3TD73 | GST class-pi | 1.258 | 0.717082789 |
| B3TD72 | GST class-pi | 1.258 | 0.717082789 |
| A0A210QDP3 | Fumarylacetoacetase | 1.258 | 0.716983586 |
| K1QVD7 | Neuronal acetylcholine receptor subunit non-alpha-2 | 1.254 | 0.724317879 |
| K1QX37 | 2-phospho-D-glycerate hydro-lyase | 1.252 | 0.727529343 |
| A0A4Y5MZ38 | Ras-related protein Rap1 | 1.244 | 0.74279959 |
| A0A210QLF5 | Kyphoscoliosis peptidase | 1.23 | 0.769673476 |
| Q9U0S7 | Myosin heavy chain (Fragment) | 1.228 | 0.773604164 |
| K1R0Y3 | Ras-related protein Rap-1b | 1.226 | 0.775890658 |
| E3VWM3 | Fructose-bisphosphate aldolase | 1.225 | 0.779505581 |

| Accession | Description | Abundance Ratio: (Infected) / (Non_infected) | Abundance Ratio P-Value: (Infected) / (Non_infected) |
|---|---|---|---|
| B7XC68 | Tropomyosin | 1.222 | 0.784309924 |
| A0A1L5JFM5 | GTP-binding nuclear protein | 1.217 | 0.793252091 |
| A0A210PFP4 | Ras-related protein Rap-1b | 1.212 | 0.80308271 |
| K1PTH4 | RING-type E3 ubiquitin transferase RAD18 | 1.212 | 0.803102213 |
| A0A344X1X2 | Arginine kinase | 1.211 | 0.805652858 |
| Q4AEC4 | Arginine kinase | 1.211 | 0.805652858 |
| K1R7V7 | Tubulin beta chain | 1.21 | 0.808036474 |
| A0A2L1TH01 | Tubulin beta chain | 1.21 | 0.808036474 |
| A0A0K0YAX9 | Filamin-like protein-1 | 1.209 | 0.809878221 |
| V9PAG9 | Thioredoxin reductase 1 (Fragment) | 1.208 | 0.812013443 |
| A0A210PSR9 | Thioredoxin reductase 3 | 1.208 | 0.812013443 |
| K1PU86 | Thioredoxin reductase 3 | 1.206 | 0.815361752 |
| K1PW06 | Filamin-C | 1.205 | 0.818266789 |
| K1QAU8 | Peptidyl-prolyl cis-trans isomerase E | 1.197 | 0.834249857 |
| A0A0G2R2A9 | Tubulin beta chain (Fragment) | 1.196 | 0.836477935 |
| K1QAJ5 | Tubulin beta chain | 1.195 | 0.837569246 |
| A0A210Q7U8 | Cystathionine beta-synthase | 1.193 | 0.841400304 |
| G8XWU1 | Tropomyosin | 1.191 | 0.845943927 |
| K1PUE0 | Cystathionine beta-synthase | 1.19 | 0.847896309 |
| K1PCV0 | Severin | 1.19 | 0.84819697 |
| A0A076KW18 | Ubiquitin C (Fragment) | 1.186 | 0.856944647 |
| A0A513U826 | Vacuolar proton pump subunit B | 1.184 | 0.860040891 |
| A0A2P0M990 | Vitelline membrane outer layer protein 1 (Fragment) | 1.184 | 0.859107829 |
| Q5GIS3 | Guanine nucleotide-binding protein subunit beta | 1.166 | 0.897852753 |
| K1R9E9 | Aldehyde dehydrogenase family 16 member A1 | 1.165 | 0.899549966 |
| C8CBN4 | Heat shock protein 22 isoform 1 | 1.164 | 0.901041568 |
| K1RJ70 | Cytosolic non-specific dipeptidase | 1.163 | 0.903930901 |

| Accession | Description | Abundance Ratio: (Infected) / (Non_infected) | Abundance Ratio P-Value: (Infected) / (Non_infected) |
|---|---|---|---|
| K1R7T2 | Isocitrate dehydrogenase [NADP] | 1.162 | 0.905678936 |
| G8XUN5 | Malate dehydrogenase | 1.16 | 0.90927776 |
| A1ILZ8 | Non muscle myosin (Fragment) | 1.158 | 0.914150276 |
| K1QFF0 | Vacuolar protein sorting-associated protein 35 (Fragment) | 1.157 | 0.915869194 |
| K1PQD4 | Phosphoglucomutase-1 | 1.157 | 0.915817736 |
| Q9U0S6 | Pedal retractor muscle myosin heavy chain (Fragment) | 1.156 | 0.919383481 |
| K1PUJ1 | Radixin | 1.152 | 0.926306864 |
| B7XC71 | Tropomyosin | 1.148 | 0.93606909 |
| A0A210PNZ3 | Vesicle-fusing ATPase | 1.142 | 0.949024994 |
| A2CI31 | Histone H2B | 1.141 | 0.950279598 |
| C0JPJ2 | Complement component C3 | 1.136 | 0.961639577 |
| E7CZG6 | Triosephosphate isomerase (Fragment) | 1.132 | 0.970635469 |
| K1PI02 | Talin-1 | 1.122 | 0.991080873 |
| A0A210R0B2 | Paramyosin | 1.121 | 0.994395473 |
| A0A023W7L5 | HSP70 protein | 1.118 | 0.998951258 |
| A0A210PNJ1 | Proteasome subunit beta | 1.117 | 0.998037591 |
| A0A194AP54 | Proteasome subunit beta | 1.117 | 0.998037591 |
| K1PNR3 | Clathrin heavy chain | 1.116 | 0.994874745 |
| K1QTV7 | Aldehyde dehydrogenase family 16 member A1 (Fragment) | 1.116 | 0.99565536 |
| K1PMP2 | Phosphoglucomutase-1 | 1.115 | 0.992923047 |
| K1Q1Z3 | Seryl-tRNA synthetase | 1.114 | 0.991797031 |
| C8CBN7 | Allograft inflammatory factor | 1.111 | 0.984085602 |
| K1QXX7 | Myosin heavy chain, non-muscle (Fragment) | 1.109 | 0.979527765 |
| A0A5J6CXN6 | Mitogen-activated protein kinase | 1.109 | 0.979590738 |
| A0A210R5E4 | Protein kinase C | 1.109 | 0.979650603 |
| K1R401 | Spectrin alpha chain | 1.107 | 0.976176758 |
| K1P339 | Vinculin | 1.1 | 0.958865455 |

| Accession | Description | Abundance Ratio: (Infected) / (Non_infected) | Abundance Ratio P-Value: (Infected) / (Non_infected) |
|---|---|---|---|
| K1QBM3 | Ras-related protein Rab-2 | 1.086 | 0.929062426 |
| A0A210PFE4 | Ras-related protein Rab-2 | 1.086 | 0.929062426 |
| D8L792 | Myosin heavy chain type II (Fragment) | 1.085 | 0.92676914 |
| A0A210PYW2 | Vinculin | 1.08 | 0.914391036 |
| K1QFR9 | Spectrin beta chain | 1.072 | 0.896356793 |
| A0A210Q886 | Serine/threonine-protein phosphatase | 1.072 | 0.896447601 |
| K1R8W3 | S-phase kinase-associated protein 1 | 1.072 | 0.895548365 |
| A0A210R0M1 | S-phase kinase-associated protein 1 | 1.072 | 0.895548365 |
| K1PJ46 | Pyruvate kinase | 1.072 | 0.895722338 |
| Q0KHB6 | Pyruvate kinase | 1.072 | 0.895722338 |
| K1QLM3 | Serine/threonine-protein phosphatase | 1.071 | 0.893314116 |
| A0A067XI81 | Ferritin | 1.069 | 0.890390759 |
| K1RSS3 | Myosin heavy chain, striated muscle | 1.068 | 0.887261597 |
| A0A210QT17 | Serine/threonine-protein phosphatase | 1.068 | 0.888068513 |
| A0A210QWL4 | Malate dehydrogenase | 1.064 | 0.878257138 |
| Q9NDL1 | Myosin (Fragment) | 1.063 | 0.875049179 |
| H6UNP2 | ATP synthase subunit beta (Fragment) | 1.05 | 0.847196606 |
| C1ITJ9 | Serine/threonine-protein phosphatase | 1.049 | 0.844378873 |
| Q9NKW8 | Serine/threonine-protein phosphatase | 1.049 | 0.844378873 |
| A9ELN4 | Serine/threonine-protein phosphatase (Fragment) | 1.049 | 0.844378873 |
| Q7YXJ8 | Tubulin beta chain (Fragment) | 1.047 | 0.838419028 |
| B3FRR7 | Ferritin (Fragment) | 1.046 | 0.836821833 |
| A0A210PR37 | Myosin regulatory light chain sqh | 1.037 | 0.816120399 |
| B7XC70 | Tropomyosin | 1.035 | 0.811207291 |
| A0A210QK95 | CCT-alpha | 1.035 | 0.812644902 |
| A0A194ALY6 | Putative myosin regulatory light polypeptide 9-like protein | 1.03 | 0.79905027 |
| K1RXA0 | cAMP-dependent protein kinase regulatory subunit | 1.03 | 0.799403685 |

| Accession | Description | Abundance Ratio: (Infected) / (Non_infected) | Abundance Ratio P-Value: (Infected) / (Non_infected) |
|---|---|---|---|
| A0A077GY59 | Beta-actin (Fragment) | 1.022 | 0.782269205 |
| A0A0P0LDM5 | Actin | 1.018 | 0.773329284 |
| K1Q122 | Myosin regulatory light chain sqh | 1.016 | 0.768229564 |
| Q962V3 | Translation initiation factor eIF4A (Fragment) | 1.015 | 0.765907108 |
| A0A1L1ZLT2 | Histone deacetylase | 1.014 | 0.762276893 |
| S4S354 | Histone H2A | 1.004 | 0.740528851 |
| A0A210PR34 | Histone H2A | 1.004 | 0.740528851 |
| Q6WV67 | Histone H2A | 1.004 | 0.740528851 |
| A2CI32 | Histone H2A | 1.004 | 0.740528851 |
| A0A3G4ZJ62 | Histone H2A | 1.004 | 0.740528851 |
| K1RXS6 | Histone H2A | 1.004 | 0.740528851 |
| K1PUM2 | Histone H2A | 1.004 | 0.740528851 |
| K1P421 | Histone H2A | 1.004 | 0.740528851 |
| Q6PTH7 | Triosephosphate isomerase (Fragment) | 1.004 | 0.740167881 |
| Q6PTL0 | Triosephosphate isomerase (Fragment) | 1.004 | 0.740167881 |
| K1PGY5 | Dihydropyrimidinase | 0.998 | 0.725842784 |
| A0A6G6CZ36 | Basic transcription factor 3 | 0.997 | 0.723762062 |
| A0A210QQE1 | U5 small nuclear ribonucleoprotein helicase | 0.996 | 0.7222714 |
| A0A194ANM3 | Septin-type G domain-containing protein | 0.996 | 0.721101093 |
| A0A210Q016 | ADP-ribosylation factor 4 | 0.993 | 0.715302073 |
| A0A210QW78 | Septin-2 | 0.988 | 0.703560762 |
| K1QLZ1 | Actin-related protein 3 | 0.986 | 0.699091188 |
| K1R282 | Adenylate kinase isoenzyme 5 | 0.982 | 0.690063938 |
| K1RH58 | Alpha-actinin, sarcomeric | 0.981 | 0.687396933 |
| P08051 | Myosin regulatory light chain, smooth muscle | 0.981 | 0.687340603 |
| Q26079 | Myosin heavy chain | 0.971 | 0.66351241 |
| A0A210QSX4 | Myosin heavy chain, non-muscle | 0.965 | 0.65081266 |

| Accession | Description | Abundance Ratio: (Infected) / (Non_infected) | Abundance Ratio P-Value: (Infected) / (Non_infected) |
|---|---|---|---|
| H6BD99 | G protein B subunit | 0.959 | 0.636856534 |
| V9PCY3 | G protein B subunit | 0.959 | 0.636856534 |
| K1PWZ3 | Guanine nucleotide-binding protein subunit beta | 0.959 | 0.636856534 |
| K1QDH9 | Myosin-11 | 0.952 | 0.620146337 |
| A0A210PU77 | DNA-directed RNA polymerases I, II, and III subunit RPABC3 | 0.951 | 0.617981137 |
| C3VUU4 | Arginine kinase | 0.949 | 0.613394552 |
| Q596I0 | Alpha-1,4 glucan phosphorylase | 0.949 | 0.614278744 |
| K1Q948 | Alpha-1,4 glucan phosphorylase | 0.949 | 0.614278744 |
| A0A210R642 | Phosphoenolpyruvate carboxykinase (GTP) | 0.924 | 0.556583286 |
| A0A210QIF0 | Arp2/3 complex 34 kDa subunit | 0.923 | 0.553678983 |
| A0A386CAF0 | Arp2/3 complex 34 kDa subunit (Fragment) | 0.923 | 0.553678983 |
| K1QCC1 | Phosphoglycerate kinase | 0.915 | 0.537119525 |
| A0A210Q0H3 | G protein-coupled receptor kinase | 0.897 | 0.497651987 |
| B8YQ57 | GST class-pi | 0.884 | 0.469019408 |
| P07290 | Myosin, essential light chain, adductor muscle | 0.881 | 0.463376895 |
| A0A210PDP3 | Myosin, essential light chain, adductor muscle | 0.881 | 0.463376895 |
| Q9U7E2 | Myosin essential light chain | 0.881 | 0.463376895 |
| Q26066 | Myosin essential light chain | 0.881 | 0.463376895 |
| P07291 | Myosin essential light chain, striated adductor muscle | 0.881 | 0.463376895 |
| K1QWX0 | 26S proteasome regulatory subunit RPN11 | 0.879 | 0.45892058 |
| A0A210QNI0 | 26S proteasome regulatory subunit RPN11 | 0.879 | 0.45892058 |
| A0A210PIS8 | Dihydropyrimidinase | 0.869 | 0.437144276 |
| A0A0K0YB28 | Transgelin-like protein-1 | 0.867 | 0.433752376 |
| A0A0K0YB23 | Transgelin-like protein-5 | 0.867 | 0.433752376 |
| A0A0K0YAZ6 | Transgelin-like protein-3 | 0.867 | 0.433752376 |
| A0A0K0YB42 | Transgelin-like protein-6 | 0.867 | 0.433752376 |
| A0A0K0YB45 | Transgelin-like protein-2 | 0.867 | 0.433752376 |

| Accession | Description | Abundance Ratio: (Infected) / (Non_infected) | Abundance Ratio P-Value: (Infected) / (Non_infected) |
|---|---|---|---|
| K1RG91 | Transgelin-2 | 0.867 | 0.433752376 |
| A0A0K0YB26 | Transgelin-like protein-4 | 0.867 | 0.433752376 |
| A0A194AJS2 | Putative eukaryotic initiation factor 4A-II | 0.853 | 0.404131615 |
| K1QBJ5 | G protein-coupled receptor kinase | 0.834 | 0.366821095 |
| A0A451ET90 | C-type lectin 5 | 0.819 | 0.337443813 |
| A0A210R727 | Ubiquitin-conjugating enzyme E2-17 kDa | 0.814 | 0.328511932 |
| K4IPB7 | Calmodulin | 0.799 | 0.301161832 |
| A0A210Q538 | Alpha-actinin cytoskeletal isoform | 0.793 | 0.289051703 |
| K1Q056 | Calpain-A | 0.791 | 0.285874256 |
| A4L694 | Beta actin (Fragment) | 0.779 | 0.265884503 |
| A0A0K0MJ14 | Heat shock protein | 0.778 | 0.263564256 |
| A0A210Q590 | Katanin p60 ATPase-containing subunit A1 | 0.765 | 0.241421472 |
| K1PY30 | Septin-2 | 0.748 | 0.214836398 |
| A0A067XHZ5 | Ferritin | 0.741 | 0.205043108 |
| K1REL9 | Disco-interacting protein 2-like protein C | 0.712 | 0.163839097 |
| O61284 | Sarcoplasmic calcium-binding protein | 0.707 | 0.157542049 |
| A0A210Q2Z7 | Disco-interacting protein 2-like C | 0.677 | 0.121804475 |
| J9UEN7 | Myosin essential light chain | 0.635 | 0.081099859 |
| A0A210PXL7 | T-complex protein 1 subunit delta | 0.611 | 0.062360692 |
| A0A067XI00 | Ferritin | 0.599 | 0.054326708 |
| K1QRU8 | Myosin heavy chain, striated muscle | 0.539 | 0.024482892 |
| K1R6N5 | Structural maintenance of chromosomes protein 3 | 0.01 | 1E-17 |

**Table S2.** GO terms protein distribution obtained from the analysis Susceptible versus Tolerant.

| GO function | Number of hits | Percentage | Associated function | Expressed |
|---|---|---|---|---|
| organic substance metabolic process | 18 | 20% | BP | Susceptible |
| primary metabolic process | 17 | 19% | BP | Susceptible |
| cellular metabolic process | 16 | 18% | BP | Susceptible |
| nitrogen compound metabolic process | 16 | 18% | BP | Susceptible |
| biosynthetic process | 10 | 11% | BP | Susceptible |
| small molecule metabolic process | 5 | 6% | BP | Susceptible |
| cellular component organization or biogenesis | 3 | 3% | BP | Susceptible |
| catabolic process | 3 | 3% | BP | Susceptible |
| cellular homeostasis | 1 | 25% | BP | Tolerant |
| regulation of biological quality | 1 | 25% | BP | Tolerant |
| protein folding | 1 | 25% | BP | Tolerant |
| establishment of localization | 1 | 25% | BP | Tolerant |
| organic cyclic compound binding | 13 | 16% | MF | Susceptible |
| ion binding | 13 | 16% | MF | Susceptible |
| heterocyclic compound binding | 13 | 16% | MF | Susceptible |
| small molecule binding | 8 | 10% | MF | Susceptible |
| carbohydrate derivative binding | 8 | 10% | MF | Susceptible |
| hydrolase activity | 8 | 10% | MF | Susceptible |
| structural constituent of ribosome | 8 | 10% | MF | Susceptible |
| transferase activity | 4 | 5% | MF | Susceptible |
| catalytic activity, acting on a protein | 4 | 5% | MF | Susceptible |
| lyase activity | 2 | 2% | MF | Susceptible |
| oxidoreductase activity | 2 | 2% | MF | Susceptible |
| ion binding | 4 | 22% | MF | Tolerant |
| small molecule binding | 2 | 11% | MF | Tolerant |
| organic cyclic compound binding | 2 | 11% | MF | Tolerant |
| carbohydrate derivative binding | 2 | 11% | MF | Tolerant |
| heterocyclic compound binding | 2 | 11% | MF | Tolerant |
| protein binding | 2 | 11% | MF | Tolerant |
| protein-containing complex binding | 1 | 6% | MF | Tolerant |
| hydrolase activity | 1 | 6% | MF | Tolerant |
| oxidoreductase activity | 1 | 6% | MF | Tolerant |
| ATP hydrolysis activity | 1 | 6% | MF | Tolerant |
| intracellular anatomical structure | 15 | 22% | CC | Susceptible |
| cytoplasm | 15 | 22% | CC | Susceptible |
| organelle | 13 | 19% | CC | Susceptible |
| ribonucleoprotein complex | 7 | 10% | CC | Susceptible |
| cytosol | 4 | 6% | CC | Susceptible |
| proteasome core complex, alpha-subunit complex | 2 | 3% | CC | Susceptible |
| supramolecular complex | 2 | 3% | CC | Susceptible |
| catalytic complex | 2 | 3% | CC | Susceptible |
| myosin complex | 2 | 3% | CC | Susceptible |

| GO function | Number of hits | Percentage | Associated function | Expressed |
|---|---|---|---|---|
| intracellular protein-containing complex | 2 | 3% | CC | Susceptible |
| proteasome core complex | 2 | 3% | CC | Susceptible |
| membrane | 1 | 1% | CC | Susceptible |
| cell periphery | 1 | 1% | CC | Susceptible |
| cell projection | 1 | 1% | CC | Susceptible |
| intracellular anatomical structure | 3 | 30% | CC | Tolerant |
| cytoplasm | 2 | 20% | CC | Tolerant |
| myosin complex | 2 | 20% | CC | Tolerant |
| organelle | 2 | 20% | CC | Tolerant |
| supramolecular complex | 1 | 10% | CC | Tolerant |

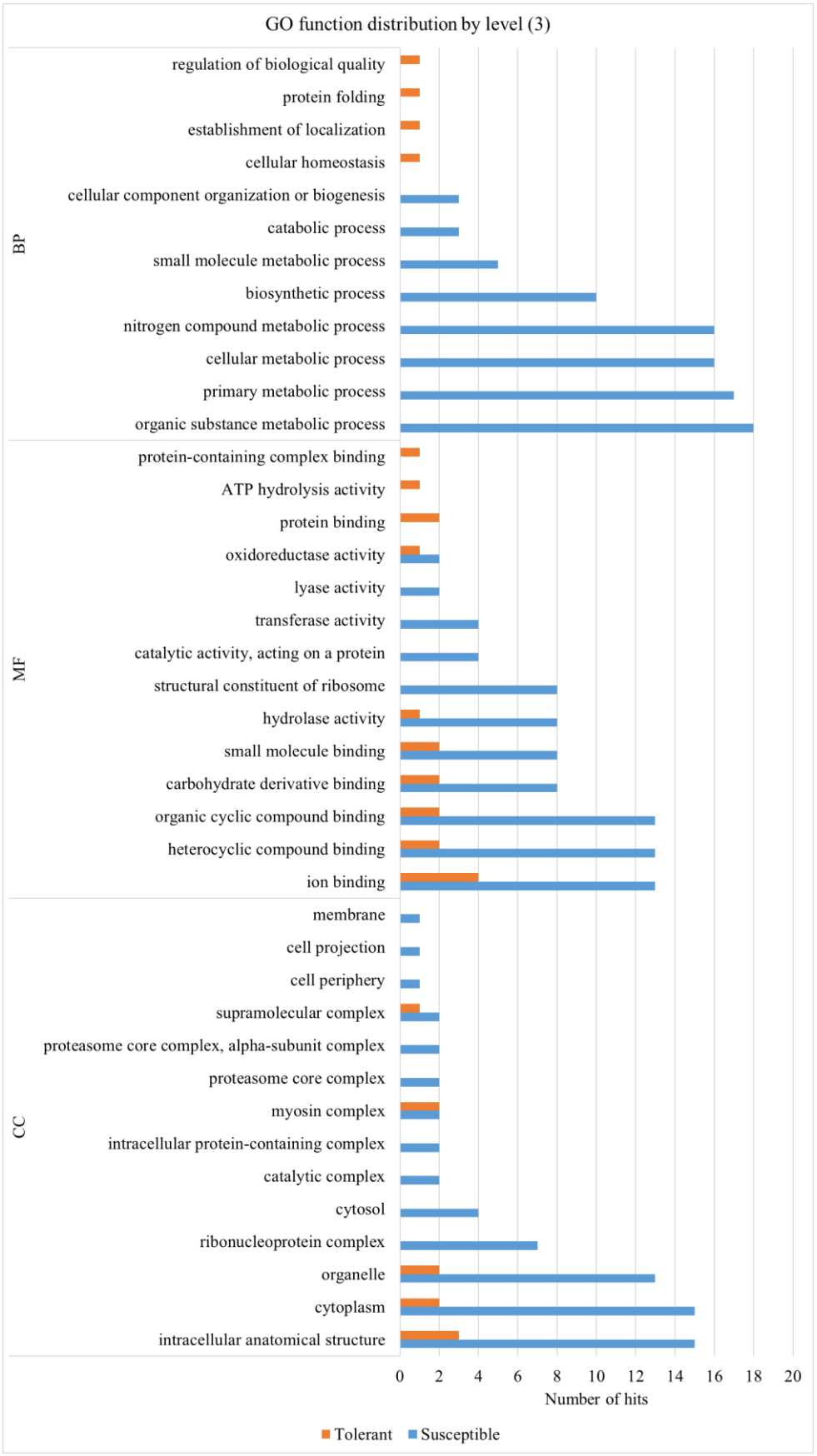


**Figure S1.** GO terms protein distribution obtained from the analysis Susceptible versus Tolerant.

# Appendix II

# Signatures of selection for resistance / tolerance to *Perkinsus olsenii* in grooved carpet shell clam (*Ruditapes decussatus*) using a population genomics approach


**Figure S1.** Bioinformatic pipeline for the assembly of the *R. decussatus* genome.

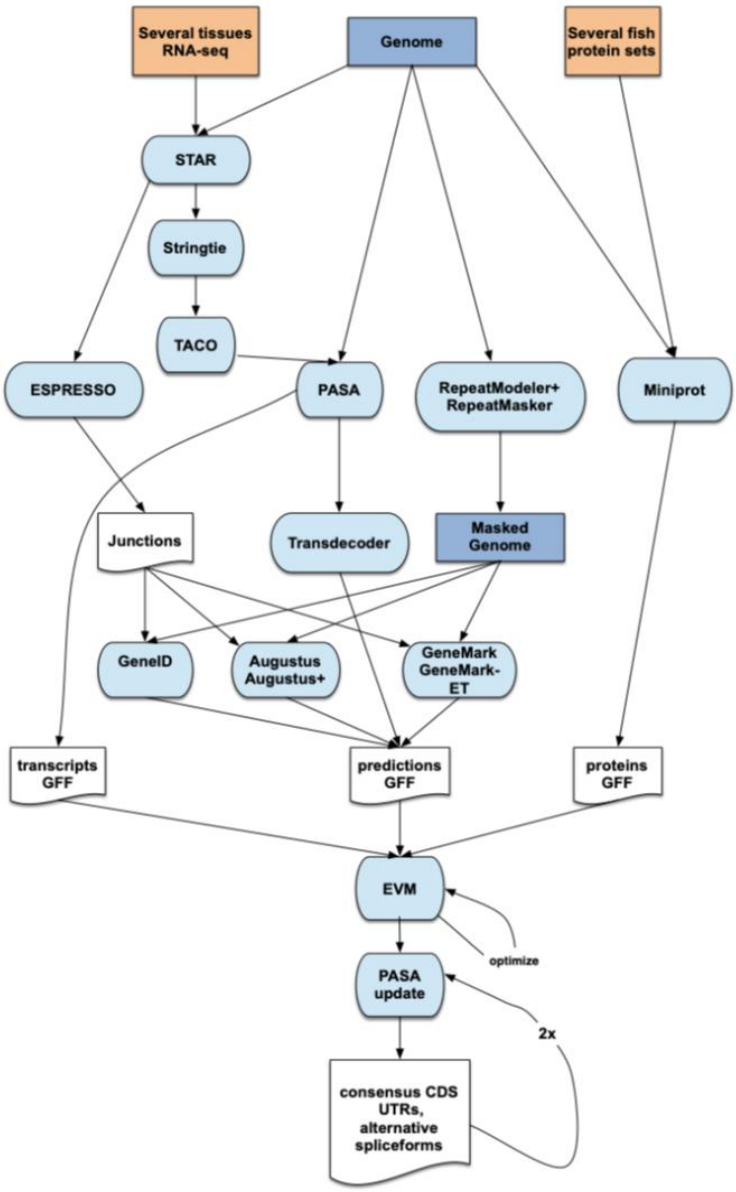


**Figure S2.** Bioinformatic pipeline for the annotation of the *R. decussatus* genome.

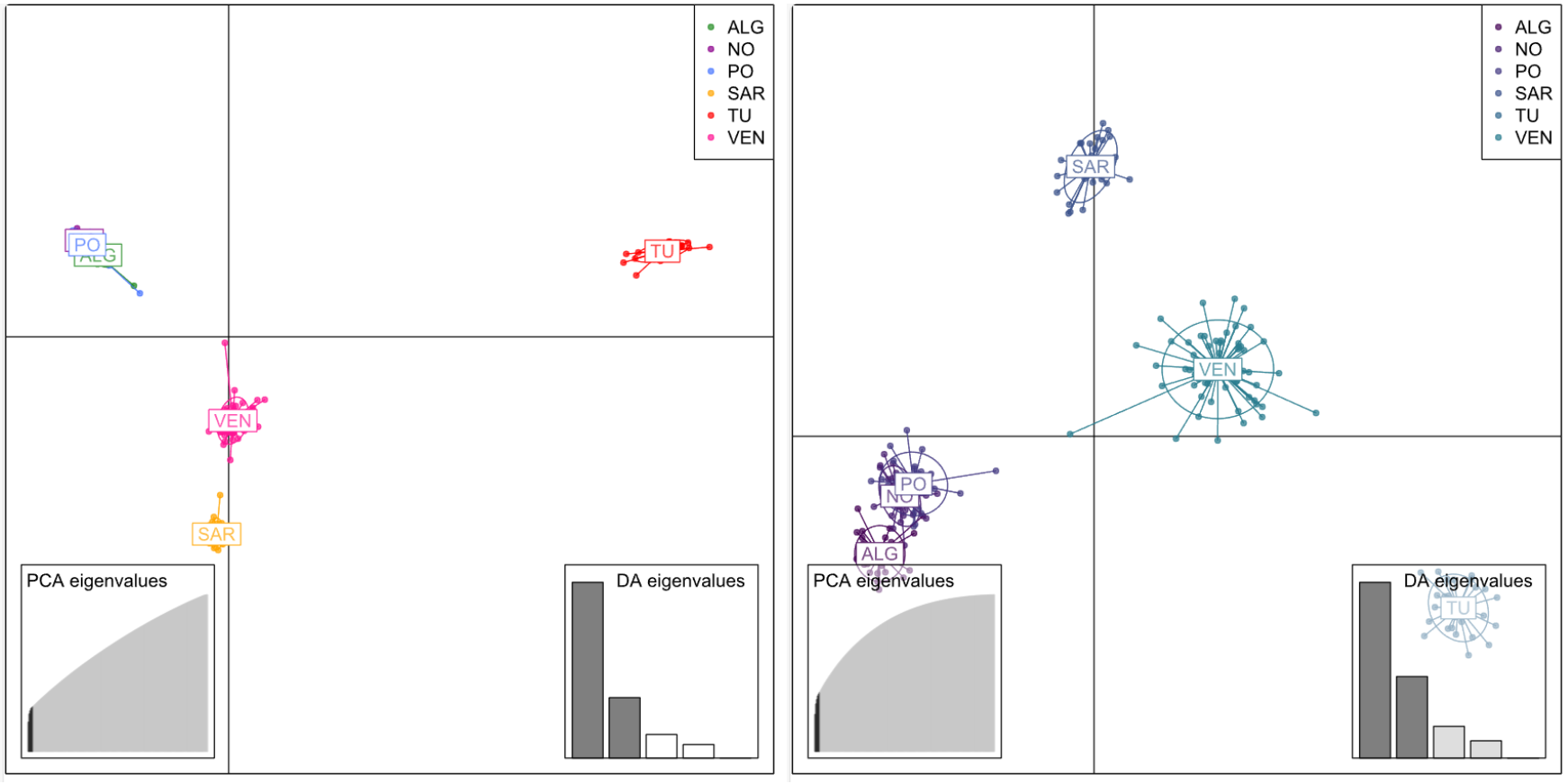


**Figure S6.** Discriminant analysis of principal components (DAPC) representation of *R. decussatus* samples: (A) whole SNP panel (B) geographic divergent outlier panel.

**Table S12.** Shared genes between this study (normal font) and that by Estevao et al. (2023) and Hasanuzzaman et al. (2018) in response to *P. olsenii* in *R. decussatus* and *R. phillipinarum*, respectively (highlighted in red font). In italic font the genes identified by Hasanuzzaman et al. (2018) as highly relevant in the response to *Perkinsus*.

| Identical or similar annotation (Estevao et al., 2023) | Identical annotation (Hasanuzzaman et al., 2018) | Similar annotation / gene families (Hasanuzzaman et al., 2018) |
|---|---|---|
| Ferritin, isoform 4 R | Asparaginyl-tRNA synthetase | Actin, cytoplasmic |
| Ferritin, isoform H | *Complement C1q-like protein 4* | actin-related protein 2 |
| Ferroxidase HEPHL1 | *Cytochrome P450 2A6* | |
| | DNA replication licensing factor mcm2 | *caprin-1* |
| Myosin essential light chain | Heat shock 70 kDa protein 12B | Caprin-2 |
| Myosin light chain kinase 3 | Long-chain-fatty-acid--CoA ligase 1 | |
| | Phosphoenolpyruvate carboxykinase [GTP] | DNA replication licensing factor mcm4 |
| Phosphoenolpyruvate carboxykinase (GTP) | Regulator of G-protein signaling 3 | DNA replication licensing factor mcm5 [Crassostrea gigas] |
| Phosphoenolpyruvate carboxykinase [GTP] | Solute carrier family 23 member 1 | DNA replication licensing factor mcm7 [Crassostrea gigas] |
| | Tetraspanin-33 | |
| Proteasome subunit alpha type | *tyrosine-protein kinase* | E3 ubiquitin-protein ligase Bre1 [Crassostrea gigas] |
| Proteasome subunit alpha type-2 | *Ubiquitin-conjugating enzyme E2 U* | E3 ubiquitin-protein ligase HECTD1 |
| Proteasome subunit alpha type-4 | | E3 ubiquitin-protein ligase RNF180 |
| | | E3 ubiquitin-protein ligase RNF185 |
| Ras-related protein Rab-1A | | E3 ubiquitin-protein ligase RNF213 |
| Ras-related protein Rab-1A | | E3 ubiquitin-protein ligase TRIM13 |
| Ras-related protein Rab-30 | | E3 ubiquitin-protein ligase TRIM33 |
| | | E3 ubiquitin-protein ligase TRIM33 |
| | | E3 ubiquitin-protein ligase TRIM33 |
| | | E3 ubiquitin-protein ligase TRIM33 |
| | | E3 ubiquitin-protein ligase TRIM33 |
| | | E3 ubiquitin-protein ligase TRIM33 |
| | | E3 ubiquitin-protein ligase TRIM33 |
| | | E3 ubiquitin-protein ligase TRIM33 |
| | | E3 ubiquitin-protein ligase TRIM45 |

| Identical or similar annotation (Estevao et al., 2023) | Identical annotation (Hasanuzzaman et al., 2018) | Similar annotation / gene families (Hasanuzzaman et al., 2018) |
|---|---|---|
| | | E3 ubiquitin-protein ligase TRIM56 |
| | | E3 ubiquitin-protein ligase TRIM56 |
| | | E3 ubiquitin-protein ligase XIAP |
| | | |
| | | Heat shock 70 kDa protein 12A |
| | | Heat shock 70 kDa protein 12B |
| | | Heat shock 70 kDa protein |
| | | Heat shock protein beta-1 |
| | | |
| | | Heterogeneous nuclear ribonucleoprotein H [Crassostrea gigas] |
| | | Heterogeneous nuclear ribonucleoprotein K |
| | | |
| | | *low-density lipoprotein receptor-related protein 2* |
| | | Low-density lipoprotein receptor-related protein 5 |
| | | Myotubularin-related protein 13 |
| | | Myotubularin-related protein 4 |
| | | Myotubularin-related protein 8 |
| | | |
| | | *palmitoyltransferase B* |
| | | Palmitoyltransferase ZDHHC3 |
| | | |
| | | Proteasome subunit alpha type-2 |
| | | Proteasome subunit alpha type-3 |
| | | Proteasome subunit alpha type-4 |
| | | Proteasome subunit beta type-2 |
| | | Proteasome subunit beta type-5 |

| Identical or similar annotation (Estevao et al., 2023) | Identical annotation (Hasanuzzaman et al., 2018) | Similar annotation / gene families (Hasanuzzaman et al., 2018) |
|---|---|---|
| | | Ras-related protein Rab-1A |
| | | Ras-related protein Rab-30 |
| | | Serine/threonine-protein kinase Chk1 |
| | | Serine/threonine-protein kinase LATS1 |
| | | Serine/threonine-protein kinase N2 |
| | | Serine/threonine-protein kinase PAK 1 |
| | | Serine/threonine-protein kinase tricornered |
| | | Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform |
| | | Serine/threonine-protein phosphatase 2A catalytic subunit beta isoform |
| | | Serine/threonine-protein phosphatase 6 regulatory ankyrin repeat subunit C |
| | | Solute carrier family 12 member 2 |
| | | Solute carrier family 12 member 6 |
| | | Solute carrier family 13 member 2 |
| | | Solute carrier family 22 member 4 |
| | | Solute carrier family 22 member 7 |
| | | Solute carrier family 23 member 2 (H) |
| | | Solute carrier family 28 member 3 |
| | | Tetratricopeptide repeat protein 16 |
| | | Tetratricopeptide repeat protein 17 |
| | | Tetratricopeptide repeat protein 38 |
| | | Tetratricopeptide repeat protein 38 |

| Identical or similar annotation (Estevao et al., 2023) | Identical annotation (Hasanuzzaman et al., 2018) | Similar annotation / gene families (Hasanuzzaman et al., 2018) |
|---|---|---|
| | | *universal stress protein A. R* |
| | | Universal stress protein MJ0531 |
| | | Universal stress protein Sll1388 |
| | | Vacuolar protein sorting-associated protein 11-like protein |
| | | Vacuolar protein sorting-associated protein 35, partial |
| | | Vacuolar protein sorting-associated protein 52 homolog |

## SUPPLEMENTARY METHODS

*Whole Genome Sequencing (WGS)*

Long-read sequencing libraries

4.0 µg of the DNA was DNA-repaired and DNA-end-repaired using NEBNext FFPE DNA Repair Mix (NEB) and the NEBNext UltraII End Repair/dA-Tailing Module NEB, respectively. Then, sequencing adaptor ligation, purification by 0.4X AMPure XP Beads and elution in Elution Buffer (SQK-LSK109) was accomplished. The sequencing runs were performed on GridION Mk1 (ONT) using a Flowcell R9.4.1 FLO-MIN106D (ONT) and the sequencing data was collected for 110 hours.

Short-read genome sequencing libraries

1.0 µg of genomic DNA was sheared on a Covaris™ LE220-Plus (Covaris) and size-selected for the fragment size of 220-550 bp with AMPure XP beads (Agencourt, Beckman Coulter). The genomic DNA fragments were then end-repaired and adenylated. Next, compatible adaptors for Illumina platforms with unique dual indexes including unique molecular identifiers (Integrated DNA Technologies) were ligated.

Hi-C sequencing

Chromatin was fixed in place with formaldehyde (Sigma Aldrich), digested with DNase I and DNA extracted. DNA ends were repaired, and a biotinylated bridge adapter was ligated followed by proximity ligation of adapter-containing ends. After reverse crosslinking, the DNA was purified and followed by the preparation of Illumina-compatible paired-end sequencing libraries (omitting the fragmentation step). Biotinylated chimeric molecules were isolated using streptavidin beads before PCR enrichment of the library.

*Genome assembly*

Preprocessing and filtering of reads

Illumina reads were trimmed using Trim-galore v0.6.6 (with options *--gzip -q 20 --paired --retain_unpaired*) (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) and the nanopore reads were filtered using FiltLong v0.2.0 (with options *--min_length* 5000 *--target_bases* 40,000,000,000) (FiltLong: https://github.com/rrwick/Filtlong). The filtering of nanopore data ensured having reads of at least 5 kb while optimizing for both length and higher mean base qualities, keeping 40 Gb (~ 65x coverage).

Filtered ONT reads were assembled with NextDenovo v2.4.0 (https://github.com/Nextomics/NextDenovo) applying the options: minimap2_options_raw = -x ava-ont,

minimap2_options_cns = -x ava-ont -k17 –w17 and seed_cutoff=10k. The resulting contigs were polished with Nextpolish v1.3.1 (Hu et al., 2020) using two rounds of long-read polishing and two rounds of short-read polishing.

The Omni-C reads were mapped to the assembly using BWA-MEM and pre-processed using the Dovetail pipeline (https://omni-c.readthedocs.io/en/latest/fastq_to_bam.html). The filtering of the alignments was done with the default minimum mapping quality of 40. After the removal of PCR duplicates, YaHS30 v1.1 was used for scaffolding with default parameters. Two rounds of assembly error correction were performed and made 15 breaks, followed by ten rounds of scaffolding from higher to lower resolution (10 Mb down to 10 Kb).

*Genome annotation*

RNA-Seq

The RNA-Seq libraries were prepared with KAPA Stranded mRNA-Seq Illumina® Platforms Kit (Roche). Briefly, 500 ng of total RNA was used for the poly-A fraction enrichment with oligo-dT magnetic beads, following the mRNA fragmentation protocol. The strand specificity was achieved during the second strand synthesis performed in the presence of dUTP instead of dTTP. The blunt-ended double stranded cDNA was 3´adenylated before Illumina platform compatible adaptors with unique dual indexes and unique molecular identifiers (Integrated DNA Technologies) were ligated.

Repetitive elements

Repeats present in the *R. decussatus* genome assembly were annotated with RepeatMasker v4-1-5-0 (http://www.repeatmasker.org) using the custom repeat library available for Mollusca. After excluding those repeats that were part of repetitive protein families (performing a BLAST search against UniProt) from the resulting library, RepeatMasker was run again with this new library to annotate the specific repeats. Next, *redmask* (https://github.com/nextgenusfs/redmask) was run on the masked genome outputted by RepeatMasker. To avoid masking certain repetitive protein families present in the genome, we performed a BLAST (Altschul et al., 1990) search of the *redmask*-produced library against Uniprot. Those repeats with significant hits (evalue $<10^{-6}$) against proteins were removed from the final repeat annotation. Bedtools v2.31.1 (Quinlan and Hall, 2010) was used to produce the final repeat-masked version of the genome.

Gene annotation

Gene annotation was done by combining transcript alignments, protein alignments and *ab initio* gene predictions following the CNAG structural genome annotation pipeline (Figure

S1; https://github.com/cnag-aat/Annotation_AAT). Firstly, RNA-Seq reads obtained from several tissues, either sequenced specifically in this study (gill, mantle, foot, haemocytes, and digestive gland) or existing in public databases, were aligned to the genome with STAR v-2.7.10a (Dobin et al., 2013). Transcript models were subsequently generated using Stringtie v2.2.1 (Pertea et al., 2015) on each BAM file and then all the models produced were combined using TACO v0.7.3 (Niknafs et al., 2017). High-quality junctions to be used during the annotation process were obtained by running ESPRESSO (Gao et al., 2023) v1.3.0 after mapping with Minimap2. Finally, PASA assemblies were produced with PASA (Haas et al., 2008) v2.5.2. The TransDecoder program, which is part of the PASA package, was run on the PASA assemblies to detect coding regions in the transcripts. Additionally, the complete proteomes of *C. virginica*, *C. gigas*, *Mytilus coruscus*, *Mytilus galloprovincialis*, and *Mytilus edulis* were downloaded from Uniprot in April 2024 and aligned to the genome using Miniprot v0.6 (Li, 2023). *Ab initio* gene predictions were performed on the repeat-masked *R. decussatus* assembly with three different programs: GeneID v1.4 (Parra et al., 2000), Augustus v3.5.0 (Stanke et al., 2006) and Genemark-ES v7.71 (Lomsadze et al., 2014) with and without incorporating evidence from the RNA-Seq data. Geneid and Augustus were specifically trained for this species with a set of 1000 gene candidates obtained from the longest Transdecoder complete models that had a significant BLAST hit against Swissprot (e-value <10-6). Genemark runs in a self-training mode and was not specifically trained with this set of gene candidates. Finally, all the data were combined into consensus CDS models using EvidenceModeler-2.1 (EVM, Haas et al., 2008). Functional annotation was performed on the annotated proteins with Blast2go (Conesa et al., 2005). First, a Diamond blastp (Buchfink et al., 2021) search was made against the nr (last accessed May 2021) and Uniprot (last accessed August 2021) databases. Then, InterProScan (Jones et al., 2014) was run to detect protein domains on the annotated proteins. All these data were combined by Blast2go, which produced the final functional annotation results. Additionally, UTRs and alternative splicing forms were annotated via two rounds of PASA annotation updates. To functionally annotate the proteins of the annotation, the Pannzer's12 online server was run (Törönen and Holm, 2022).

The annotation of ncRNAs was obtained by running the following steps on the repeat-masked version of the genome assembly. First, the program cmsearch (Cui et al., 2016) v1.1 that is part of the Infernal package (Nawrocki and Eddy, 2013) was run against the RFAM database of RNA families v12.0. Additionally, tRNAscan-SE (Chan et al., 2019) v2.11 was run in order to detect the transfer RNA genes present in the genome assembly. Identification of lncRNAs was done by first filtering the set of PASA-assemblies that had not been included in the annotation of protein-coding genes to retain those longer than 200bp

and not covered more than 80% by a small ncRNA. The resulting transcripts were clustered into genes using shared splice sites or significant sequence overlap as criteria for designation as the same gene.

The non-coding RNA annotation required several steps. First, those expressed transcripts that had been assembled by PASA but that had not been annotated as Protein-Coding genes were tagged as long-non-coding RNAs. The reason for this step is that it helps to have putative lncRNAs annotated before using annotation for downstream analysis. However, due to the poor lncRNAs conservation between species, no function was assigned to these lncRNA genes. Moreover, to remove false positives, transcripts overlapping with other Protein-coding genes or repeats were not included into the lncRNA annotation. Finally, only transcripts longer than 200 bp were considered lncRNAs.

The final non-coding annotation contains the lncRNAs and the sncRNAs. The resulting transcripts were clustered into genes using shared splice sites or substantial sequence overlap as criteria for designation as the same gene.

# Appendix III

# Insights on the susceptibility of three commercial clam species to *Perkinsus olsenii* infection and its response to infection

**Supplementary table 1.** Differentially expressed genes in *R. decussatus* at 48 hours post exposure between control and low infection conditions.

| Gene ID | log2Fold Change | padj | Gene.description | GO terms |
|---|---|---|---|---|
| RDEC1A043316 | -12.64 | 9.71E-22 | NA | NA |
| RDEC1A025899 | -8.81 | 4.41E-08 | NA | NA |
| RDEC1A004286 | -7.40 | 1.95E-04 | NA | NA |
| RDEC1A026495 | -6.93 | 1.01E-03 | NA | NA |
| RDEC1A034200 | -6.62 | 4.39E-03 | NA | NA |
| RDEC1A017500 | -6.51 | 0.01 | NA | NA |
| RDEC1A002880 | -4.31 | 5.95E-49 | NA | NA |
| RDEC1A023589 | -4.27 | 1.27E-80 | NA | NA |
| RDEC1A044303 | -4.03 | 4.40E-10 | NA | NA |
| RDEC1A022360 | -3.72 | 1.22E-12 | NA | NA |
| RDEC1A013947 | -2.95 | 4.39E-03 | Teneurin-3 | NA |
| RDEC1A022246 | -2.83 | 2.00E-03 | Thrombospondin-2 | NA |
| RDEC1A035649 | -2.56 | 0.01 | Apolipophorins | NA |
| RDEC1A053117 | -2.50 | 1.20E-03 | Hemicentin-1 | NA |
| RDEC1A052230 | -2.47 | 1.57E-05 | Highly reducing polyketide synthase tstA | NA |
| RDEC1A036442 | -2.13 | 7.05E-05 | Ganglioside GM2 activator | NA |
| RDEC1A043955 | -2.13 | 0.04 | Complement C1q-like protein 4 | NA |
| RDEC1A038691 | -2.13 | 0.01 | NA | NA |
| RDEC1A014180 | -2.12 | 0.01 | Hemicentin-1 | NA |
| RDEC1A010771 | -1.87 | 0.01 | NA | NA |
| RDEC1A012924 | -1.62 | 1.18E-06 | EMILIN-1-A | NA |
| RDEC1A018338 | -1.53 | 3.69E-04 | Large neutral amino acids transporter small subunit 2 | GO:1902475,GO:0015804 |
| RDEC1A009007 | -1.53 | 0.03 | Antiviral innate immune response receptor RIG-I | NA |
| RDEC1A015818 | -1.53 | 0.01 | Methionine aminopeptidase 1 | NA |
| RDEC1A026498 | -1.50 | 0.02 | NA | NA |

| Gene ID | log2Fold Change | padj | Gene.description | GO terms |
|---|---|---|---|---|
| RDEC1A007679 | -1.46 | 6.98E-04 | Gamma-soluble NSF attachment protein | GO:0006886 |
| RDEC1A034458 | -1.45 | 0.01 | Mitochondrial uncoupling protein 4 | GO:0009409,GO:0055085 |
| RDEC1A027838 | -1.27 | 2.35E-03 | Temptin | NA |
| RDEC1A017621 | -1.24 | 0.01 | Apolipophorins | NA |
| RDEC1A044632 | -1.23 | 7.15E-04 | NA | NA |
| RDEC1A023725 | -1.20 | 1.04E-03 | N-acetylneuraminate lyase | NA |
| RDEC1A005470 | -1.17 | 0.02 | Perlucin-like protein | NA |
| RDEC1A049181 | -1.10 | 0.03 | ATP-dependent DNA helicase PIF1 | GO:0045944,GO:0051301 |
| RDEC1A035975 | -1.08 | 0.01 | NA | NA |
| RDEC1A053263 | -1.08 | 0.03 | Solute carrier family 23 member 2 | GO:0071702,GO:0055085,GO:0030324,GO:0007420,GO:0051180 |
| RDEC1A021263 | -1.03 | 0.01 | Monocarboxylate transporter 13 | GO:0015718,GO:0055085 |
| RDEC1A014712 | -0.74 | 0.05 | Glucosidase 2 subunit beta | GO:0006491 |
| RDEC1A014354 | -0.71 | 3.12E-03 | Perlucin-like protein | NA |
| RDEC1A005928 | -0.62 | 0.05 | Serine--tRNA ligase, cytoplasmic | GO:0070158 |
| RDEC1A021032 | 0.93 | 0.02 | Polycystin-2 | NA |
| RDEC1A044367 | 1.02 | 0.01 | NA | NA |
| RDEC1A025186 | 1.11 | 4.33E-03 | Temptin | GO:0006589,GO:0042420,GO:0042421 |
| RDEC1A028359 | 1.14 | 0.01 | PHD finger-like domain-containing protein 5A | GO:0000398,GO:0006413 |
| RDEC1A033901 | 1.40 | 0.01 | NA | NA |
| RDEC1A037960 | 1.71 | 0.02 | Fibrillin-1 | NA |
| RDEC1A001660 | 2.23 | 0.01 | Centrosomal protein of 70 kDa | GO:0070507,GO:0060271 |
| RDEC1A051884 | 2.34 | 9.77E-07 | Glucose dehydrogenase [FAD, quinone] | GO:0019285 |
| RDEC1A015804 | 2.49 | 0.04 | Ubiquitin-ribosomal protein eL40 fusion protein | NA |
| RDEC1A002787 | 2.73 | 0.04 | NA | NA |
| RDEC1A040166 | 2.98 | 0.04 | 17-beta-hydroxysteroid dehydrogenase type 6 | NA |
| RDEC1A052065 | 3.81 | 0.03 | NXPE family member 4 | NA |
| RDEC1A022044 | 4.04 | 3.78E-04 | von Willebrand factor C domain-containing protein 2-like | NA |

| Gene ID | log2Fold Change | padj | Gene.description | GO terms |
|---|---|---|---|---|
| RDEC1A022935 | 4.06 | 2.85E-04 | NA | NA |
| RDEC1A021011 | 4.13 | 1.54E-04 | NA | NA |
| RDEC1A028319 | 4.46 | 0.03 | Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1 | NA |
| RDEC1A032628 | 4.58 | 0.01 | E3 ubiquitin-protein ligase HERC2 | NA |
| RDEC1A002658 | 4.60 | 4.24E-08 | NA | NA |
| RDEC1A044222 | 4.73 | 0.01 | NA | NA |
| RDEC1A010192 | 5.15 | 0.01 | Interferon-induced protein 44 | GO:0006955 |
| RDEC1A014108 | 5.85 | 0.01 | NA | NA |
| RDEC1A006871 | 5.99 | 0.04 | NA | NA |
| RDEC1A011615 | 6.22 | 0.03 | Hemicentin-1 | NA |
| RDEC1A050736 | 6.61 | 1.44E-04 | Synaptonemal complex protein 3 | NA |
| RDEC1A035529 | 6.85 | 0.03 | Calcium-binding and coiled-coil domain-containing protein 2 | NA |
| RDEC1A011086 | 7.89 | 2.36E-03 | Pathogenesis-related protein 5 | GO:0006952 |
| RDEC1A006883 | 8.15 | 4.41E-08 | NA | NA |
| RDEC1A003856 | 8.19 | 0.01 | NA | NA |
| RDEC1A008077 | 20.63 | 1.95E-04 | Cleavage and polyadenylation specificity factor subunit 3 | GO:0006397 |
| RDEC1A049879 | 20.65 | 1.95E-04 | NA | NA |

**Supplementary table 2.** Differentially expressed genes in *R. decussatus* at 4 weeks post exposure between control and low infection conditions.

| Gene ID | log2FoldChange | padj | Gene.description | GO terms |
|---|---|---|---|---|
| RDEC1A018218 | -12.24 | 7.22E-11 | NA | NA |
| RDEC1A021867 | -8.79 | 2.65E-07 | Complement C1q tumor necrosis factor-related protein 4 | NA |
| RDEC1A015573 | -8.74 | 1.40E-03 | Guanylate-binding protein 1 | NA |
| RDEC1A030282 | -7.78 | 6.52E-04 | NA | NA |
| RDEC1A012214 | -7.69 | 0.05 | NA | NA |
| RDEC1A036020 | -5.69 | 0.01 | Complement C1q-like protein 4 | NA |
| RDEC1A020476 | -5.09 | 3.11E-03 | NA | NA |
| RDEC1A035815 | -4.46 | 0.02 | NA | NA |
| RDEC1A026480 | -4.46 | 0.01 | NA | NA |
| RDEC1A024585 | -4.20 | 0.03 | FMRFamide receptor | GO:0007186 |
| RDEC1A035559 | -3.82 | 0.01 | Monocarboxylate transporter 3 | GO:0009086,GO:0032259 |
| RDEC1A045007 | -3.76 | 1.54E-03 | NA | NA |
| RDEC1A043096 | -3.52 | 0.01 | 2-oxoglutarate-dependent dioxygenase htyE | NA |
| RDEC1A047531 | -2.96 | 0.01 | NA | NA |
| RDEC1A000316 | -2.73 | 0.02 | NA | NA |
| RDEC1A045103 | -2.60 | 0.01 | Tryptase gamma | NA |
| RDEC1A001853 | -2.56 | 0.03 | T-complex protein 1 subunit gamma | NA |
| RDEC1A053273 | -1.77 | 0.01 | NA | NA |
| RDEC1A002343 | -1.74 | 0.02 | Growth arrest and DNA damage-inducible protein GADD45 gamma | NA |
| RDEC1A021321 | -1.35 | 0.03 | NA | NA |
| RDEC1A018060 | -1.27 | 0.04 | Cytochrome P450 2J4 | NA |
| RDEC1A028953 | -1.21 | 0.03 | IgGFc-binding protein | NA |
| RDEC1A022246 | 2.18 | 0.02 | Thrombospondin-2 | NA |
| RDEC1A014824 | 2.22 | 2.44E-03 | Tenascin-R | NA |
| RDEC1A014714 | 2.30 | 1.24E-04 | NA | NA |
| RDEC1A024625 | 2.76 | 7.71E-05 | Chitotriosidase-1 | GO:0006032,GO:0005975,GO:0002532,GO:0032722 |

| Gene ID | log2FoldChange | padj | Gene.description | GO terms |
|---|---|---|---|---|
| RDEC1A046837 | 2.77 | 0.01 | NA | NA |
| RDEC1A048371 | 3.74 | 2.24E-04 | NA | NA |
| RDEC1A015288 | 6.12 | 3.46E-04 | NA | NA |
| RDEC1A015275 | 7.35 | 1.24E-04 | ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase | NA |
| RDEC1A002803 | 7.69 | 0.05 | Ectin | NA |
| RDEC1A043816 | 7.90 | 8.99E-05 | Baculoviral IAP repeat-containing protein 7-A | NA |
| RDEC1A013950 | 8.15 | 9.52E-04 | NA | NA |
| RDEC1A007148 | 10.88 | 9.18E-14 | NA | NA |

**Supplementary table 3.** Differentially expressed genes in *R. decussatus* at 48 hours post exposure between control and high infection conditions.

| Gene ID | log2Fold Change | padj | Gene.description | GO terms |
|---|---|---|---|---|
| RDEC1A021848 | -11.06 | 1.13E-15 | Complement C1q tumor necrosis factor-related protein 6 | NA |
| RDEC1A026773 | -9.05 | 6.42E-09 | Gamma-interferon-inducible lysosomal thiol reductase | NA |
| RDEC1A043581 | -8.29 | 2.46E-07 | PKS-NRPS hybrid synthetase swnK | NA |
| RDEC1A008556 | -8.11 | 6.92E-06 | NA | NA |
| RDEC1A015710 | -7.59 | 5.78E-05 | NA | NA |
| RDEC1A024688 | -7.56 | 4.29E-05 | NA | NA |
| RDEC1A038706 | -6.50 | 0.01 | NA | NA |
| RDEC1A032389 | -6.49 | 0.01 | NA | NA |
| RDEC1A008919 | -6.37 | 0.01 | Highly reducing polyketide synthase phiA | NA |
| RDEC1A037849 | -5.64 | 0.02 | Hemicentin-1 | NA |
| RDEC1A011409 | -5.18 | 0.02 | Hemicentin-1 | NA |
| RDEC1A025374 | -4.41 | 0.01 | Dermonecrotic toxin LhSicTox-alphaIA2aviii | NA |
| RDEC1A028846 | -3.79 | 1.38E-04 | E3 ubiquitin-protein ligase TRIM33 | NA |
| RDEC1A030232 | -3.54 | 2.83E-06 | NA | NA |
| RDEC1A011800 | -3.48 | 0.05 | NA | NA |
| RDEC1A004472 | -3.13 | 4.40E-07 | NA | NA |
| RDEC1A024005 | -2.94 | 8.54E-04 | Cartilage intermediate layer protein 1 | NA |
| RDEC1A002880 | -2.24 | 1.80E-16 | NA | NA |
| RDEC1A022378 | -2.14 | 0.05 | Bis(monoacylglycero)phosphate synthase CLN5 | GO:0007040,GO:0007035,GO:0042147,GO:0007420,GO:0006465 |
| RDEC1A004797 | -2.04 | 0.02 | Protein NO VEIN | GO:0010305,GO:0009793,GO:0048364 |
| RDEC1A040538 | -2.03 | 0.02 | Death-associated inhibitor of apoptosis 1 | NA |
| RDEC1A002659 | -1.97 | 4.61E-03 | Sodium- and chloride-dependent glycine transporter 2 | GO:0015812,GO:0035725,GO:1903804,GO:0060012,GO:0006836 |
| RDEC1A042959 | -1.93 | 5.78E-05 | Lysosomal Pro-X carboxypeptidase | GO:0060055,GO:0003085,GO:0043535,GO:0006508,GO:0002353 |
| RDEC1A018405 | -1.89 | 1.58E-03 | Glycerophosphocholine cholinephosphodiesterase ENPP6 | NA |
| RDEC1A034457 | -1.88 | 3.58E-03 | Sorbitol dehydrogenase | NA |

| Gene ID | log2Fold Change | padj | Gene.description | GO terms |
|---|---|---|---|---|
| RDEC1A052934 | -1.81 | 0.02 | A disintegrin and metalloproteinase with thrombospondin motifs 14 | NA |
| RDEC1A007264 | -1.74 | 1.38E-05 | Cytidine deaminase | NA |
| RDEC1A032462 | -1.66 | 0.02 | Matrilin-2 | GO:0048513 |
| RDEC1A006292 | -1.64 | 1.31E-05 | Probable tubulin polyglutamylase TTLL9 | GO:0000226,GO:0036211,GO:0030317 |
| RDEC1A017665 | -1.63 | 0.05 | Lambda-crystallin homolog | GO:0006631 |
| RDEC1A044303 | -1.54 | 4.05E-05 | NA | NA |
| RDEC1A004356 | -1.49 | 0.03 | NA | NA |
| RDEC1A023767 | -1.47 | 0.03 | NA | NA |
| RDEC1A023589 | -1.41 | 9.51E-06 | NA | NA |
| RDEC1A040910 | -1.38 | 0.03 | Solute carrier family 35 member C2 | GO:0015786,GO:0036066,GO:0045747,GO:0010629 |
| RDEC1A020532 | -1.37 | 0.05 | CD209 antigen-like protein C | NA |
| RDEC1A012390 | -1.36 | 0.03 | NA | NA |
| RDEC1A025032 | -1.23 | 0.03 | NA | NA |
| RDEC1A012108 | -1.16 | 0.02 | Collagen alpha-1(X) chain | NA |
| RDEC1A007463 | -1.16 | 0.02 | Fucolectin-1 | NA |
| RDEC1A038211 | -1.02 | 0.02 | Cyclic GMP-AMP synthase-like receptor 1 | NA |
| RDEC1A012461 | -0.98 | 0.01 | Neuropeptides capa receptor | NA |
| RDEC1A001010 | -0.97 | 0.05 | Stearoyl-CoA desaturase | GO:1903966,GO:0006636,GO:0070542,GO:0060613 |
| RDEC1A004575 | -0.92 | 5.74E-06 | NA | NA |
| RDEC1A012924 | -0.81 | 0.01 | EMILIN-1-A | NA |
| RDEC1A013808 | 0.58 | 4.04E-03 | Histone-lysine N-methyltransferase SETD2 | NA |
| RDEC1A008835 | 0.69 | 0.03 | Tubulin beta chain | GO:0000226,GO:0000278 |
| RDEC1A014084 | 0.80 | 0.04 | Dynein light chain Tctex-type 5 | NA |
| RDEC1A018911 | 0.90 | 0.03 | NA | NA |
| RDEC1A011180 | 0.90 | 5.45E-05 | Complement C3 | NA |
| RDEC1A044993 | 0.93 | 0.03 | Protein MEMO1 | NA |
| RDEC1A011407 | 0.93 | 0.02 | Protein Wnt-8a | GO:0060070,GO:0045165,GO:0030182 |

| Gene ID | log2Fold Change | padj | Gene.description | GO terms |
|---|---|---|---|---|
| RDEC1A004677 | 1.00 | 0.03 | All trans-polyprenyl-diphosphate synthase PDSS2 | NA |
| RDEC1A034350 | 1.01 | 0.03 | Hydroxylamine reductase | GO:0042542,GO:0098869 |
| RDEC1A051808 | 1.04 | 0.01 | Cadherin-related tumor suppressor | GO:0007156,GO:0016339,GO:0044331,GO:0034332,GO:0007043 |
| RDEC1A032114 | 1.10 | 4.51E-03 | NA | NA |
| RDEC1A002066 | 1.33 | 0.05 | ATP-binding cassette sub-family C member 2 | NA |
| RDEC1A019026 | 1.38 | 4.40E-03 | Cytochrome P450 2D14 | NA |
| RDEC1A039110 | 1.40 | 3.58E-03 | NA | NA |
| RDEC1A013944 | 1.42 | 0.01 | Calmodulin-like protein 3 | GO:1903475 |
| RDEC1A040973 | 1.63 | 1.16E-11 | Cell surface hyaluronidase | NA |
| RDEC1A014178 | 1.66 | 0.01 | Multidrug resistance-associated protein 1 | GO:0042908,GO:0055085 |
| RDEC1A051703 | 1.69 | 5.27E-10 | GTP cyclohydrolase 1 | GO:0006729,GO:0046654,GO:0036269,GO:0070050,GO:0045088 |
| RDEC1A047281 | 1.71 | 0.01 | NA | NA |
| RDEC1A015015 | 1.77 | 0.02 | Cell division control protein 42 homolog | GO:0007264,GO:0099563,GO:0051489,GO:0007163,GO:0035006 |
| RDEC1A051205 | 1.83 | 0.03 | Stimulated by retinoic acid gene 6 protein-like | GO:0071939,GO:0034633 |
| RDEC1A018383 | 1.97 | 2.25E-03 | Cytochrome P450 27C1 | GO:0006700,GO:0034650,GO:0006704,GO:0071375,GO:0008203 |
| RDEC1A039009 | 2.37 | 0.01 | Interferon-gamma-inducible GTPase 10 | NA |
| RDEC1A046274 | 2.55 | 0.01 | NA | NA |
| RDEC1A008333 | 2.56 | 3.46E-08 | Sodium- and chloride-dependent glycine transporter 1 | NA |
| RDEC1A046177 | 2.70 | 7.65E-04 | NA | NA |
| RDEC1A015179 | 2.75 | 4.20E-03 | Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1 | NA |
| RDEC1A038171 | 2.80 | 3.58E-03 | Sodium- and chloride-dependent GABA transporter 2 | NA |
| RDEC1A034169 | 3.11 | 6.12E-05 | Leucine-rich repeat-containing protein 74A | NA |
| RDEC1A053900 | 4.35 | 0.01 | Interleukin-6 receptor subunit beta | NA |
| RDEC1A004671 | 5.96 | 0.03 | NA | NA |
| RDEC1A033395 | 6.37 | 1.30E-03 | Serum paraoxonase/arylesterase 2 | NA |

| Gene ID | log2Fold Change | padj | Gene.description | GO terms |
|---|---|---|---|---|
| RDEC1A011086 | 7.14 | 0.01 | Pathogenesis-related protein 5 | GO:0006952 |
| RDEC1A015076 | 9.59 | 6.25E-11 | Receptor-type tyrosine-protein phosphatase mu | NA |

**Supplementary table 4.** Differentially expressed genes in *R. decussatus* at 4 weeks post exposure between control and high infection conditions.

| Gene ID | log2Fold Change | padj | Gene.description | GO terms |
|---|---|---|---|---|
| RDEC1A025759 | -15.15 | 1.19E-25 | NA | NA |
| RDEC1A011964 | -12.35 | 7.36E-14 | Cartilage intermediate layer protein 1 | NA |
| RDEC1A044718 | -10.84 | 3.01E-10 | E3 ubiquitin-protein ligase RNF213 | NA |
| RDEC1A001813 | -10.82 | 3.38E-09 | CD109 antigen | NA |
| RDEC1A019711 | -10.00 | 4.40E-11 | NA | NA |
| RDEC1A037692 | -9.90 | 2.26E-11 | NA | NA |
| RDEC1A042541 | -9.55 | 3.76E-05 | Sperm axonemal maintenance protein CFAP97D1 | NA |
| RDEC1A011901 | -9.45 | 4.23E-04 | NA | NA |
| RDEC1A046391 | -9.33 | 2.01E-09 | NA | NA |
| RDEC1A034496 | -9.04 | 8.64E-04 | Sperm axonemal maintenance protein CFAP97D1 | NA |
| RDEC1A050395 | -8.83 | 1.24E-05 | CD109 antigen | NA |
| RDEC1A026104 | -8.19 | 0.04 | NA | NA |
| RDEC1A043458 | -7.65 | 7.14E-04 | Atrial natriuretic peptide receptor 1 | GO:0007168,GO:0006182,GO:0035556,GO:0007601,GO:0055085 |
| RDEC1A049825 | -7.45 | 0.05 | Sterile alpha motif domain-containing protein 15 | NA |
| RDEC1A045415 | -7.20 | 0.04 | Germ cell-less protein-like 1 | NA |
| RDEC1A021685 | -7.19 | 5.80E-04 | NA | NA |
| RDEC1A036020 | -7.06 | 1.35E-03 | Complement C1q-like protein 4 | NA |
| RDEC1A013669 | -6.90 | 0.02 | Histone deacetylase 9 | NA |
| RDEC1A029839 | -6.66 | 0.02 | Serine/threonine-protein kinase/endoribonuclease IRE2 | NA |
| RDEC1A048358 | -6.51 | 0.02 | NA | NA |
| RDEC1A023540 | -6.31 | 0.05 | NA | NA |
| RDEC1A028172 | -6.07 | 0.02 | NA | NA |
| RDEC1A054070 | -6.00 | 1.69E-08 | Transcription intermediary factor 1-alpha | NA |
| RDEC1A027894 | -5.80 | 0.05 | Protocadherin Fat 4 | NA |
| RDEC1A028815 | -5.31 | 0.03 | Caprin-2 | NA |
| RDEC1A041710 | -4.65 | 0.02 | NA | NA |

| Gene ID | log2Fold Change | padj | Gene.description | GO terms |
|---|---|---|---|---|
| RDEC1A010158 | -4.41 | 0.02 | Outer dynein arm-docking complex subunit 3 | NA |
| RDEC1A019961 | -4.12 | 1.83E-06 | NA | NA |
| RDEC1A017051 | -3.76 | 8.47E-07 | RTX-III toxin determinant A from serotype 8 | NA |
| RDEC1A026828 | -3.38 | 4.23E-04 | Meprin A subunit alpha | NA |
| RDEC1A038975 | -2.89 | 3.45E-11 | Meprin A subunit beta | NA |
| RDEC1A050200 | -2.86 | 4.17E-03 | Meprin A subunit beta | NA |
| RDEC1A028783 | -2.81 | 8.59E-04 | MAM and LDL-receptor class A domain-containing protein 2 | NA |
| RDEC1A008823 | -2.15 | 9.72E-04 | NA | NA |
| RDEC1A030190 | -1.68 | 4.04E-03 | NA | NA |
| RDEC1A013162 | 1.17 | 0.03 | NA | NA |
| RDEC1A043533 | 1.49 | 0.02 | Transcription elongation factor B polypeptide 3 | NA |
| RDEC1A053004 | 2.42 | 0.01 | NA | NA |
| RDEC1A014824 | 2.45 | 0.04 | Tenascin-R | NA |
| RDEC1A014714 | 2.52 | 0.03 | NA | NA |
| RDEC1A002394 | 2.71 | 6.65E-07 | Large ribosomal subunit protein bL36m | NA |
| RDEC1A000896 | 3.14 | 2.54E-03 | NA | NA |
| RDEC1A044100 | 3.29 | 2.69E-03 | Galaxin | NA |
| RDEC1A053900 | 5.01 | 2.26E-11 | Interleukin-6 receptor subunit beta | NA |
| RDEC1A015288 | 5.67 | 2.37E-11 | NA | NA |
| RDEC1A049559 | 7.27 | 0.03 | Atrial natriuretic peptide receptor 3 | NA |
| RDEC1A015275 | 7.37 | 0.02 | ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase | NA |
| RDEC1A029236 | 7.62 | 3.52E-03 | Complement C1q-like protein 4 | NA |
| RDEC1A012085 | 8.17 | 1.29E-06 | NA | NA |
| RDEC1A039391 | 9.28 | 2.00E-09 | NA | NA |
| RDEC1A027049 | 10.25 | 2.26E-11 | Deoxynucleoside triphosphate triphosphohydrolase SAMHD1 | GO:0006203,GO:0045088,GO:0051607 |
| RDEC1A042537 | 10.35 | 7.79E-13 | 3'-5' exoribonuclease HELZ2 | NA |

**Supplementary table 5.** Differentially expressed genes in *R. philippinarum* at 4 weeks post exposure between control and low infection conditions.

| Gene ID | log2Fold Change | padj | Gene.description | Go terms |
|---|---|---|---|---|
| RPHI1A006818 | -8.56 | 8.43E-06 | NA | NA |
| RPHI1A039586 | -8.43 | 3.62E-19 | NA | NA |
| RPHI1A039047 | -7.46 | 6.35E-04 | Monomeric sarcosine oxidase | GO:0008115,GO:0050660 |
| RPHI1A029548 | -6.78 | 0.05 | NA | NA |
| RPHI1A005855 | -6.74 | 0.01 | NA | NA |
| RPHI1A018835 | -6.73 | 0.01 | NA | NA |
| RPHI1A032191 | -6.31 | 0.03 | Complement C1q tumor necrosis factor-related protein 3 | NA |
| RPHI1A016946 | -5.33 | 0.03 | HORMA domain-containing protein 1 | GO:0051321,GO:0005694,GO:0005634 |
| RPHI1A015414 | -4.68 | 0.01 | Sarcoplasmic calcium-binding protein | NA |
| RPHI1A010104 | -3.51 | 5.16E-04 | Major facilitator superfamily domain-containing protein 4A | NA |
| RPHI1A003430 | -3.37 | 0.03 | Nuclear nucleic acid-binding protein C1D | GO:0000178,GO:0000460,GO:0003723,GO:0003677,GO:0005730,GO:0010468 |
| RPHI1A040252 | -3.03 | 6.35E-04 | Prominin-1-A | NA |
| RPHI1A004043 | -2.88 | 0.03 | Probable serine/threonine-protein kinase roco4 | NA |
| RPHI1A021596 | 2.30 | 0.04 | RNA-binding protein RO60 | NA |
| RPHI1A022380 | 2.66 | 0.03 | Aplysianin-A | NA |
| RPHI1A000065 | 2.76 | 0.03 | Integrin alpha-4 | NA |
| RPHI1A001424 | 2.83 | 3.80E-03 | NA | NA |
| RPHI1A042951 | 2.88 | 0.03 | Ectonucleoside triphosphate diphosphohydrolase 1 | GO:0005886,GO:0005524,GO:0016787 |
| RPHI1A008793 | 3.16 | 4.50E-04 | NA | NA |
| RPHI1A022885 | 3.36 | 1.73E-05 | Protocadherin Fat 4 | NA |
| RPHI1A024918 | 3.48 | 0.04 | NA | NA |
| RPHI1A005493 | 3.72 | 0.01 | NA | NA |
| RPHI1A039681 | 5.25 | 0.01 | NA | NA |
| RPHI1A038371 | 9.08 | 3.05E-07 | NA | NA |

**Supplementary table 6.** Differentially expressed genes in *R. philippinarum* at 48 hours post exposure between control and high infection conditions.

| Gene ID | log2Fold Change | padj | Gene.description | GO terms |
|---|---|---|---|---|
| RPHI1A025065 | -12.82 | 1.25E-10 | NA | NA |
| RPHI1A036384 | -10.35 | 3.21E-08 | Hemicentin-2 | NA |
| RPHI1A007497 | -9.34 | 2.19E-03 | Galaxin | NA |
| RPHI1A045619 | -8.98 | 1.35E-03 | Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1 | NA |
| RPHI1A010296 | -8.91 | 1.99E-04 | Guanylate-binding protein 1 | NA |
| RPHI1A045077 | -8.55 | 1.97E-03 | Gastrin/cholecystokinin type B receptor | NA |
| RPHI1A017669 | -8.34 | 2.87E-03 | NA | NA |
| RPHI1A045078 | -8.05 | 0.03 | NA | NA |
| RPHI1A040408 | -7.96 | 0.01 | WSC domain-containing protein ARB_07867 | NA |
| RPHI1A042216 | -7.83 | 3.18E-03 | Tigger transposable element-derived protein 6 | NA |
| RPHI1A038198 | -7.64 | 4.97E-03 | Alpha-protein kinase vwkA | GO:0004674 |
| RPHI1A010691 | -7.49 | 0.01 | Perlucin-like protein | NA |
| RPHI1A009839 | -7.27 | 3.98E-04 | Ciliary microtubule associated protein 1A | GO:0005856 |
| RPHI1A044904 | -7.05 | 0.02 | Neurocan core protein | NA |
| RPHI1A008267 | -6.99 | 1.55E-03 | NA | NA |
| RPHI1A039389 | -6.30 | 2.54E-06 | Apolipophorins | NA |
| RPHI1A006077 | -6.24 | 4.21E-05 | NA | NA |
| RPHI1A023500 | -6.24 | 9.27E-04 | NA | NA |
| RPHI1A042931 | -6.22 | 0.01 | Ankyrin-3 | NA |
| RPHI1A044792 | -6.18 | 0.03 | Tenascin-X | NA |
| RPHI1A003029 | -5.87 | 0.02 | Probable ATP-dependent DNA helicase RecS | GO:0009378,GO:0006268,GO:0005694,GO:0043138,GO:0000724,GO:0005634,GO:0005524,GO:0003676 |
| RPHI1A035046 | -5.72 | 0.04 | Type-2 ice-structuring protein | NA |
| RPHI1A010610 | -5.70 | 0.02 | SCO-spondin | NA |
| RPHI1A034828 | -5.49 | 1.18E-04 | Complement C1q-like protein 4 | NA |
| RPHI1A024564 | -5.02 | 1.80E-03 | Guanylate-binding protein 2 | NA |

| Gene ID | log2Fold Change | padj | Gene.description | GO terms |
|---|---|---|---|---|
| RPHI1A042346 | -4.36 | 0.01 | Inositol phosphoceramide mannosyltransferase 3 | NA |
| RPHI1A009683 | -4.23 | 0.01 | Glutaredoxin-like protein C5orf63 homolog | NA |
| RPHI1A039586 | -4.04 | 0.01 | NA | NA |
| RPHI1A007096 | -3.81 | 0.02 | NA | NA |
| RPHI1A032829 | -3.68 | 3.64E-03 | Ciliary microtubule inner protein 2B | GO:0042995,GO:0005856 |
| RPHI1A031943 | -3.28 | 4.97E-03 | NA | NA |
| RPHI1A019722 | -3.24 | 0.01 | NA | NA |
| RPHI1A036101 | -3.17 | 0.04 | Probable serine/threonine-protein kinase pats1 | NA |
| RPHI1A011342 | -2.62 | 0.02 | NA | NA |
| RPHI1A025017 | 3.36 | 0.02 | NA | NA |
| RPHI1A019942 | 4.20 | 0.01 | Uncharacterized protein K02A2.6 | GO:0015074,GO:0003676 |
| RPHI1A008037 | 4.49 | 0.05 | Cyclic GMP-AMP synthase-like receptor 2 | NA |
| RPHI1A037783 | 5.03 | 6.04E-04 | NA | NA |
| RPHI1A008606 | 5.32 | 0.02 | NA | NA |
| RPHI1A024160 | 5.74 | 0.03 | Cartilage matrix protein | NA |
| RPHI1A045021 | 5.77 | 0.02 | Methyltransferase-like protein 27 | NA |
| RPHI1A008220 | 5.91 | 3.21E-08 | NA | NA |
| RPHI1A024086 | 6.65 | 2.12E-03 | NA | NA |
| RPHI1A003101 | 6.75 | 0.05 | G2/M phase-specific E3 ubiquitin-protein ligase | NA |
| RPHI1A043121 | 6.90 | 0.03 | Zinc finger protein 862 | GO:0046983 |
| RPHI1A005021 | 6.94 | 0.04 | Uncharacterized protein R871 | NA |
| RPHI1A045995 | 6.95 | 1.33E-10 | Complement factor B | NA |
| RPHI1A044170 | 7.24 | 0.03 | Uncharacterized protein CXorf38 homolog | NA |
| RPHI1A036090 | 7.32 | 0.03 | Glycine-rich domain-containing protein 1 | NA |
| RPHI1A039405 | 7.84 | 9.27E-04 | Hemagglutinin/amebocyte aggregation factor | NA |
| RPHI1A005139 | 8.47 | 2.60E-03 | Interferon-inducible GTPase 1 | NA |
| RPHI1A014944 | 8.50 | 9.27E-04 | NA | NA |

| Gene ID | log2Fold Change | padj | Gene.description | GO terms |
|---|---|---|---|---|
| RPHI1A013695 | 8.81 | 2.07E-03 | Protein yellow | NA |
| RPHI1A009734 | 9.16 | 0.01 | Trophoblast Kunitz domain protein 1 | NA |
| RPHI1A032528 | 10.88 | 3.66E-05 | CD109 antigen | NA |
| RPHI1A040535 | 20.29 | 3.38E-03 | NA | NA |
| RPHI1A043365 | 20.61 | 2.67E-03 | NA | NA |

**Supplementary table 7.** Differentially expressed genes in *R. philippinarum* at 4 weeks post exposure between control and high infection conditions.

| Gene ID | log2FoldChange | padj | Gene.description | GO terms |
|---|---|---|---|---|
| RPHI1A011370 | -7.90 | 1.76E-03 | Death-associated inhibitor of apoptosis 1 | NA |
| RPHI1A014989 | -5.59 | 0.01 | NA | NA |
| RPHI1A043394 | -4.81 | 0.04 | Bacterial dynamin-like protein | NA |
| RPHI1A006164 | -4.26 | 4.04E-03 | Isoaspartyl peptidase/L-asparaginase | GO:0004067,GO:0033345,GO:0008798 |
| RPHI1A010432 | -3.85 | 0.04 | FMRFamide receptor | GO:0004930,GO:0007186 |
| RPHI1A029696 | -3.52 | 0.02 | Extracellular protease inhibitor 10 | NA |
| RPHI1A042229 | -2.60 | 0.04 | Serine protease inhibitor dipetalogastin | NA |
| RPHI1A010104 | -2.49 | 0.04 | Major facilitator superfamily domain-containing protein 4A | NA |
| RPHI1A003321 | 3.46 | 0.04 | Cytochrome P450 4F2 | NA |
| RPHI1A040764 | 3.81 | 0.04 | Beta-1,3-glucosyltransferase | GO:0008375 |
| RPHI1A030378 | 4.56 | 0.04 | Bile salt-activated lipase | NA |
| RPHI1A027262 | 6.76 | 0.04 | NA | NA |
| RPHI1A002136 | 21.11 | 1.43E-03 | NA | NA |

**Search for new biomarkers of resistance to *Perkinsus olsenii* parasite in *Ruditapes decussatus* clams**

ICBAS-UP