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Development of genomic tools in the Manila clam, *Ruditapes philippinarum*, to investigate resistance to major pathogens and potential selection for aquaculture.

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“Experience is simply the name we give our mistakes”

- Oscar Wilde.

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ABSTRACT

The Manila clam is a bivalve species of particular commercial interest, currently representing about a quarter of total bivalve aquaculture worldwide. European production of this species has suffered greatly from the negative impact of infectious diseases, namely due to the protozoan parasite *Perkinsus olseni* and the Gram-negative bacteria *Vibrio tapetis*. These pathogens induce chronic infections that have been associated with reduced growth, fecundity, spat production, and overall health, as well as triggering mass mortality in cultured clam populations. The difficulties inherent in treating diseases in commercial bivalve production have led to the search for alternative solutions to limit the impact of disease by 1) improving our understanding of virulence, resistance, and host-pathogen interactions and 2) developing the tools necessary for hatcheries to initiate selection for traits of commercial interest.

This PhD, in joint agreement between the University of Padova and the University of Brest, and within the framework of the EU Horizon2020 project VIVALDI, seeks to investigate the potential for selection to increase resistance to disease in the Manila clam, as well as examine the functional mechanisms associated with resistance. In order to lay the basis for understanding the genetic factors of resistance a novel tool for parentage assignment was developed and applied for the first time to several populations of clams from hatchery broodstock. This made it possible to estimate genetic parameters for multiple traits including resistance to disease in large-scale field experiments, revealing the high potential for multiple trait selection in this species for the first time, and raising interesting questions regarding impact of genotype-by-environment on selection strategies. Furthermore, the molecular basis for resistance was investigated through a relevant proteomics approach, shedding light on the metabolisms at play in long-term resistant juvenile clams.

Overall, the work carried out in these studies highlights the feasibility of future selection methods based on both genetic information and clear molecular markers as a solution for increasing disease resistance in the Manila clam.

KEYWORDS: *Ruditapes philippinarum*, disease, resistance, selection, genetics, proteomics

RÉSUMÉ

La palourde japonaise est une espèce bivalve d'intérêt économique qui représente aujourd'hui environ un quart de la production mondiale de mollusques en aquaculture. La production européenne de cette espèce a beaucoup souffert de l'impact des maladies infectieuses, notamment celles induites par le parasite protozoaire *Perkinsus olseni* et par la bactérie Gram-négative *Vibrio tapetis*. Ces pathogènes induisent des maladies chroniques qui ont été associées à des mortalités de masse dans les parcs de production de palourde, ainsi qu'à une diminution de la croissance, de la fécondité, de la production de semis naturel, et de l'état de santé global des animaux. Les difficultés intrinsèques au traitement des maladies dans le contexte de la production de mollusques bivalves ont souligné l'importance d'orienter la recherche vers des stratégies alternatives pour limiter l'impact de ces maladies par 1) l'amélioration des connaissances sur la virulence, la résistance, et les interactions hôte-pathogène et 2) le développement des outils nécessaires aux écloseries pour initier la sélection pour des caractères d'intérêt commercial.

Cette thèse, en cotutelle entre l'Université de Padova et l'Université de Brest, et au sein du projet EU Horizon2020 VIVALDI, cherche à évaluer le potentiel de sélection pour l'amélioration de la résistance aux maladies chez la palourde japonaise, ainsi qu'à étudier les mécanismes fonctionnels associés à la résistance. Afin de pouvoir examiner les facteurs génétiques associés à la résistance, un nouvel outil génétique pour l'assignation de parenté a été développé et appliqué pour la première fois à des groupes de palourdes produits en écloserie et élevés dans des conditions commerciales. Cela a permis par la suite d'estimer les paramètres génétiques pour la résistance et pour des caractères morphologiques suite à des études de terrain de grande échelle, révélant un fort potentiel de sélection pour de multiples caractères chez cette espèce pour la première fois, et soulevant des questions sur l'importance de l'effet génotype-par-environnement pour les stratégies de sélection. De plus, les mécanismes moléculaires liés à la résistance ont été étudiés par une approche de protéomique, soulignant les métabolismes potentiellement impliqués dans la résistance à long-terme de palourdes juvéniles.

Dans l'ensemble, les travaux effectués lors de cette thèse soulignent la faisabilité d'une future sélection qui intégrerait à la fois des facteurs génétiques et des marqueurs moléculaires pour l'amélioration de la résistance aux maladies chez la palourde japonaise.

MOTS CLÉS : *Ruditapes philippinarum*, maladies, résistance, sélection, génétique, protéomique

GENERAL INTRODUCTION

INTRODUCTION

The Manila clam, *Ruditapes philippinarum* (=*Tapes philippinarum*, *Tapes japonica*, *Venus philippinarum*, *Venerupis philippinarum*; Adams & Reeve, 1850), also known as the Japanese littleneck and Japanese carpet shell clam, owes its names to its Asian origins and to the colorful and "carpet"-like patterns on its shell (from the Ancient Greek "tápēs" for "carpet, rug"). Appreciation for this species as a food source has rendered it into one of the largest bivalve productions on the global scale (see below: "Aquaculture and genetic resources"), despite continued negative impact of disease in a number of production zones (see below: "Transmissible diseases in the Manila clam").

This bivalve species lives buried several centimeters into the sediment of the intertidal zone, filtering water through its siphons for both food and respiration. Clams consume a range of suspended particles including numerous phytoplankton species, benthic microplankton, nanoplankton, bacteria, and macroalgae debris (Gosling, 2003). The Manila clam is a dioecious species (i.e. separate sexes) that generally reaches sexual maturity at 20-25 mm in length, or 1-3 years of age depending on growth rate, and maximum shell length reaches up to 75 mm. Due to its ability to tolerate fluctuating abiotic factors, the Manila clam can grow and reproduce in a broad range of costal environments with different sediment types, water salinity (15–50 PSU), and water temperatures (6-30ºC) (Breber, 2002). In natural environments, reproduction occurs in summer months when water temperatures rise rapidly and induce spawning, at which point mature adult clams release their gametes into the water column (Laruelle et al., 1994). Within 12 hours after fertilization, ciliated, planktonic larvae form and drift for up to 4 weeks before falling to the seafloor in search of appropriate sediment. Spat attaches to sediment granules using a byssus until it reaches roughly 5 mm in length, at which point the juvenile clam settles in its permanent environment. Like many mollusk species, the Manila clam is characterized by a very high fecundity (ca. 1.8 million eggs per female at commercial size of 40 mm), adding to its ability to rapidly colonize new environments (Gosling, 2003).

ORIGIN AND TRANSFER OF THE MANILA CLAM

The original geographic range of the Manila clam is along the Pacific coasts of Japan, Korea, China, Vietnam and the Philippines, where coastal water temperatures can vary seasonally between 12 ºC and 32 ºC depending on the region, and salinity is between 30 and 36 PSU (NOAA Satellite and Information Service, 2018). Firstly introduced to Hawaii as a food source in 1918, subsequent transfer of oysters from Japan to British Columbia in the early 1930s led to the transport of Manila clam juveniles and the introduction of this species into the Puget Sound (Carlton &

Figure 1: Current global distribution of the Manila clam. Dates refer to the first recorded introduction. Grey highlights indicate the Manila clam's original distribution zone; Blue highlights indicate Pacific coast introductions from Japan (Hawai'i in 1918, North American west coast in the 1930's, southern limit of California in 1960); Orange highlights indicate introductions to the EU from the North American populations (France in 1972, UK in 1980, Italy in 1983, Portugal in 1984, Spain between 1983-85). Adapted from Cordero et al. 2017.

Eldredge, 2009; Cohen & Carlton, 1995; Quayle, 1963). The clams rapidly established self-sustaining populations in British Columbia and along the North-west coasts of North America, gaining popularity as a cultured species for their adaptability and resilience, and expanding as far as the Californian coast by the 1960s (Chiesa et al., 2017; Cordero et al., 2017) (Figure 1).

European clam aquaculture was initially focused on the Manila clam's close cousin, *Ruditapes decussatus*, though production of this native species encountered limitations namely due to overfishing and disease (Savini et al., 2010). In order to diversify clam aquaculture in Europe,

France imported several batches of Manila clams between 1972-1974, which presented the advantage of being more resistant to disease, having a higher growth rate, and a longer reproductive period (Flassch & Leborgne, 1992; Laruelle et al., 1994). The French population then spread both intentionally and accidentally to Spanish and Portuguese coasts (Perez-Camacho & Cuna, 1985; F. Ruano & Sobral, 2000), while in the UK and Italy the Manila clam was deliberately introduced for aquaculture purposes in the 1980s from the Puget Sound (BC) population and the French population, respectively (Breber, 1985; Utting & Spencer, 1992) (Figure 1).

AQUACULTURE AND GENETIC RESOURCES

The Manila clam can currently be found along the Atlantic coasts of Europe and North Africa, the Pacific coasts of North America and Asia, and Mediterranean coasts of Europe and North Africa. It has become one of the most produced mollusk species worldwide, representing 25 % of the global mollusk production in 2014 (FAO, 2018). The vast majority of this production takes place in China, which produces over 4 million tons of clams annually, accounting for 99% of the worldwide share. The European market is the second most important worldwide. Production peaked at 68 000 tons in 2005, declining however since 2010 to about 35 000 tons in 2015. The instability of clam production is linked to a number of factors, including unsustainable aquaculture practices, coastal land use disputes, and mass mortality events brought on by extreme environmental conditions and infectious diseases.

Over 95 % of European clam production takes place in the North Adriatic lagoons (Venice lagoon, Sacco di Goro) and the Po' river delta in Italy. Though the Manila clam was initially introduced to the French Atlantic coast in the early 1970s, the French production has remained limited due to coastal zoning disputes, mortality events, and a subsequent loss of the initial market share when the species was introduced for aquaculture in Italy, after which the Italian production steadily took over. Today, France produces about 1 000 tons of Manila clam per year, mainly in the Arcachon and Morbihan bays of the Atlantic coast. Italy, on the other hand, currently produces over 33 000 tons of Manila clam per year (Figure 2).

Traditional clam farming uses seed collected from beaches and sandy areas, which involves the selection and preparation of seed collection beds, eradication of predators, and routine maintenance. While many production zones continue to use these traditional methods, the

economic importance of this species and the high degree of uncertainty regarding the availability of natural seed eventually led to a significant amount of research on the improvement of production methods. A number of techniques have since been developed to increase clam production, especially in China: 1) early spawning and over-wintering indoors (greenhouses); 2) optimized larval culture conditions and techniques; 3) juvenile rearing in shallow, fertilized (with suitable microalgae) nursery ponds; 4) optimized stocking size and substrate for mudflat grow out. At about 1 year of age, juvenile clams from hatcheries (10-15 mm) or pre-growing ponds are seeded in grow-out areas until they reach market size (35-40 mm in China, compared to 40-50 mm in Europe), 1-2 years later.

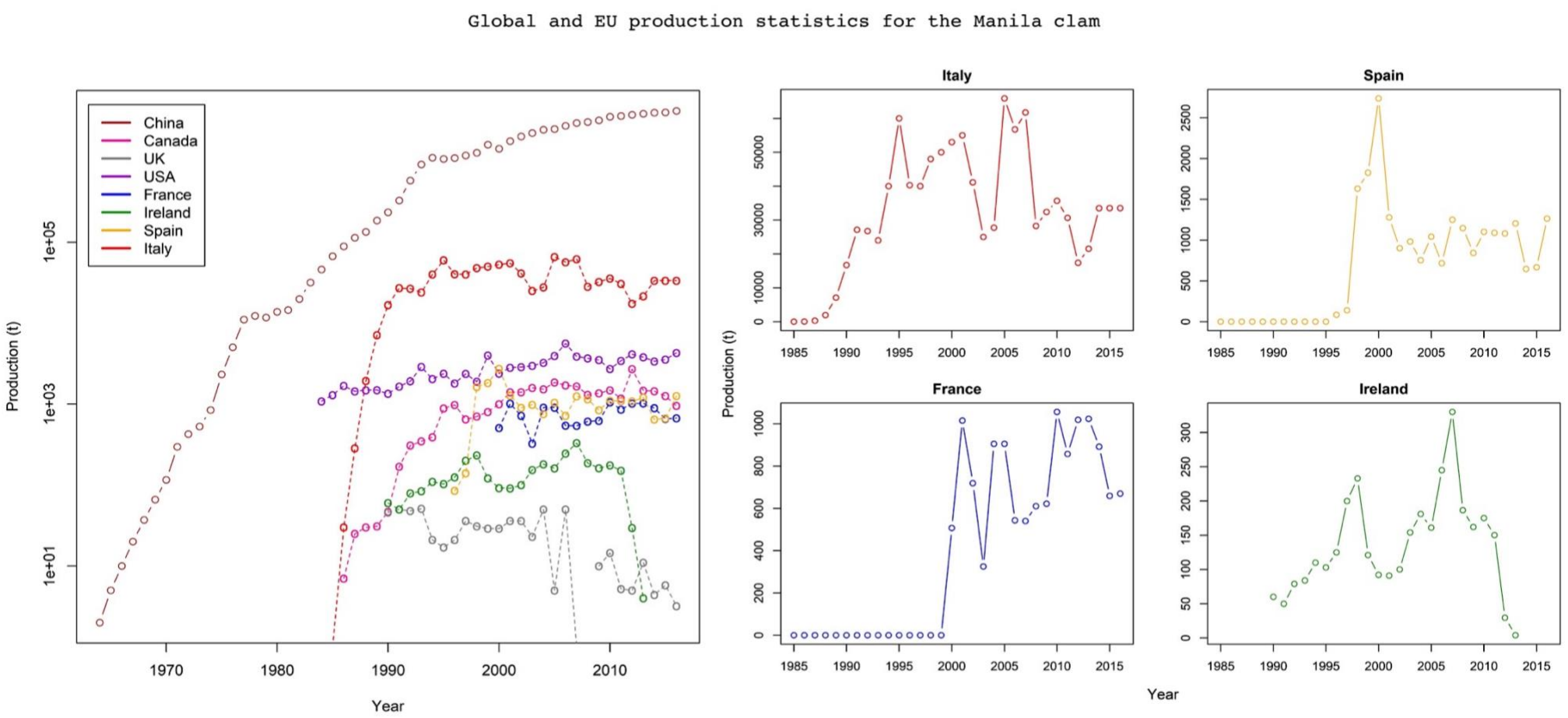

Figure 2: Production statistics worldwide (left) and zoom for four main European producers (right) of Manila clam. Italy as the main EU producer clearly dominates the market for this species, with about 35 000 tons in 2014 and 2015. While there are major differences in terms of scale, the recurrent trend is a clear lack of stability of this aquaculture sector, with many of the stark falls associated to mortality episodes (either linked to environmental stressors or disease). FAO 2016.

GENETIC BACKGROUND OF MANILA CLAM POPULATIONS

The increase in production and economic importance of the Manila clam has been accompanied by frequent transfers of spat between hatcheries, juveniles to grow-out zones, and adults to international markets for human consumption. This has led to investigations regarding the genetic background of populations to trace back their origins and the links between them, as well as research aiming to characterize the provenance of clams to ensure market traceability. While there is a significant body of literature tracing imports for aquaculture trials, such as those carried out in Europe starting in the 1970s, the development of genetic markers has opened the door to assessing genetic variability and differentiation of populations worldwide. A recent study aiming to understand the global genetic background of the Manila clam analyzed the genetic structure of nine populations from Asia, North America, and Europe, supporting the recorded population transfers between the three continents, and highlighting interesting differences between Japanese and Chinese populations that may be representative of the type of aquaculture (i.e. traditional or intensive) carried out in these zones (Cordero et al., 2017). In addition, several European populations have been compared to Asian and North American populations, indicating a loss of genetic diversity such as a bottleneck effect, and suggesting that expansion of the species in Europe has been largely mediated by hatchery-produced spat (Chiesa et al., 2017; Cordero et al., 2017). A loss of genetic diversity within a population can be of concern, namely in species that are likely to benefit from high genetic variability in order to counter the negative impacts of disease (see below: "Diseases in the Manila clam" and "Bivalve immunity").

TRANSMISSIBLE DISEASES IN THE MANILA CLAM

Bivalves such as the Manila clam filter vast quantities of the surrounding seawater for both food and oxygenation, and by doing so they come in frequent contact with both the benthic and planktonic microbial communities of their environment. While microorganisms are an essential component of the host's diet as well as of its core microbiome, the marine environment is home to a wide range of microbial pathogens including bacteria, fungi, viruses, and protozoan parasites (Zannella et al., 2017). In both fish and shellfish, pathogens are a major limiting factor for aquaculture production, costing over US$ 6 billion per annum in losses alone, and far more in

treatment such as vaccinations, cleaning, and antibiotics (Stentiford et al., 2017; World Bank, 2014).

The pathogenicity of these microorganisms depends on the pathogen's virulence, the host's defense mechanisms, and the environmental factors that may hinder host defense mechanisms or favor pathogen proliferation. It is the interaction between the host, pathogen, and environment that ultimately determine the impact of a pathogen on a host (Figure 3). Intensive production systems and environmental changes undoubtedly contribute to increasing the spread of diseases in farmed bivalve species by stressing the host and thus impacting its ability to respond to infectious agents (Guo & Ford, 2016). Limiting the impact of diseases in farmed aquatic species is thus a multifaceted issue that is rendered all the more delicate in the case of invertebrates by the fact that they rely solely on their innate immune system (see below: "BIVALVE IMMUNITY"), as opposed to vertebrate species that have an adaptive immune system capable of being stimulated by vaccination (Zannella et al., 2017).

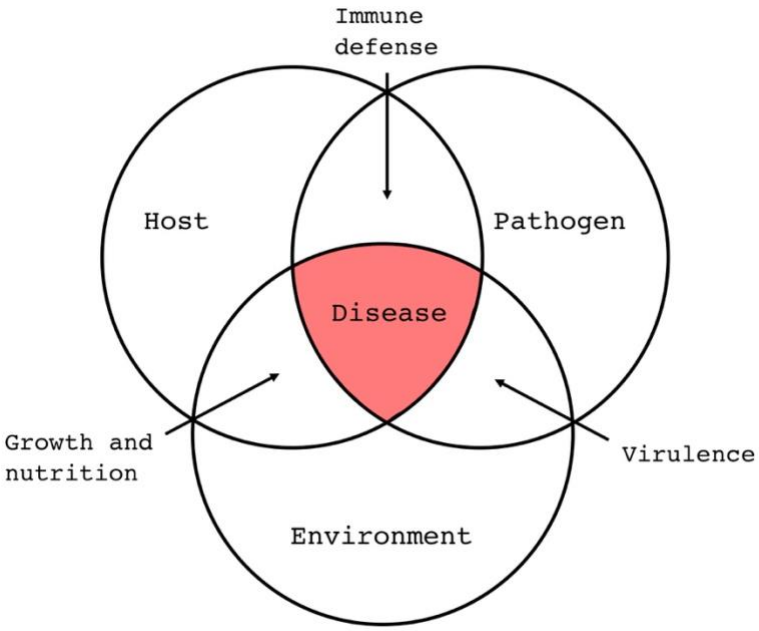

Figure 3: Disease occurs and can be more or less severe depending on the complex interactions between host immune capacity, pathogen virulence, and environmental factors that modulate both of these.

While a number of pathogens affect the Manila clam worldwide, the bacterium *Vibrio tapetis* and the protozoan parasite *Perkinsus olseni* have been identified as the two major pathogens responsible for negatively impacting growth, reproduction, and overall health of this species in aquaculture, as well as occasionally inducing mass mortality episodes. While *V. tapetis* is most virulent in colder waters and at lower salinities such as along the Atlantic coast of Europe, *P. olseni* has optimal development at warmer temperatures and higher salinities, making it more problematic in Mediterranean aquaculture zones (Christine Paillard et al., 2004; Ramilo et al., 2015; Reid et al., 2003; Antonio Villalba et al., 2005)

THE PROTOZOAN PARASITE, *PERKINSUS OLSENI*

Protozoan parasites of the genus *Perkinsus* cause severe infections in a wide variety of molluscan hosts (Goggin et al., 1989), inducing a chronic and generally sub-lethal disease termed Perkinsosis (Philippe Soudant et al., 2013; Antonioa Villalba et al., 2004). Of the seven currently accepted species (*P. marinus, P. olseni, P. qugwadi, P. cheasapeaki, P. mediterraneus, P. honshuensis, and P. beihaiensis*), all infect only bivalve mollusks, with the exception of *P. olseni* which is also a pathogen of abalone (Antonioa Villalba et al., 2004). *Perkinsus marinus* and *P. olseni* have been associated with mass mortalities in their host species, and have thus been extensively studied in an attempt to mitigate the severe economic impact on cultured mollusks, namely *P. marinus* in the oyster *Crassostrea gigas* and *P. olseni* in the clams *R. philippinarum* and *R. decussatus* (Philippe Soudant et al., 2013).

There are four general life stages for parasites of the genus *Perkinsus*: trophozite (meront), hypnospore (preezoosporangia), zoospore and zoosporangia (Figure 4). Trophozites (or meronts) are the vegetative multiplication stage residing in the host connective tissues, eventually creating nodules and lesions in heavily infected animals. In moribund animal tissues, trophozites enlarge into hypnospores (preezoosporangia, or “growth stage”) which, when in seawater, undergo multiple cell divisions within the hypnospore membrane. This forms a zoosporangia containing hundreds of bi-flagellated zoospores which are subsequently released through an opening in the hypnospore into the water column and sediments, where they are known to persist (Chambouvet et al., 2014). All life stages of *Perkinsus* spp. are infective (Antonioa Villalba et al., 2004).

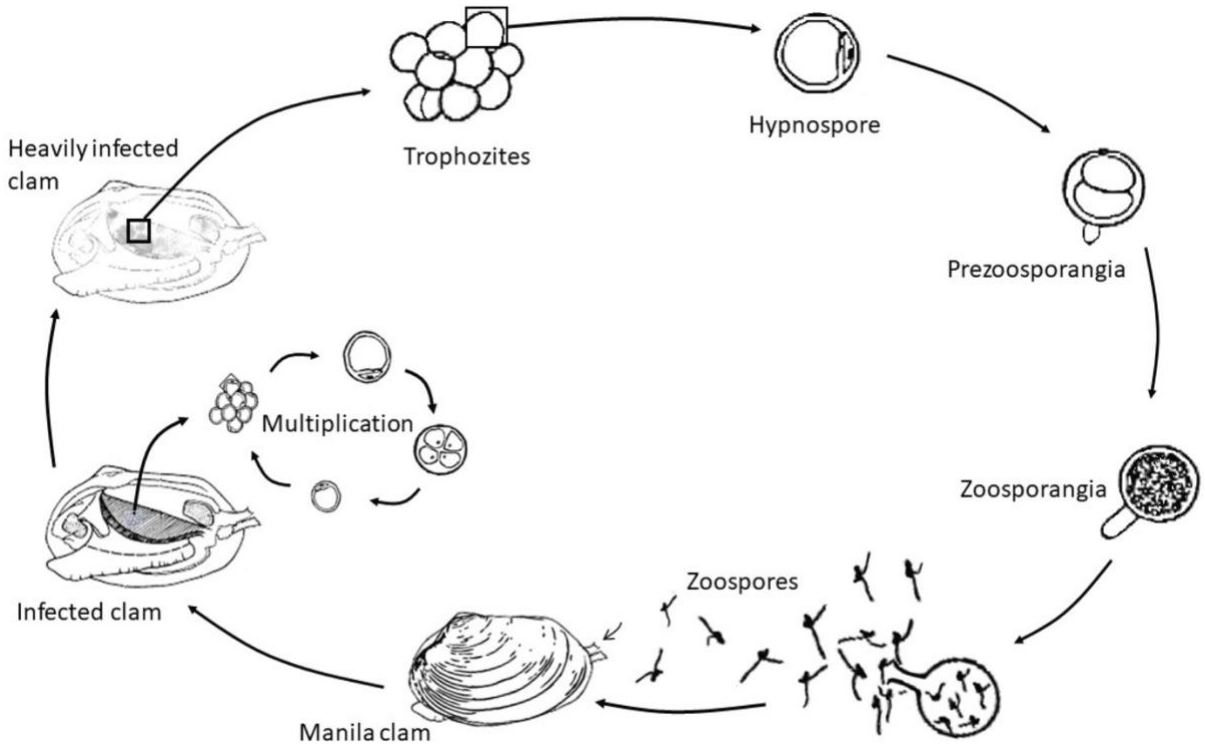

Figure 4: Infection cycle of Perkinsus olseni in the Manila clam (adapted from Garcia et al. 2008). Perkinsus zoospores enter the host through the siphons and penetrate host tissues, starting with the gills. Trophozites multiply vegetatively and spread to the gut, mantle, and foot. In heavily infected individuals, trophozites transform into hypnospores, which undergo karyokinesis and cytokinesis to become zoosporangia which release zoospores into the water column and sediment.

Italian production of the grooved carpet shell clam (*R. decussatus*) has traditionally taken place in the Northern Adriatic lagoons, where parasites of the *Perkinsus* genus were first detected in moribund clams from the Venice lagoon in 1978, as well as in other bivalve species (Da Ros & Canzonier, 1985). Following the import of *R. philippinarum* for aquaculture in the Northern Adriatic lagoons, *Perkinsus* infections were reported in the Murano lagoon in 1991 (Ceschia et al., 1991), where a prevalence of over 90 % was detected in 1996 (Da Ros et al., 1998). Spanish clam landings of the Ebro Delta also suffered severe mortality episodes associated to the species *P. olseni* (=*atlanticus*) (Sagristà et al., 1996). *P. olseni* was the second *Perkinsus* species to be described following increasing levels of infection in the blacklip abalone, *Haliotis ruber*, in Australia (Lester & Davis, 1981). In clams, *P. olseni* induces the formation of nodules within the gills that gradually spread to other host tissues. In advanced stages of the disease, the parasite causes lesions and affects

the host's respiration and other physiological processes, leading to a reduction in growth, reproduction, and condition index (Park et al., 2006).

Currently, the standard method for detection and intensity ranking of Perkinsosis in the Manila clam (and other host species) is Ray's Fluid Thioglycollate Medium (RFTM) assay (S. m. Ray, 1966; S. M. Ray, 1952). This method consists in incubating infected host tissue, either whole body or gill tissue, in thioglycollate medium (which mimics the conditions of moribund host tissues) to enlarge *Perkinsus* sp. hypnospores. Enlarged cells are then stained with Lugol's iodine to visualize the parasitic cells and diagnose the infection. While this method and its subsequent infection intensity ranking system (Mackin's scale and adaptations, (Mackin, 1962)) have been largely standardized, RFTM is time-consuming, not species-specific, and considered unreliable for quantifying low-intensity infections (Ramilo et al., 2015; Antonioa Villalba et al., 2004).

Molecular methods such as PCR have also been applied for diagnosis of Perkinsosis, at both the genus and species level. Species-specific PCR now allows for the discrimination between numerous *Perkinsus* pathogens in infected bivalves, and specific primers targeting the ITS and 5.8S regions of the genome have been developed for the rapid detection and quantification (qPCR) of *P. olseni*. Recently, real-time PCR assays for the discrimination and quantification of *Perkinsus* spp. in the Manila clam have been developed with primers amplifying a region spanning from the ITS1 to the ITS2, and the quantification results have been compared to RFTM in naturally infected clam and for six strains of *P. olseni* (4 Japanese, 1 Spanish, and 1 New Zealand) (Umeda & Yoshinaga, 2012). Further studies investigating the Perkinsea lineage to which the *Perkinsus* genus belongs to have revealed a high diversity and wide distribution of these marine parasites (Chambouvet et al., 2014).

THE BACTERIAL AGENT OF BROWN RING DISEASE, *VIBRIO TAPETIS*

Soon after the introduction of the Manila clam in Europe, in the early 1970s, a bacterial disease began affecting clam beds and spat in hatcheries, causing mortalities along the west coast of France. In 1987, the Aber basin on the French Atlantic coast experienced mass mortalities in cultured clam beds, with dead and moribund clams exhibiting characteristic brown deposits on the inner surface of their shells (C. Paillard et al., 1989). Further investigation of the causative agent of the disease called "Brown Ring disease" (BRD) lead to the identification and characterization

of *Vibrio tapetis* (Borrego et al., 1996; C. Paillard et al., 1994; Christine Paillard & Maes, 1990). *V. tapetis* is a Gram-negative bacterium present in the sediment as well as in the water column, infecting several clam and fish species. Growth of *V. tapetis* and infection in the Manila clam have been shown to be dependent on temperature and salinity, with an "optimal temperature" for the development of BRD at 14ºC (Christine Paillard et al., 2004). Temperatures below 3ºC and above 27ºC inhibit *V. tapetis* pathogenicity, and it has been shown that low salinities favor the development of BRD (Reid et al., 2003). To date, 17 strains of *V. tapetis* have been isolated in

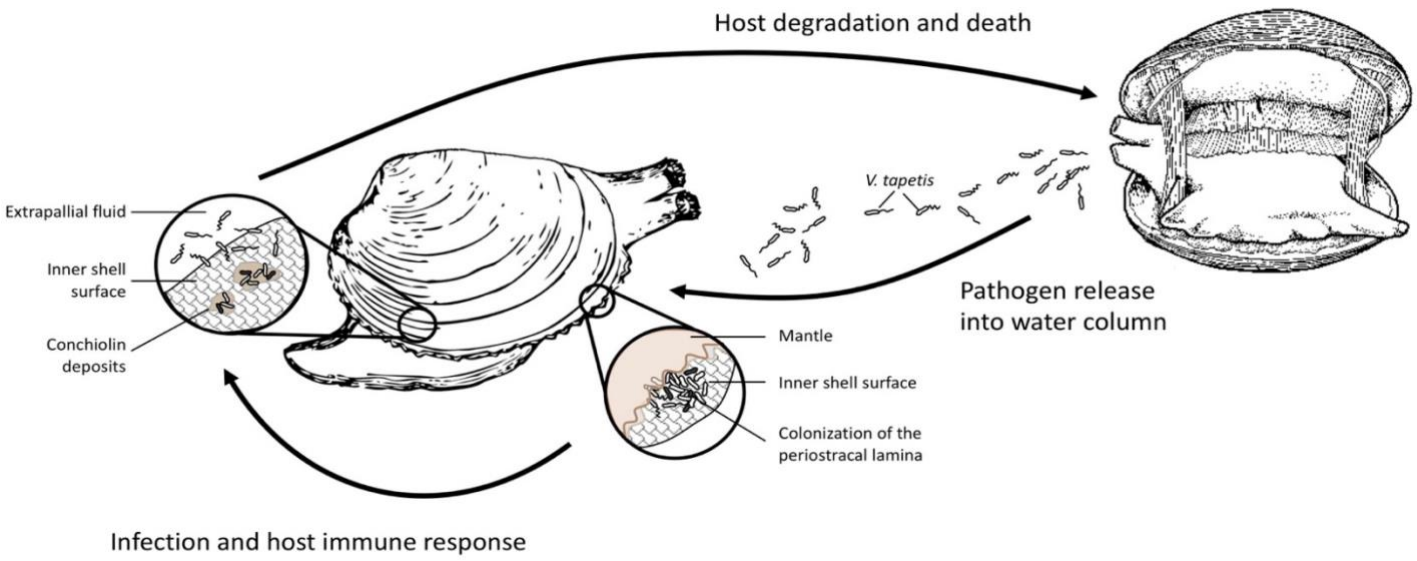

Figure 5: Infection and colonization of the Manila clam extrapallial compartment by *Vibrio tapetis*. Adapted from T. Ben-Horin et al. 2015.

European and Asian populations of various clam and fish species (Balboa et al., 2011; Dias et al., 2018).

In the Manila clam, the bacteria come into direct contact with the periostracal lamina, which is the biomineralization site where the outer limit of the mantle attaches to the edge of the shell (Figure 5; Figure 6). *V. tapetis* then penetrates the periostracal lamina and colonizes the extrapallial compartment by adhering to the inner surface of the shell using numerous pili (C Paillard & Maes, 1995), thus disrupting normal calcification of the shell. This, in turn, induces the depositing of conchiolin by the host in an attempt to re-calcify over the conchiolin matrix. *V. tapetis* has also been shown to produce toxins which may be associated with the inhibition of hemocyte functions

such as pseudopod formation and adherence capacity (Choquet et al., 2003; Madec et al., 2014). Because the bacteria proliferate in this extra pallial compartment, it is in the extra pallial fluids contained between the inner shell and the mantle that they are detected. The brown-colored conchiolin deposited by the host on the affected areas of the inner shell gives BRD its name, and the extent to which it can be observed (microscopy or by the naked eye) on the inner surface of the shell has allowed for the establishment of a classification system for the visual diagnosis of the disease and shell repair stages (Figure 7) (C. Paillard & Maes, 1994). In some cases, clams are able to completely repair the shell by covering the bacterial biofilm in conchiolin and to re-calcifying normally over the new matrix (C Paillard & Maes, 1995; Trinkler et al., 2011). In cases where the clam is unable to repair over the bacterial biofilm, *V. tapetis* proliferates and eventually penetrates into the clam hemolymph. This quickly transports the bacteria throughout the host tissues and leads to septicemia and host death. When the host dies, virulent bacteria are released into the water column as the tissues degrade, able to infect other nearby hosts.

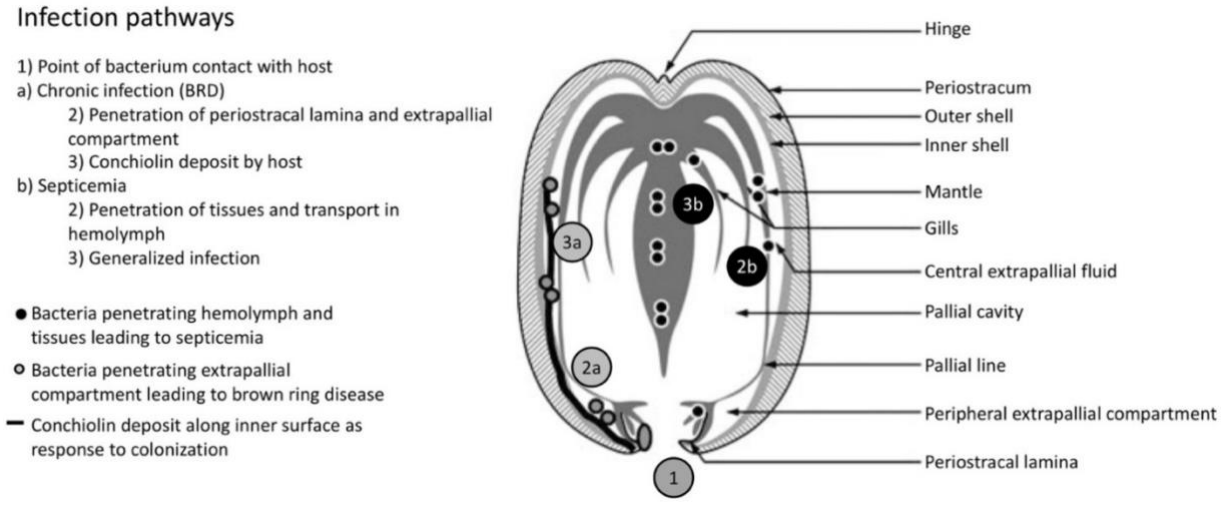

Figure 6: Representation of the main infection pathways of *Vibrio tapetis* in the Manila clam. 2a and 3a: chronic infection leading to brown ring disease; 2b and 3b: rapid septicemia and host death. Adapted from Paillard 2004.

Diagnosis of BRD is typically carried out by observing the extent of conchiolin deposits on the inner surface of the shell as well as their localization, which can be characterized according to a classification system established in 1994 (C. Paillard & Maes, 1994). Identification of the bacteria

was initially carried out through serological procedures and biochemical criteria, which are unfortunately rather time-consuming and far from practical for screening individual clams (Allam et al., 2000; Borrego et al., 1996; Noël et al., 1992). These methods did, however, lead to the understanding that clams could be asymptomatic (no conchiolin deposits on the shell) while still being infected, a situation that was further elucidated through molecular techniques able to detect bacterial DNA in host tissues and compartments (Christine Paillard et al., 2006; Romalde et al., 2014). More recently, molecular diagnosis by real-time PCR was developed to quantify bacteria in the extra pallial fluids of clams, detecting as few as 10_1 cells.mL^{-1} (Bidault et al., 2015). Today it is generally accepted that the use of both the visual and molecular techniques is necessary in order to establish the diagnosis of BRD caused by *V. tapetis* (Drummond et al., 2006; OIE, 2019; Christine Paillard, 2016).

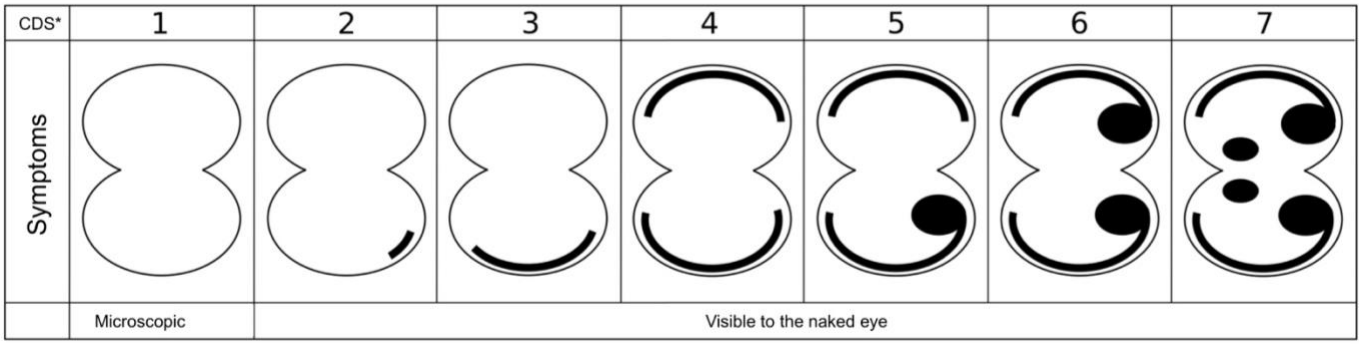

Figure 7: Example of the classification system established by Paillard & Maes, 1994. *CDS: Conchiolin Deposit Stage.

BIVALVE IMMUNITY

Bivalve immunity relies on an innate immune system, which is made up of functional molecules (essentially proteins and enzymes) which are coded for in the animal's genetic material. A bivalve's first defense against biological and physio-chemical intrusions is undoubtedly the physical protection provided by its shell, which is covered by the periostracum, a hard calcium carbonate structure secreted by the mantle which the animal can retract into and clamp shut thanks to its anterior and posterior adductor muscles. The second barrier to pathogens is the mucosal lining that can be found namely on the surface of the mantle, gills, and labial palps. Mucus represents not only a physical barrier that allows for the entrapment of particles, but it also functions as a matrix

containing numerous immune effectors such as hemocytes and the antimicrobial compounds that they excrete (Russell et al., 2015). Research focusing on the immune functions of mucus in bivalves was recently been reviewed by Allam and Espinosa (Allam & Pales Espinosa, 2016), highlighting the importance of mucus in host-pathogen interactions and the initial immune response to pathogen stimuli. There is also a vast body of research regarding the compartments associated with innate immunity in bivalves: the open circulatory system, the hemolymph pumped through sinuses and soft tissues by a systemic heart, and the extra pallial fluids situated between the mantle and the inner surface of both valves. Both the hemolymph and extra pallial fluids are composed of hemocytes and immune effectors (Allam & Paillard, 1998; C Paillard et al., 1996). Hemocytes in mollusks are frequently divided into two categories: granulocytes, which are the most implicated in microbial defense and contain a larger number of lysosomal granules within the cell, and hyalinocytes (or agranulocytes), which contain few or no granules (Dyachuk, 2016). The innate immune system in bivalves is made up of two major components: the cellular (hemocyte-associated) and humoral (pathway-associated) mechanisms (Allam & Raftos, 2015; Oubella et al., 1994).

The immune response is initiated when the pathogen is detected by specific cellular and humoral pattern recognition receptors (PRRs), which are present as soluble molecules within the plasma of hemolymph or bound on hemocyte membranes and are capable of recognizing pathogen-associated molecular patterns (PAMPs). The lipopolysaccharides present on the membranes of Gram-negative bacteria such as the genus *Vibrio*, as well as the glycans on the surface of parasites such as *P. olseni*, are examples of molecular patterns recognized by specific PRRs from the galectin family such as MCGal, a tandem-repeat galectin characterized in the Manila clam (Kim et al., 2008; Malagoli, 2016; Song et al., 2010). Pathogen recognition by PRRs triggers a cascade of molecular interactions in the host which lead to additional hemocyte recruitment, production of antimicrobial compounds and/or their release into the extracellular matrix, and phagocytosis or encapsulation of the pathogen for its neutralization.

Like other bivalves, Manila clam hemocytes produce and secrete a range of humoral immune effectors for pathogen elimination including antimicrobial peptides (AMPs), lectins, cytokines, protease inhibitors, and hydrolytic enzymes such as phenoloxidases (Allam & Auffret, 2000; Allam et al., 2014; Park et al., 2012; Richard et al., 2015). AMPs, a certain number of which have been described in *R. philippinarum*, play an essential role in destabilizing and lysing the

membranes of microbial pathogens and are released into the plasma following specific pathogen recognition (Destoumieux-Garzón et al., 2016). *VpBD*, *MCdef*, and *VpDef* are three known defensin-type AMPs present in the Manila clam which have been shown to have strong antimicrobial activity through suggested membrane-destabilizing activity (Adhya et al., 2012; Kim et al., 2008; L. Zhang et al., 2015). Lectins, a diverse group of PRRs such as *MCL-3* from the Manila clam, are also involved in immobilizing and agglutinating pathogens, and play a role in chemotaxis, the directed migration of host cells towards pathogens (Chatterjee & Adhya, 2013). Interestingly, lectins have been found to be not only extremely abundant in expressed-sequence tag studies of Manila clam hemocytes, but their expression appears to be specific to the pathogen with which the host is challenged (Kang et al., 2006). Recruitment, chemotaxis, phagocytosis, effector secretion, apoptosis, and a number of other immune response cascades are set off by the action of cytokines, a broad repertoire of small signaling proteins present within the extracellular matrix as well as within hemocytes (Rosani et al., 2019). Cytokines mediate, among others, the liberation of protease inhibitors, hydrolytic enzymes, and initiate the phenoloxidase system (Pei et al., 2012; Richard et al., 2015; Venier et al., 2016).

Phagocytosis is the internalization of pathogens within hemocytes, an essential process during which hemocyte membranes engulf microbial agents through endocytosis and contain them within an internal membrane called the phagosome. The cytoskeleton, an intracellular network of microtubules responsible for the cell structure and movement of cellular components, is implicated in the migration of lysosomes toward the phagosome, allowing for the fusion of these two membranes so that the lysosomal contents can initiate pathogen neutralization and digestion (Canesi et al., 2002). Bivalve pathogens, including *V. tapetis* and *P. olseni* in the Manila clam, can be intracellular, and some have been shown to develop mechanisms to evade phagocytosis and inhibit the fusion of lysosomes with the phagosome (Carella et al., 2015; Flannagan et al., 2009). *V. tapetis*, for example, has been shown to induce the rounding of hemocytes, a process suggested to occur through reorganization of the actin cytoskeleton which is responsible the formation of pseudopods implicated in cellular adhesion and phagocytosis (Allam et al., 2014; Brulle et al., 2012; Choquet et al., 2003; Rahmani et al., 2019). Oyster hemocytes are able to actively phagocytose *Perkinsus marinus*, though the parasite is able to evade degradation by either producing antioxidant compounds to counter the reactive oxygen species (ROS) intended to neutralize the pathogen, or by inhibiting ROS-production by the host (Philippe Soudant et al.,

2013). This has led to the suggestion that *Perkinsus* parasites may use a "Trojan horse"-like strategy by remaining protected from other host immune effectors, and this may also be a means of accelerating parasite dissemination to other host tissues through migration of infected hemocytes via the circulatory system (Hasanuzzaman et al., 2018; LaPeyre et al., 1995; Lau et al., 2018). Encapsulation of the parasite by polypeptide-bound layers of hemocytes has been recorded to be more frequent than phagocytosis in clams infected with trophozites of *P. olseni*, possibly a means to limit the diffusion of the infection throughout the host (Ramilo et al., 2015; Antonio Villalba et al., 2005).

Contrary to vertebrates, there is no such thing as an adaptive (or acquired) immune system in bivalves. Although, growing knowledge regarding the immune system of bivalves has also raised the question of immune memory, a feature characteristic of adaptive immunity which has nonetheless been demonstrated in invertebrate species through "recall" response and immune shift (Melillo et al., 2018). The "recall" response is when an initial immune response to a pathogen is detected and then diminishes, and a second challenge with the same pathogen elicits a stronger and more rapid response through the same molecular processes (Milutinović & Kurtz, 2016). Immune shift, on the other hand, describes the process through which the host's secondary response shifts to implicate different molecular mechanisms which may be more efficient in pathogen neutralization. Immune priming of the Pacific oyster *C. gigas* with heat-killed *Vibrio splendidus* showed an efficient "recall" response with an increase in both hemocyte count and specific phagocytosis during a second challenge with live bacteria, similar to the increased phagocytosis and acid phosphatase activity recorded in the Zikong scallop *Chlamys farrer*i sequentially challenged with *Vibrio anguillarum* (Cong et al., 2008; T. Zhang et al., 2014). A shift in immune response in mollusk species was thus far described only in the snail *Biomphalaria glabrata*, which demonstrated a shift from cellular to humoral immune response mechanisms for elimination of the parasite *Schistosoma mansoni* (Coustau et al., 2015; Pinaud et al., 2016). More recently, research on the immune response of the mussel *Mytilus galloprovincialis* suggests another sort of immune shift in reaction to sublethal injections with *Vibrio splendidus,* whereby the host appears to temper its inflammatory response during secondary infection, possibly in order to lessen tissue damage as a result of its own bactericidal mechanisms (Figueras et al., 2019; M. Rey-Campos et al., 2019). While our current understanding of immune memory in invertebrate species remains limited and the phenomenon is still controversial and rather poorly-characterized, it does raise a number of

important questions about the nature of immune memory and its ties to genetic variability (Figueras et al., 2019).

SELECTION FOR RESISTANCE TO DISEASE IN BIVALVES

Research regarding bivalve diseases has highlighted remarkable variability in the immune response not only between tissues and compartments of the host, but also between individuals of the same species (Allam & Pales Espinosa, 2016; Gerdol et al., 2011; Magalí Rey-Campos et al., 2019). The latter has been demonstrated in numerous bivalve species including the Manila clam, demonstrating interindividual variability in hemolymph composition (Ford & Paillard, 2007), numerous hemocyte parameters (Flye-Sainte-Marie et al., 2009), shell repair during BRD (Trinkler et al., 2011), and especially disease intensity and host survival to infection within a given population (Jeffroy, 2011; Christine Paillard et al., 2014; Francisco Ruano et al., 2015; Antonio Villalba et al., 2005; Yang et al., 2010). Advances in molecular techniques have greatly improved our ability to characterize and diagnose infection, evaluate pathogen virulence, and investigate host responses. This has, in turn, made it possible to establish that there is high interindividual variability regarding host response within bivalve populations, raising the question as to whether there are genetic factors underlying resistance to bivalve diseases.

Resistance to disease is a term that groups together several concepts surrounding the processes and outcomes of infection and disease, and its definition thus varies slightly depending on authors. Resistance has been defined as the ability of a host to avoid becoming infected or diseased when exposed to infectious material, as opposed to endurance, which defines the ability of a diseased host to survive infection, and tolerance, the ability to reduce the consequences of infection on the host's fitness (Saura et al., 2019). Others define resistance more broadly as the ability of a host to exert control over the life cycle of the pathogen, for example by limiting infectivity or restricting proliferation within the host, considering resistance as a relative term instead of an absolute measurement (Bishop & Woolliams, 2014). While resistance is often measured as survival, such as for extremely virulent viral and protozoan pathogens affecting oysters, measuring resistance becomes more complex in the case of chronic diseases which, while they do not necessarily induce high mortality, have a severe negative long-term impact on growth, reproduction, and overall health, leading to immunodepression and increased risk of mortality depending on environmental factors (Bishop & Woolliams, 2014; Hofmann et al., 2009). One of

the first attempts at selection for disease resistance in bivalves was carried out in the 1970s in oysters in Delaware Bay, New Jersey (USA): oysters (*C. virginica*) from imported, native, and laboratory-reared resistant broodstock was selected and deployed in production zones, and mortality due to MSX (the protozoan parasite *Minchinia nelsoni*) was recorded over three generations, resulting in significantly reduced mortality in first, second, and third generation resistant oysters (Haskin & Ford, 1979). The European flat oyster, *O. edulis*, was also the subject of selective breeding for resistance to *Bonamia ostreae*, a protozoan parasite that induces a chronic disease, and selected lines showed four times higher survival rates as well as lower parasite prevalence compared to unselected lines, leading to the suggestion that genetically-based resistance may be difficult to acquire in natural populations due to the fact that susceptible (infected) oysters could still reach sexual maturity and thus contribute to the gene pool of the following generations (Naciri-Graven et al., 1998). Selection for increased survival has also been carried out in the Sydney rock oyster, *Saccostrea glomerata*, for resistance to QX disease caused by *Marteilia sydneyi*, resulting in a 22% reduction in mortality accompanied by a 21% increase in weight for selected lines after two generations, suggesting that selective breeding was not only feasible but also largely beneficial to a secondary trait of interest (Nell & Hand, 2003). Selective breeding for disease resistance (survival) in the Sydney rock oyster was pursued over numerous generations and eventually included resistance to a second pathogen, *Bonamia roughleyi*, demonstrating that selection for dual resistance is effective (mortality reduced by more than half in selected lines), although selection for resistance to one disease was not effective in conferring resistance to the other disease (Dove et al., 2013).

In the Manila clam, resistance to BRD was assessed in terms of the prevalence of symptoms (characteristic conchiolin deposits) in four populations from the same hatchery broodstock over a 15-month period, resulting in significantly less BRD symptoms in hatchery stock compared to natural populations (P. Soudant et al., 2004). Resistance to BRD in this same stock has also been assessed in terms of shell repair capacity, which indicates the pathogen was able to elicit a host response and thus a certain level of infection, but that the host subsequently was able to calcify over the initial infection. In a study that followed three generations of hatchery selected Manila clams, the comparison between low-susceptibility and high-susceptibility stocks showed that not only was shell repair more frequent and advanced in the low-susceptibility stock, but cumulative mortality was also significantly lower in that group (albeit not directly attributed to *V. tapetis*)

(Trinkler et al., 2010). While the study was not able to measure estimated heritability for resistance (shell repair), it did suggest that some level of genetic variability may be responsible for different shell repair capacity between the two selected stocks, and ultimately the differences in environment between studied sites were likely the most influential factor.

Today, selection programs are beginning to couple both phenotypic and genotypic measurements for breeding, mostly in oyster species for which there is a longer history of selective breeding as well as considerably more genomic resources than in other bivalve species (Hollenbeck & Johnston, 2018; Saavedra & Bachère, 2006). Markers for resistance/susceptibility (survival) to *Bonamia ostrea* have been investigated for use in marker-assisted selection in the European flat oyster, highlighting nine potential loci associated with resistance and six associated with susceptibility (Lallias et al., 2009). Survival to the oyster Herpes virus (OsHV-1) in selectively bred Pacific oysters over four generations increased by over 60%, and heritability has also been shown to be promisingly high, between 0.34 to 0.63, though it should be noted that the size of individuals exposed to the virus had a significant impact on this result, and that this study also highlighted the importance of environmental pressure driving the differential survival of oyster families from selected lines (Dégremont, Garcia, et al., 2015; Dégremont, Nourry, et al., 2015).

While phenotypic selection for survival clearly suggested early on the existence of genetic factors governing resistance to disease in bivalves, untangling environmental factors from host genetics requires the use of genomic tools applied to cohort studies to investigate the genetic parameters of resistance traits in selectively bred families (Hedgecock et al., 2007). The shift towards genomic selection in aquaculture, i.e. the calculation of breeding values independent of the underlying loci affecting the trait of interest, has already shown promising results in finfish species and, with the development of genomic tools such as high-density genetic marker panels for parentage assignment, has the potential to greatly improve hatchery stock in farmed bivalve species (Gutierrez et al., 2017; Ross D. Houston, 2017; Yáñez et al., 2014; Zenger et al., 2019).

GENOMIC RESOURCES

Deciphering the genetic basis behind the innate immune system of bivalves depends on the availability of genomic resources for these species, a field which has only recently begun to develop significantly. The first investigations regarding the genetic and molecular basis of traits such as

disease resistance and growth in the Manila clam were initiated in the early 2000s in response to mass mortality events and in the hopes of supporting aquaculture of this species (Saavedra & Bachère, 2006). Later, the specie's tolerance of a wide range of environmental stressors prompted the assembly of an annotated transcriptome using microarray technology as a tool for studying gene expression profiles in response to stress factors (Milan et al., 2011). Over the past decade, the rapid decrease in sequencing costs has spurred a significant amount of transcriptomic data for this species, shedding light on gene expression for a number immune-related pathways and molecules discussed above (Hasanuzzaman et al., 2016, 2017; Moreira et al., 2012).

A recent review on molluscan genomics highlighted not only the significant gap in the amount of genomic resources available for mollusks compared to all other cultured species, but also the high potential for genetic improvement in cultured mollusks (Takeuchi, 2017). As genome sequencing becomes increasingly cost-effective, even for non-model species, several whole genome assemblies of farmed bivalve species have recently been published: the Pacific oyster (*C. gigas*) (G. Zhang et al., 2012), pearl oyster (*Pinctada fucata*) (Du et al., 2017), Yesso scallop (*Patinopecten yessoensis*) (Wang et al., 2017), Zhikong scallop (*Chlamys farreri*) (Li et al., 2017), Eastern oyster (*Crassostrea virginica*) (Gómez-Chiarri et al., 2015), Mediterranean mussel (*Mytilus galloprovincialis*) (Murgarella et al., 2016), Sydney rock oyster (*Saccostrea glomerate*) (Powell et al., 2018), and the Manila clam (*R. philippinarum*) (Mun et al., 2017; Yan et al., 2019) have all been sequenced over the past few years. A number of characteristics are shared in these bivalve genomes, namely their high levels of polymorphism, existence of long repeat sequences, and immune- and stress-related gene family expansions which have been hypothesized to derive from the sessile nature of most bivalve species (Mun et al., 2017; Nie et al., 2017; Takeuchi et al., 2016; Wang et al., 2017). Another common characteristic is a tendency for deviation from expected Hardy-Weinberg equilibrium and Mendelian ratios across generations, an important phenomenon to account for in population genetics and selective breeding (Hollenbeck & Johnston, 2018).

The reasons for the high genetic diversity observed in mollusk species are yet to be elucidated, namely due to the lack of genome-wide studies carried out on this taxonomic group. One explanation lies in their high fecundity and open-water spawning, as it has been suggested that they may have higher mutation rates due to the more numerous meiosis events necessary for the production of millions of gametes for reproduction (Plough, 2016). High mutation rates may also be one of the underlying causes of lethal alleles, as observed in bivalves such as the oyster, which

can greatly influence the viability of early life stages and thus lead to distortion of the expected Mendelian segregation ratios (Plough & Hedgecock, 2011).

Hardy-Weinberg equilibrium (HWE) is the expected balance of alleles or genotypes within a population, from one generation to another, in the absence of influences such as selection, mutation, inbreeding, genetic hitchhiking, etc. That is to say that, in theory, the proportion of homozygous and heterozygous genotypes remain constant assuming a random mating scheme, which is the basis for genetic variability. Mendelian ratio describes the expected phenotypes and genotypes of descendants based on the parental genotypes for traits that follow Mendelian inheritance, a framework that assumes perfect segregation of diallelic alleles during gametogenesis and their independent assortment within a given gamete. As mentioned earlier, numerous genetic studies using microsatellite markers and single nucleotide polymorphisms have demonstrated that bivalve species deviate from expected HWE and Mendelian ratios, and the hypotheses behind this rely partly in the frequent occurrence of deleterious alleles and early selection during larval stages, such as lethal alleles leading to "viability selection" (Hollenbeck & Johnston, 2018).

The sequencing of the Manila clam genome was prompted in part by a notable decline in aquaculture due to infectious disease and the hope that increased genomic resources could help initiate marker-assisted and genomic selection to breed more resistant stocks for aquaculture (Yan et al., 2019). In keeping with other sequenced bivalve genomes, the current publicly available genome assembly demonstrated notoriously high heterozygosity as well as over 26% of repeat sequences. Similarly, another genome draft for this species, assembled in order to study the evolutionary basis for Double Uniparental Inheritance, a particular mitochondrial DNA inheritance scheme seen in a limited number of bivalve species, reported high heterozygosity and numerous tandem repeats, many of which were longer than the actual read sequences, an additional hurdle in genome assembly (Ghiselli et al., 2017; Passamonti & Ghiselli, 2009). A newly assembled Manila clam genome has been available as of August 2019, in which the authors confirmed the high heterozygosity observed in previous versions, and suggested the expansion of immune- and stress-related gene families in this species (Yan et al., 2019).

The Manila clam has been bred in hatcheries since the 1970s, allowing for the control and optimization of food sources, reproductive cycle, and larval and spat handling techniques through to management of grow-out for commercial size (Perez-Camacho & Cuna, 1985; Qiuzheng et al.,

1981; Utting & Spencer, 1992). More recently, a number of studies have applied genetic markers such as microsatellites and single nucleotide polymorphisms to study differences among various clam populations and to initiate selection for desirable traits in the Manila clam (Jiang et al., 2018; Nie et al., 2017). These traits typically include growth, shell color, and yield at commercial size, which are phenotypic traits that can easily be recorded and selected without necessarily sacrificing the animal, which is a major limit when it comes to assessing resistance or tolerance to disease, especially for chronic diseases. Diseases such as BRD and Perkinsosis tend to negatively impact condition index (a measure of overall health based on dry tissue mass) and reproduction of the host over the long term, and early diagnosing these diseases requires either sacrificing the animal or applying invasive, time-consuming, and delicate biopsy techniques which are incompatible with large-scale studies.

A 2014 review on the available genetic information in mollusk aquaculture species found that, at the time, only about 30 papers discussed the genetics of the Manila clam. Of those, the majority pertained to population structure and genetic differentiation (42%), followed by genetic response and gene expression associated with the immune system (21%), research regarding molecular markers (17%), species hybridization (13%) and finally 2 papers discussing heritability and selection (8%) (Astorga, 2014). Since then research regarding selection has expanded somewhat for this species, though disease resistance remains difficult to evaluate and has yet to be put in place effectively in breeding programs. Growth rate has shown to have a genetic basis in the Manila clam, as divergent selection for fast-growing and slow-growing clams from the same base population showed an increase in shell length of over 18% in fast-growing lines compared to control lines (Huo et al., 2016). Shell color in the Manila clam is both extremely variable and highly heritable, as demonstrated by the mass selection of the orange-colored shell phenotype in Chinese aquaculture which naturally occurs in only about 0.4% of the population (Peignon et al., 1995; G. F. Zhang & Yan, 2010). Selection for the orange-shell strain has shown up to 97.3% of progeny carry the orange phenotype, and additional selection for increased shell length within this selected orange-shell strain demonstrated a realized heritability of 0.61 at one year of age, suggesting that over 60% of the increase in shell length is genetically determined (Huo et al., 2017). A recently constructed linkage map using SNP markers established 18 linkage groups, four of which appear to be candidate loci for genetic factors underlying shell length, shell width, wet weight, and shell color/striation (Nie et al., 2017). Regarding disease resistance, an attempt at phenotypic and

microsatellite-validated selection of Manila clam lines resistant to BRD was carried out in 2011, leading to the identification of several differentially expressed cytoskeleton-associated genes potentially associated with resistance, although the small number of families used in the study were insufficient for large-scale genomic analysis (Brulle et al., 2012; Jeffroy, 2011).

Genomic selection rests on the assumption that the effect of markers across the genome (i.e. SNPs, microsatellites) is individually small, but by increasing the number of markers and assessing them overall it becomes possible to evaluate the effect of the genotype on the phenotype (visibly expressed trait, such as shell length or color), as opposed to marker-assisted selection (MAS) which, while based on genomic information, focuses on one or a few markers known to be associated with traits of interest (called quantitative trait loci, or QTL) and assuming those markers have a significant effect on the phenotype. Many of the production traits sought out in animal breeding are controlled by multiple genes or loci, each of which has a small effect on the desired trait, thus greatly limiting the efficiency of MAS (Meuwissen et al., 2016). The reduction in costs for sequencing and high-throughput genotyping has opened the door to using vast numbers of genetic markers such as SNPs, which are estimated to be present every 10-30 bp in mollusks, for parentage assignment and genomic selection in non-model species, including those for which high-quality annotated genomes are not yet available.

OVERALL OBJECTIVES AND APPROACH

OBJECTIVES

In light of the current state of the art regarding the potential for selective breeding for resistance to disease in bivalve species, it appears evident that the lack in genomic resources for selection in the Manila clam is one of the major limits for genetic improvement of this species in aquaculture. While production of the Manila clam continues to grow on the global scale, European aquaculture of this species continues to suffer from consequential losses and instability, due in part to the negative impact of its two major pathogens, *P. olseni* and *V. tapetis*. To date, there remains a significant knowledge gap regarding not only the genetic factors underlying resistance in the Manila clam, but also the molecular mechanisms of the innate immune system that represent the functional response of the host to the diseases caused by these pathogens.

This PhD presents the development of a panel of genetic markers for genotyping and parentage assignment in an experimental population of Manila clam (Chapter I), the use of the tool developed to carry out the first estimations of genetic parameters for disease resistance in the experimental population subjected to field challenges in commercial grow-out conditions (Chapter II), and a novel functional study of resistance to Brown Ring disease in the experimental population through a proteomics approach (Chapter III).

EXPERIMENTAL POPULATION

In April 2016, the SATMAR hatchery (Société Atlantique de Mariculture) in France bred an experimental population from 53 female and 56 male clams, producing an F1 generation (n > 1 million) representing 1479 potential half-sib families (2 factorials mixed of 31 ♀ x 25 ♂ (775 families) and 22 ♀x 32 ♂ (704 families). The two mating groups (factorials) were mixed immediately after fertilization and raised as a single group to avoid any tank bias.

Batches from this F1 population are subjected to natural and controlled challenge experiments with the two main pathogens of the Manila clam, the protozoan parasite *P. olseni* and the Gram-negative bacteria *V. tapetis*. Using batches from the same genetic background makes it

possible to measure the heritability of and correlations between genetic parameters associated to production traits and resistance to disease at different life stages.

GENERAL WORKFLOW

In order to understand the genetic factors associated with disease resistance, batches (of at least 1000 individuals) of the F1 population described above are used for challenge experiments. Following these challenge experiments, clams are diagnosed using molecular and phenotypic methods that are either previously published diagnostic techniques or protocols developed within the VIVALDI Horizon2020 European project. The development of a parentage assignment tool and the availability of information regarding the genetic parameters for resistance and growth, as well as the evaluation of the functional mechanisms underlying long-term resistance to disease, will ultimately allow for the development of efficient breeding programs focused on improving the genetic basis of Manila clam stocks used in aquaculture.

Development of the Manila clam parentage assignment tool, and all experimental activities and data analysis of Perkinsosis-infected clams took place at the Comparative Biomedicine and Food Science (BCA) department of the University of Padova (UNIPD), under the supervision of Prof. Luca Bargelloni.

Controlled and natural challenge experiments with *V. tapetis* and associated proteomic analyses benefit from the experimental infrastructures present at the Laboratoire des Sciences de l'Environnement Marin (LEMAR) at the University of Western Brittany (UBO), under the supervision of CNRS Dr. Christine Paillard.

CHAPTER 1: DEVELOPMENT OF A SNP PANEL FOR PARENTAGE ASSIGNMENT IN THE MANILA CLAM

DEVELOPMENT OF A SNP PANEL FOR PARENTAGE ASSIGNMENT IN THE MANILA CLAM

ABSTRACT

SNP arrays are powerful tools for high-resolution studies addressing population genetics or selection for complex traits in farmed and wild animals. SNP arrays are widely used in a number of species, however mollusks, and more specifically bivalves, are known to be highly polymorphic. This peculiarity makes the design and application of genetic tools more complex. The aim of this work was to design the first parentage assignment panel for the Manila clam, *Ruditapes philippinarum*. Sequence data from 2b-RAD and PoolSeq technology was used to identify SNPs in Manila clam and select highly variable genetic markers shared across two populations.

This new SNP chip allows for the first large-scale parentage assignment test this highly polymorphic bivalve species. SNP selection in bivalves requires more stringent filter steps compared to other organisms. Concordance between PoolSeq and 2b-RAD sequencing was 90% for > 66 000 SNPs. The sequence data was aligned against a draft reference genome, and a pipeline developed for PoolSeq was applied to a hatchery broodstock population and a natural population, providing 117.9 million and 121.2 million candidate SNPs, respectively. A series of stringent filter steps was defined for common SNPs ultimately provided a list of 844 markers from which the 250 final SNPs were chosen, for mounting on an Illumina chip. Validation of genotyping and assignment capacity for the panel was carried out using 82 F1 individuals from 7 families of known pedigree, resulting in 81.2% assignment capacity. Furthermore, 96 F1 individuals produced from the hatchery broodstock population were genotyped, and 74.7% of individuals were assigned to a parent-pair.

While assignment capacity of the current SNP panel is lower than for panels developed in other bivalve species, it can still serve as a novel tool for analyzing population variability and tracing pedigree in order to estimate the genetic parameters for various traits of commercial interest in this major farmed bivalve species.

1. INTRODUCTION

Obtaining pedigree information is an essential step for designing selective breeding programs for bivalve hatcheries, a sustainable and feasible solution to mitigate the impact of infectious diseases and improve genetic gain for traits of commercial interest. Genetic selection for improvement of animal stocks remains poorly exploited in the aquaculture sector, while it has long since revolutionized the efficiency of terrestrial plant and livestock production. While selective breeding in plants and livestock has been in place since the beginning of the 20th century, the first large-scale breeding programs for fish weren't initiated until the 1970s in salmonids [1]. Since then, a number of breeding programs have been introduced for fish and shellfish, and many aquaculture breeding programs have obtained high genetic gains due to a combination of high heritability for economically favorable traits, high fecundity, and short generation time of many of the studied species [2–4]. Despite the promising results in genetic gain for aquaculture species, it has been estimated that less than 10% of aquaculture production is based on genetically improved stocks [5].

Breeding programs for bivalves, both commercial and experimental, are relatively new, with the most notable industrial-scale programs including the Pacific oyster (*Crassostrea gigas*), Mediterranean mussel (*Mytilus galloprovincialis*), Greenlip mussel (*Perna canaliculus*), Sydney rock oyster (*Saccostrea glomerata*), and Bay scallop (*Argopecten irradians*). In addition, selection experiments have been conducted for numerous other species including pearl oysters (*Pinctada fucata*, *Pinctada margaritifera*, and *Pictada maxima*), the freshwater pearl mussel (*Hyriopsis cumingii*), clams (*R. philippinarum, Meretrix sp.,* and *Cyclina sinesis*), and scallops (*Argopecten nucleus*, *Argopecten ventricosus*, and *Chlamys farreri*) [6–15]. Most programs for bivalves are experimental and small-scale, with few lines and limited numbers of parents within each line. Furthermore, the objectives of trait selection are not always well defined and most often depend on economic constraints. To date, the only studies carried out for the Manila clam have focused on shell length (divergent selection of extreme phenotypes) [16], shell color (also divergent selection) [16], and heritability for larval and juvenile growth has been assessed through separate family rearing [17].

In the case of most farmed aquatic animals, juveniles are too small at hatching (from a few micrograms in mollusks and crustaceans to ca. 100 mg in salmonid fish) to be physically tagged, which makes traceable pedigree information difficult and costly to obtain and continues to be a limit for the development of optimized selective breeding schemes, particularly for shellfish. Thus, distinguishing between families would evidently require raising large numbers of them

separately, a method that comes with major disadvantages including high costs, extensive infrastructure, and the risk of confounding factors such as the “batch effect”, which can significantly skew the accuracy of the measured breeding values [18]. In Manila clam, first publication to report estimates of genetic parameters used separate family rearing, thus running the risk of potentially overestimating the heritability due to confounding tank and family effects [17].

The use of genetic marker panels using microsatellites or single nucleotide polymorphisms (SNPs) to trace relationships between full-sib families can greatly facilitate the development of family-based breeding schemes [19]. Microsatellite markers have been widely exploited for studies on both genetic diversity and parentage assignment in fish and shellfish species, but come with a number of limitations including a high potential for null alleles and higher laboratory costs compared SNPs. Diallelic SNPs are stable, abundant, evenly distributed markers that can be multiplexed to provide high-accuracy parentage assignment, including in non-model species [20].

SNP chips containing hundreds to thousands of markers already allow for accurate, high-throughput, low-cost genotyping for parentage assignment in many animal and plant species.

Genotyping panels are harder to develop in marine mollusks mainly due to high punctual polymorphism, large insertions, deletions, structural variations, and genomic plasticity (spontaneous auto-polyploidy or aneuploidy) [6,21]. This, in turn, leads to Mendelian segregation distortion as well as numerous null alleles, which are well documented in the literature [22–25]. In addition, parentage assignment supposes that all loci have no physical linkage and are statistically independent, though the risk of loci being linked is higher when the SNP count surpasses the number of chromosomes. Thus, when linkage maps or genome assemblies to the chromosome level are available, SNPs are selected based on distance so as to strengthen statistical independence [26].

The aim of this study was to develop a parentage assignment tool that is able to discriminate between Manila clam families produced within the context of the VIVALDI Horizon2020 EU project as well as more broadly in hatchery populations in the EU with both industrial and academic scopes.

2. MATERIALS AND METHODS

2.1. *Validation of the sequencing approach for SNP identification:*

Prior to the identification of SNPs, a comparison was carried out between two sequencing approaches, 2b-RAD (individual) sequencing and PoolSeq using pooled samples consisting of 50 Manila clams collected from a farmed population in the Venice lagoon. This comparison allowed us to validate PoolSeq as a cost-effective and time-efficient sequencing method for further application to a hatchery population of 109 broodstock Manila clams from the commercial hatchery SATMAR (Barfleur, France).

2.2. *Comparison between 2b-RAD sequencing and PoolSeq on Manila clams from Venice lagoon*

A sample of 50 individuals of *R. philippinarum* was collected in 2011 from a clam production site in the Venice lagoon. Total genomic DNA was extracted in 2015 using a commercial Invisorb® Spin Tissue Kit (STRATEC Biomedical), following the manufacturer's recommendations. DNA was quantified by spectrophotometry (NanoDrop™) and quality was assessed on a 1% agarose gel. For 2bRAD, DNA digestion was done using the restriction enzyme *AlfI* according to the protocol described in [27] with minor modifications to the PCR protocol, as described in [28]. Post-PCR library quantification was carried out by fluorometry with Qubit® 2.0 to increase homogeneity of sequencing output among multiplex libraries. The library pool was sequenced on a HighSeq 2500 (Illumina, San Diego, CA, USA) with a single 1*50 bp setup using 'Version2' chemistry at the UC Davis Genome Center (University of California, California, USA).

The reads produced from 2bRAD sequencing were analyzed in 2017 using the Genome Analysis Tool Kit (GATK). Firstly, raw reads were trimmed (TRIMMOMATIC, minimum length = 34) and aligned to a reference genome of *R. philippinarum* provided by colleagues at the University of Bologna [29] using BWA aln with default parameters. Only unique mapping reads (tag XT:U) with no suboptimal alternative mapping (tag X1:0) were conserved. Within GATK, the HaplotypeCaller tool was used for filtration process of variant calling (cluster threshold at 2 SNP/read; individual DP>10; 0.1<MAF<0.9) [30–32].

For PoolSeq, the original total DNA from the same 50 Manila clams was fragmented using Covaris and prepared for sequencing in 2017 to create two equimolar pools of 25

individuals/pool. Libraries for the two pools were prepared in triplicate using TruSeq® DNA Library Prep (Illumina). Triplicate libraries were re-quantified using a Qubit 2.0 fluorometer, and library fragment size (300-500bp) was evaluated using Agilent 2100 Bioanalyzer. The triplicate libraries were pooled (equimolar) and sequenced using Illumina HiSeq 4000 for 150-bp paired-end reads at the Genome Center of the University of California Davis. Reads were trimmed to remove low-quality reads using TRIMMOMATIC, and CLC Genomics Workbench was used for aligning raw reads to the *R. philippinarum* draft reference genome. SNP identification was carried out using PoPoolation2, a pipeline for analyzing pooled next generation sequencing data, which builds on open source tools [33].

2.3. PoolSeq of parental DNA from VIVALDI experimental population

Individual samples of mantle tissue (stored in ethanol) from a population of 109 hatchery Manila clams used as broodstock by the SATMAR hatchery were sent from the Syndicat des Sélectioneurs Avicoles et Aquacoles Français (SYSAAF) to the BCA laboratory for DNA extraction in March 2018.

Extractions were carried out individually using Invisorb® DNA Tissue Kit (STRATEC Biomedical). Total DNA was quantified by Qubit® 2.0 fluorometer and quality was evaluated on a 1% agarose gel. Based on DNA quality (molecular weight as visualized by electrophoresis), the samples were allocated to a "lower quality" (LQ) and "higher quality" (HQ) group.

Library preparation and sequencing was carried out in the same way as described previously for the Venice lagoon population. Two equimolar pools were prepared (LQ and HQ pools) and libraries for each pool were prepared in triplicate using TruSeq® DNA Library Prep (Illumina). After Qubit 2.0 quantification and quality evaluation using Agilent 2100 Bioanalyzer, the triplicate libraries were pooled (equimolar) and sent for Illumina HiSeq 4000 for 150-bp paired-end sequencing at the Genome Center of the University of California – Davis. Reads were trimmed, aligned, and analyzed using the pipeline described above for the Venice lagoon PoolSeq approach.

2.4. SNP filtering by selection criteria

Starting from the two sets of SNPs in the PoolSeq datasets of Venice lagoon and hatchery broodstock groups, the following list of criteria was defined to select 250 SNPs of interest for the development of the parentage assignment tool:

- Minor allele frequency
    - Only SNPs with a minor allele frequency greater than or equal to 0.3 were considered.
- Minimum and maximum coverage
    - In order to avoid selecting SNPs present in long tandem-repeat regions, candidate markers were selected within genomic regions showing a sequencing coverage ranging between 25x and 100x.
- Minimum stable flanking region
    - In order to design specific primers to amplify each genetic marker on the chip, each SNP must have a non-variable up- and down-stream flanking region of at least 100 bp.
- Presence in both hatchery broodstock and Venice lagoon populations
    - This criterion was chosen in order to provide a panel that could assign parentage not only for the cohort produced from the hatchery population, but also more broadly in other Manila clam populations.
- Number of alternative alleles
    - Considering that diallelic SNPs are easier to design probes for, multiallelic markers were not considered.
- Contig proximity
    - In order to decrease the risk of using linked genetic markers, no more than one SNP per contig of the *R. philippinarum* reference genome was selected.
- Mirror SNPs
    - Mirror SNPs are those targeting complementary bases (i.e. A/T or C/G) for which single probes present the same fluorescence, hence they require a secondary probe to distinguish between them. These complementary base SNPs were removed from the list to avoid requiring secondary probes and introducing additional signal analysis complexity.

2.5. SNP-chip design

The 100 bp flanking sequences for each SNP were shared with Labogena genotyping company, which designed probe sequences using Illumina DesignStudio® Sequencing Assay Designer, following supplier recommendations. From the initial 250 probes designed, single probes for 245 SNPs were placed on an Illumina Infinium® genotyping chip able to genotype 96 samples / chip.

2.6. Hatchery families of known pedigree

The SATMAR hatchery produced 7 independent full-sib families of known pedigree in September 2018, which were raised in separate nursery tanks until March 2019. The 14 sires and dams and 89 offspring were sampled by opening the animal with a clean scalpel and taking a 2 x 2 mm piece of mantle tissue, which was stored in 90 % ethanol. Total DNA was extracted in 96-well plates using the Qiasymphony® automated extraction from Qiagen, following supplier recommendations, at the Labogena genotyping company (Jouy-en-Josas, France). Lysis with proteinase K was carried out overnight at 56°C and no purification step was necessary in the protocol. DNA was eluted in 100 uL of the elution buffer (TE) and stored at 4°C until genotyping.

2.7. F1 cohort bred from hatchery broodstock

The population of 109 broodstock clams from the SATMAR hatchery was composed of 56 sires and 53 dams. In April 2016, the broodstock was conditioned for mating at the SATMAR nursery and by controlled temperature adjustments mass spawning according to two full-factorial designs was carried out: 31 ♀ x 25 ♂ (775 potential families) and 22 ♀x 32 ♂ (704 potential families). Larvae from the two pools were mixed together and reared as a single batch representing potentially 1479 families. In July 2017, 20,000 individuals from this F1 experimental population were transferred from the nursery to a grow-out zone production unit within the hatchery. In order to test assignment capacity of the SNP panel on this population, 96 offspring were collected at random from the F1 cohort in November 2018, sampled for mantle tissue (3 x 3 mm) conserved in 90 % ethanol, and sent to the Labogena genotyping company for DNA extraction and genotyping according to the same procedures described previously for the seven families of known pedigree.

2.8. Genotyping and parentage analysis

DNA samples were genotyped (4 uL of genomic DNA with a minimum concentration 10 ng.uL-1) using the Infinium chip and analyzed on a fully automated Illumina iScan® platform, following supplier recommendations.

Genotype quality control was carried out GenomeStudio® software, according to the following standard Labogena clustering procedure:

- Run of Illumina automated clustering algorithm [34]

- Call rate calculation for every sample
- Exclusion of outlier samples
- Second run of Illumina automated clustering algorithm
- Readjustment of clustering on every SNP, according to Labogena internal protocol
- Exclusion of SNPs presenting ambiguous graphical cluster profiles
- Second evaluation of call rate per sample, using last clustering
- Exclusion of samples with call rates under NorM R threshold value of 0.2

Genotypes that met the above criteria were analyzed using AccurAssign® software from Labogena, which carries out parentage assignment based on both maximum likelihood and exclusion methods, using the following parameters: 1% of genotyping errors, minimum of 70% of positive markers for each sample, maximum of 3 mismatches between progeny and parent couple. Furthermore, the mating plan used to produce the populations tested (7 hatchery families and 109 hatchery parents including 96 F1 descendants) was included in the software parameters in order to correct assignments lying outside of the mating plan [35].

3. RESULTS

3.1. Individual 2b-RAD sequencing of Venice lagoon population

2b-RAD sequencing of 50 Manila clams produced an average of 4.9 million 36-bp raw reads per individual. Of these, 30% mapped to the genome uniquely and with no alternative (suboptimal) match. These were retained for downstream analyses. HaplotypeCaller tool identified 130,721 SNPs from 2b-RAD sequences aligned against the reference genome.

3.2. *Pooled sequencing of Venice lagoon population*

Sequences from the two pools were merged and trimmed, accounting for a total of 1 217 486 430 reads. 68.07 % of reads were successfully mapped to the reference genome with average read length of 121 bp using CLC Genomics Workbench. As for the individual sequencing approach, only uniquely mapping reads with no alternative best match (598 113 399 reads) were retained for SNP identification.

PoPoolation identified 121,206,047 SNPs from the pooled sequencing data (min-qual = 20; min- count = 3; min-coverage = 15; max-coverage = 200).

3.3. Comparison between 2b-RAD and PoolSeq data for the Venice lagoon population

All SNPs identified through PoolSeq and 2b-RAD were compared. Based on their position in the genome, 66,078 SNPs were shared between both lists, 90 % of which presented identical alternative alleles for which the average difference in minor allele frequency is 0.19 (Figure 1).

3.4. Pooled sequencing of hatchery broodstock population

Extracted DNAs for the clam broodstock were of variable quality, therefore “lower quality” (LQ) and “higher quality” (HQ) individual DNAs were processed separately. The two pools were composed of 48 (LQ) and 61 (HQ) samples, respectively. Illumina HiSeq 4000 sequences were trimmed, producing 629,419,546 (LQ) and 582,816,122 (HQ) reads, which were subsequently merged for mapping. A total of 67.69 % of reads were mapped to the reference genome, with average read length 124 bp, using CLC Genomics Workbench.

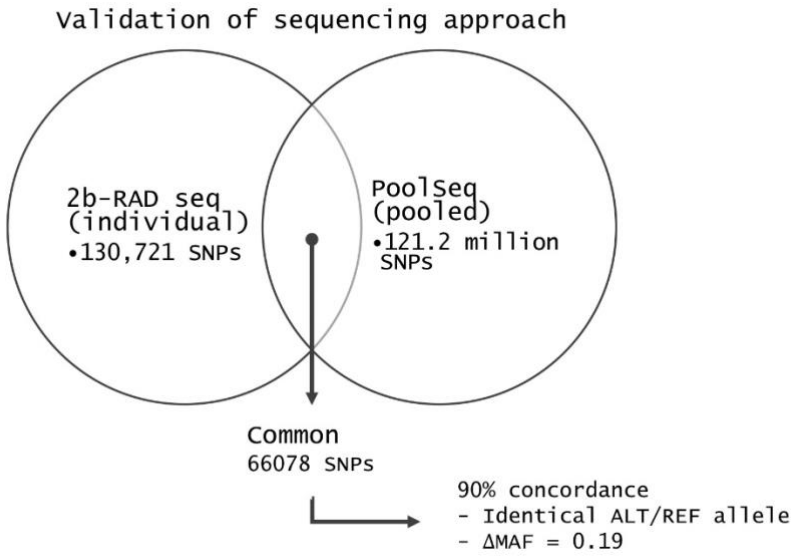

Figure 1: Comparison of SNPs detected in 2b-RAD (individual) and pooled DNA sequencing from the same 50 individuals. Allele type (ALT/REF) and Minor Allele Frequency (MAF) were compared for common SNPs, showing 90% identical results between the sequencing approaches.

Variant calling analysis was carried out using the pipeline developed for the Venice lagoon population PoolSeq, 281,651,950 reads were retained for variant analysis, from which PoPoolation identified 117,976,460 SNPs (parameters were kept identical to those in the Venice lagoon PoolSeq analysis).

3.5. SNP candidate selection

Variants called from PoolSeq of the Venice lagoon population and of the parental DNA from the VIVALDI experimental population were filtered based on the previously described selection criteria, retaining 32,914 and 32,004 SNPs, respectively (Figure 2). These lists were then compared to find 2,111 SNPs present in both of these populations. Of these, only one SNP per contig was kept, leaving 1,165 SNPs that fit all the selection criteria.

SNPs and their 100 bp flanking regions were provided to LABOGENA, which then eliminated all "mirror" SNPs, leaving 844 candidate markers from which 250 SNPs were selected for probe designing prior to mounting on the chip.

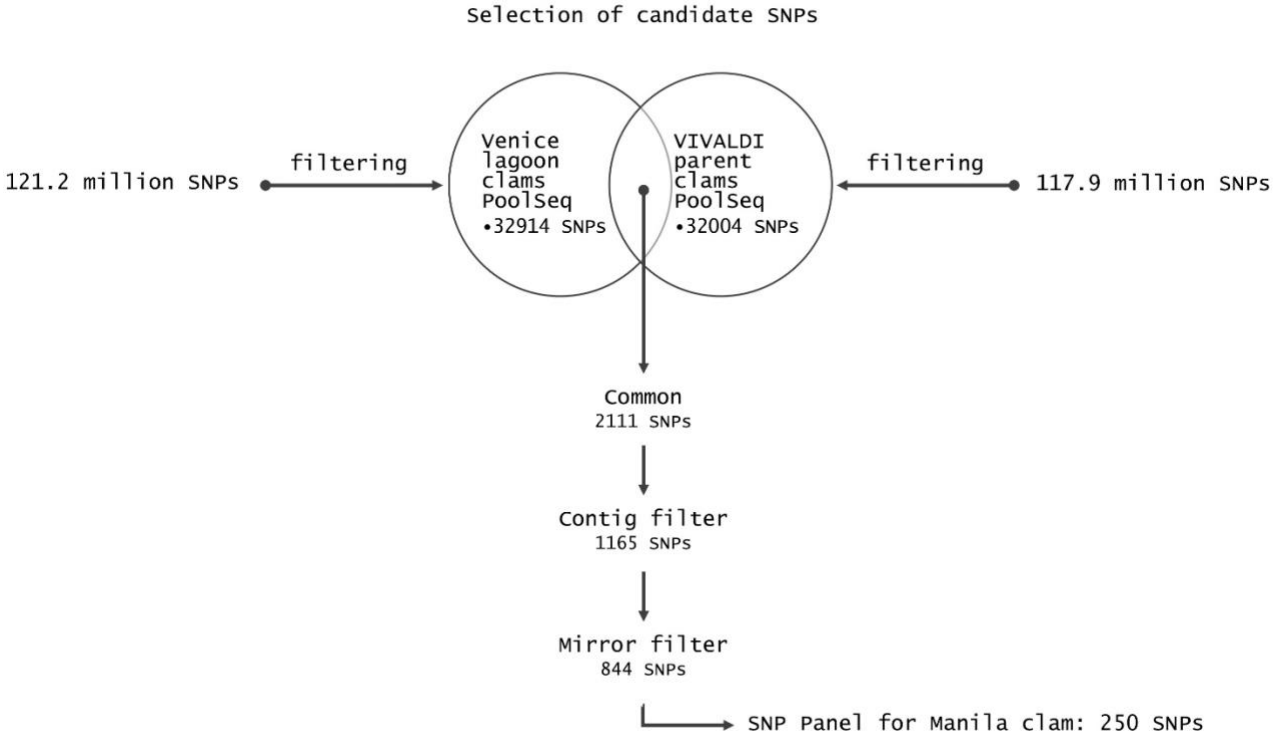

Figure 2: Representation of the filter processes in the SNP candidate selection pipeline used for analysing PoolSeq output from the Venice lagoon population and the hatchery broodstock ("VIVALDI parent clams").

3.6. SNP panel assignment capacity

The ability of the selected SNPs to correctly assign individuals to their parents was assessed in seven families of known pedigree produced by the SATMAR hatchery according to the mating design in Figure 3. Parents (7♂and 7♀) and all offspring (n=79) from each of the seven families were genotyped and parentage assignment was carried out using the 245-SNP panel. The mating design provided seven families produced for the panel from SATMAR (Table 1) was compared to observed assignments from the AccurAssign software (Table 2). Overall, one sample had insufficient genotype quality for assignment, and 68 out of the 78 genotyped descendants from the 7 families were correctly assigned, resulting in 88.4% assignment capacity.

A second genotyping and assignment test was carried out with 96 individuals from the F1 cohort produced using the hatchery broodstock (109; 56♂and 56♀). Genotyping was successful for 95 out of 96 samples (98.9%). Assignment to one parent couple was successful for 78 out of the 95 genotyped samples (82.1%), however correction based on the 2-factorial mating plan excluded 7 assignments, representing an overall assignment rate of 74.7 % (Table 3). From the broodstock population, 63 parent couples were observed, of which 53 were represented by a

single offspring (F1); 7 were represented by two F1s; 2 were represented by three F1s; and 1 family was represented by 5 F1s. The 56 sires and 53 dams used in the mating plan were represented in the F1 population by 32 sires and 30 dams (Table 3).

Table 1: Mating design and number of descendants for each of the seven families produced with unique parent crosses.

Dams ♀ / Sires ♂	Dam 1	Dam 2	Dam 3	Dam 4	Dam 5	Dam 6	Dam 7
Sire 1	Fam 1 (n=7)						
Sire 2		Fam 2 (n=12)					
Sire 3			Fam 3 (n=12)				
Sire 4				Fam 4 (n=12)			
Sire 5					Fam 5 (n=12)		
Sire 6						Fam 6 (n=12)	
Sire 7							Fam 7 (n=14)

Table 2: Assignment results for the 7 hatchery families of known pedigree used to evaluate assignment capacity of the SNP panel.

Family	**Genotyped offspring**	**Assigned offspring**	**Not assigned**
Fam1	5	4	1
Fam2	12	12	0
Fam3	11	10	1
Fam4	12	6	6
Fam5	12	12 (+1 wrongly assigned from Fam7)	0
Fam6	12	11	1
Fam7	14	13	1*
Total	78 (98.7%)	68 (88.4%)	

* The sample not assigned in Fam7 was assigned to Fam5

Table 3: Overall results of genotyping and parentage assignment for known-pedigree families and for unknown pedigree families (mixed families, 96 F1 individuals).

	Families of **known pedigree** (seven hatchery families)	Families of **unknown pedigree** (109 hatchery brood stock families)
Number of families	7	1479 potentially from mating plan
Number of parents	14	109
Number of F1	79	96
Genotyped	78 (98.7%)	95 (98.9%)
Assigned	69 (88.4%)	74 (74.7%)
Sires represented	7 (100%)	32 (57%)
Dams represented	7 (100%)	30 (56%)
Total markers	245	245
Number of activated markers	231	231
Number of polymorphic markers used for assignment	169	110

Of the 250 markers initially proposed for the SNP panel, probes were successfully designed for 245 markers, of which 231 showed a positive fluorescence signal. Polymorphic markers used to assign parentage were 169 in the seven test families and 110 in the hatchery F1 population (Table 3).

4. DISCUSSION

Genetic and genomic resources in bivalves remain relatively scarce despite the growing importance of this aquaculture sector, and the currently available resources often lack the ability to be applied to large-scale industrial production. This trend can in part be explained by the complex features of bivalve genomes, such as extremely high polymorphism and distortion from Mendelian inheritance ratios. This study provides, to our knowledge, a novel tool for parentage assignment of the Manila clam (*Ruditapes philippinarum*), the second most produced bivalve species worldwide, using SNP markers selected from pooled sequencing (PoolSeq) data from two distinct European populations and filtered according to stringent criteria. In addition, a PoolSeq pipeline was successfully validated against individual 2b-RAD sequencing and then applied for SNP candidate selection across both populations, representing the first use of this technology to this aim in a bivalve species.

While less SNPs were found in the 2b-RAD sequence data compared to PoolSeq, this difference can be attributed mainly to two factors. First, 2bRAD as other RAD-like methods produces a "reduced representation" of the genome, selecting only genomic regions flanking the restriction site. Second, 2bRAD sequencing produces short sequence length reads (36 bp), which have a higher tendency to align to several locations within the genome, thus many reads presenting multiple best-matches were discarded from mapping and subsequent variant discovery. Although this does not represent a major problem in most species, in the case of Manila clam, ultimately only 30% of 2bRAD reads were uniquely aligned to the genome, compared to over 68% of PoolSeq (average length of reads 121 bp).

A comparison between SNPs found in mapped reads of both PoolSeq and individual 2b-RAD showed that over 90% of the 66,078 common SNPs had identical alleles and similar MAF, leading us to consider the PoolSeq method and apply it to analyze the DNA from the 109 hatchery broodstock clams.

PoolSeq of the 50 Venice lagoon individuals and the 109 hatchery broodstock individuals showed very similar outputs, with 1.217 and 1.212 billion reads, respectively. Mapping and variant discovery (carried out with identical parameters) also produced similar results, with 68.07% alignment and 121 million SNPs for the Venice lagoon population, and 67.69% alignment with 117 million SNPs for the hatchery broodstock, showing the high reproducibility of this approach.

Of the 245 SNPs for which probes were mounted on the Illumina Infinium chip, 231 SNPs showed clustering profiles, and the number of polymorphic SNPs that assigned parentage was

169 in the seven hatchery families (73.1%) and 110 in the 96 F1 individuals (47.6%), which fall within the ballpark of other panels in mollusk species such as the mussel *Mytilus galloprovincialis* (52.8%), and the silver-lipped pearl oyster, *Pinctada maxima* (55.7%) [36,37]. Similarly, a 384-SNP panel for the Pacific oyster (*Crassostrea gigas*) and the European flat oyster (*Ostrea edulis*) showed about 60 % of successfully genotyped markers for both species [38], and more recently a higher-density SNP panel for the same two species reported successful genotyping of 68.2% of SNPs (n = 27,697) for *C. gigas* and 74.6% of SNPs (n = 11,151) in *O. edulis* [39].

Parentage assignment in the seven hatchery families and the F1 cohort from the broodstock population both showed a relatively limited assignment capacity (88.4% and 74.7%, respectively), considering the stringent filtering applied for candidate marker selection in our study. Most existing SNP panels for parentage assignment in bivalve species filter for coverage over 20x, MAF over 0.1 or occasionally 0.05, and stable flanking regions between 60 and 120 bp. We limited SNP selection in both of our candidate populations to select only markers with high coverage (> 25x, although keeping in mind an upper limit of 100x to avoid sequences in potential repeat regions), minor allele frequencies over 0.3, and stable flanking regions of over 100 bp. That said, the variable success rate of parentage assignment in non-model species for which fully annotated genomes are not available is a known problem, most often mitigated by the use of several hundred to thousand markers during panel development trials, followed by subsequent analysis to select informative SNPs and develop lower-density panels. The list of filtered candidate markers represented 2,111 SNPs and may have allowed for far more accurate and efficient parentage assignment were it not for the 250-marker capacity limit imposed by the genotyping company Labogena based on their array designs. Arrays developed in species for which there is significantly more knowledge have been able to build on previous studies to reach higher accuracy for parentage assignment in mixed-family groups, such as a 227-SNP panel for *M. galloprovincialis* able to assign 92.5% of offspring to their parent pairs using 179 polymorphic markers [37], and a 2x58-SNP panel that could assign 99.37% of 160 offspring in a hatchery population of *C. virginica* [40].

5. CONCLUSION

While the assignment capacity of the current panel (in both known-pedigree families and offspring from a complex broodstock cross) is lower than what is currently observed for some other cultured bivalve species such as oysters and mussels, it is a novel tool with which to initiate the first estimations of genetic parameters in mixed family designs in this species. In future panel constructions, it appears necessary, in addition to stringent filtering, to test a far larger number of markers than the 250 initially proposed in this study. The availability of an assembled and annotated genome for the Manila clam in the near future will also be an invaluable resource that will aid the selection of informative genetic markers for applications not only in selective breeding programs but also more broadly in fields such as population genetics.

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CHAPTER 2: FIRST ESTIMATIONS OF GENETIC PARAMETERS FOR RESISTANCE TO DISEASE AND PRODUCTION TRAITS IN THE MANILA CLAM

FIRST ESTIMATIONS OF GENETIC PARAMETERS FOR RESISTANCE TO DISEASE AND PRODUCTION TRAITS IN THE MANILA CLAM

ABSTRACT

Bivalve aquaculture is a rapidly growing sector that already represents a significant part of global aquaculture production, and while open-water grow-out techniques allow producers to benefit from a natural supply of food and oxygen, this method also exposes bivalves to potential pathogens. Diseases in represent a major limitation for bivalve production, namely reduced growth and reproduction, and mass mortality episodes, all of which are exacerbated in the case of shellfish by the difficulty in treating these diseases using the techniques which have been put in place (with variable success) for finfish species.

Genetic selection to improve desirable traits for aquaculture is relatively new in shellfish species, and it represents a sustainable alternative for mitigating the impact of diseases in farmed bivalve species. In order to balance selective breeding across multiple traits, phenotypes for traits of interest are recorded in a large number of related individuals and genetic parameters are calculated across traits, making it possible to estimate heritability for and genetic correlations between growth-related traits and resistance to disease.

A panel of 245 genetic markers (SNPs) was designed and mounted onto an Illumina SNP-chip and used assign parentage of two batches of F1 Manila clams from a single cohort produced from 109 parents using mass spawning. The cohort was sent to two production sites for grow-out in France and Italy for at least one year to assess the genotype by environment (GxE) interaction on production traits. Sampling (>1000 individuals per site) was carried out at approximately 2.5 years of age to record phenotypes (resistance, growth traits, sex) and collect DNA samples for parentage assignment. F1 samples were genotyped and assigned to their parent pairs using AccurAssign software. Despite limited assignment efficiency (54% and 64%), heritability for traits of commercial interest were 0.52 for resistance to Perkinsosis, and 0.23 and 0.46 for total weight in Italy and France, respectively. In addition, genetic correlations between biometric traits and resistance suggest that selecting for growth traits and disease resistance will not negatively affect other traits. The results of this first estimation of genetic parameters in the Manila clam in a mixed family breeding design indicate that developing a selective breeding program for growth traits and/or resistance will be possible without artificial fertilization methods and beneficial to the production industry, though the assignment rate of the current panel should be improved to increase accuracy of the current estimations.

1. INTRODUCTION

Genetic selection has long since revolutionized the efficiency of terrestrial plant and livestock production, in mollusk aquaculture, however, selection for the improvement of desirable traits remains poorly exploited. Bivalve aquaculture represents a rapidly growing aquaculture sector with high potential for expansion, having already increased its production at a rate of 7% between 2000 and 2014 [1,2].

Large-scale breeding programs for fish weren't initiated until the 1970s [3]. Since then, a number of breeding programs have been started for fish and shellfish, many of which have obtained high genetic gains due to a combination of high heritability for economically favorable traits, high fecundity, and short generation time of many of the studied species [4,5]. Despite the promising results in genetic gain for aquaculture species, it was estimated that in 2010 only 8.2 % of aquaculture production was based on genetically improved stocks [6].

Infectious diseases continue to be a major limit to aquaculture production, leading to the development of a number of techniques to control the spread of disease including vaccination, antibiotics, and cleaning methods in fish. However, pathogens continue to be a major limiting factor for aquaculture production, costing over US$ 6 billion per annum in losses alone, and far more in treatment [7,8]. While several pathogens have been reported to infect the economically relevant Manila clam, *Ruditapes philippinarum*, the protozoan parasite *Perkinsus olseni* has been identified as a major pathogen responsible for mass mortality of this species in Europe, namely in Italy [9]. The parasite induces the formation of nodules within the clam's gills that gradually spread to other tissues. In advanced stages of the disease, *P. olseni* causes lesions throughout various tissues that negatively affect the respiration and other physiological processes of the clam, leading to a reduction in growth, reproduction, and condition index [10]. While this pathogen is consistently present in clam production zones such as the Venice Lagoon in Italy, with disease prevalence regularly reaching 80-100% in clams, uninfected Manila clams are detected in exposed populations during routine monitoring carried out by the Italian health authority and research organization for animal health and food safety (IZSVe). It should also be noted that mortality is not always observed within the two-year grow-out period typically seen in Italy, as the parasite induces a chronic infection leading to varying degrees of infection throughout a given clam population.

Shellfish lack the adaptive immune system necessary for classical vaccination, and the open-water systems in which they are cultured make the use of antibiotics understandably controversial. Genetic selection for disease resistance in fish and shellfish, which is already in place for certain species, represents a feasible and long-term alternative to the current treatment

solutions. Recent estimates have shown that resistance could be improved by as much as 15% per generation in mollusks by implementing individual or family-based selection [11]. The few existing breeding programs in bivalve hatcheries are recent and mostly small-scale (few selected lines and limited broodstock size). Another significant bottleneck is the lack of genomic resources for most bivalve species, namely the Manila clam, which make it difficult to implement breeding programs despite having clearly defined objectives. Although Manila clam aquaculture represents roughly a quarter of all cultured bivalve species produced worldwide, European production currently accounts for only about 2% [12]. Most of the studies carried out so far seeking to evaluate the potential for genetic gain in bivalves have focused on traits of commercial interest, such as disease resistance, growth rate, meat yield, and visual aspects such as shape and shell color, and even fewer have tackled the estimation of genetic parameters across all traits simultaneously [13]. Balancing genetic gain for disease resistance with genetic gain for production traits is an essential step in designing rational breeding programs in order to evaluate the potential for genetic improvement across traits and, more importantly, to avoid selecting for a trait that may negatively affect other traits of interest.

Pedigree information is essential to evaluate the expected breeding values for measurable traits and develop sustainable breeding programs, however most aquatic species are impossible to physically tag at the larval stage due to their small size, which makes pedigree information difficult and costly to obtain. In order to distinguish between families, it was until recently unavoidable to raise large numbers of them separately, a logistical issue has long been among the many limits for the development of optimized breeding schemes in aquatic species. Individual tagging and separate family rearing also come with major disadvantages including high costs, extensive infrastructure, and the risk of confounding factors such as a “batch effect” which can significantly skew the accuracy of the estimated breeding values.

The use of genetic markers to trace the relationships between individuals within a large number of families greatly enhances the efficiency of family-based breeding schemes [14]. Furthermore, as genotyping costs continue to decrease it is becoming increasingly feasible to carry out large-scale studies with dense genotypic marker panels, allowing for more an accurate prediction of breeding values [15]. Bivalve genomes are notoriously difficult to assemble due to particularly high rates of heterozygosity, large insertions, and complex structural variation [16]. Elevated levels of polymorphism, namely de novo mutations, can lead to Mendelian errors and null alleles which make it difficult to reconstruct pedigree through genetic analyses [17–19]. Notwithstanding, several recent studies have successfully applied SNP panels for parentage assignment in order to estimate genetic parameters of traits of commercial interest in mollusks

[20–23]. A 245-SNP panel was conceived for parentage assignment in the Manila clam. In addition, a number of programs have been developed to be able to integrate phenotypic, pedigree and genomic information in prediction of breeding values, such as the BLUPF90 program family, under which fall several programs for variance component estimation such as GIBBSfF90 and THRGIBBSxf90 [24,25].

The objective of this study is to carry out a first estimation of the genetic parameters of production traits and resistance to infection with *P. olseni* in the commercially important Manila clam, *R. philippinarum*, in order to evaluate the feasibility of carrying out rational genetic selection of multiple production traits and lay the basis for designing balanced breeding programs in hatcheries.

2. MATERIALS AND METHODS

2.1. Experimental Clams

An experimental group of Manila clam *R. philippinarum* was produced by mass spawning in May 2016 at the French hatchery SATMAR (Barfleur, France) with 109 adult clams of hatchery broodstock, according to a two-factorial mating design: 31 dams x 25 sires (775 putative families) and 22 dams x 32 sires (704 putative families), providing potentially 1479 families. A piece of mantle from each of the 109 parent clams was kept in 99% ethanol for genotyping. The F1 cohort (or progenies) was reared at the SATMAR hatchery in a single mixed-family tank in order to avoid differences between rearing conditions, or "tank effect". In July 2017, a batch of circa 20 000 progenies was seeded in Marennes, a commercial grow-out site in France (as a control), and in September another batch of about 10 000 progenies was seeded in Chioggia, a commercial grow-out site in Italy (as a field challenge with *Perkinsus olseni*) (Table 1).

Table 1: Summary of grow-out conditions and F1 batch numbers at the two field sites.

Site name	***n* =**	**Grow-out conditions**	**Date of seeding**	***P. olseni* prevalence**
Chioggia (Italy)	10 000	Under nets in pre-vacated sand plots	September 2017	87% in 2016 (pers. comm, IZSVe)
Marennes (France)	20 000	In protected grow-out ponds	July 2017	NA (not detected in ponds)

2.2. Sampling strategy and data collection

In Chioggia, prevalence and intensity of *P. olseni* in the gill tissues of clams was evaluated monthly from the time of seeding during one year. Each month, about 30 clams were sampled for whole weight, shell weight, and shell length, and the gills were dissected and frozen in liquid nitrogen for subsequent DNA extraction. Biometric measurements allowed us to follow growth parameters of the clams. Disease intensity was measured based on quantification of parasite DNA in the total DNA from the gill sample (protocol described below), and prevalence was considered as the percentage of quantifiably infected clams. This monthly sampling strategy was employed in order to monitor disease prevalence, bearing in mind the annual variability of disease intensity and the historical risk of mass mortality in the area. Mass sampling was carried out at commercial size, when about 50% of the group showed quantifiable levels of infection.

In Chioggia, the mass sampling event was carried out in September 2018, and then in November 2018 for the Marennes batch. Mass sampling entailed gathering the measurements for biometric traits in both sites (Table 2). The number of samples was set at $n > 1\ 000$ in order to expect a mean of 18-20 progenies per parent, for a relevant estimation of genetic parameters in a mixed family design which accounts for a variable number of sibs per parent [26]. based on the estimations described by Dupont-Nivet et al. (2006) who demonstrated effective population size (N_e) and genetic variability to be maintained in a total population size of $n = 1\ 000$ for full factorial mating designs considering 50 x 50 parents [27]. Over 1 000 clams at each site were individually weighed, and length, width and height were recorded, as depicted in Figure 1 [28]. Then, meat and shell were separated and weighted after draining. Sex was determined by visualization under a microscope of a gonad smear during Chioggia sampling. For Marennes clams it was not possible to determine sex as individuals were not mature due to lower water temperatures at the time of sampling.

Both gills were collected from each of the Chioggia clams and a piece of mantle tissue was collected from the Marennes clams. Gills were selected for the samples from Chioggia as they are the first tissue to become infected with *P. olseni*, and thus it was possible to carry out total DNA extraction from the same sample for both parasite quantification and host genotyping (see below: *Perkinsus olseni* quantification). Mantle tissue was preferred for Marennes clams due to ease of sampling. Gill samples were stored in 70% ethanol, and mantle sample in 99% ethanol until DNA extraction (see below: DNA extraction and genotyping)

All data were recorded in the INFAQUA database system dedicated to data storage and provided by SYSAAF.

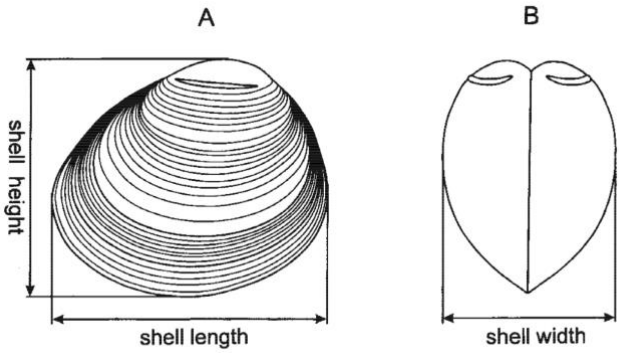

Figure 1: Shell dimensions measured for all F1 clams from both Chioggia and Marennes clam groups, adapted from Grigorovich et al. 2000 [28].

Table 2: Description of recorded traits, units, and number of measurements.

Trait	**Unit**	**Description**
Total weight	g	Whole individual weight
Shell length	mm	Maximum length between the posterior and anterior parts (see Figure 1)
Shell width	mm	Shell width at the center (see Figure 1)
Shell height	mm	Maximum length between the top (superior shell) and the bottom (inferior shell) (see Figure 1)
Tissue weight	g	Meat separated from the shell was drained on absorbing paper during a few seconds, then weighed
Shell weight	g	Weight of cleaned shell, without meat
Tissue yield	ratio	Ratio of tissue weight measurement over total weight for each individual
Gill weight	g	Wet weight of the two gills together
Sex		Male (1), female (2), or unknown (0)
Parasite load	copies.uL^{-1}	Number of copies of *Perkinsus olseni* DNA in 1uL of total extracted DNA (concentrations normalized at 10ng/uL of total DNA).

2.3. Perkinsus olseni quantification

Perkinsus olseni load in individual clams was established by real-time PCR using specific primers for *P. olseni* to amplify 1µL total DNA extracted from one whole clam gill. Individual total DNA extraction was carried out by UNIPD-BCA using Invisorb® DNA Tissue HTS 96 Kit according to the manufacturer's instructions. Briefly, samples were homogenized with a benchtop TissueLyser® in 80 µL PBS buffer with silica beads. Lysis with proteinase K was

carried out overnight at 56ºC prior to isopropanol precipitation, and DNA was eluted in 100 µL MilliQ water. Total DNA concentration was measured by spectrophotometry (Nanodrop®) and quality of the DNA was assessed by electrophoresis on 1% agarose gel.

Total DNA samples were normalized at 10 ng.µL^{-1} and transferred to the facilities of the National Reference Laboratory for Fish, Crustacean and Mollusk Pathology at the IZSVe, who collaborated with the Instituto de Investigaciones Marinas (CSIC, Vigo, Spain) to optimize the *P. olseni* quantification protocol. Briefly, PCR assays were performed in a total volume of 25 µL containing 1 µL genomic DNA, 12.5 µL Sybergreen PCR Master mix, 10.5 µL clean H2O and 0.5 µL both forward and reverse primers (10 µM). PCR cycles were performed using a 7300 Real Time PCR system from Applied Biosystems as follows: 95ºC for 10 min; 40 cycles at 95ºC for 30 sec and 60ºC for 1 min followed by 72ºC for 5 min and a malting curve at 95ºC for 15 sec; 60ºC for 30 sec and 95ºC for 15 sec. (CSIC, in prep).

2.4. Genotyping and parentage assignment

Two DNA extraction protocols using Qiasymphony® automated extraction from Qiagen, were used following supplier recommendations, with minor modifications based on the size of the tissue samples: the first included a simple lysis with proteinase K at 56°C overnight (for small punches, i.e. 3 x 3 mm pieces of tissue); the second included a lysis and purification step (for big samples, i.e. > 3 x 3 mm pieces).

DNA quality and quantity were assessed by measuring DNA absorbance with a Nanodrop® spectrophotometer, following supplier recommendations, and by running 10 µL total extracted DNA on 1% agarose gels by electrophoresis.

DNA samples were analyzed using the Manila clam SNP-chip developed within the EU Horizon2020 project VIVALDI. Briefly, the chip was constructed using Illumina Infinium® chemistry, based on 245 SNP sequences provided by partner UNIPD-BCA and initially analyzed on DesignStudio® following supplier recommendations.

Genotyping analyses were carried out using an Illumina iScan® fully automated platform, following supplier recommendations. Quality control was carried out using GenomeStudio® software, according to the Labogena clustering procedure (see Chapter 1 of the PhD thesis).

Samples were then assigned using AccurAssign® from Labogena [29], considering the mating plan used to produce the F1 clams, and applying the following parameters:

- 1% of genotyping errors
- Minimum of 70% of markers with results for each sample
- Maximum of 3 mismatches between progeny and couple.

Parameters were adjusted depending on the quality of certain samples (up to 5% of genotyping errors and 5 mismatches between progeny and couple were accepted).

Assignment results were calculated based on maximum likelihood and exclusion, and the mating plan used to produce the F1 Manila clams was included in the software parameters in order to correct assignments lying outside of the mating plan [29].

2.5. Genetic variability

Estimations of effective population size (N_e) are used to estimate the number of parents necessary to maintain genetic diversity, or allele frequencies, within a population. N_e can be calculated assuming random mating and equal parent representation among the F1 clams, according to:

$$N_e = \frac{4}{\frac{1}{N_{em}} + \frac{1}{N_{ef}}}$$

Where N_{em} and N_{ef} are the number of effective male (sire) and female (dam) parents, respectively.

As equal family representation is rarely observed in mass spawning of marine fish and shellfish [14], in which there is often a tendency towards strong overrepresentation in the F1 generation of only a few of the parents spawned, there is a high risk of inbreeding and loss of genetic diversity. Estimated N_e can give an indication of the risk of inbreeding, and in order to increase the accuracy of inbreeding estimations, the variability of family representation can be considered when calculating Ne, according to:

$$\frac{4}{N_e} = \frac{\left(K_m + \frac{V_m}{K_m}\right) + \left(K_f + \frac{V_f}{K_f}\right) - 2}{N - 2}$$

Where N is the total number of individuals, K_m and K_f are the average number of progenies per sire and dam, and V_m and V_f are the variance of the number of progeny between sires and dams. The rate of inbreeding per generation (F_t) can be calculated for a population and a number of generations (t) based on the N_e, according to [30]:

$$F_t = 1 - [1 - \frac{1}{2N_e}]^t$$

2.6. Estimating Genetic Parameters

Data were checked before analyses to eliminate outliers: measurements that were less than or greater than the mean + 4 standard deviations were systematically eliminated. Meat yield was calculated as tissue weight divided by total weight, as high phenotypic correlations were observed for these measurements with the residual of tissue weight to total body weight.

Genetic parameters were estimated using two programs derived from the software BLUPF90: GIBBSF90 for continuous traits and the THRGIBBSF90 threshold model in cases where at least one non-continuous trait (in our study this pertains to resistance to *P. olseni* and sex-ratio) was included in the model. Genetic parameters were estimated using a generalized mixed linear animal model [31,32] that includes both fixed and random effects using pedigree information, calculated as follows:

$$Y = \mathbf{X}\beta + \mathbf{Z}u + \boldsymbol{\varepsilon}$$

Where Y is the studied phenotype matrix of n observations. **X** is a $(n \times p)$ matrix that links the observations to the vector β of p fixed effects; in our case we considered only one fixed effect for the batch (Chioggia or Marennes). **Z** is a $(n \times r)$ matrix which links the observations to the vector u of r random effects. As we consider only one random effect, the additive genetic effect, **Z** can be considered as the genetic variance-covariance matrix G = **A** × V_a, where **A** is the additive relatedness matrix derived from the pedigree data [33]. Residual errors ($\boldsymbol{\varepsilon}$) are assumed to be independent between individuals, with the variance-covariance matrix for r being **R** = **I** × V_r .

Heritability (*h2*) was calculated according the following formula:

$$h2 = \frac{{\sigma^2}_a}{{\sigma^2}_t} = \frac{{\sigma^2}_a}{{\sigma^2}_a + {\sigma^2}_e}$$

Where σ_{2a} is the genetic additive variance related to pedigree, and σ_{2t} is the total phenotypic variance, defined as the sum of genetic additive variance σ_{2a} and residual variance σ_{2e}. In the case of analyses which included multiple traits, genetic additive covariance was estimated and genetic correlations (Rg) between two traits were calculated as follows:

$$R_{g_{(trait1;\, trait2)}} = \frac{Cov_{a_{(trait1;\, trait2)}}}{\sigma_{a_{(trait1)}} \times \sigma_{a_{(trait2)}}}$$

Where $Cov_{a\ (trait1;\ trait2)}$ is the genetic additive covariance between trait 1 and trait 2, $\sigma_{a\ (trait1)}$ and $\sigma_{a\ (trait2)}$ are the standard deviation of additive genetic effect for trait 1 and trait 2, respectively. When calculating genetic correlations between traits on the same individuals on same site, then we estimate variance and co variance residuals, however when calculating the genetic variance between sites for a trait, then only the residual variance matrix can be estimated, and co variance residuals cannot be estimated.

Heritability was estimated using a mono-trait analysis. Then, genetic correlations were computed using multi-trait analyses.

3. RESULTS

3.1. Phenotyping of biological material from experimental clams.

In Chioggia during the mass sampling event 1088 clams were sampled for biometric traits, sex, and parasite load. Descriptive statistics for these F1 individuals are reported in Table 3.

Table 3: Full summary of traits recorded in Chioggia F1 group.

Chioggia	***n*=**	**Mean ± SD**	**Min**	**Max**
Total Weight (g)	1087	11.89 ± 3.63	2.8	26.9
Shell Length (mm)	1088	36.94 ± 3.78	23.18	50.55
Shell Height (mm)	1088	26.45 ± 2.69	16.69	37.86
Shell Width (mm)	1088	17.74 ± 2.08	11.23	23.46
Shell Weight (g)	1058	6.725 ± 2.09	1.86	15.21
Tissue Weight (g)	1057	1.538 ± 0.52	0.39	3.78
Tissue Yield (ratio)	1056	0.129 ± 0.02	0.07	0.29
Gill Weight (g)	1056	0.335 ± 0.1	0.11	0.82
Log10 P. olseni (copies.µL-1))	544	3.2 ± 0.67	2.01	5.23

For the Chioggia group, real-time PCR on DNA extracted from clam gills revealed that 244 clams were negative for *P. olseni* DNA, 268 were below the limit of quantification (LOQ: > 30 threshold cycle; eq. 100 copies of *P. olseni* DNA), and 544 samples presented a quantifiable amount of *P. olseni* DNA (Table 4).

Table 4: Means and SD for measured traits, presented in three clam groups depending on the parasite load (negative; below LOQ; quantifiable for parasite DNA) for experimental F1 clams in the Chioggia field challenge.

Parasite load class	***n* =**	**Copies.uL-1**	**Shell width**	**Shell height**	**Shell length**	**Gill weight**	**Tissue weight**	**Shell weight**	**Total weight**
Quantifiable	544	5265 ± 1189	17,3 ± 2,11	25,9 ± 2,83	36,2 ± 3,88	0,31 ± 0,09	1,44 ± 0,52	6,33 ± 2,09	11,2 ± 3,60
<LOQ	268	NA	18,2 ± 1,97	26,8 ± 2,48	37,3 ± 3,70	0,35 ± 0,09	1,59 ± 0,51	7,10 ± 2,10	12,5 ± 3,54
NEG	244	NA	18,2 ± 1,75	27,2 ± 2,16	38,1 ± 3,05	0,36 ± 0,09	1,68 ± 0,50	7,18 ± 1,85	12,7 ± 3,21

Sex, as a qualitative trait, could be determined for 890 individuals out of 1059 (Figure 2). 169 individuals were indeed not mature enough for sex determination. Male to female ratio was 47:53.

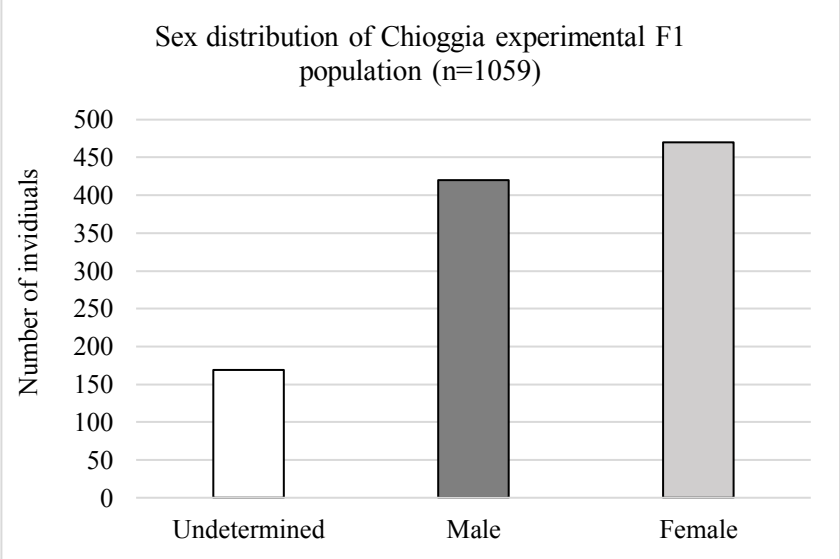

Figure 2: Sex distribution of Chioggia experimental F1 clams (n=1059).

In Marennes, 1016 clams were sampled for biometric traits. Descriptive statistics are reported in Table 5. Gill samples for *P. olseni* quantification were also taken on 30 of the sampled clams (by real-time PCR following identical protocol as described previously). None of the samples tested positive for the parasite, consistent with the fact that Marennes was considered as a control sight regarding disease prevalence.

Table 5: Descriptive statistics for all traits measured in the Marennes F1 clams.

Marennes	***n*=**	**Mean ± SD**	**Min**	**Max**
Total Weight (g)	1028	22.84 ± 4.73	9.9	49.7
Shell Length (mm)	1028	45.05 ± 3.27	32.28	58.73
Shell Height (mm)	1028	34.50 ± 2.49	26.17	44.45
Shell Width (mm)	1028	22.81 ± 1.79	16.8	29.94
Shell Weight (g)	1018	12.57 ± 2.71	5.26	29.22
Tissue Weight (g)	1018	5.94 ± 1.49	2.04	12.58
Tissue Yield (ratio)	1016	0.259 ± 0.03	0.135	0.371

3.2. Genotyping and parentage assignment of experimental clam groups.

Of the 1088 samples analyzed for parasite load, 992 samples had sufficient remaining biological material to be sent to Labogena from the Chioggia F1 clam group, and 601 samples were successfully genotyped with the SNP-chip (61% success rate). Labogena was able to successfully assign parentage to 324 (54%) of the genotyped samples.

For the 1016 samples from the Marennes F1 clam group, genotyping was far more successful with 946 (93%) samples genotyped, however only 64% of these F1 individuals were assigned to one parent couple.

3.3. Genetic variability

Of the 56 sires and 53 dams used to produce the F1 clam groups, parentage assignment showed that most were represented in the F1 clams at commercial size in both rearing sites (Table 6; Figure 3). Despite the low number of assignments for the Chioggia batch (N=246), 163 out of 1479 potentially expected full-sib families from 42 sires and 45 dams were observed. In samples from Marennes, the greater number of successful assignments (N=604) allowed a better view of variability with 283 full-sib families from 49 sires and 44 dams. While more families were represented in Marennes compared to Chioggia, there is no significant difference in parent representation between the two sites (Table 6). Pearson correlation on the number of offspring by sire was 0.94, and 0.79 for dams.

Table 6: Genetic variability from control site and challenge site: parent representation and effective size (N_e).

	Chioggia	**Marennes**
Assigned offspring	246	604
Expected sires	56	56
Expected dams	53	53
Expected families	1479	1479
Observed sires	42	49
Observed dams	45	44
Observed families	163	283
Expected N_e	108.9	108.9
Observed N_e	86.9	92.7
Observed N_e with offspring variance	39.0	38.7

The initial expected effective population size (N_e) based on number of parents that were used in the mass spawning was 108.9. After parentage assignment of the F1 clam groups for both sites, the observed N_e (calculated based on the number of parents that were actually represented in the F1 groups) was 86.9 for Chioggia and 92.7 for Marennes. When considering the variability in representation of the parents, i.e. the variance of the number of offspring per sire and dam, (Table 6; "Observed Ne with offspring variance"), the N_e drops down to 39.0 in Chioggia and 38.7 in Marennes.

Inbreeding rates per generation based on the observed N_e are 0.58% for Chioggia and 0.54% for Marennes, while inbreeding rates considering N_e with offspring variance are 1.28% for Chioggia and 1.29% for Marennes.

3.4. Estimation of genetic parameters.

Estimates of heritability (h2) for traits are detailed per trait and per site (Table 7). Genetic correlations (Rg_{site}) between sites were calculated per trait (Table 7). Genetic correlations (Rg_{trait}) between traits within sites were also calculated pairwise for Chioggia (Table 8) and Marennes (Table 9).

Table 7: Estimated heritability (h2) of each trait for the two experimental clam groups ("Chioggia" and "Marennes"). The last column ("Correlation $R_{g\ site}$") represents the correlation between the two sites for each measured trait. The standard deviation of heritability is presented in brackets.

TRAIT	**CHIOGGIA *h2* *(n=246)***	**MARENNES *h2* *(n=595)***	**CORRELATION $R_{g\ site}$ *(n=841)***
Total weight	0.23 [0.11]	0.46 [0.11]	0.54 [0.26]
Shell length	0.29 [0.13]	0.42 [0.1]	0.67 [0.21]
Shell height	0.25 [0.13]	0.41 [0.11]	0.62 [0.67]
Shell width	0.3 [0.12]	0.39 [0.1]	0.51 [0.43]
Shell weight	0.35 [0.13]	0.51 [0.11]	0.66 [0.42]
Tissue weight	0.19 [0.1]	0.33 [0.1]	0.04 [0.36]
Tissue yield	0.18 [0.08]	0.29 [0.1]	0.30 [1.87]
Gill weight	0.2 [0.11]	*NA*	*NA*
Sex	0.42 [0.19]	*NA*	*NA*
Parasite load	0.52 [0.22]	*NA*	*NA*

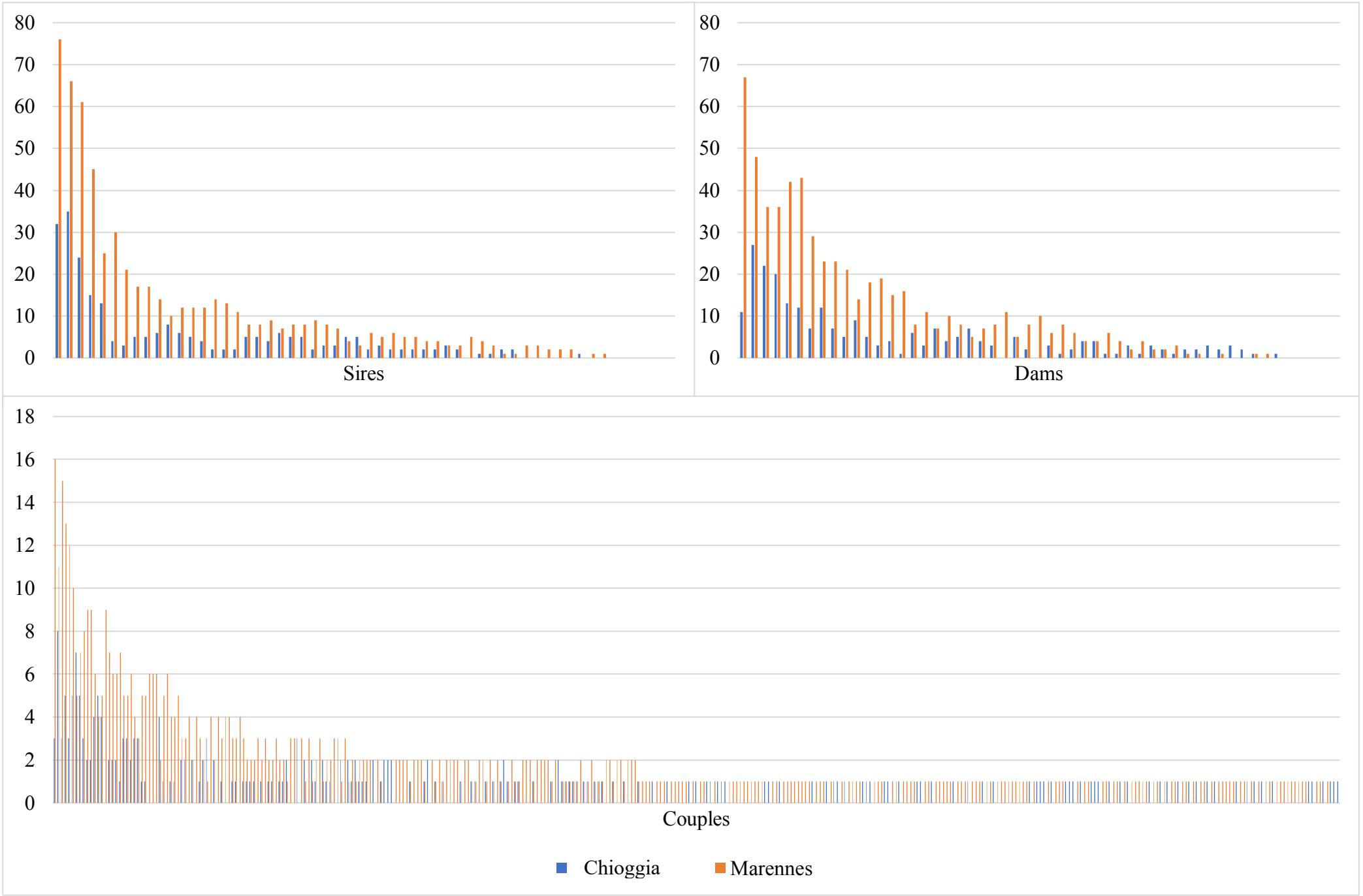

Figure 3: Assignment of the F1 group of Chioggia and Marennes to parental group. Top 2 graphs: Individually represented assignment per sire (left) and per dam (right). Lower graph: Assignments to parent couples.

Individually estimated heritability for biometric traits in the Chioggia F1 group range from 0.18 ± 0.08 (tissue yield) to 0.35 ± 0.13 (shell weight). Heritability for both sex (0.42 ± 0.19) and parasite load (0.52 ± 0.22) is high. In Marennes, estimated heritability for all biometric traits was higher than in Chioggia, ranging from 0.39 ± 0.1 (shell width) to 0.51 ± 0.11 (shell weight). Shell weight is the most heritable trait measured in both sites. The higher heritability and lower error (standard deviation) observed in the Marennes group compared to Chioggia can likely be associated to the higher number of assigned individuals in Marennes. The genetic correlations between sites for each trait are not significant, ranging from 0.51 [0.43] for tissue weight to 0.67 [0.21] for shell length. The high variances of these measurements make it difficult to accurately evaluate the correlation, and for this reason the Chioggia and Marennes F1 groups were considered separately for the estimations of heritability and genetic correlations between traits. The lack in significant genetic correlation between sites for all of the traits measured suggests there may be an important GxE interaction.

Table 8: Correlations between traits measured for Chioggia F1 clams, with standard deviation in brackets.

Correlations between traits ($R_{g\ trait}$) for Chioggia experimental group									
	Shell length	Shell height	Shell width	Shell weight	Tissue weight	Tissue yield	Gill weight	Sex	Parasite load
Total weight	0.9 [0.07]	0.76 [0.28]	0.97 [0.03]	0.97 [0.03]	0.84 [0.14]	-0.19 [0.39]	0.22 [0.46]	0.06 [0.65]	-0.26 [0.4]
Shell length		0.81 [0.24]	0.8 [0.12]	0.78 [0.13]	0.79 [0.16]	-0.1 [0.38]	0.16 [0.45]	0.11 [0.72]	0.16 [0.41]
Shell height			0.87 [0.11]	0.86 [0.12]	0.9 [0.11]	0.06 [0.37]	0.87 [0.13]	0.07 [0.62]	-0.15 [0.4]
Shell width				0.96 [0.04]	0.86 [0.12]	-0.08 [0.37]	0.92 [0.1]	0.08 [0.56]	-0.35 [0.31]
Shell weight					0.8 [0.16]	-0.25 [0.36]	0.8 [0.19]	0.05 [0.28]	-0.27 [0.32]
Tissue weight						0.46 [0.32]	0.91 [0.11]	0.11 [0.18]	-0.14 [0.19]
Tissue yield							0.12 [0.41]	0 [0.09]	-0.02 [0.58]
Gill weight								0 [0.1]	-0.07 [0.1]
Sex									0.22 [0.26]

Table 9: Correlations between measured traits for the Marennes F1 clams, with standard deviation in brackets.

Correlations between traits ($R_{g\ trait}$) for Marennes experimental clams						
	Shell length	*Shell height*	*Shell width*	*Shell weight*	*Tissue weight*	*Tissue yield*
Total weight	0.92 [0.03]	0.96 [0.02]	0.91 [0.04]	0.98 [0.01]	0.9 [0.05]	-0.26 [0.25]
Shell length		0.94 [0.03]	0.75 [0.1]	0.87 [0.05]	0.83 [0.08]	-0.2 [0.25]
Shell height			0.82 [0.08]	0.92 [0.04]	0.94 [0.04]	-0.08 [0.26]
Shell width				0.87 [0.05]	0.83 [0.09]	-0.19 [0.25]
Shell weight					0.82 [0.08]	-0.38 [0.23]
Tissue weight						0.19 [0.26]

For the Chioggia dataset, correlations between biometric traits were high, ranging between 0.76 ± 0.28 (shell height vs. total weight) and 0.97 ± 0.03 (shell height and width vs. total weight). The average measured values for biometric traits between negative, <LOQ, and quantifiably infected clams (Table 2) tended to decrease with increasing parasite quantity. This is supported by the fact that the genetic correlations between parasite load and biometric traits, except shell length, tend to be negative (Table 4). Similarly, sex-ratio does not appear to be correlated to any other measured trait, though it is interesting to note that the strongest (albeit insignificant) positive correlation is with parasite load (0.22 ± 0.26).

Genetic correlations between all measured biometric traits in Marennes were high, ranging from 0.75 ± 0.1 (shell width and shell length) to 0.98 ± 0.01 (total weight and shell weight).

For both sites, tissue yield did not appear correlated with any other trait, besides a slight positive but insufficiently precise correlation with tissue weight in both sites (Chioggia, 0.46 ± 0.32; Marennes, 0.19 ± 0.26).

The highest estimated heritability in both Chioggia and Marennes appears to be shell weight, which in both sites is most strongly correlated with total weight. Also, shell length and shell width are among the least correlated measured biometric traits in both sites.

4. DISCUSSION

Here we present the first estimate of genetic parameters of resistance to disease and growth traits in the Manila clam, *Ruditapes philippinarum*, using DNA parentage assignment for pedigree reconstruction in a breeding design with mixed family rearing and mass spawning. Previously, only one publication has reported estimations of genetic parameters for larval growth in the Manila clam, although using separate family rearing [34]. The use of a mixed-family breeding design to estimate genetic parameters in mollusks has previously been investigated in the pearl oyster, blue mussel, Mediterranean mussel, freshwater pearl mussel, and European abalone [23,35–39]. In our study, biometric traits, sex, and parasite load were recorded at commercial size in mixed-family batches of F1 Manila clams from the same parents, reared in two different clam grow-out sites.

The low genotyping rate for Chioggia compared to Marennes is surprising, and possibly due to differences in DNA quality due to the tissue sampled, although this was unexpected as previous tests carried out by Labogena on total DNA extracted from both clam gills and mantle showed no difference in quality, and significantly higher DNA concentrations in gill-tissue extractions (see Supplementary File 1).

Of these, parentage assignment to one parent couple was successful for 54% of the Chioggia samples and 64% of the Marennes samples. During the development of the SNP panel used for the parentage assignment of the Manila clam F1 groups in this project, an assignment capacity test was carried out using seven distinct full-sib families of known pedigree produced in the SATMAR hatchery in France, which showed 81.2% assignment rate. The assignment rates observed in the Chioggia and Marennes F1 groups are thus significantly lower compared to results obtained in the seven hatchery families, as well as in other fish and shellfish species such as the Pacific oyster, *C. gigas*, in which assignment rates varied from 72% to 94% [40]. As mollusks are highly polymorphic species, SNP panels are notoriously difficult to design. That said, parentage assignment using SNPs has been demonstrated in the Pacific oyster, leading us to believe that with the availability of a higher- quality genome assembly for the Manila clam it will be feasible to improve the current panel. Improvement of the panel would allow for a better evaluation of the genetic parameters of phenotypes recorded in the field.

Despite the low number of assigned individuals in comparison with the number of parents used, a majority of the 109 parents are represented in offspring after 2.5 breeding years in both sites: 75% sires and 85% dams in the Chioggia group, and 88% sires and 83% dams in the Marennes group. This indicates that mass spawning using a large number of broodstock seems to be a suitable solution to keep genetic variability for the next generation, as parent representation for

sires and dams in Chioggia and Marennes (75 and 85%, and 87.5 and 83%, respectively) are similar, and in some cases higher, than those for the Pacific oyster *C. gigas* (64-100% for sires and 68-96% for dams in 4 hatchery populations) [40].

When N_e with parent representation is considered (without offspring variance), the N_e decreased from 108.9 (expected N_e) to 86.9 and 92.7 in Chioggia and Marennes (observed N_e), respectively. When offspring variance was taken into account, the N_e dropped to 39.0 in Chioggia and 38.7 in Marennes. These N_e calculations represent the same number of parents (both consider the real number of parents having participated in producing the assigned offspring, assuming correct assignment) and allow for an estimation of the inbreeding rate after one generation ($\Delta F = 1/ (2*N_e)$). In the first case, the inbreeding rates are lower than 0.6 % and in the second case equivalent to 1.3 % per generation, for both sites. Even with the lower N_e observed when taking into account the offspring variance, the inbreeding rate is much lower than what has previously been reported in rainbow trout, which were between 1 and 2.2 % per generation [41], and in the banana shrimp, which showed an average inbreeding rate of about 4% per generation [42]. In the first case (N_e without offspring variance), inbreeding estimations for the Manila clam are in agreement with recognized accepted maximum inbreeding rate per generation in animal breeding (under 1% per generation). In the second case (N_e with offspring variance), they remain within the internationally proposed maximums suggested by the FAO for fish breeding in order to assure long term management of genetic variability [43,44]. It is important to highlight that the cohort assessed throughout this study represents only one third of the SATMAR breeding program, which subdivides its production into three yearly cohorts. This means that the actual N_e could in reality be much higher at the hatchery level than what we have reported in this study for only one cohort. More generally, these results strongly support the use of a mating design with mass spawning and mixed family rearing, as even with no control on initial equal representation of the offspring per parents or per family, there appears to be a relatively low risk of inbreeding.

It is clear however that further investigations should be carried out with a higher number of assigned individuals in order to more precisely evaluate family representation with this mating process. Despite the low numbers of assigned offspring, this first investigation appears favorable for estimations of genetic parameters and individual breeding values in mixed family design [14].

Heritability of traits showed that shell weight is the most heritable biometric trait in both sites, with 0.35 ± 0.13 for Chioggia and 0.51 ± 0.11 for Marennes. In contrast, results regarding the Pacific oyster measured a heritability of 0.15 ± 0.04 for total shell weight, as well as 19 ± 0.04

for inferior shell weight and 0.12 ± 0.04 for superior shell weight, using the same calculations as described here for the Manila clam [40]. While shell weight appears less heritable in the Pacific oyster, this may be partially attributed to the variable morphology of oyster shells as opposed to the symmetric and more predictable dimensions of Manila clam shells. Interestingly, genetic correlations between shell weight and total weight, shell length, width, and height were very similar (though slightly higher in the Manila clam) between the Pacific oyster and both Chioggia and Marennes clam groups, ranging from 0.69 ± 0.20 to 0.98 ± 0.05 in the oyster, 0.78 ± 0.13 to 0.97 ± 0.03 in Chioggia clams, and 0.87 ± 0.05 to 0.98 ± 0.01 in Marennes clams. Overall, selection for growth would be efficient in the Manila clam and show notable improvement in only a few generations. Moreover, these intermediate heritability estimates suggest an important additive genetic effect on clam morphology.

That said, genetic correlations between sites for a same trait were not significant, hence the two environments were considered as different ($R_{g\ site} < 0.85$). These values should be considered with caution as the errors of the genetic correlations are very high (Table 7). One of the explanations for the high error is likely the limited number of progenies assigned (n=246 for Chioggia, and n=604 for Marennes). A higher rate of assignment would have both reduced the error and, in doing so, would have provided more precise estimations of the family or parent ranking across environments. At this stage and with the available genetic data from the two sites, we have to consider the traits as different. Lack of genetic correlations between sites for the same trait suggests an important genotype-by-environment effect, which may be attributed not only to the different environmental conditions (temperature, salinity, etc.) but also to the presence of *P. olseni* in the Chioggia site, in that the parasite may negatively influence growth traits to a higher degree in some families than in others, much like an environmental parameter. The lack of significant genetic correlations for growth traits between the two sites may also mean that the genetic progress created in on site (e.g. Marennes) would not be expressed to the same extent in the other site (Chioggia). In order to estimate the genetic progress expected by selection carried out in one site, and the expected genetic response in either the same site or in the other, simulations were done for total body weight, a common trait of interest for shellfish producers, considering a selection pressure of 5 % (i = 2.063) and using the heritability and genetic correlations estimated from our data sets (Figure 4). It was concluded that genetic selection in Marennes could improve total weight by 16.9% per generation. With the same selection pressure, total weight could be improved by 14.5 % for clams in Chioggia. If broodstock was selected in Marennes and the resulting cohort reared in Chioggia, the expected gain per generation should be +11.1 % and inversely + 6.3%. Based on the genetic parameters

and genetic correlations estimated in this study, selection in Marennes (+16.9% gain) would engender almost 20% more genetic gain in total weight in Marennes than in Chioggia (+14.5% gain). Our results also indicate that the rearing of clams in Chioggia in using spat from broodstock selected in Marennes (+11.1% gain) would limit potential genetic progress by only about 23 %, when compared to the potential genetic gain for selection and rearing in Chioggia (+14.5%). As mentioned previously, these first estimations of the genetic parameters should be considered with caution as the errors for our estimations are high, and different and more accurate conclusions may be drawn with the availability of more data.

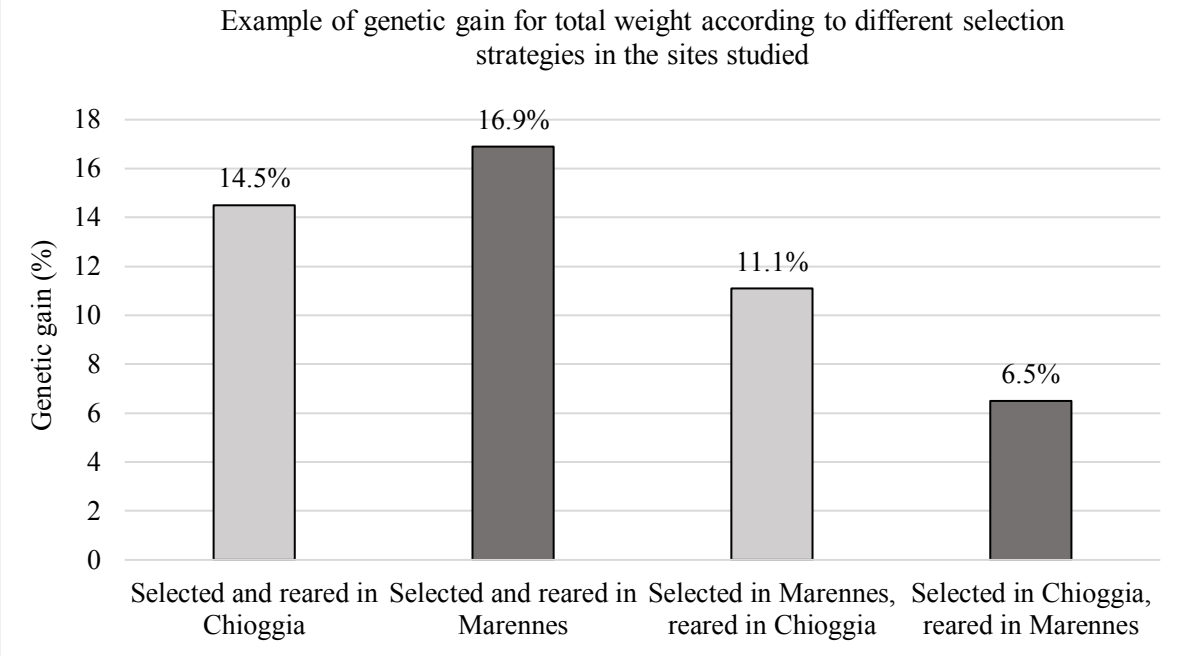

Figure 4: Based on the heritability and variance calculated for total weight in Chioggia and Marennes F1 clams, selection and rearing in the same site could amount to a 14.5% and 16.9% gain in total weight for Chioggia and Marennes, respectively. If clams were to be selected in Chioggia and then reared in Marennes, this would lessen the gain in total weight by 10.4%. If the situation were inverted and clams were selected in Marennes and then reared in Chioggia, the loss in potential total weight gain for clams in Chioggia would be less, going from 14.5% to 11.1%.

Heritability estimates for sex-ratio was high (0.42 ± 0.19), and it should be noted that this study provides the first evaluation of heritability of sex for the Manila clam. In the case of strict genetic determinism for this trait (i.e. XX/XY or ZZ/ZW), heritability is null as there is no variance in the number of sons or daughters produced by sires, dams, or families. Our estimated heritability suggests there is a variation in the number of sons and daughters produced by the parents, and one of the underlying reasons for this variation may be linked to environmental factors influencing the sex-ratio between parents or families. It should be noted that the Manila clam also has the peculiarity of Double Uniparental Inheritance (DUI) of mitochondrial DNA

(mtDNA), an inheritance model so far seemingly specific to a number of bivalve species, in which both male and female mtDNA is transferred to the offspring, and the resulting male and female offspring transfer the male or female mtDNA based on their respective sex [45–48]. While this reaches beyond the scope of the present study and was thus not investigated further, the sequencing of parental and offspring mtDNA may provide a complementary approach with which to investigate the genetic factors underlying sex determinism in this species. In addition, the models used in our study to estimate genetic parameters did not account for maternal or paternal effects, which may explain in part the high heritability for sex observed in our results.

Finally, heritability for parasite load with *P. olseni* was estimated at 0.52 ± 0.22, indicating high potential for genetic improvement regarding resistance to this disease. That said, resistance to disease was measured as the parasite load of individual clams after one year in the grow-out zone (Chioggia). This can be considered somewhat of an indirect measure of resistance, especially in the case of chronic infections such as Perkinsosis which, with time and given the right environmental conditions, are likely to affect up to 100% of the population. Another interesting result was the estimated genetic correlation between resistance and body weight which tended to be negatively correlated with biometric traits (length, weight, etc.), however the high standard error due to the lack of sufficient parentage assignments means that this result must be considered with caution. In addition, as body weight was not individually recorded at the beginning of the experiment we cannot differentiate between a real negative correlation between these two traits and an indirect effect in which diseased individuals suffer from lower growth rate. The existence of a negative genetic correlation between disease and growth may be quantified in the future by measuring the resistance capacity of clams selected for growth over several generations. Regardless, this study provides novel results that indicate high heritability associated with the level of infection by the parasite *P. olseni*. It provides promising results regarding the possibility of limiting the impact of this disease through selection, not only in European clam aquaculture but also more broadly in Asia, where mass infestation and mortality has previously been reported in China, Korea, and Japan [49–51].

This work remains the first estimate of genetic parameters in Manila clams reared in mixed-family batches. The results presented here indicate that selection for growth and resistance to *P. olseni* could be implemented today in hatcheries and has the potential to improve these traits of interest for the aquaculture sector.

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CHAPTER 3: A PROTEOMIC STUDY OF RESISTANCE TO BROWN RING DISEASE IN THE MANILA CLAM, *Ruditapes philippinarum*.

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A PROTEOMIC STUDY OF RESISTANCE TO BROWN RING DISEASE IN THE MANILA CLAM, *Ruditapes philippinarum*.

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ABSTRACT

Marine mollusk aquaculture has more than doubled over the past twenty years, accounting for over 15% of total aquaculture production in 2016. Infectious disease is one of the main limiting factors to the development of mollusk aquaculture, and the difficulties inherent to combating pathogens through antibiotic therapies or disinfection have led to extensive research on host defense mechanisms and host-pathogen relationships. It has become increasingly clear that characterizing the functional profiles of response to a disease is an essential step in understanding resistance mechanisms and moving towards more effective disease control. The Manila clam, *Ruditapes philippinarum*, is a main cultured bivalve species of economic subject to Brown Ring Disease (BRD), an infection induced by the bacterium *Vibrio tapetis*. In this study, juvenile Manila clams were subjected to a 28-day controlled challenge with *Vibrio tapetis*, and diagnosis was carried out to distinguish two extreme phenotypes: uninfected ("RES", resistant) and infected ("DIS", diseased) post-challenge. Total protein extractions were carried out for resistant and diseased clams, and proteins were identified using LC-MS/MS. Protein sequences were matched against a reference transcriptome of the Manila clam, and protein intensities based on label-free quantification were compared to reveal 49 significantly accumulated proteins in resistant and diseased clams. Proteins with known roles in pathogen recognition, lysosome trafficking, and various aspects of the energy metabolism were more abundant in diseased clams, whereas those with roles in redox homeostasis and protein recycling were more abundant in resistant clams.

Overall, the comparison of the proteomic profiles of resistant and diseased clams after a month-long controlled challenge to induce the onset of Brown Ring disease suggests that redox homeostasis and maintenance of protein structure by chaperone proteins may play important and interrelated roles in resistance to infection by *Vibrio tapetis* in the Manila clam.

1. INTRODUCTION

Mollusks represent over a fifth of the global aquaculture market, accounting for USD 29.2 billion in 2016 of which the most heavily traded species are oysters, clams, scallops, and mussels. Originating from the Asian Pacific coast, the Manila clam, *Ruditapes philippinarum* has become the second major cultured bivalve in the world, with over 4.4 million tons per year produced worldwide [1]. This species was introduced to the French Atlantic coast for aquaculture diversification in the 1970s [2], and following a rapid increase in clam production, mortality events became increasingly frequent and severe, eventually leading to the closure of many clam production parks [3]. The mass mortality episodes were subsequently associated to Brown Ring disease (BRD) [4,5], a chronic extra-pallial infection caused by *Vibrio tapetis*. After initial proliferation of the bacteria in the extra-pallial compartment, diseased clams manifest abnormal conchiolin deposits along the inner surface of the shell. In severe infections, the pathogen may cause lesions in the mantle and penetrate the hemolymph, in which case septicemia and death occur within 4-5 days [6,7].

In France, BRD continues to negatively impact production and prevalence can reach 80 - 100 % [8] along the Northern Atlantic coast. While the severity of the disease and the virulence of its etiological agent are known to be largely dependent on a number of environmental factors, namely temperature and salinity, bivalves have a number of sophisticated stress and immune response mechanisms, as well as a highly specific innate immune system on which they rely to directly combat infection [9,10]. As marine bivalves lack an adaptive immune system, the innate genomic component of their immune system plays an essential role in mitigating the host response to pathogens.

Invertebrate innate immunity relies on a number of pathogen recognition factors that trigger signaling pathways involved in hemocyte recruitment, phagocytosis, and the production of a wide range of antimicrobial compounds for host defense. While resistance to infection in bivalves initially depends on the ability of mucosal interfaces to impede pathogen entrance into the host, circulating hemocytes from fluids (such as hemolymph and extra-pallial fluids) and bioactive molecules in the plasma are responsible for mediating the secondary host response through phagocytosis and direct bacterial neutralization by antimicrobial effectors [11,12]. Interestingly, clams have been shown to recover from BRD through shell repair processes, leading to the

investigation of this resistant phenotype in several populations [13]. Previous gene expression and transcriptomic studies on the Manila clam have led to the assembly of a transcriptome and have shed light on several factors such as pathogen recognition and killing, modulation of hemocyte cytoskeleton, regulation of apoptosis, and bio-mineralization that are likely to play key roles in the innate immune response against bacterial infections [4–8,14–19]. The factors influencing virulence and highlighting the dynamics of the infection process leading to Brown Ring disease in the Manila clam are increasingly well described [8,20–23], and it is has become clear that the interactions between host and pathogen during infection leads to a complex remodeling of the molecular framework of both organisms, highlighting the importance of understanding the changes in gene expression as well as those occurring on the proteomic level. In addition, several studies focusing on *Vibrio*-induced expression of immune-related genes in *R. philippinarum* suggest a tendency towards a downregulation of the inflammatory response and an upregulation of genes related to homeostasis in this species, insisting on the importance of investigating the molecular mechanisms at play to identify markers of resistance [24,25]. The growing number of genomic and transcriptomic resources available for this host species have unveiled particularly high levels of polymorphism, a factor that may mitigate the observed functional variability in the immune response to pathogens [26,27], though to date there remains a significant knowledge gap surrounding the functional response of the Manila clam to infection with *Vibrio tapetis*, particularly in disease-resistant clams.

The present study aims to characterize the proteomic profiles of resistant and susceptible Manila clams following infection with *Vibrio tapetis*. By comparing the functional response in these two extreme phenotypes, we seek to shed light on the factors responsible for resistance to pathogens in this invertebrate species.

2. MATERIALS AND METHODS

2.1. Experimental design and sampling

Juvenile *R. philippinarum* (average shell length 12.37 mm) from a cohort of mixed families produced at the SATMAR hatchery (Marennes, France) were acclimated for 12 days in aerated seawater tanks at 14ºC. An experimental group (n=1200) was exposed to air for 8 hours at 14ºC to facilitate valve opening, then placed in a shallow tank and injected into the pallial cavity (without damaging the mantle epithelium) with 50 µL of *V. tapetis* suspension (strain CECT4600$_T$) prepared in filter-sterilized sea water (FSSW) containing 8.2 x10_6 bacteria.mL^{-1} (4.1 x 10_5 bacteria injected per clam). A first control group (C1; *n*=300) was injected with 50 µL of FSSW, and a second control group (C2; *n*=300) received no treatment. All clams (injected as well as not injected) were then kept in dry conditions for at least 6 hours to ensure that the injected clams retained the fluid, then returned to separate tanks in a thermoregulated room at 14ºC with no food and constant aeration for four weeks, according to a standardized protocol established by Paillard & Maes [28]. As BRD is a chronic infection localized in the extra-pallial compartment, injection into the pallial cavity mimics the natural infection process, whereas injecting tissues results in rapid septicemia for *V. tapetis* injections, or tissue disruption in the case of sterile filtered sea water injection into the tissues. Dead clams, when they occurred, were recorded daily and removed from the tanks. The seawater remained unchanged throughout the duration of the experiment. After the four-week incubation period, all clams were sampled for whole weight, shell weight, shell length, and each clam was individually opened with a cleaned scalpel over a tube, allowing whole tissues and fluids to be collected together, after which the sample was flash frozen in liquid nitrogen and stored at -80ºC.

2.2. Diagnostic methods

For visual diagnosis, shells were observed under a binocular magnifier to identify and quantify the extent index of Brown Ring disease according to the classification system described by Paillard & Maes (1994). For molecular diagnosis, DNA from whole-body tissue and fluid samples was analyzed. Briefly, the samples were homogenized in a volume of phosphate buffer saline (PBS; pH = 7) based on tissue weight, for a final concentration of 0.25 mg/µL tissue in the buffer. Ceramic beads were added to each sample and mechanical tissue disruption was done using 2 cycles of 20 seconds beating (10 sec. pause) at room temperature at 6.5 m/sec on a FastPrep-24 benchtop

homogenizer (MP-Bio). Total DNA extractions were carried out using 80 µL (eq. 20 mg) of the homogenate and the Nucleospin 96 Tissue Kit (Macherey-Nagel) according to the manufacturer's protocol with minor adaptations (see detailed protocol in supplementary file 1). The remaining homogenate was flash frozen and stored at -80ºC for subsequent protein extractions. PCR mix was prepared with 1 µL template DNA, 5 µL GoTaq G2 Flexi buffer, 0.15 µL GoTaq polymerase enzyme (1 U/µL), 0.5 µL dNTP mix (each 10 mM), 17.35 µL H_20, and 0.5 µL of forward and reverse primers specific to the *virB4* gene of the *V. tapetis* strain CECT4600T (final volume 25 µL, adapted from Bidault et al. [27b]). Initial denaturation was done at 94ºC for 5 min, followed by 40 cycles of denaturation (94ºC), annealing (54ºC), and extension (72ºC) for 30 seconds each, and a final extension step at 72ºC for 3 min. PCR products were deposited on 1% agarose gel and electrophoresis was carried out at 110 V for 45 min. In total DNA samples containing *V. tapetis* DNA, a 173 bp amplicon was then visible by fluorescence.

2.3. Total protein extraction and digestion

Based on both visual and molecular diagnoses, samples were assigned to one of four categories representative of the disease kinetics: BRD-/PCR- (0); BRD-/PCR+ (1); BRD+/PCR- (2); BRD+/PCR+ (3). Total proteins were extracted from three samples from category 0 and three samples from category 3 (presenting the same extent index of conchiolin deposit according to Paillard & Maes [28]), representing the extreme phenotypes hereafter referred to as "RES" (Category 0) and "DIS" (Category 3). Sample homogenates were defrosted on ice and 10 µL protease inhibitor mix (GE Healthcare) was added. After mixing by vortex, the samples were centrifuged at 15 000 x g for 10 min at 4ºC. The supernatant was transferred to a clean tube and proteins were quantified according to the Bradford method [29]. Based on protein concentration, the volume necessary for 50 µg of total proteins was transferred to a clean tube and volume was adjusted to 50 µL with an ammonium bicarbonate (AmBic) solution (100 mM). Samples were reduced with 5 µL dithiothreitol (10 mM) for 40 minutes at 56ºC, then alkylated with 10 µL iodoacetamide (20 mM) for 30 min in the dark. Protein digestion was carried out at 37ºC overnight with 10 µL trypsin buffer (0.1 µg/µL). After digestion, 5% formic acid was added and peptide samples were dehydrated using a SpeedVac™ concentrator.

2.4. LC-MS/MS analyses

Peptide quantification and identification was carried out through nano-LC MS/MS to allow for the comparison of the proteomic profiles of resistance to Brown Ring disease in the Manila clam.

2.4.1. Sample Preparation for Mass Spectrometry Analysis

For nano-LC fragmentation, peptide samples were first desalted and concentrated onto a µC18 Omix (Agilent) before analysis. The chromatography step was performed on a NanoElute (Bruker Daltonics) ultra-high pressure nano flow chromatography system. Peptides were concentrated onto a C18 pepmap 100 (5 mm x 300 µm i.d.) precolumn (Thermo Scientific) and separated at 50°C onto a Aurora reversed phase Reprosil column (25 cm x 75 µm i.d.) packed with 1.6 µm C18 coated porous silica beads (Ionopticks). Mobile phases consisted of 0.1% formic acid, 99.9% water (v/v) (A) and 0.1% formic acid in 99.9% ACN (v/v) (B). The nanoflow rate was set at 400 nl/min, and the gradient profile was as follows: from 2 to 15% B within 60 min, followed by an increase to 25% B within 30 min and further to 37% within 10 min, followed by a washing step at 95% B and reequilibration.

2.4.2. Mass Spectrometry Analysis

MS experiments were carried out on a TIMS-TOF pro mass spectrometer (Bruker Daltonics) with a modified nano electrospray ion source (CaptiveSpray, Bruker Daltonics). The system was calibrated each week and mass precision was greater than 1 ppm. A 1400 spray voltage with a capillary temperature of 180°C was typically employed for ionizing. MS spectra were acquired in the positive mode in the mass range from 100 to 1700 m/z. In the experiments described here, the mass spectrometer was operated in PASEF mode with exclusion of single charged peptides. A number of 10 PASEF MS/MS scans was performed during 1.25 seconds from charge range 2-5.

2.4.3. Peptide Sequencing and Protein Precursor Identification

The fragmentation pattern was used to determine the sequence of the peptide. Database searching was performed using the Peaks X software. A custom database was used, consisting in the translated sequences of loci from the digestive gland transcriptome of the Manila clam (unpublished data; a FASTA file containing loci sequences for significantly differentially accumulated proteins can be sound in supplementary file 3, with the corresponding annotations in

supplementary file 2). The variable modifications allowed were as follows: C-Carbamidomethyl, K-acetylation, methionine oxidation, and Deamidation (NQ). “Trypsin” was selected as Semispecific. Mass accuracy was set to 30 ppm and 0.05 Da for MS and MS/MS mode respectively. Data were filtering according to a FDR of 0.5% and the elimination of protein redundancy on the basis of proteins being evidenced by the same set or a subset of peptides.

2.5. Data analysis

Label-free quantitative data from Peaks X software were imported into Perseus in which statistical analyses were performed [30]. Data were log2-transformed and only proteins identified in every sample of at least one of the conditions tested were kept for further analysis. Data were then compared using a t-test between conditions “RES” and “DIS”, a threshold of significance of 0.05 was applied, below which proteins were considered as statistically differentially accumulated.

3. RESULTS AND DISCUSSION

3.1. Identification of RES vs DIS individuals following experimental infection

A major goal of this study was to compare the proteomic changes in *R. philippinarum* individuals, from a single population, showing contrasted susceptibilities to BRD. Overall, a low mortality of 2.4 % was observed during the four-week incubation period, as it often observed when *V. tapetis* is injected into the pallial fluids. Moreover, this low mortality occurred mostly on days 5-6, as was previously described by authors which suggested this is due to septicemia following accidental injections of *V. tapetis* in the tissues [31]. The highest mortality per group (5.5 %) occurred in group C1 (inoculated with FSSW), whereas group C2 (no treatment) and the experimental group (inoculated with *V. tapetis*) showed 1.8 % and 1.9 % total mortality, respectively, comparable to the results obtained in other BRD studies [13]. Shell length (12.37±0.14 mm) and total weight (0.411±0.011 g) were measured for all individuals, and dual diagnosis was carried out for 430 experimental clams. Dual diagnosis of the experimental clams showed that 59 % showed varying degrees of conchiolin deposits, and 42 % were PCR-positive for the strain-specific *virB4* 173 bp amplicon. Overall, the experimental population was relatively evenly distributed in the four categories (Table 1). Control groups C1 (n=66) and C2 (n=49) showed conchiolin deposits in 1.5 % and 2 % of individuals, respectively, and none of the control samples were positive for molecular diagnosis.

The four diagnostic categories in which the experimental clams were placed represent four stages in the infection process as it occurs within the extra pallial compartment, summarized in Figure 1. Clams from the two extreme phenotypes, i.e. category 0 (BRD-/PCR-) and category 3 (BRD+/PCR+), were chosen for the following proteomic study.

3.2. Differential shotgun proteomics of resistant (RES) vs diseased (DIS) clams

In all, we could identify 2093 proteins, of which 2021 were present in at least 2 out of 3 samples in one or both condition(s) (termed “RES” for category 0/resistant and “DIS” for category 3/diseased). Spectral counts were used to calculate the relative abundance of proteins. A Student T-test was used to identify proteins for which abundance was significantly modified in either of the two categories, yielding a list of 102 proteins significantly more abundant in either RES or DIS clams ($p\text{-}val < 0.05$; a complete list of these proteins can be found in the supplementary file 2, and

a FASTA file containing the corresponding sequences of the loci can be found in supplementary file 3). Of these, 49 proteins had a fold-change of at least 1.5: seventeen proteins were accumulated in the RES group and thirty-two proteins in the DIS group, four of which could not be characterized (*C. gigas* protein IDs: EKC23703, EKC34161, EKC41442, EKC37917). These 49 proteins are presented in Figure 2 and their annotations are further detailed in Table 2; they were functionally annotated by examining their associated COG categories, biological process GO terms, and literature review, and discussed below according to their potential roles in different aspects of Brown Ring disease, i.e. the "Immune response", "Energy production" and "Protein metabolism".

Table 1: Dual diagnosis through visual inspection of the inner surface of both valves (BRD+ or BRD-) and PCR amplification of the virB4 gene region of 173 bp (PCR+ or PCR-) allows for distinction between 4 categories, ranging from CAT 0 (uninfected post-challenge, termed "RES") to CAT 3 (infected according to both diagnostic methods, termed "DIS"). The 430 samples tested show that 24 % and 26 % of samples fall in CAT 0 and CAT 3, respectively. The link between these categories and the kinetics of infection in the extra pallial compartment with *Vibrio tapetis* is described in Figure 1.

DIAGNOSTIC CATEGORIES:

Total experimental clams n= 430	**BRD-**	**BRD+**
PCR-	**CAT 0** *n= 105*; 24%	**CAT 2** *n= 142*; 33%
PCR+	**CAT 1** *n= 70*; 16%	**CAT 3** *n= 113* ; 26%

1

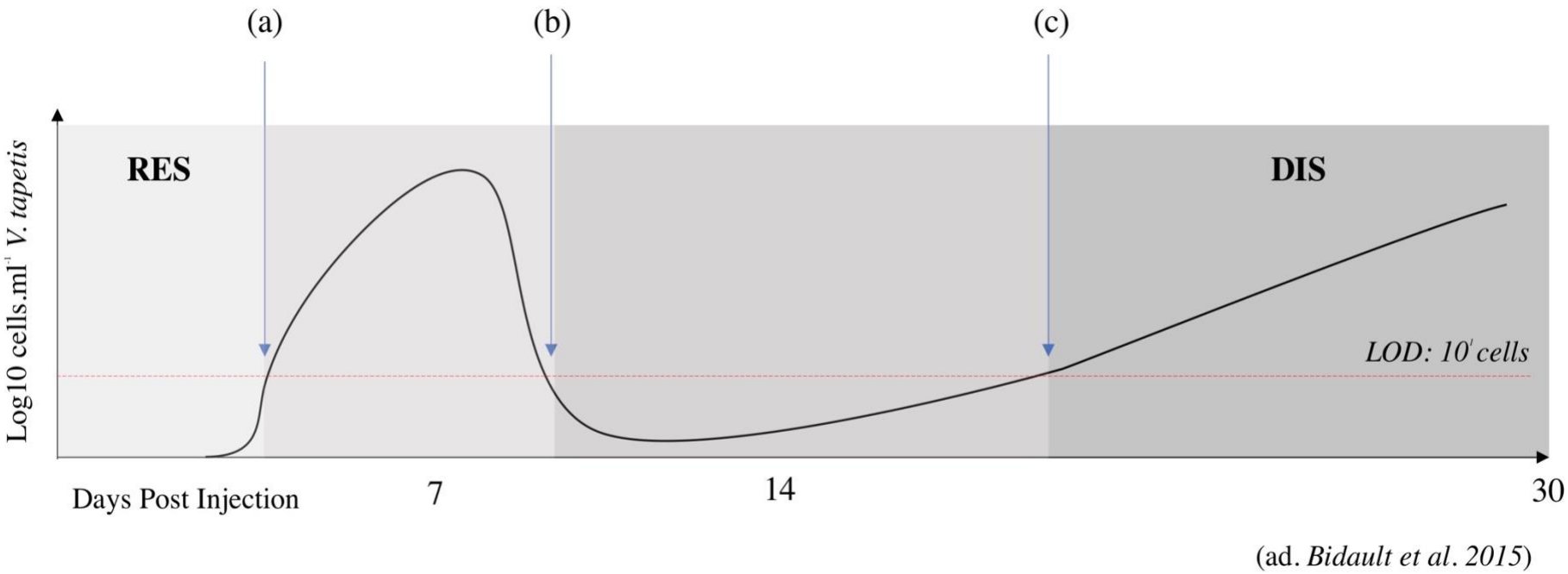

Figure 1: Schematic representation of the kinetics of Brown ring disease development during 30 days post injection (DPI) in a controlled challenge, adapted from Bidault et al. 2015, showing concentration of V. tapetis in extrapallial fluids (log10 cells.mL-1) and limit of detection (LOD) at 1x101 cells. "RES" clams are negative for both visual and molecular diagnosis. Bacteria enter the extrapallial compartment and become quantifiable at point (a), then proliferate rapidly (clams at this stage are termed “CAT 1” in our study), with highest concentrations generally observed around 7 DPI. The host then begins to trap the bacteria within the conchiolin deposits characteristic of Brown ring disease, thus leading to a decrease in the concentration of bacteria in the extrapallial compartment (clams at this stage are termed “CAT 2” in our study), represented by point (b). Clams can thus be positive for the visual diagnosis and negative for molecular diagnosis whilst the bacteria remain trapped against the inner surface of the shell, a process during which the host attempts to recalcify over the bacterial biofilm. In the case of “DIS” clams, conchiolin deposits are present but insufficient in limiting the pathogen, which will continue to proliferate (point (c), also termed “CAT 3” for shit study) and reach high concentrations in the extrapallial compartment once more.

2

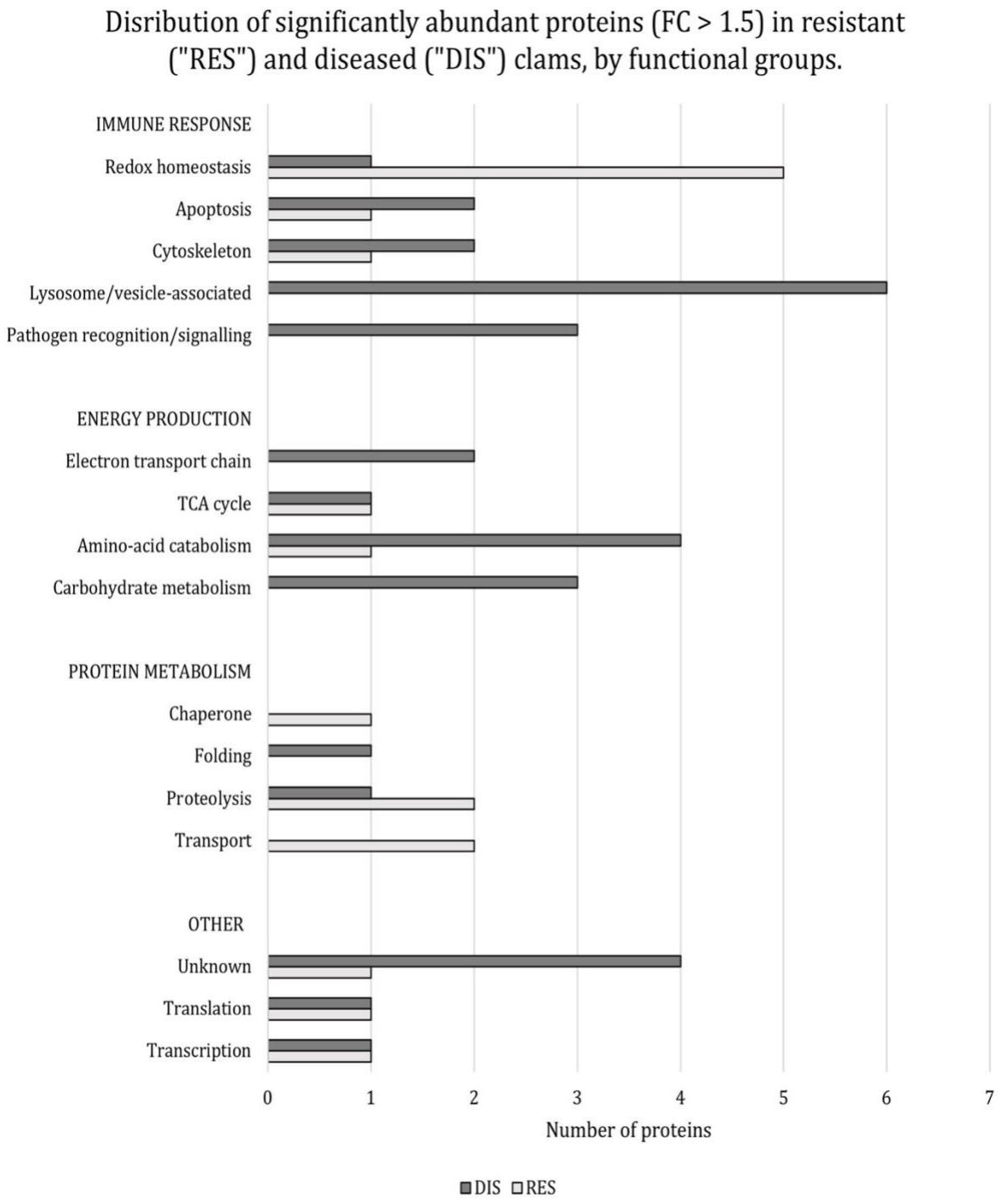

Figure 2: Number of proteins per functional group (i.e. “immune response”, “energy production”, “protein metabolism”) that were more abundant in DIS clams (dark bars) and in RES clams (light bars).

Table 2: Proteins significantly abundant (p-val < 0.05) in DIS and RES clams, with fold-change > 1.5 ("group ratio"). Based on Clusters of Orthogous Groups (COG), Biological Process GO terms, and literature review regarding the roles of these proteins in the context of disease, the proteins are grouped into three main functional roles described in the discussion; “Immune response”, “Energy production”, and “Protein metabolism”.

DIS Intensity	RES Intensity	Group Ratio+	Student's T-test p-value	Unique peptides	*R. philippinarum* locus ID	*C. gigas* protein ID	Protein name	COG	*C. gigas* gene description	Biological process GO term	Functional role discussed
1440	948	1.51:1.00	0.002	3	Locus 6868673	EKC40501	LGALS9	W	Galectin-9	GO:0006954 inflammatory response	Immune response - pathogen recognition
1250	421	2.97:1.00	0.041	1	Locus 5288240	EKC31577	BGBP	G	Beta-1,3-glucan-binding protein 1	GO:0002752 cell surface pattern recognition receptor signaling pathway	Immune response - pathogen recognition
384	244	1.57:1.00	0.026	1	Locus 4334179	EKC24393	C3	O	Complement C3	GO:0006955 immune response	Immune response - signalling
647	1770	2.73:1.00	0.005	3	Locus 615620	EKC23268	IQGAP1	Z	Ras GTPase-activating-like protein	GO:1903829 positive regulation of cellular protein localization	Immune response - cytoskeleton
1270	832	1.53:1.00	0.025	3	Locus 2955238	EKC27178	DCTN2	Z	Dynactin subunit 2	GO:0006888 endoplasmic reticulum to Golgi vesicle-mediated transport	Immune response - cytoskeleton
14900	4540	3.29:1.00	0.018	9	Locus 7235177	EKC29122	SCP	S	Sarcoplasmic calcium-binding protein	GO:0051480 regulation of cytosolic calcium ion concentration	Immune response - cytoskeleton
3200	2010	1.59:1.00	0.025	4	Locus 2215912	EKC27269	CPVL	O	Putative serine carboxypeptidase CPVL	GO:0051603 proteolysis involved in cellular protein catabolic process	Immune response - lysosomal
910	486	1.87:1.00	0.005	1	Locus 2384090	EKC25290	EPDR1	S	ependymin related 1	GO:0007160 cell-matrix adhesion	Immune response - lysosomal
903	446	2.03:1.00	0.045	1	Locus 2964637	EKC26355	OVCH1	O	Ovochymase-1	GO:0006508 proteolysis	Immune response - lysosomal
2660	531	5.02:1.00	0.029	2	Locus 3062641	ENSDARP00000139466*	PPT1	S	palmitoyl-protein thioesterase 1	GO:0007042 lysosomal lumen acidification	Immune response - lysosomal
680	374	1.82:1.00	0.014	1	Locus 665720	EKC31469	RAB43	U	Ras-related protein Rab-43	GO:0090382 phagosome maturation	Immune response - vesicles

1780	716	2.49:1.00	0.027	6	Locus 2853765	EKC32573	VPS35	U	Vacuolar protein sorting-associated protein 35	GO:0007040 lysosome organization	Immune response - vesicles
296	2550	8.61:1.00	0.003	3	Locus 6888709	EKC26119	ACE	C	Angiotensin-converting enzyme	GO:0001817 regulation of cytokine production	Immune response - redox
323	669	2.07:1.00	0.019	1	Locus 2057997	EKC35339	TXNDC5	O	Thioredoxin domain-containing protein 5	GO:0045454cell redox homeostasis	Immune response - redox
4480	8140	1.82:1.00	0.045	3	Locus 355976	EKC36585	ACE	E	Angiotensin-converting enzyme	GO:0001817 regulation of cytokine production	Immune response - redox
152	245	1.61:1.00	0.048	1	Locus 4589062	EKC37227	ADH5	Q	Alcohol dehydrogenase class-3	GO:0051775 response to redox state	Immune response - redox
399	638	1.60:1.00	0.036	1	Locus 2688502	EKC36531	XDH	F	Xanthine dehydrogenase/oxidase	GO:2000379 positive regulation of reactive oxygen species metabolic process	Immune response - redox
2250	783	2.87:1.00	0.006	2	Locus 6590720	EKC20036	GPX3	O	Glutathione peroxidase	GO:0034599 cellular response to oxidative stress	Immune response - redox
448	753	1.68:1.00	0.003	2	Locus 3201607	EKC33267	SH3GLB1	T	Endophilin-B1	GO:0006915 apoptotic process	Immune response - apoptosis
1030	593	1.73:1.00	0.007	1	Locus 1202748	EKC29685	PPP3CC	T	Serine/threonine-protein phosphatase	GO:0035970 peptidyl-threonine dephosphorylation	Immune response - apoptosis
749	351	2.13:1.00	0.002	2	Locus 1979842	EKC21473	CECR1	F	Adenosine deaminase CECR1	GO:0006154 adenosine catabolic process	Immune response - apoptosis
3500	2120	1.65:1.00	0.036	1	Locus 1236277	EKC20480	AGL	G	Glycogen debranching enzyme	GO:0005980 glycogen catabolic process	Energy production – carbohydrate metabolism
2860	1720	1.67:1.00	0.007	3	Locus 8380444	EKC18570	PGM1	G	Phosphoglucomutase-1	GO:0006006 glucose metabolic process	Energy production - carbohydrate metabolism
11100	6930	1.60:1.00	0.037	7	Locus 381335	EKC27095	PCK2	C	Phosphoenolpyruvate carboxykinase [GTP]	GO:0006094 gluconeogenesis	Energy production - carbohydrate metabolism
1130	1770	1.57:1.00	0.031	2	Locus 2128190	EKC32958	AUH	I	Methylglutaconyl-CoA hydratase	GO:0006552 leucine catabolic process	Energy production - AAs
581	298	1.95:1.00	0.013	1	Locus 2982362	EKC42273	SLC1A1	E	Excitatory amino acid transporter 1	GO:0006537 glutamate biosynthetic process	Energy production - AAs

6150	1990	3.09:1.00	0.041	10	Locus 8587681	EKC33186	GPT2	E	Alanine aminotransferase 2	GO:0042851 L-alanine metabolic process	Energy production - AAs
21400	6060	3.54:1.00	0	19	Locus 2044774	EKC40669	GOT1	E	Aspartate aminotransferase, cytoplasmic	GO:0006107 oxaloacetate metabolic process	Energy production - AAs
4660	2820	1.65:1.00	0.034	1	Locus 7730822	EKC43060	PCCB	EI	Propionyl-CoA carboxylase beta chain	GO:0006552 leucine catabolic process	Energy production - AAs
9940	24000	2.41:1.00	0.038	8	Locus 4823168	EKC21276	PDHA1	C	Pyruvate dehydrogenase E1 subunit alpha	GO:0006086 acetyl-CoA biosynthetic process from pyruvate	Energy production - TCA
38700	24600	1.57:1.00	0.001	26	Locus 2348137	EKC25158	MDH1	C	Malate dehydrogenase	GO:0006099 tricarboxylic acid cycle	Energy production - TCA
1120	662	1.69:1.00	0.026	1	Locus 5269440	EKC19854	REBM	I	3-demethylubiquinone-9 3-methyltransferase	GO:0006744 ubiquinone biosynthetic process	Energy production - ETC
42700	24200	1.77:1.00	0.041	34	Locus 4313121	EKC39329	ATP5A1	C	ATP synthase subunit alpha	GO:0006754 ATP biosynthetic process	Energy production - ETC
1190	2290	1.92:1.00	0.049	3	Locus 1397283	EKC35325	TTN	T	Titin	GO:0007155 cell adhesion	Protein metabolism - transport
1940	2980	1.54:1.00	0.041	3	Locus 5145028	EKC29146	YWHAE	O	14-3-3 protein epsilon	GO:0035556 intracellular signal transduction	Protein metabolism - transport
1600	3590	2.24:1.00	0.034	10	Locus 3208485	EKC19309	CAND1	S	Cullin-associated NEDD8-dissociated protein 1	GO:0016567 protein ubiquitination	Protein metabolism – proteolysis
498	1630	3.27:1.00	0.044	2	Locus 4231109	EKC28114	PSMC1	O	26S proteasome non-ATPase regulatory subunit 7	GO:0000209 protein polyubiquitination	Protein metabolism - proteolysis
440	235	1.88:1.00	0.035	1	Locus 4900110	EKC29780	PSMD7	O	26S protease regulatory subunit 4	GO:0000209 protein polyubiquitination	Protein metabolism - proteolysis
4860	2510	1.94:1.00	0.012	4	Locus 2922613	EKC25378	FKBP14	O	FK506 binding protein 14	GO:0046716 muscle cell cellular homeostasis	Protein metabolism - protein folding
2550	4140	1.62:1.00	0.014	16	Locus 2939806	EKC25687	HSP90AB1	O	Heat shock protein HSP 90-alpha 1	GO:0050821 protein stabilization	Protein metabolism - chaperone

633	1200	1.90:1.00	0.048	2	Locus 2389731	EKC42074	PCBP3	A	Poly(RC)-binding protein 3	GO:0000122 negative regulation of transcription by RNA polymerase II	Other - transcription
1300	563	2.31:1.00	0.023	1	Locus 995640	EKC39351	PURA	K	Transcriptional activator protein Pur-alpha	GO:0006268 DNA unwinding involved in DNA replication	Other - transcription
1860	2940	1.58:1.00	0.003	1	Locus 453724	EKC20816	NARS	J	Asparaginyl-tRNA synthetase	GO:0006421 asparaginyl-tRNA aminoacylation	Other - translation
1300	752	1.73:1.00	0.024	2	Locus 454248	EKC31246	RPL27A	J	60S ribosomal protein L27a	GO:0006412 translation	Other - translation
818	1560	1.91:1.00	0.021	2	Locus 3238632	EKC23703	-	-	Uncharacterized - Calycin superfamily	- - -	Other - unknown
584	357	1.64:1.00	0.018	1	Locus 4959443	EKC41849	ABHD14A	S	Abhydrolase domain-containing protein 14A	GO:0006656 phosphatidylcholine biosynthetic process	Other - unknown
670	396	1.69:1.00	0.033	1	Locus 1004421	EKC34161	-	-	si:ch73-250a16.5	- - -	Other - unknown
3520	1910	1.85:1.00	0.042	6	Locus 6596965	EKC41442	-	-	Uncharacterized - SH3-like domain	- - -	Other - unknown
1060	350	3.03:1.00	0.028	3	Locus 4498780	EKC37917	-	-	Uncharacterized - Carbohydrate esterase 4 (CE4) family	- - -	Other - unknown

✝ Ratios are presented as RES:DIS for those more abundant in RES clams, and as DIS:RES for those more abundant in DIS clams.

* Danio rerio protein ID and protein description

3

3.3. IMMUNE RESPONSE-ASSOCIATED PROTEINS

3.3.1. *Pathogen recognition and immune-pathway activation*

The ongoing infection process in clams from the DIS group is supported by the presence of a number of proteins whose primary functions are associated with pathogen recognition and the subsequent triggering of signaling pathways, such as Galectin-9 (Gal9), 1-3-β-glucan-binding protein (BGBP), and Complement component C3 (C3). Proteins such as Galectins, which have previously been demonstrated as upregulated in the extra pallial fluids of *V. tapetis*-infected clams, and BGBP act as pattern recognition receptors (PRRs) by recognizing β-galactoside and lipopolysaccharide residues found on bacterial membranes [18,32,33]. This initiates the immune response by activating signaling pathways for chemotaxis, phagocytosis, and opsonization and induces antimicrobial peptide (AMP) synthesis through the prophenoloxidase and complement cascade systems [34,35]. Interestingly, selective breeding for parasite resistance in the Sydney rock oyster found that resistance was directly related to the loss of a specific form of phenoloxidase enzymes, supporting the presence of this particular enzyme as a marker of susceptibility [36,37]. The C3 protein identified in our dataset belongs to the complement cascade system, an essential component of the invertebrate immune response leading to the opsonization and lysis of pathogens [38–41]. Upregulation of proteins associated to the complement system have previously been described in *R. philippinarum* through several transcriptomic studies investigating response to disease, highlighting the importance of this pathway in host response to *Vibrio* pathogens [18,24]. The significantly high abundance of proteins specifically involved in pathogen recognition in the DIS group supports a strong acute response to the ongoing infection with *V. tapetis* in these animals.

Interestingly, the RES clam group showed high abundance of Ras GTPase-activating-like protein 1 (IQGAP1), a pathway-activating protein that is implicated in a number of immune-associated functions. Namely, during infection by microbial pathogens that target the host microtubule network, similar to the way in which *V. tapetis* inhibits pseudopod formation in the hemocytes of *R. philippinarum*, IQGAP1 has been shown to bind to and modulate the activity of proteins involved in bacterial invasion, ultimately interacting with the actin cytoskeleton [19,42].

3.3.2. *Pathogen-associated lysosomal activity*

Other proteins highly abundant in the DIS group are involved in immune response through antimicrobial and lysosomal activity. The putative serine carboxypeptidase (CPLV) and the serine protease Ovochymase (OVCH1) identified in the DIS group are known to have antibacterial activity and can be involved in proteolytic digestion of lysosomal components [43,44]. As lysosomes contain the hydrolytic enzymes necessary for degradation of cellular components as well as encapsulated pathogenic agents, it is also interesting to note the higher abundance of Ependymin-related protein 1 (EPDR1) in DIS samples, which has been suggested to function as a lysosomal activator protein, and Palmitoyl-protein thioesterase-1 (PPT1), which is associated with lysosomal degradation of proteins [45,46]. An uncharacterized protein in the DIS clam group belonging to the carbohydrate esterase 4 (CE4) family may also play a role in degrading phagocytosed bacteria as well as inhibiting biofilm formation, as certain enzymes of the CE4 family, whose main function is to de-acetylate polysaccharides, specifically degrade the essential peptidoglycan polymers of bacterial cell walls [47]. In addition, active intracellular membrane trafficking (ex. phagosomes) in DIS clams is suggested by the high abundance of proteins such as the Ras-related protein RAB43, which regulates vesicular movement following immune system activation by microbial infections, and a vacuolar-sorting protein VPS35, which directs transmembrane cargo proteins to the lysosomal degradation pathway [48,49].

3.3.3. *Cytoskeleton-associated immune response*

The cytoskeleton is a network of filaments that plays an essential role in certain aspects of immunity through cell structure maintenance, transport, phagocytosis, and communication between cellular components. As such, it is also known to be one of the targets of invading pathogens such as *Perkinsus olseni* and *V. tapetis* in the Manila clam [19,50,51]. Interestingly, a dynactin subunit (DCTN2) and a sarcoplasmic calcium-binding protein (SCP) were highly abundant in DIS clams, both of which play a role in cytoskeleton function. DCTN2 is part of a dynein/dynactin complex which coordinates the microtubule movement of vacuoles towards lysosomes and plays an important role in the biogenesis and transportation of pathogen-containing vacuoles in rabbit cells infected by obligate intracellular bacteria [52,53]. This may reflect the internalization of *V. tapetis* by hemocytes in the case of DIS clams, activating microtubule motors such as the dynein/dynactin complex in an attempt to fuse bacteria-containing vacuoles with

lysosomal membranes. SCPs, which are known to interact with the cytoskeleton by regulating the calcium balance, have been shown to be upregulated in Manila clam hemocytes in response to both parasitic and bacterial infections [18,22,50]. Bacteria of the genus *Vibrio* have been suggested to inhibit intracellular trafficking and the fusion of bacteria-containing phagosomes with lysosomes in order to avoid neutralization, a relatively common immune-evasion technique seen in a number of pathologies [54]. In this light, the elevated abundance of lysosome-associated proteins in clams of the DIS group may reflect an attempt to counter the bacteria's inhibitory processes.

3.3.4. *Apoptotic processes*

Apoptosis of host immune cells during an infection can represent one of the ultimate defense strategies against invading pathogens, whereby the host cell is sacrificed so as to eliminate the internalized pathogenic agent [55]. Induction of apoptosis has been suggested to be one of the mechanisms of survival put in place by resistant oysters, *Ostrea edulis*, perhaps in response to anti-apoptotic mechanisms that are a known survival strategy for some intracellular pathogens, such as the protozoan parasite *Bonamia ostreae* [56]. Clams from the RES group in our study demonstrated higher abundances of Endophilin B1 (Bif-1), a protein associated with the formation of pores in the outer mitochondrial membrane that leads to apoptosis through the caspase pathway [56–58]. In contrast, DIS clams showed high abundance of adenosine deaminase (CECR1) a protein that, while primarily associated with the purine metabolism, also plays an important role in reducing the concentration of the toxic derivatives of adenosine and deoxyadenosine to protect cells from apoptosis [59,60]. A serine/threonine protein phosphatase PPP3CC, also more abundant in DIS clams, bears close resemblance with the protein phosphatase 3 catalytic subunit beta, PPP3CB, which was recently suggested to promote cell-proliferation and may play an anti-apoptotic role in tumorous human kidney cells [61]. While apoptosis is a complex process that can benefit immune defense, anti-apoptosis is also a mechanism by which the host may maintain cellular functions and continue combatting infection, though seemingly at a cost given the relatively high abundance of proteins involved in energy metabolism in the DIS clam group (see part 3.4).

3.3.5. *Redox homoeostasis*

Host production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) is a known mechanism of defense and pathogen neutralization, though this process requires a delicate balance

as the accumulation of ROS/RNS can also lead to oxidative damage to the host [62]. Glutathione peroxidase (GPX3), a member of the cellular antioxidant system, was more abundant in the DIS clam group. Interestingly, a previous studies on BRD-infected Manila clams reported a decrease in enzymatic activity of GPX at 7 dpi, though the study did not measure enzyme abundance nor activity at 30 dpi [21]. Oysters subjected to bacterial infection, however, have shown an upregulation of glutathione peroxidase gene expression in pathogen-challenged larvae, suggesting that GPX may be an indicator of oxidative stress that may have been occurring in DIS clams from our study as a result of the ongoing antibacterial processes discussed in part 3.3.2 [21,63].

The RES clam group was characterized by a higher abundance of proteins more or less directly associated with ROS/RNS production and scavenging. Thioredoxin domain-containing protein 5 (TXNDC5) is a member of the protein disulfide isomerase family whose expression has been associated with oxidative stress and cellular pathology [64,65]. At least two shorter-term (max 7 days) studies have investigated the role of thioredoxin in response to *V. tapetis*, pointing towards an increase in activity of thioredoxin in *V. tapetis*-challenged clams compared to controls or to resistant clams, thus the higher abundance of thioredoxin observed in RES clams of this study may reflect a possible reversal of this mechanism in the case of long-term resistance (30 days) [21,66]. Similarly, xanthine oxidoreductase (XOR), an enzyme implicated in the purine metabolism, has previously been associated with a number of innate immunity processes including redox homeostasis through the production of ROS and RNS by xanthine oxidase (XO), as well as indirect free radical sequestration by xanthine dehydrogenase (XD) through the synthesis the antioxidant uric acid [62,67–69]. RES clams also presented a significantly higher abundance of two Angiotensin-converting enzymes (ACE), which were recently demonstrated to induce superoxide production and thereby enhance bactericidal activity in mouse neutrophils [70], and class-III alcohol dehydrogenase (ADH3). ADH3 uses the reducing power glutathione to eliminate the organic compounds formaldehyde and S-nitrosothiols (SNO), ultimately limiting their decomposition into the RNS nitric oxide [71,72]. Nitric oxide is a known toxic agent produced by immune cells of invertebrates for pathogen defense, and NO production by *R. philippinarum* in response to *V. tapetis* has been correlated to hemocyte rounding and pseudopod loss [73], highlighting the possible importance of NO-sequestration by proteins such as ADH3 in order to counter the negative effects of this compound on the host.

These findings support previous hypotheses suggesting that ROS/RNS production by hemocytes may represent an alternative anti-bacterial response against pathogens such as *V. tapetis*, which is known to evade host immune responses such as phagocytosis by inhibiting pseudopod formation [73,74]. In addition, the higher abundance of the redox homeostasis-associated enzymes RES clams supports findings from previous studies indicating that resistance may be associated with a greater ability to balance ROS/RNS production and scavenging, allowing them to rapidly neutralize pathogens before they are able to colonize the host while simultaneously protecting host cells from oxidative damage, a dual process previously suggested using enzyme activity assays in the Manila clam exposed to *V. tapetis* [21].

3.4. PROCESSES ASSOCIATED WITH ENERGY PRODUCTION

Proteins potentially associated with energy production through carbohydrate and amino acid catabolism represent the second largest group in our dataset, most of which (10 out of 12) are more abundant in the DIS clam group. This section groups together the proteins implicated in the degradation of glycogen and amino acids that generate essential metabolites for the tricarboxylic acid (TCA) cycle, leading to the production of high-energy electron donors such as NADH which can integrate the electron transport chain (Figure 3).

3.4.1. *Amino acid degradation for energy production*

Propionyl-CoA carboxylase beta chain (PCCB) and Methylglutaconyl-CoA hydratase (AUH), enzymes involved in the degradation of branched-chain amino acids (BCAAs) such as leucine, were significantly more abundant in DIS and RES clams, respectively. Demand for BCAAs as energy metabolites or for the synthesis of immune-related molecules has been demonstrated to increase during disease [75,76]. Decreased levels of a number of other amino acids during *Vibrio* infection in mussels have also been reported, suggesting that the significantly higher abundance of Alanine aminotransferase (GPT2), Aspartate aminotransferase (GOT1), and Excitatory amino-acid transporter-1 (SLC1A1) in DIS clams may also be associated with the degradation and/or conversion of amino acids into metabolites such as pyruvate, oxaloacetate, and α-ketoglutarate for the TCA cycle [77–80].

3.4.2. *Carbohydrate metabolism and TCA cycle*

Our dataset showed a high abundance of proteins linking glycolysis with the TCA cycle for the aerobic production of ATP such as Pyruvate dehydrogenase (PDH) E1 subunit (alpha type III) in RES clams, as well as Glycogen debranching enzyme (AGL) and Phosphoglucomutase-1 (PGM1) in DIS clams, which may reflect the degradation of carbohydrates for the production of pyruvate, namely in the DIS clam group [81]. This group is also characterized by a greater number of proteins associated with various aspects of the immune response, a process known to be energetically demanding [82]. More importantly, weight loss, decreased glycogen reserves, and condition index, which are indicative of energy imbalance and poor health, have previously been attributed to the negative impact of BRD on energy balance in the Manila clam [8,83,84]. Malate dehydrogenase (MDH1), which participates in the TCA cycle by oxidizing malate to form oxaloacetate, was also more abundant in DIS clams, supporting the hypothesis of increased carbohydrate degradation for energy production. Our dataset also indicated a significantly higher abundance of the mitochondrial Phosphoenolpyruvate carboxykinase (PEPCK-M) in the DIS group, which is generally associated with catalyzing the irreversible conversion of oxaloacetate to phosphoenolpyruvate (PEP) for gluconeogenesis. While this appears to contradict the hypothesis of glycogen and glucose degradation, recent studies have demonstrated that overexpression of PEPCK-M (as opposed to the cytosolic form PEPCK-C) may play a role in antiviral immunity in insects, and the accumulation of PEP in infected ticks was suggested to be an antibacterial mechanism against the bacterial pathogen *Anaplasma phagocytophilum* [85,86].

3.4.3. *Electron transport chain*

The end products of the TCA cycle ultimately convey their electrons to the electron transport chain (ETC), composed of molecules within the inner membrane of the mitochondria, including the essential proteins ubiquinone (coenzyme Q10) and ATP synthase. In the DIS group, the high abundance of 3-demethylubiquinone-9 3-O-methyltransferase, which participates in the final step of ubiquinone synthesis, suggests that there is a high demand for electron acceptor molecules which increase the proton gradient in the inter membrane space of the mitochondria [87].

1

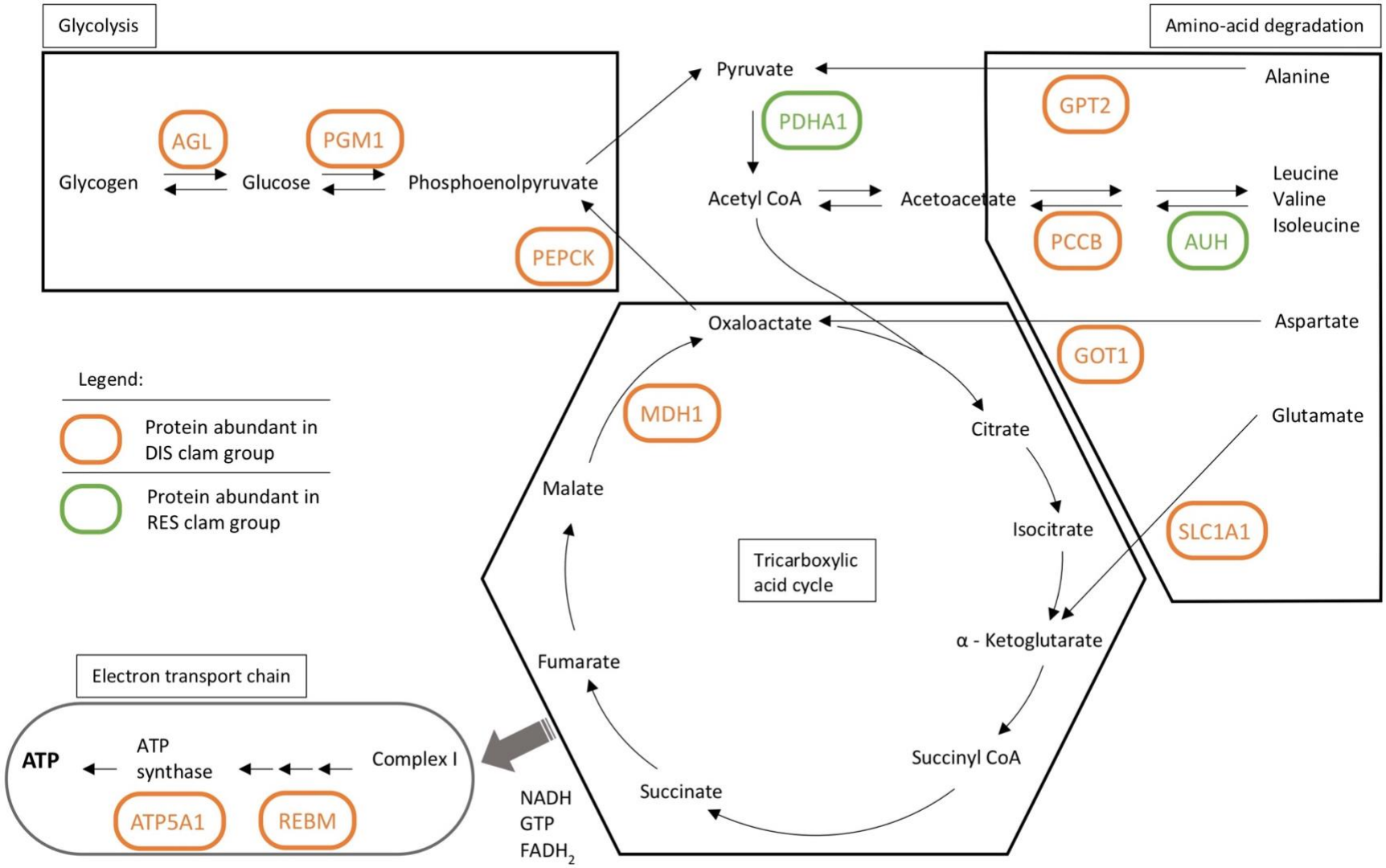

Figure 3: Schematic representation of energy production and the carbohydrate and amino acid metabolisms, including glycolysis, the TCA cycle, amino-acid degradation pathways, and the electron transport chain. The processes in which highly abundant proteins in DIS (orange) and RES (green) clams are implicated are annotated with the abbreviated protein name. Glycogen debranching enzyme (AGL), Phosphoglucomutase-1 (PGM1), and Phosphoenolpyruvate carboxykinase (PEPCK) are associated to the carbohydrate metabolism (both glycolysis and gluconeogenesis). Alanine aminotransferase (GPT2), Propionyl-CoA carboxylase beta chain (PCCB), Methylglutaconyl-CoA hydratase (AUH), Aspartate aminotransferase (GOT1), and Excitatory amino-acid transporter-1 (SLC1A1) all participate in the degradation of amino acids that can play a role in replenishing metabolites of the TCA cycle. Malate dehydrogenase 1 (MDH1) is an essential enzyme of the TCA cycle, the high-energy products of which are then shuttles to the electron transport chain where proteins such as 3-demethylubiquinone-9 3-methyltransferase (REBM) and ATP synthase subunit alpha (ATP5A1) participate in the production of ATP.

Overall, proteins associated with the energy metabolism, including ATP synthase subunits and ETC-associated proteins, as well as proteins associated with energy production from both amino acids and sugars, are more abundant in the DIS group than in the RES group, reflecting a high energy demand likely as a result of the active immune response described previously.

3.5. PROTEIN METABOLISM

Seven highly abundant proteins in the dataset were associated with various aspects of protein metabolism, namely proteolysis, transport, and chaperones.

3.5.1. *Proteolysis*

Proteolytic processes are represented in our dataset by the proteins cullin-associated NEDD8-dissociated protein 1 (CAND1) and subunit 4 of the 26S proteasome complex (PSMC1) in the RES group, and by the 26S proteasome non-ATPase regulatory subunit 7 (PSMD7) in the DIS group (Table 2). In eukaryotic cells, proteolysis, or the degradation of proteins and recycling of their components, is a process mediated by the conjugation of polyubiquitin chains to proteins which are then recognized by the 26S proteasome complex, a multi-subunit enzyme responsible for proteolysis [88]. CAND1 binds to unneddylated CUL1, one of the three major components of an E3 ubiquitin ligase playing an essential role in protein degradation by regulating ligase ubiquitination [89]. Interestingly, ubiquitin ligase complexes were found to be one of the targets of pathogenic bacteria during infection, whereby inhibiting factors produced by pathogens may be able to effectively bind to CUL1, preventing it from correctly forming the ligase complex [90]. PSMC1 coordinates substrate recruitment and translocation into the proteolytic chamber of the proteasome, and is essential for rapid proteolysis [91]. Similarly, PSMD7 is another component of the proteasome important in mediating the recognition of polyubiquitin chains and cleavage of ubiquitin from degraded proteins (Shi et al. 2018). In a previous gene-expression study of *P. olseni*-infected Manila clams, proteasome subunits were found to be downregulated in diseased animals, indicating decreased proteolytic activity [40]. In that respect, the elevated abundance of CAND1 and PSMC1 in RES clams may indicate a trend towards ubiquitin tagging of damaged proteins and more active protein degradation in RES clams than in DIS clams.

High proteolytic activity ion RES clams may be linked to the digestion of phagocytosed and neutralized bacteria, and may also reflect the presence of a pro-apoptotic protein in this group. In addition, it may be possible that RES clams were able to sustain the cellular functions necessary for effective elimination of the pathogen, after which proteins damaged by oxidative stress during the immune response are degraded.

3.5.2. *Transport, folding, and chaperone functions*

Our dataset also contained proteins associated with various aspects of protein synthesis, including protein transport (Table 2). Titin (TTN), a large structural protein thought to function as a scaffold protein, has been shown to interact with actin and filamin, proteins of the cytoskeleton implicated in the movement of cellular components and proteins [92]. In vertebrate striated muscle, TTN was specifically shown to recruit E3 ubiquitin-ligase [93], thus its high abundance in RES clams may reflect transport associated with proteolysis, consistent with the fact that this group also showed higher abundance of CAND1 and a proteasome subunit. Another protein implicated in protein transport is 14-3-3 epsilon (YWHAE), a binding protein suggested to play a role in protein transport to the secretory pathway, namely through interaction with GTPase-activating proteins such as IQGAP1 (see 3.3.3 *Cytoskeleton-associated immune response*), also found highly abundant in RES clams [94].

DIS clams showed a high abundance of FK506 binding protein 14 (FKBP14), which is thought to accelerate protein folding as it belongs to a family of peptidyl-prolyl cis-trans isomerases that play a role in the folding of newly synthesized proteins [95]. The higher abundance of this protein in the DIS group may reflect an increase in the synthesis of immune-related proteins, which were also more abundant in DIS clams. Furthermore, it is also interesting to note that this family of proteins (also called immunophilins) has been shown to inhibit early establishment and intracellular infection by bacteria [96]. Though not directly implicated in protein metabolism, the DIS group also presented high abundance of an Abhydrolase domain-containing protein associated with biosynthesis of phosphatidylcholine, an essential class of membrane phospholipids, possibly reflecting an increase in membrane synthesis due to the internalization of bacteria in phagosomes [97].

Heat-shock protein 90 (HSP90), a chaperone protein that plays a crucial role in protecting protein structure in response to stress conditions, was highly abundant in RES clams. Due to its interaction with the major histocompatibility complex (MHC) and the antigen processing pathways in vertebrates [98], HSP90 has been suggested to play a role in the innate immune system response and resistance to infection in invertebrates [99]. As HSP synthesis is promoted by protein denaturation, the trend towards proteolytic activity in RES clams discussed in part 3.5.1 may be partially responsible for activating HSP synthesis. That said, the higher abundance of HSP90 in RES clams may also indicate that resistant clams have a lower threshold for protein denaturation and thus perhaps more rapidly activate the synthesis of protective chaperone proteins, granting them an advantage over DIS clams when it comes to cellular protection.

3.6. OTHER

The transcriptional activator protein Pur-alpha (PURA), a protein involved in controlling DNA replication and gene transcription processes [100,101], was highly abundant in the DIS group, while a Poly (rc)-binding protein 3 (PCBP3) associated with negative regulation of transcription [102] was highly abundant in the RES group [102].

Proteins associated with translation include RPL27A, a structural component of the 60S ribosome subunit whose upregulation has previously been reported in white-spot infected shrimp and in hypoxia-stressed oysters [103,104], and asparaginyl-tRNA synthetase (NARS), whose primary function in translation is to catalyze the attachment of asparagine to its corresponding tRNA.

Finally, four significantly abundant proteins were uncharacterized; three were highly abundant in the DIS group and one in the RES group.

4. CONCLUSION

Despite being one of the fastest-growing sectors of aquaculture worldwide, mollusk production continues to suffer significant losses due in part to the impact of infectious diseases. The study of proteomic profiles offers the possibility of better understanding the complex functional mechanisms at play during host response to disease, and may shed light on factors associated with resistance to disease.

The aim of the present study was to investigate the proteomic profiles of resistance to controlled infection with *Vibrio tapetis*, the etiological agent of Brown Ring Disease, in the Manila clam, *Ruditapes philippinarum*. The comparison of proteomic profiles of two extreme phenotypes (RES and DIS) observed in juvenile Manila clams shows a number of functional differences in highly abundant proteins implicated in the immune response-associated processes, energy production, and protein metabolism. Twice as many significantly abundant proteins associated with the immune response were accumulated in the DIS group compared to the RES group, reflecting the ongoing infection as established by the presence of both visual and molecular signs of disease. That said, the function of immune-associated proteins in the RES group was almost consistently associated with redox homeostasis, whereas in DIS group the abundant proteins were mostly involved in pathogen recognition, signaling, and neutralization. This may suggest that disease resistant clams are better equipped to manage ROS production and scavenging in order to simultaneously eliminate the pathogen and protect host cellular components from oxidative stress. Protein degradation as well as protection by chaperones were another process highly represented in resistant clams, with degradation possibly as a result of successful elimination of the pathogen which may nonetheless have left a number of cellular components damaged by oxidative stress.

The fact that only resistant clams showed significantly high abundance of a chaperone-associated protein suggests that this may be an important factor of resistance to disease. In contrast, diseased clams showed a higher abundance of proteins involved in protein synthesis and functional modifications, possibly in response to activation by the immune system in order to continue fighting infection. Both immune response and protein synthesis are energy demanding processes, which is further supported by the presence of proteins involved in glycolysis, TCA cycle, and the electron transport chain. Overall, the comparison of the proteomic profiles of resistant and sick clams suggests that redox homeostasis and maintenance of protein structure by chaperone proteins may play important and interrelated roles in resistance to infection by *Vibrio tapetis* in the Manila clam.

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GENERAL DISCUSSION AND CONCLUSIONS

DISCUSSION

The work carried out in this PhD seeks to lay the foundations for developing a long-term strategy for disease management in Manila clam aquaculture through selective breeding for resistance to disease and a better understanding of the functional mechanisms associated with this resistance.

Traditional breeding methods rely on the definition of desirable traits, and on crosses between individuals that carry those traits, leading to a gradual increase in the frequency of the selected trait, or phenotype, over the course of sequential generations. While this is undoubtedly the basis of domestication of most plant and farmed animal species, selection based exclusively on phenotypic criteria is not efficient or even feasible for traits such as disease resistance that are costly and difficult to measure in selection candidates (Hollenbeck & Johnston, 2018). The efficiency of breeding programs, measured for example as the rate of increase of the selected trait within the population, depends largely on the ability to follow pedigree and on knowledge regarding the genetic architecture underlying desirable traits, and both of these factors rely in turn on the availability of genomic data regarding the species in question (Gjedrem & Rye, 2018; Meuwissen et al., 2016).

Traits of interest to the aquaculture sector vary depending on the species, though in general the most sought-after improvements include growth, meat yield, morphology, robustness, fecundity, and resistance to disease. Traits such as growth and morphology tend to show moderate to high heritability, and selective breeding in aquaculture species has shown that the genetic gain per generation is up to 5 times higher than for terrestrial livestock (Zenger et al., 2019). Physical traits regarding shell dimensions and weight also come with the advantage of being easy to measure, which is not the case for traits such as fecundity or disease resistance, which are harder to select for namely because they involve destructive sampling methods, which means that selecting candidates for mating requires accurate pedigree information on the siblings of the individuals measured for those traits. However, maintaining separate family lines to record pedigree information involves costly and extensive infrastructure, and as sib selection can only benefit from between-family genetic variability (and cannot utilize within-family variance) it

entails the rearing of numerous lines in order to maximize genetic gain and limit inbreeding (Dupont-Nivet et al., 2006; Hollenbeck & Johnston, 2018; Yáñez et al., 2015).

Inbreeding in molluscan species is notoriously difficult to avoid in selectively bred lines, namely because of the high variance in reproductive success (i.e. while several broodstock may be mated together, the progeny is often the result of far fewer crosses than initially expected) and high rates of lethal alleles (i.e. mutations at loci that block development to the adult stage), both of which likely derive from the polymorphic nature of molluscan genomes (Plough, 2016). In order to both maintain and benefit from the genetic diversity of bivalve populations, it is thus essential that selective breeding programs for complex traits integrate genomic information to distinguish ideal breeding candidates and optimize genetic gains.

Genomic selection, which associates pedigree information and genome-wide genetic markers with measured phenotypic information for large numbers of individuals, has the potential to limit many of the risks associated with family or lineage-based breeding schemes, while also increasing the accuracy and effectiveness of selection. The basic principles of genomic selection rest on the use of high-density marker panels (ex. SNPs) for parentage assignment within mixed family groups, the estimation of genomic breeding values in "test" populations (i.e. association of phenotypic and genotypic information), and the selection of candidates from a "selection" population based on their genotypes (Zenger et al., 2019). This allows for selection of traits that are difficult to measure from a far greater genetic pool than when selection is carried out between separate lines.

As mentioned in the introduction, the Manila clam, *R. philippinarum*, currently lacks the genomic resources necessary to establish genomic selective breeding programs, despite being among the most produced bivalve species on the global market. One of the major limits thus far has been the ability to accurately and reliably establish pedigree and estimate breeding values for desirable traits within large mixed-family cohorts. With the decrease in sequencing and high-throughput genotyping costs, genetic marker panels have allowed us to move away from separate family rearing towards mixed family rearing, which eliminates the need for individual tagging, avoids rearing bias such as "tank effects", and substantially decreases hatchery and grow-out infrastructure (Ross D. Houston, 2017).

The first chapter of this PhD demonstrated the development of a SNP panel for parentage assignment in the Manila clam, aiming to provide a tool that would make it possible to evaluate the genetic parameters of resistance to disease and growth traits (discussed in chapter II) for future application to the aquaculture industry. Marker selection requires the sequencing of DNA and alignment of the resulting reads to a reference genome in order to identify polymorphisms such as SNPs. To obtain a reliable panel of highly informative, i.e. highly variable, genetic markers for parentage assignment, DNA sequence information is required from a large number of individuals, which can be time-consuming as well as economically challenging. Pooled sequencing (Pool-seq), in which DNA from multiple individuals is mixed together for sequencing, offers a more cost-effective and efficient alternative method for investigating polymorphism which also requires less DNA from each single individual. A growing number of studies have demonstrated the accuracy of allele frequency calculations in Pool-seq studies in research areas ranging from population genetics to investigations on the genetic basis for complex traits. In the case of the Manila clam, a highly polymorphic species for which genomic information is still relatively scarce, the accuracy of Pool-seq as a method for SNP identification had yet to be established. The first chapter of this PhD, in which the SNP panel for parentage assignment was developed, the allele frequency and variant type of over 66,000 SNPs detected through both individual genotyping-by-sequencing (2b-RAD) and Pool-seq were compared, resulting in high concordance and allowing us to validate the application of this novel method for candidate SNP identification in the Manila clam.

In bivalve species such as the Mediterranean mussel (*Mytilus galloprovincialis*), high-accuracy parentage assignment has been demonstrated using low-density SNP panels of as few as 227 markers, of which only 179 successful and informative SNPs were necessary for correct assignment (Nguyen et al., 2014), and the same holds true for the Eastern oyster, *C. virginica*, for which only 116 informative markers (2x58 SNPs) were shown to be necessary for accurate parentage assignment (Thongda et al., 2018). This contrasts with the results of the panel developed for the Manila clam and raises some questions regarding the SNPs chosen for our array, in which too few SNPs were successful or informative enough to allow for accurate parentage assignment. One of the initial constraints for the development of this tool was the size of the panel, which was limited to 250 markers. Given this limitation, our candidate markers were selected following a long list of stringent criteria, though at the time we lacked an annotated genome and information regarding polymorphism in coding regions. While it would have been possible to select SNPs from

coding regions by aligning reads to the transcriptome of the Manila clam, the fact that coding regions are generally more conserved lead to uncertainty regarding the risk of selecting our limited number of SNPs based on this criterion. One of the known challenges associated with the use of Pool-seq is the decrease in accuracy for alleles present at low frequency (AF < 0.01), which can lead to false identification of variants and thus be problematic, especially when marker density is limited (Kofler et al., 2016; Schlötterer et al., 2014). This potential problem was however minimized in our study by applying filters to select SNPs with high minor allele frequencies, in addition to which coverage, flanking regions, contig proximity, and a number of other parameters were meticulously examined in the aim of selecting informative markers.

Despite the filters put in place in the hopes of selecting markers which would be polymorphic and present in the F1 generation, a number of markers resulted in unclear genotypes. A possible explanation for this may be the occurrence of mutations, an extremely frequent phenomenon in bivalve species which leads to null alleles. It appears clear following the results of this first SNP panel that, in order to have a sufficient number of reliable SNPs in a panel, higher-density panels are necessary to initially screen markers for parentage assignment. That said, the ability to assign parentage using molecular markers, and thus raise numerous families or lines in the same environment, is essential to establishing a genetic selection strategy in the Manila clam, and the results presented in the first part of this PhD provide a promising first step towards developing breeding programs for this species (Figure 1, boxed in blue).

The F1 clams from Chioggia (Italy) and Marennes (France) for which we were able to obtain pedigree information enabled us to carry out the first estimations of genetic parameters for disease resistance, sex, weight, and shell dimensions. One of the major advantages of this study was the number of measurements recorded, or phenotypes, within the populations assessed. Accurate, high-throughput, cost-effective phenotype recording is essential for accurate estimation of breeding values in commercial breeding programs, and this is all the more pertinent for complex traits such as disease resistance. To assess resistance, our study quantified the pathogen using molecular diagnostics as opposed to histological techniques in order to reduce the inherent costs, material, and time necessary (Balseiro et al., 2010). Furthermore, our study decided to sample clams after one year in the field, when about 50 % of the population had a quantifiable amount of

pathogen DNA, which balances this trait across our sample set and provides us with a sufficient number of "resistant" samples across multiple parent pairs. It should be noted that prevalence would likely have been far higher had the clams spent a second summer in the grow-out zones, as local prevalence of *P. olseni*-infected clams is notoriously high at commercial size in the Venice lagoon (*pers. comm.* Giuseppe Arcangeli, Istituto Zooprofilatico Sperimentale delle Venezie). This raises questions regarding the use of the term "resistance" in the case of our study, and it may indeed be more accurate to qualify this trait as "resistance to early infection" instead. While complete, or absolute, resistance is possibly non-existent in the context of commercial clam production in the Venice lagoon, selection for resistance to early infection is a viable solution that would not only reduce infection intensity in the long run, but may also contribute towards the production of natural spat. Clam production in Chioggia traditionally relies on a natural supply of spat which is collected in defined zones and then transferred to grow-out plots, but there has been a significant decline in natural spat despite continued production of commercial-sized clams, a problem that has been suggested to be linked to chronic infection with *P. olseni* (Boscolo Papo et al., 2014; Pretto et al., 2014). The ability to select clams more resistant to early development of the disease may favor gametogenesis and the production of natural spat, providing a more stable supply for the local industry. That said, many factors such as pollution and more frequent extreme temperatures associated with climate change certainly contribute to the decline in natural spat, and the impact of these stressors on fecundity and fertilization in the Manila clam are poorly, if at all, described.

The influence of environmental parameters on selection strategy was highlighted in our study through the fact that, while both the Chioggia and Marennes sites showed promising heritability for all biometric traits evaluated, the genetic correlations between the two F1 clam groups were low. This suggests that within the same F1 cohort of common genetic background, clams with the best performance for growth traits at one site were not from the same parent pairs as those that performed best at other sites. While this indicates that selection for growth traits would be more beneficial if it were carried out in a site-specific manner, simulations for estimated genetic gain regarding total weight showed that if selection were carried out based on the genetic parameters from the Marennes (disease-free) site and the clams were then reared in Chioggia (*P. olseni*-affected site), there would still provide a gain in total weight in Chioggia, and this is an important factor to consider for hatcheries seeking to establish a breeding program, especially for

spat export. Similarly, mass selection for resistance to MSX and Dermo as well as growth was investigated in the Eastern oyster *C. virginica* in Virginia, USA, demonstrating that mass selection was efficient in increasing resistance to disease in the oyster, but that selected lines with the best performance varied greatly between sites, and concluding that selection models cannot exclude the influence of genotype-by-environment (GxE) interaction (Dégremont, Garcia, et al., 2015; Frank-Lawale et al., 2014; Proestou et al., 2016). The high estimated heritability for parasite load in the Manila clam suggests that implementing a similar approach to the one used for the Eastern oyster has the potential to create genetic progress, however the results of our study regarding the potential GxE effect are to be interpreted with caution given the low assignment rate and inherently high standard errors of our estimations.

Dual selection potential for both BRD and Perkinsosis in the Manila clam has yet to be investigated, though it would likely be of great benefit in large-scale production sites where these diseases co-occur, as is the case along the Korean peninsula. Given the high estimated heritability observed for resistance to Perkinsosis in our study, as well as demonstrated more broadly for disease resistance in other bivalve species, we have reason to believe that resistance to BRD will also show high potential for genetic selection in the Manila clam. In order to estimate the genetic parameters of resistance to infection with *V. tapetis*, a batch of F1 clams from the same cohort as those used in our study were subjected to a field challenge in a production area in Chausey, France, which has a history of BRD prevalence, following the same experimental design as in Chioggia and Marennes (Trinkler et al., 2010). While the results from this experiment are not yet available and thus were not included in this PhD thesis, this experiment will provide a third set of ca. 1000 individuals that were measured for all of the same biometric traits as those in Marennes and Chioggia, and for which disease intensity will be evaluated using both molecular (quantitative PCR) and visual diagnostic methods.

For both Perkinsosis and BRD, the internal nature of the infection makes it impossible to carry out non-lethal sampling, thus positive correlations between measurable biometric traits (i.e. shell dimensions, weight) and resistance to disease would have been an ideal facilitator for selection. Had this been the case, genotyping of test populations for candidate selection would not have been necessary, and resistance would have increased by default in lines selected for growth. The calculated genetic correlations between biometric traits and resistance in our study suggest, on the contrary, that these traits do not have the same genetic basis, meaning that while selecting for

one will not benefit the other, selection for both growth and resistance can be carried out in parallel without one of the traits negatively affecting genetic gain for the other trait.

The major advantage of genomic selection rests on the fact that phenotyping test populations to select candidates is not necessary, as selection is based solely on genotype information (Hollenbeck & Johnston, 2018). This, in turn, implies that DNA be sampled from the test population and that genotyped individuals be assessed for their potential as broodstock. Non-lethal genotyping of clams for candidate selection is feasible, albeit slightly more complicated than in other organisms by the fact that tissues are difficult to access across the physical barrier of the shell. Hemolymph sampling, which can be carried out in the adductor muscle without opening the two valves, is a relatively simple (although slightly invasive) sampling strategy which can be applied to collect biological material for DNA genotyping. The tagging or labelling of individuals sampled for DNA between the time of sampling and the genotype result is, however, essential to retrieve selection candidates post-genotyping, representing an additional hurdle in the selection process which has thus far been eased by the use of small pet-scan chips which can be set on the clam shell. Both the non-lethal sampling technique and the individual tagging method have been tested in clams and, while not as simple as in fish, the procedures are feasible. If the time interval between sampling and genotype results is shortened, non-lethal sampling could be applied to future breeding programs.

The most encouraging results of the second chapter of this PhD thesis are the high estimated heritability for resistance to Perkinsosis as well as the moderate-to-high heritability for biometric traits pertaining to growth, though it should be noted that our results regarding heritability and genetic correlations between sites may be overestimated due to the limited number of progenies successfully assigned in the Chioggia site. Despite the high variability in our estimations due to the lack of sufficient parentage assignment in our datasets, these first estimations of the genetic parameters for traits of commercial interest strongly support the importance of investing in high-density SNP panels for the Manila clam, as demonstrated already in other bivalve species (Gutierrez et al., 2017; Zenger et al., 2019). Another incentive for developing high-density panels is that increasing the number of markers on a panel also increases the chances that certain markers may be significantly associated with the recorded phenotypes such as growth, resistance, and sex, thus opening the door to developing weighted prediction models which have the potential to further improve genetic gain in breeding programs (Karaman et al., 2018). Given the previous lack of an

annotated genome for the Manila clam, there is still very little known regarding genetic regions associated to disease resistance, though a recent genetic linkage map was able to highlight several QTLs significantly associated with shell dimensions, weight, and shell color, using over 349,000 polymorphic SNPs (Nie et al., 2017). The fact that genetic regions have been associated with these traits in the aforementioned linkage map, and that our study highlighted genetic correlations between these same traits in both populations investigated, strongly supports the potential for genetic improvement of aquaculture stock (Figure 1, boxed in orange). Furthermore, the currently available biological samples (extracted DNA) and measurements provide a remarkable resource for further investigations regarding the development of future breeding programs.

Including weighted markers within a prediction model requires not only the identification of the genetic regions associated with traits of interest, but also benefits significantly from knowledge regarding the functional molecules underlying those traits. While there have been some exceptional examples of resistance-related QTLs being rapidly integrated in marker-assisted selection programs (e.g. IPN in the Atlantic salmon, (R. D. Houston et al., 2008)), the genetic architecture for disease resistance is rarely consists in only a few major contributing loci. Most often, resistance to a disease is a highly polygenic trait relying on a large number of loci and the complex interactions between them. In the case of the Manila clam, the genetic markers we used were far from numerous enough to identify resistance-associated polymorphisms, however the hypothesis that numerous genes are involved is likely. The third chapter of this PhD thesis touches on the functional mechanisms underlying resistance to Brown ring disease through a proteomics approach, suggesting that the host's ability to mediate the effects of oxidative stress and maintain protein integrity may be associated to increased resistance. Redox balance and protein stability are essential to normal cellular function that implicate numerous proteins, many of which have several functions and can participate in various metabolisms, making it all the more difficult to target specific genes associated with resistance. While our study does not pinpoint any single protein or enzymatic activity, it does shed light on the possible proteins and metabolisms implicated in resistance, which is an important step towards identifying the specific molecules and the genes regulating them in order to improve prediction models for selective breeding.

The experimental design used for the controlled challenge described in chapter 3 also provides a valuable resource for future studies seeking to identify the genomic factors underlying resistance. Currently, the DNA of over 450 diagnosed samples is available for genetic analyses, and overall more than 1000 tissue samples and shells from the experiment are available for DNA extraction and genotyping. As the clams used in this study were from the same F1 cohort as in all the other experiments described in this PhD thesis, genotyping samples presenting extreme phenotypes has the potential to provide an interesting comparison with results from the field challenge carried out in Chausey, France. There may be variation in the observed family representation between the two groups (control vs field challenge), possibly due to the differences in experimental conditions, infection procedures, age of the clams at the time of infection, and duration of the challenge. That said, controlled challenges come with the major advantage of being able to measure and regulate a number of influential parameters such as water temperature, salinity, clam density, feeding rate, and oxygenation, thus eliminating (or at least significantly reducing) the inter-individual variability in infection intensity due to variation in environmental stressors in addition to infection. Due to the differences between conditions in field and controlled challenges, the results obtained in juvenile clams from the latter are not always directly applicable to the field, further supporting the need to validate the genetic parameters for resistance calculated in controlled challenges with those obtained through realistic field challenges such as those described in chapter 2 of this PhD thesis (Dubert et al., 2017; Zannella et al., 2017). In order to characterize the genotypes of the available samples from the controlled challenge, however, the SNP panel would have to be improved to increase the rates of genotyping and parentage assignment and allow for more accurate estimation of genetic parameters. Finally, the sampling strategy applied to the controlled challenge was conceived in order to allow for potential gene expression analyses through a transcriptomics approach, providing another angle from which to investigate the genomic basis for resistance. Transcriptomic studies would complement the genomic and proteomic investigations carried out thus far, and could contribute substantially to understanding the link between the genetic blueprint and the final active molecules produced to counter infection (Figueras et al., 2019; Moreira et al., 2014, 2015).

With the identification of proteins (and hence, genes) associated with resistance mechanisms, future prediction models for the Manila clam may be able to integrate information from functional studies by applying weighted models for the selection of broodstock (Zenger et al.,

2019). While basic genomic selection with SNP panels assumes that each SNP throughout the genome has the same effect with regard to measured phenotypes, weighted prediction models can assign greater importance to markers based on their location within a gene or genomic region of interest (Karaman et al., 2018). The results provided by the proteomic study discussed in chapter 3 contribute to our understanding of the molecular processes playing a role in disease resistance and may one day serve as a resource for breeding program refinement in which functional information can be integrated in the prediction model (Figure 1, boxed in green).

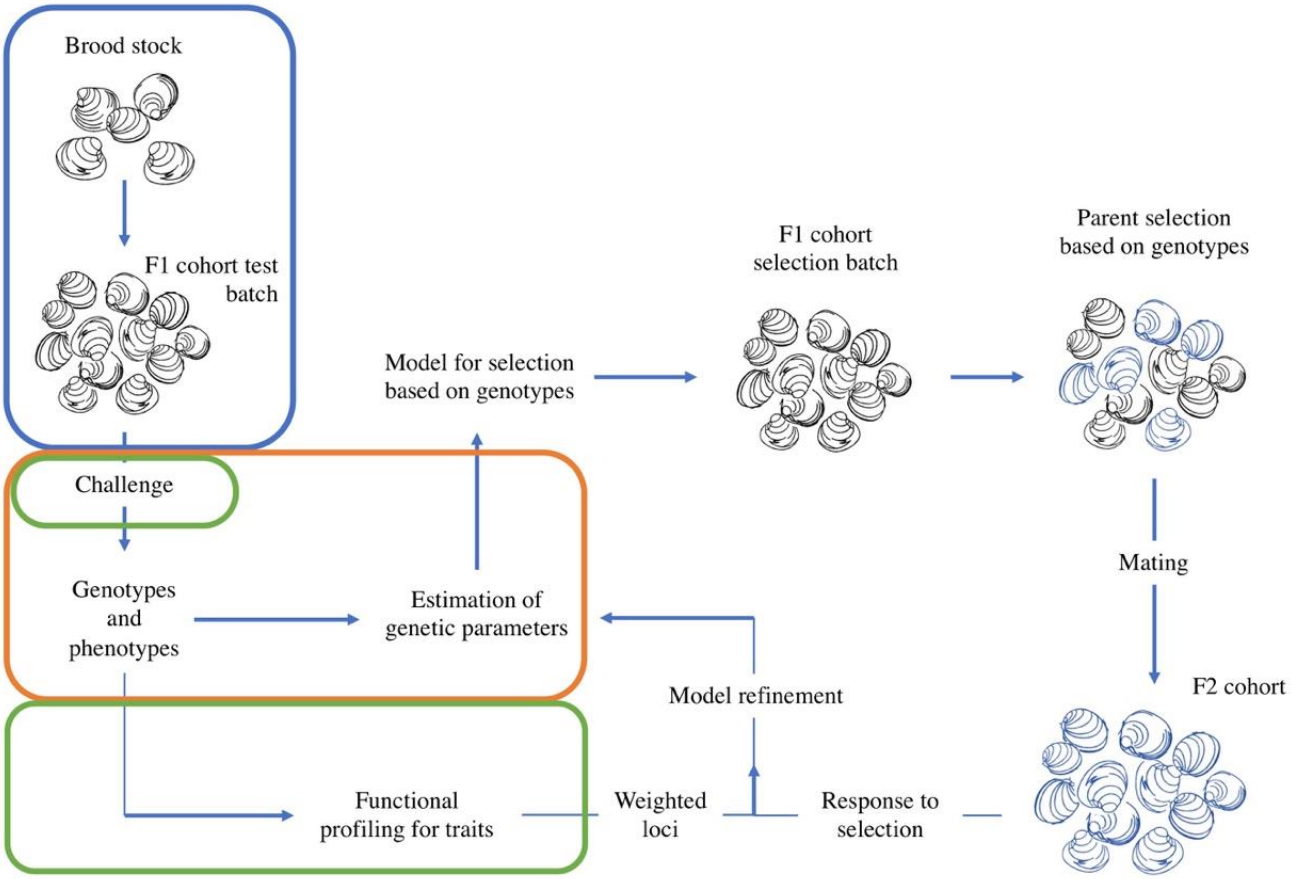

Figure 1: An overview of how the studies carried out in this PhD fit in with a hypothetical prediction model in the Manila clam. Chapter 1, which describes the development of a genomic tool to define pedigree in mixed batch families, is represented in blue. Chapter 2, which focuses on estimating the genetic parameters associated with resistance and other commercial traits of interest, is represented in orange. Chapter 3, which investigates resistance mechanisms through proteomic profiling, is represented in green. Overall, this PhD has made progress in the essential first steps necessary for the establishment of future breeding programs for the Manila clam.

CONCLUSIONS

The aquaculture of marine bivalves has seen a remarkable increase in production over the past 50 years, currently accounting for about 15% of global marine aquaculture production, yet this sector continues to struggle with infectious diseases and the risks associated with them. This raises concerns for the stability of the industry and has been the subject of recent research initiatives aimed at finding sustainable solutions such as the development of breeding programs. This is namely the case for the Manila clam, *R. philippinarum*, which has rapidly become one of the most intensively produced bivalves worldwide, but for which genomic resources have thus far been insufficient to allow investigation of the genetic parameters for commercial traits of interest, namely resistance to disease.

This PhD thesis presents the first large-scale investigation of the potential for selection in this species, approaching the problem from both a quantitative genetics angle as well as from a functional perspective, with the aim of providing further resources to move towards developing breeding programs for Manila clam hatcheries. With the development of a tool for parentage assignment using genetic markers (Chapter 1), we were able to carry out extensive field challenges in relevant production conditions to estimate the genetic parameters for numerous traits in the Manila clam (Chapter 2). Using biological material from the same cohort, we were also able to carry out a controlled challenge in the aim of understanding the molecular mechanisms at play in resistant clams (Chapter 3), providing a more comprehensive view of the proteomic profiles associated with resistance. Overall, the results from these studies highlight a strong potential for site-specific selection for both disease resistance and growth traits, and suggest that proteins involved in maintaining cellular component integrity may be key actors in combating the onset of infectious disease at the individual level. In addition, the difficulties encountered throughout these studies provide a better understanding of how to further develop genomic tools and selection strategies to increase their accuracy and resolution, and support the need for continued investment and collaboration from both research facilities and the aquaculture industry.

While we currently still lack many pieces of the puzzle in order to establish multi-trait selection for the Manila clam, selection based on both genetic information and molecular markers could be a solution for improving disease resistance in this bivalve species.

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To those who encouraged,
And those who warned,
Those who inspired,
Those who judged,

To those who guided,
Those who questioned,
Those who offered,
Those who accepted,

To those who listened,
Those who spoke,
Those who stood by,
And those who stepped back,

To those who laughed,
Those who looked on,
Those who followed,
Those who fell away,

To those who just smile,
To those who try.

Thank you.

SUPPLEMENTARY FILES

CHAPTER 2; SUPPLEMENTARY FILE 1

DNA extractions compared between gill and mantle tissue of the same individuals (results from Labogena, Jouy-en-Josas, France). ANOVA test shows that there is a significant difference in the DNA concentration, with gill tissue providing a higher concentration than mantle tissue.

Table a: DNA concentrations from extractions performed at Labogena, using gill and mantle tissue from the same individuals and using the same extraction method.

DNA ng/uL	**Tissue**	
Individual	Gill	Mantle
Clam 1	97.09	72.63
Clam 2	91.15	47.24
Clam 3	141.21	56.79
Clam 4	127.12	51.26
Clam 5	141.23	48.63
Clam 6	125.54	64.44
Clam 7	142.9	62.06
Clam 8	124.33	70.48
Clam 9	75.26	47.33
Clam 10	146.36	67.43
Clam 11	129.91	50.24
Clam 12	109.62	85.94
Clam 13	112.96	70.34
Clam 14	152.77	81.82
Clam 15	105.82	70.87

Table b: ANOVA test results showing a significant difference between gill and mantle tissue for the DNA concentration.

Result Details One-Way Repeated Measures ANOVA				
Source	SS	df	MS	
Between-treatments	25565.7698	1	25565.77	F = 89.3778
Within-treatments	9279.9235	28	331.4258	p-val < 0.05
Error	4004.5824	14	286.0416	

CHAPTER 3; SUPPLEMENTARY FILE 1: DETAILED PROTOCOL

Sample preparation and DNA extraction protocol.

1. Whole clams (including fluids) are sampled individually in 2 mL test tubes and immediately frozen in liquid nitrogen
2. 1X PBS (pH 7) is added depending on weight of tissue, to obtain a concentration of 0.25 mg/µL tissue in PBS for each sample
3. Circa 500 µL mix of ceramic beads (1.4 mm and 2.8 mm, Ozyme) is added to each tube
4. FastPrep-24 MP benchtop, 2x20 seconds at room temperature is used to homogenize in buffer
5. 80 µL of homogenate from each sample is transferred to the 96-well plate of the NucleoSpin-96 Tissue kit (Macherey Nagel), manufacturer's instructions are then followed
    a. Modifications to the manufacturer's protocol:
        i. After lysis, volume of ethanol for precipitation is increased to at least 400 µL
        ii. Spin-down cycles are done at 1000 xg
        iii. Centrifugations are done at 2000 xg
        iv. Elution is done using clean water heated to 70ºC
        v. Eluate is recovered in PCR plate
        vi. Eluate is passed through column a second time (double elution)
6. DNA concentrations as measured by spectrophotometry (Nanodrop) range from 80-150 ng/µL

CHAPTER 3; SUPPLEMENTARY FILE 2: ALL IDENTIFIED LOCI

DIS Intensity	RES Intensity	Group ratio DIS/RES	Student's T-test p-value	Coverage (%)	Unique peptides	*R. philippinarum* transcriptome locus	Frame	*C. gigas* protein ID	C. gigas gene description	UniProtKB/ TrEMBL acces.
2.96E+02	2.55E+03	1.16E-01	0.003863371	8	3	Locus_6888709	2	EKC26119	Angiotensin-converting enzyme	K1Q3Z9
4.98E+02	1.63E+03	3.06E-01	0.044157274	15	2	Locus_4231109	3	EKC28114	26S proteasome non-ATPase regulatory subunit 7	K1Q9Z6
6.47E+02	1.77E+03	3.66E-01	0.005195361	3	3	Locus_615620	1	EKC23268	Ras GTPase-activating-like protein IQGAP1	K1PNK5
9.94E+03	2.40E+04	4.14E-01	0.038861271	16	8	Locus_4823168	3	EKC21276	Pyruvate dehydrogenase E1 component subunit alpha type II, mitochondrial	K1QI11
1.60E+03	3.59E+03	4.46E-01	0.03461866	11	10	Locus_3208485	-1	EKC19309	Cullin-associated NEDD8-dissociated protein 1	K1PK85
3.23E+02	6.69E+02	4.83E-01	0.019981752	3	1	Locus_2057997	-3	EKC35339	Thioredoxin domain-containing protein 5	K1QVS2
1.19E+03	2.29E+03	5.20E-01	0.049892706	34	3	Locus_1397283	1	EKC35325	Titin	K1R2G8
6.33E+02	1.20E+03	5.28E-01	0.048952877	6	2	Locus_2389731	-1	EKC42074	Poly(RC)-binding protein 3	K1RED7
8.18E+02	1.56E+03	5.24E-01	0.021344037	4	2	Locus_3238632	-1	EKC23703	Unknown	NA
8.18E+02	1.56E+03	5.24E-01	0.021344037	10	2	Locus_4644207	-3	EKC23703	Unknown	NA
4.48E+03	8.14E+03	5.50E-01	0.045077782	2	3	Locus_355976	-3	EKC36585	Angiotensin-converting enzyme	K1R5X4
4.48E+02	7.53E+02	5.95E-01	0.003002961	10	2	Locus_3201607	-3	EKC33267	Endophilin-B1	K1Q963
2.55E+03	4.14E+03	6.16E-01	0.014952908	20	16	Locus_2939806	-1	EKC25687	Heat shock protein HSP 90-alpha 1	K1PNQ5
1.52E+02	2.45E+02	6.20E-01	0.04829303	3	1	Locus_4589062	-1	EKC37227	Alcohol dehydrogenase class-3	K1QKF8

3.99E+02	6.38E+02	6.25E-01	0.036287292	2	1	Locus_2688502	-1	EKC36531	Xanthine dehydrogenase/oxidase	K1RQC1
1.86E+03	2.94E+03	6.33E-01	0.003473597	5	1	Locus_453724	1	EKC20816	Asparaginyl-tRNA synthetase, cytoplasmic	K1QGR8
1.13E+03	1.77E+03	6.38E-01	0.031595166	13	2	Locus_2128190	3	EKC32958	Methylglutaconyl-CoA hydratase, mitochondrial	K1QGC5
1.94E+03	2.98E+03	6.51E-01	0.041428934	38	3	Locus_5145028	-3	EKC29146	14-3-3 protein epsilon	K1R5F2
1.03E+03	1.53E+03	6.73E-01	0.042360434	13	3	Locus_32267	1	EKC31248	Major egg antigen	K1QBC3
2.84E+02	4.18E+02	6.79E-01	0.044512152	7	1	Locus_118477	1	EKC41956	High mobility group protein B3	K1S3G2
3.04E+02	4.46E+02	6.82E-01	0.039419008	4	1	Locus_4810952	1	EKC41455	Methionine aminopeptidase	K1RIM7
1.19E+03	1.74E+03	6.84E-01	0.031382208	26	3	Locus_857065	3	EKC33590	Coactosin	K1QXD4
1.57E+03	2.24E+03	7.01E-01	0.009655976	3	10	Locus_3338093	-3	EKC21066	Dynein heavy chain, cytoplasmic	K1QHK9
1.24E+03	1.72E+03	7.21E-01	0.048553894	12	2	Locus_2395784	3	EKC24481	26S protease regulatory subunit 6B	K1QSB2
7.59E+02	1.06E+03	7.16E-01	0.027897	15	3	Locus_3187173	2	EKC25494	Unknown	NA
7.59E+02	1.06E+03	7.16E-01	0.027897	23	3	Locus_7886543	-1	EKC25494	Unknown	NA
1.69E+03	2.27E+03	7.44E-01	0.030574334	2	1	Locus_2134092	1	EKC20392	Tripartite motif-containing protein 56	K1P9D6
1.69E+03	2.27E+03	7.44E-01	0.030574334	2	1	Locus_2134197	1	EKC20392	Tripartite motif-containing protein 56	K1P9D6
1.69E+03	2.27E+03	7.44E-01	0.030574334	2	1	Locus_4558084	1	EKC20392	Tripartite motif-containing protein 56	K1P9D6
1.69E+03	2.27E+03	7.44E-01	0.030574334	9	1	Locus_6068810			Unknown	NA
1.51E+03	1.99E+03	7.59E-01	0.036193551	23	10	Locus_154300	-2	EKC22875	Seryl-tRNA synthetase, cytoplasmic	K1Q1Z3

2.36E+03	2.94E+03	8.03E-01	0.01248782	12	2	Locus_6269162	1	EKC20816	Asparaginyl-tRNA synthetase, cytoplasmic	K1QGR8
3.19E+03	3.89E+03	8.20E-01	0.013332718	25	6	Locus_7585742	-1	EKC29122	Sarcoplasmic calcium-binding protein	K1PY28
7.99E+02	9.63E+02	8.30E-01	0.02295491	14	2	Locus_11440055	-3	EKC38084	Protein tyrosine phosphatase type IVA 2	K1R3S5
1.16E+03	1.38E+03	8.41E-01	0.026526515	12	3	Locus_993081	-3	EKC23357	Unknown	NA
6.38E+03	6.39E+03	9.98E-01	0.04069191	34	11	Locus_654267	-1	EKC43227	Four and a half LIM domains protein 2	K1R1Q9
2.41E+03	2.36E+03	1.02E+00	0.009251365	39	7	Locus_3897176	-2	EKC29123	Sarcoplasmic calcium-binding protein	K1Q5G7
3.78E+03	3.68E+03	1.03E+00	0.006521013	10	5	Locus_5499761	3	EKC20321	Apoptosis-inducing factor 3	K1QFI3
4.86E+03	4.36E+03	1.11E+00	0.027120849	27	12	Locus_3184590	3	EKC33663	78 kDa glucose-regulated protein	K1QIR8
3.63E+03	3.08E+03	1.18E+00	0.037881364	10	3	Locus_4988945	1	EKC42336	2-oxoisovalerate dehydrogenase subunit alpha, mitochondrial	K1S4J4
1.59E+04	1.32E+04	1.20E+00	0.036852613	41	12	Locus_6492742	3	EKC33793	Mammalian ependymin-related protein 1	K1QJ28
3.34E+03	2.65E+03	1.26E+00	0.030119383	12	8	Locus_2375117	2	EKC21880	Dihydropyrimidine dehydrogenase [NADP+]	K1PZ93
1.08E+04	8.58E+03	1.26E+00	0.024335735	43	17	Locus_5934801	2	EKC34306	Cytosolic non-specific dipeptidase	K1RJ70
2.08E+03	1.60E+03	1.30E+00	0.004509178	12	5	Locus_2639797	3	EKC40259	Succinyl-CoA:3-ketoacid-coenzyme A transferase 1, mitochondrial	K1R9C2
1.01E+03	7.78E+02	1.30E+00	0.008713769	6	1	Locus_2027321	-2	EKC35648	Growth factor receptor-bound protein 2	K1QP30
3.33E+03	2.55E+03	1.31E+00	0.04975621	19	4	Locus_1912089	-1	EKC20972	Propionyl-CoA carboxylase alpha chain, mitochondrial	K1PAY7
2.32E+03	1.77E+03	1.31E+00	0.013009977	19	7	Locus_802380	1	EKC35014	Leukotriene A-4 hydrolase	K1QUS2

1.33E+03	9.90E+02	1.34E+00	0.033599693	16	3	Locus_3118257	3	EKC34207	Zinc-binding alcohol dehydrogenase domain-containing protein 2	K1QBN3
5.87E+03	4.24E+03	1.38E+00	0.020314463	32	5	Locus_1630758	2	EKC23413	Mammalian ependymin-related protein 1	K1PP20
9.42E+03	6.82E+03	1.38E+00	0.020229858	35	13	Locus_2801107	2	EKC43060	Propionyl-CoA carboxylase beta chain, mitochondrial	K1RNB5
1.57E+03	1.14E+03	1.38E+00	0.041062057	5	3	Locus_6501655	3	EKC42344	Peroxisomal multifunctional enzyme type 2	K1RF31
6.23E+02	4.50E+02	1.38E+00	0.010846366	5	1	Locus_1912805	1	EKC36164	Trans-1,2-dihydrobenzene-1,2-diol dehydrogenase	K1QYD8
6.98E+02	4.93E+02	1.42E+00	0.000903546	2	1	Locus_2777729	2	EKC31685	Dystroglycan	K1QS28
6.97E+02	4.86E+02	1.43E+00	0.005574703	7	1	Locus_2513624	-1	EKC40501	Galectin-9	K1RZS5
1.23E+03	8.53E+02	1.44E+00	0.010745339	21	2	Locus_6165846	-3	EKC23652	40S ribosomal protein S26	K1PI50
1.09E+04	7.43E+03	1.47E+00	0.014883739	26	9	Locus_5155129	-2	EKC31815	Fructose-1,6-bisphosphatase 1	K1QSB0
1.09E+04	7.43E+03	1.47E+00	0.014883739	26	9	Locus_6761836	2	EKC31815	Fructose-1,6-bisphosphatase 1	K1QSB0
1.09E+03	7.37E+02	1.48E+00	0.003052002	7	2	Locus_833244	-3	EKC39307	Major vault protein	K1QQR1
1.44E+03	9.48E+02	1.52E+00	0.002260222	9	3	Locus_6868673	3	EKC40501	Galectin-9	K1RZS5
1.27E+03	8.32E+02	1.53E+00	0.025337791	14	3	Locus_2955238	-1	EKC27178	Dynactin subunit 2	K1PZS6
3.87E+04	2.46E+04	1.57E+00	0.001911968	38	26	Locus_2348137	1	EKC25158	Malate dehydrogenase	K1PU26
3.84E+02	2.44E+02	1.57E+00	0.026614707	10	1	Locus_4334179	1	EKC24393	Complement C3	K1PRU2
3.84E+02	2.44E+02	1.57E+00	0.026614707	4	1	Locus_8101751	2	EKC24393	Complement C3	K1PRU2

3.20E+03	2.01E+03	1.59E+00	0.025779348	14	4	Locus_2215912	1	EKC27269	Putative serine carboxypeptidase CPVL	K1Q7K0
1.11E+04	6.93E+03	1.60E+00	0.037834579	52	7	Locus_381335	2	EKC27095	Phosphoenolpyruvate carboxykinase [GTP]	K1QEA6
5.84E+02	3.57E+02	1.64E+00	0.018994293	4	1	Locus_2642049	3	EKC41849	Abhydrolase domain-containing protein 14A	K1RDS5
5.84E+02	3.57E+02	1.64E+00	0.018994293	5	1	Locus_4959443	3	EKC41849	Abhydrolase domain-containing protein 14A	K1RDS5
3.50E+03	2.12E+03	1.65E+00	0.03692546	13	1	Locus_1236277	1	EKC20480	Glycogen debranching enzyme	K1PW08
4.66E+03	2.82E+03	1.65E+00	0.034006285	9	1	Locus_7730822	-1	EKC43060	Propionyl-CoA carboxylase beta chain, mitochondrial	K1RNB5
2.86E+03	1.72E+03	1.66E+00	0.007062156	38	3	Locus_8380444	-3	EKC18570	Phosphoglucomutase-1	K1PQD4
6.70E+02	3.96E+02	1.69E+00	0.033377431	8	1	Locus_1004421	3	EKC34161	Unknown	NA
6.70E+02	3.96E+02	1.69E+00	0.033377431	8	1	Locus_2059043	-3	EKC34161	Unknown	NA
1.12E+03	6.62E+02	1.69E+00	0.026551314	4	1	Locus_5269440	-1	EKC19854	3-demethylubiquinone-9 3-methyltransferase	K1PLX6
1.12E+03	6.62E+02	1.69E+00	0.026551314	4	1	Locus_6546419	-1	EKC19854	3-demethylubiquinone-9 3-methyltransferase	K1PLX6
1.03E+03	5.93E+02	1.74E+00	0.007722812	7	1	Locus_1202748	1	EKC29685	Serine/threonine-protein phosphatase	K1QLM3
1.30E+03	7.52E+02	1.73E+00	0.02448731	14	2	Locus_454248	-1	EKC31246	60S ribosomal protein L27a	K1RB07
4.27E+04	2.42E+04	1.76E+00	0.041549616	36	34	Locus_4313121	3	EKC39329	ATP synthase subunit alpha	K1R6Z7
6.80E+02	3.74E+02	1.82E+00	0.014390615	13	1	Locus_665720	-2	EKC31469	Ras-related protein Rab-43	K1QJY5
3.52E+03	1.91E+03	1.84E+00	0.042624796	27	6	Locus_6596965	1	EKC41442	Unknown	NA
3.52E+03	1.91E+03	1.84E+00	0.042624796	26	6	Locus_6596980	1	EKC41442	Unknown	NA

3.52E+03	1.91E+03	1.84E+00	0.042624796	26	6	Locus_6596996	1	EKC41442	Unknown	NA
9.10E+02	4.86E+02	1.87E+00	0.005464252	6	1	Locus_2384062	-3	EKC29415	Unknown	NA
9.10E+02	4.86E+02	1.87E+00	0.005464252	7	1	Locus_2384090	-2	EKC25290	Unknown	NA
9.10E+02	4.86E+02	1.87E+00	0.005464252	7	1	Locus_4921255	1	EKC29415	Unknown	NA
4.40E+02	2.35E+02	1.87E+00	0.035491592	3	1	Locus_4900110	-3	EKC29780	26S protease regulatory subunit 4	K1QLT5
4.86E+03	2.51E+03	1.94E+00	0.012540244	15	4	Locus_2922613	1	EKC25378	Unknown	NA
4.86E+03	2.51E+03	1.94E+00	0.012540244	15	4	Locus_6924033	-3	EKC25378	Unknown	NA
5.81E+02	2.98E+02	1.95E+00	0.013569856	3	1	Locus_2982362	2	EKC42273	Excitatory amino acid transporter 1	K1R8Z9
9.03E+02	4.46E+02	2.02E+00	0.045912107	4	1	Locus_2964602	-1	EKC26355	Ovochymase-1	K1QC16
9.03E+02	4.46E+02	2.02E+00	0.045912107	4	1	Locus_2964637	-1	EKC26355	Ovochymase-1	K1QC16
7.49E+02	3.51E+02	2.13E+00	0.002530096	7	2	Locus_1979842	-1	EKC21473	Adenosine deaminase CECR1	K1PIS0
1.30E+03	5.63E+02	2.31E+00	0.02331922	6	1	Locus_995640	3	EKC39351	Transcriptional activator protein Pur-alpha	K1RWS2
1.78E+03	7.16E+02	2.49E+00	0.027363921	12	6	Locus_2853765	-3	EKC32573	Vacuolar protein sorting-associated protein 35	K1QFF0
2.25E+03	7.83E+02	2.87E+00	0.006433069	14	2	Locus_6590720	-1	EKC20036	Glutathione peroxidase	K1QEW1
1.25E+03	4.21E+02	2.97E+00	0.041636137	3	1	Locus_5288240	-2	EKC31577	Beta-1,3-glucan-binding protein 1	K1Q4U8
1.25E+03	4.21E+02	2.97E+00	0.041636137	3	1	Locus_7622663	-1	EKC31577	Beta-1,3-glucan-binding protein 1	K1Q4U8
1.25E+03	4.21E+02	2.97E+00	0.041636137	3	1	Locus_7622701	-1	EKC31577	Beta-1,3-glucan-binding protein 1	K1Q4U8

1.06E+03	3.50E+02	3.03E+00	0.028139147	13	3	Locus_4498780	-3	EKC37917	Unknown	
6.15E+03	1.99E+03	3.09E+00	0.041175252	37	10	Locus_8587681	-3	EKC33186	Alanine aminotransferase 2	K1RGF4
1.49E+04	4.54E+03	3.28E+00	0.018065777	35	9	Locus_7235177	1	EKC29122	Sarcoplasmic calcium-binding protein	K1PY28
2.14E+04	6.06E+03	3.53E+00	0.000209326	34	19	Locus_2044774	3	EKC40669	Aspartate aminotransferase, cytoplasmic	K1RAL0
2.66E+03	5.31E+02	5.01E+00	0.029017038	12	2	Locus_3062641		ENSDARP00000139466*	palmitoyl-protein thioesterase 1*	ZDB-GENE-040426-2653

* *Danio rerio* protein ID and protein description

CHAPTER 3; SUPPLEMENTARY FILE 3: LOCI SEQUENCES

➔ The FASTA file submitted with the article for publication is available upon request.

ANNEXES: OTHER PUBLICATIONS

1) M. Milan*, M. Smits*, G. Dalla Rovere, S. Iori, A. Zampieri, L. Carraro, C. Martino, C. Papetti, A. Ianni, N. Ferri, M. Iannaccone, T. Patarnello, R. Brunetta, C. Ciofi, L. Grotta, G. Arcangeli, L. Bargelloni, B. Cardazzo, G. Martino, Host-microbiota interactions shed light on mortality events in the striped venus clam *Chamelea gallina*, Mol. Ecol. (2019) 1–14. doi:10.1111/mec.15227.

   * *Equal contribution*

2) M. Milan, G. Dalla Rovere, M. Smits, S. Ferraresso, P. Pastore, M.G. Marin, S. Bogialli, T. Patarnello, L. Bargelloni, V. Matozzo, Ecotoxicological effects of the herbicide glyphosate in non-target aquatic species: Transcriptional responses in the mussel *Mytilus galloprovincialis*, Environ. Pollut. 237 (2018) 442–451. doi:10.1016/j.envpol.2018.02.049.

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Host-microbiota interactions shed light on mortality events in the striped venus clam *Chamelea gallina*

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Abstract

Mass mortalities due to disease outbreaks have recently affected a number of major taxa in marine ecosystems. Climate- and pollution-induced stress may compromise host immune defenses, increasing the risk of opportunistic diseases. Despite growing evidence that mass mortality events affecting marine species worldwide are strongly influenced by the interplay of numerous environmental factors, the reductionist approaches most frequently used to investigate these factors hindered the interpretation of these multifactorial pathologies. In this study, we propose a broader approach based on the combination of RNA-sequencing and 16S microbiota analyses to decipher the factors underlying mass mortality in the striped venus clam, *Chamelea gallina*, along the Adriatic coast. On one hand, gene expression profiling and functional analyses of microbial communities showed the over-expression of several genes and molecular pathways involved in xenobiotic metabolism, suggesting potential chemical contamination in mortality sites. On the other hand, the down-regulation of several genes involved in immune and stress response, and the over-representation of opportunistic pathogens such as *Vibrio* and *Photobacterium* spp. indicates that these microbial species may take advantage of compromised host immune pathways and defense mechanisms that are potentially affected by chemical exposure, resulting in periodic mortality events. We propose the application of our approach to interpret and anticipate the risks inherent in the combined effects of pollutants and microbes on marine animals in today's rapidly changing environment.

KEYWORDS

bivalve, host-microbiota interactions, mass mortality, molluscs, pollutant–pathogen interactions, transcriptomics

Milan and Smits contributed equally to this work.

1 | INTRODUCTION

For decades, the study of mortality events affecting marine species has been restricted to simplified experimental plans focused on investigating the response to environmental stressors through biomarkers or gene expression analyses. While recent technological advances (e.g., next-generation sequencing) have made it possible to obtain increasingly relevant information regarding the response to various environmental factors, even in nonmodel species, the interactions between hosts, their associated micro-organisms and biotic and abiotic environmental factors have been minimally explored. These reductionist approaches have led to difficulties in deciphering multifactorial pathologies and mass mortality events affecting nonmodel species worldwide (de Lorgeril et al., 2018).

Currently, there remains a lot to elucidate about how the host and its microbial component can cooperate or interact in response to various environmental factors, how these interactions can undergo changes due to anthropogenic environmental stress and finally how infections can arise from opportunistic pathogens following a series of changes in both the host and its environment. This lack of knowledge concerns in particular nonmodel species of ecological and/or economic interest as they are increasingly affected by recurrent mass mortalities. The objective of the present study was to investigate mass mortality events occurring in the striped venus clam, *Chamelea gallina*, by applying a holistic approach able to concomitantly characterize host transcriptional profiles and the dynamics of their associated microbiota. The striped venus clam *C. gallina* (Linnaeus, 1758) is a bivalve venerid mollusk distributed throughout the Mediterranean and Black Sea (Moschino & Marin, 2006), inhabiting sandy bottoms from the lower shore to depths of approximately 15 m (Morello, Froglia, Atkinson, & Moore, 2005). Commercial landings of *C. gallina* have an important economic role along the central and northern Adriatic coasts of Italy (Moschino & Marin, 2006; Ramon & Richardson, 1992), and consumer interest in this clam species has recently increased (Orban et al., 2006). The number of natural beds of the striped venus clam in the Adriatic Sea has significantly declined over the last decade due to unexplained mortality events, forcing national and regional authorities to introduce new regulations to preserve juveniles by forbidding the fishery of clams less than 25 mm in diameter. Multiple factors have been proposed to be involved in venus clam mass mortalities (Del Piero, Piero, Fornaroli, & Balzo, 1998; Froglia, 2000), such as pollution (Visciano et al., 2015), alterations in water temperature and salinity (Ezgeta-Balić et al., 2011; Matozzo et al., 2012; Monari, Foschi, Rosmini, Marin, & Serrazanetti, 2011; Sobral & Widdows, 1997), summer phytoplankton blooms (Romanelli, Cordisco, & Giovanardi, 2009), increased predation, fishery pressure (Moschino, Deppieri, & Marin, 2003) and infectious diseases (Milan, Matozzo, et al., 2016; Milan, Palazzo, et al., 2016; Torresi et al., 2011). Among the most affected sites, the Abruzzo coast has experienced significant fluctuations in fishing yields over the last six years and has observed a severe demographic decline of the locally available *C. gallina* stock. A first study (Milan, Matozzo, et al., 2016; Milan, Palazzo, et al., 2016) focused on comparing gene expression profiles of *C. gallina* collected at different sampling times (from June 2014 to September 2014) along the Abruzzo coast in a control site (T7; Francavilla) and in a site subjected to mortality events (T4; San Vito) through a DNA microarray approach. This study highlighted (a) similar chemical concentrations of heavy metals, polycyclic aromatic hydrocarbons (PAHs) and pesticides (OCPs, OPPs) in sediments between investigated sites, suggesting that chemical pollution was not the singular primary cause of differences in mortality observed between sites T4 and T7, although *Artemia fraciscana* toxicity tests showed higher mortality in T4; (b) consistent up-regulation of several genes involved in immune response at all sampling times; (c) reduced energy metabolism in T4 clams, likely related to metabolic depression affecting the energy budget available for immune response. The substantial number of overexpressed genes involved in the immune response strongly supported the hypothesis of a bacterial and/or viral infection as the major cause of clam mortality in T4 (Milan, Matozzo, et al., 2016; Milan, Palazzo, et al., 2016). Additional stressors such as transitory episodes of acute chemical contamination or reduced salinity could not be excluded as possible factors contributing to increased susceptibility to infection. Since the summer of 2015, a dramatic reduction in landings was also reported in Francavilla (T7) by fishing cooperatives (mainly affecting juvenile individuals), resulting in a severe stock reduction that raised the concerns of fishermen and control authorities (personal communications by fishermen and control authorities).

Here, a novel approach combining the microbiome characterization (16S) and gene expression profiles (RNA-seq) has been applied to this case study. This holistic approach allows us to shed light on the potential stressors involved in mortality events, demonstrating the potential of this approach to investigate the responses of marine species to environmental stressors and to prevent risks associated with pollutant-pathogen interactions.

2 | MATERIAL AND METHODS

2.1 | Sampling of *Chamelea gallina*

Chamelea gallina specimens were collected from the following three sites: T7 (near Francavilla; 42°16′59″N, 14°29′41.6″E), T4 (near San Vito; 42°25′37.8″N 14°17′28.8″E) and Cupra Grottamare (CG; 43°2′3.026″N 13°51′53.894″E; Figure 1) considered as a "control" site. Sampling was performed at 0.25 mi (ca. 0.4 km) from the coast in collaboration with local fishing cooperatives. No dead *C. gallina* individuals were found in the CG control site, while in T4 and T7 sites, it was extremely difficult to find live individuals. Individuals were collected at commercial size (min. 25 mm) by hydraulic dredge. All individuals considered for microbiological, gene expression and microbiota analyses appeared to be in good health as confirmed by histological analyses (see below), with no apparent differences in reproductive status between the control site and sites subject to mortalities. The sampling took place in July (T4 and CG) and September (T4, T7 and CG) 2016. After fishing, clams were kept on ice until their

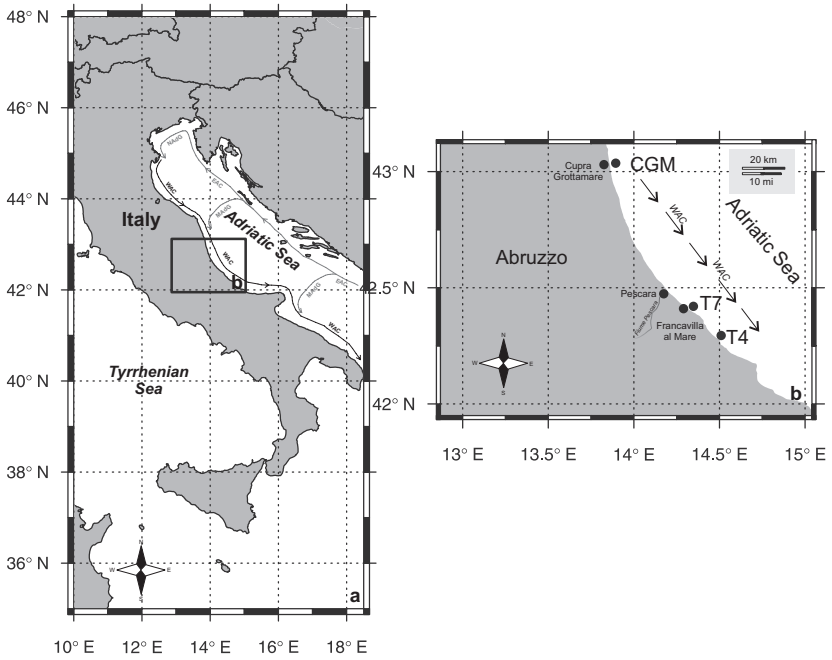

FIGURE 1 *Chamelea gallina* sampling sites along the Abruzzo coast and circulation pattern in the Adriatic Sea

arrival at the laboratory, within 6 hr. At each sampling site and time, the digestive gland of approximately 80 clams was dissected, frozen in liquid nitrogen and stored at −20°C for gene expression and microbiota analyses. In addition, approximately 100 clams for each sampling site/time were transferred to the Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe) for microbiological analyses. All sample sites, sampling times and analyses performed are summarized in Table S1.

2.2 | Histological, microbiological and electron microscope analyses

The whole soft tissue of 30 specimens for each sampling time and site was fixed in Carson's solution for 48 hr. The sample was then reduced to obtain transverse whole-body sections. The tissue was dehydrated and embedded in paraffin, and 3 µm sections were stained with Harri's haematoxylin and eosin-phloxine. For each sample, a pool of 50 g (about 15 clams) containing flesh and intravalvular liquid was transferred to a stomacher bag, then homogenized for 1 min. From the homogenate, 50 µl was directly plated in duplicate on marine salt agar for the total viable bacteria count, and another 50 µl was plated on thiosulfate-citrate-bile-sucrose (TCBS). Random colonies of *Vibrio* spp. from TCBS plates were identified to the species level through MALDI-TOF mass spectrometry. To do so, first, the plates were incubated at 22°C for 4 days. Preliminary identification of *Vibrio* spp. strains was performed on the basis of colony morphology on TCBS, oxidase test, sucrose fermentation and salt requirements. The suspected colonies were analysed with API 20E (modified adding 2.5% NaCl in the microtubes) and vibriostatic O129 (10 µg and 150 µg). To confirm the preliminary identification, strains were identified using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). MALDI-TOF target plates were inoculated into the spots by picking a freshly grown overnight colony and overlaid with 1 ml of 70% formic acid (Sigma-Aldrich). Each spot was allowed to dry and subsequently overlaid with 1 ml of matrix (a-cyano-4-hydroxycinnamic acid). Mass spectra were acquired using the MALDI-TOF MS spectrometer in a linear positive mode (Microflex LT, BrukerDaltonics). The MALDI BIOTYPER BDAL Library 5627 MSPs (v. 4.0.0.1) and the MALDI BIOTYPER software version 3.1 were used for bacterial identification. The similarity of patterns was represented as a score (≥2.000, identification at the species level; 1.700 to 1.999, identification at the genus level; <1.700, no reliable identification). Supernatants were put onto Formvar/carbon-coated grids, ultra-centrifuged for 15 min in a Beckman Air-fuge at 20 psi (125,000 g) using an A-100 rotor, stained with 1% phosphotungstic acid solution and subjected to direct transmission electron microscopy (TEM; Philips EM208S operating at 80 kV, at a magnification of 19,000–45,000).

2.3 | qPCR for total bacteria and Vibrio quantification

In order to define the total bacteria and *Vibrio* community load, DNA extraction was performed using DNeasy PowerSoil Kit (Qiagen) from the pools of *C. gallina* digestive gland employed for gene expression analyses and microbiota characterization (see below). In detail, each pool was composed of five individuals pooled together based on their DNA concentrations to obtain five pools for each site and each time point. After extraction, the quality of DNA was assessed by agarose gel electrophoresis 1%. Quantification of total 16S rDNA and 16S rRNA genes specific to the *Vibrio* genus were performed using quantitative PCR (qPCR). All amplification reactions were

analysed using a Roche LightCycler 480 Real-Time thermocycler. The total qPCR reaction volume was 10 μl and consisted of 2.5 μl DNA (10–50 ng/μl) and 7.5 μl LightCycler 480 SYBR Green I Master mix (Roche) containing 10 μM PCR primer (Eurofins). Total bacteria specific primer pairs were the 16S_Fw—TCCTACGGGAGGCAGCAGT and 16S_Rev—GGACTACCAGGGTATCTAATCCTGTT, targeting the variable V3V4 loops for bacterial communities (Nadkarni, Martin, Jacques, & Hunter, 2002). Total *Vibrio* specific primer pairs were Vib1-Fw GGCGTAAAGCGCATGCAGGT and Vib2-Rev GAAATTCTACCCCCCTCTACAG (Siboni, Balaraju, Carney, Labbate, & Seymour, 2016), targeting the 16S rRNA of *Vibrio* genus. As a reference gene, we considered the universal 18S ribosomal RNA gene, using the specific primer pairs bivalve_uni_Fw CCGATAACGAACGAGACTC and bivalve_uni_Rev CACAGACCTGTTATTGCTC.

2.4 | RNA extraction and microbiota analyses

Total RNA was extracted from the digestive gland of individual samples with RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Each extraction cycle included a sterility control. Pools of total RNA were composed of five individuals pooled together based on their RNA concentrations to obtain five pools for each site and each time point. Samples collected in September 2016 were considered for both gene expression (RNA-Seq) and microbiota analyses (16S) using the same RNA pools. Microbiome analysis was also performed for samples collected in July in T4 and CG. A single sterility control pool was created by mixing 1 μl of each sterility control, and the resulting pool was included in the microbiome analysis. RNA integrity number (RIN) index was calculated for each sample using AGILENT 2100 EXPERT software. The RIN provides a numerical assessment of the integrity of RNA, facilitating the standardization of sample quality interpretation. In order to reduce experimental biases in RNA-Seq analysis due to poor RNA quality, only RNA samples with a RIN > 7 were further processed.

For microbiota analyses, 1 μg of RNA was retro-transcribed to cDNA using the Superscript IV kit (Invitrogen, Life Technologies). cDNA was diluted to 0.2 ng/μl and amplified in a 50 μl reaction including 5 μl diluted DNA and 1.5 μl of both reverse and forward primers (10 μM) that specifically target the V3–V4 gene region of the bacterial 16S rRNA as described by Milan et al. (2018). Libraries were then pooled together based on their concentrations, and the final pool was quantified using a Bioanalyzer 2100 (Agilent Technologies) and sequenced by GENOMIX4LIFE s.r.l. with a Miseq Illumina 2x300 (Illumina). The microbiome sequencing generated 5 million reads, averaging about 170,000 reads per pool (sequences available in NCBI Sequence Read Archive SRA https://www.ncbi.nlm.nih.gov/sra; BioProject PRJNA494711). Raw reads were trimmed using CLC Genomic WorkBench version 10.1.1 to eliminate adaptors and select high-quality sequences (https://www.qiagenbioinformatics.com/). The same software was used to merge forward and reverse sequences of the same fragment. Sequences were analysed with QIIME 1 (Quantitative insights into microbial ecology; Caporaso et al., 2010) to explore the microbial communities in each pool. First, merged reads were clustered by OTU (Operational Taxonomic Unit) based on their sequence similarity and a taxa assignment text file was created by blasting clusters to Greengenes 13_5 (http://greengenes.secondgenome.com), a 16S full-length database with a minimum match value of 0.8. Data were normalized by random sampling based on the number of OTUs in the least represented pool. Rare sequences were filtered out of the final OTU list by applying a 0.005% cut-off. In order to analyse the microbial diversity in a single sample (richness), alfa-rarefaction data were used to calculate different metric statistics. β-diversity was calculated using the UNIFRAC method for comparing biological communities in order to estimate the variability of microbial communities between pairs of samples. The first statistical analyses on microbiota composition were performed using the software CALYPSO, version 8.20 (Zakrzewski et al., 2017) using the OTU table produced in QIIME. Data were normalized using the cumulative sum scaling (CSS + log) as explained in Paulson, Colin Stine, Bravo, and Pop (2013). Every group of samples was organized by Principal Coordinates Analysis (PCoA), and a two-way ANOVA was carried out in order to identify different taxa between sample groups (i.e., sites subjected to mortality events vs. control site). The OTU table produced in QIIME was also used for PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) in Galaxy (http://huttenhower.sph.harvard.edu/galaxy/; Langille et al., 2013) to carry out a functional analysis of detected microbial communities. The PICRUSt output was visualized with STAMP (Statistical Analysis of Metagenomic Profiles (http://kiwi.cs.dal.ca/Software/STAMP).

2.5 | RNAseq library preparation and sequencing

As mentioned above, RNA-Seq analyses were performed for samples collected in September in T4, T7 and CG sites (Table S1). The cDNA libraries were constructed starting from five pools for each sampling site using a SureSelect Strand-Specific mRNA Library (Agilent Technologies) according to the manufacturer's protocol. Briefly, before fragmentation, oligo d(*T*) beads were used to purify poly(A) mRNA from total RNA. First-strand cDNA was synthesized from the fragmented mRNA using random hexamer primers, and the cDNA libraries were prepared in accordance with the Illumina protocol. After a purification step, the libraries were quantified with a Qubit Fluorometer (Invitrogen) and pooled together according to their relative concentrations. The concentration and quality of the pool was assessed by Agilent 2100 Bioanalyzer. Library pools were sequenced by HiSeq 4000 (Illumina) with a 150 bp paired-end approach (University of California) yielding a total of 780,364,422 reads (details are reported in Table S2, sequences available in NCBI SRA, BioProject PRJNA494495).

2.6 | *Chamelea gallina* transcriptome assembly

A FastQC report was used to perform an initial quality check of the raw sequencing data (Babraham Bioinformatics). Adapter trimming was

carried out on CLC GENOMICS WORKBENCH v.10.1.1. *C. gallina* transcriptome assembly was performed with CLC GENOMIC WORKBENCH version 10.1.1 with the following parameters: Minimum contig length = 250; Perform scaffolding; Auto-detect paired distances; word size: 18; Bubble size: 45; map reads back to contigs. A total of 266,178 transcripts were obtained (reported in File S1) and used as the reference transcriptome for RNA-seq reads mapping. Transcriptome annotation was performed by Blastx similarity search on Swissprot (Uniprot), *Homo sapiens* protein Ensembl database, *Danio rerio* protein Ensembl database, *Crassostrea gigas* protein Ensembl and *Drosophila melanogaster* protein Ensembl database (Evalue < 0.0001). Of 266,178 unique sequences, 64,315 (24.16%) showed at least one significant match. Details are reported in Table S3, while the annotation of each contig is reported in File S2. Trimmed RNA-Seq reads of each sample were then mapped against the reference transcriptome to obtain gene expression profiles of each pool.

2.7 | Gene expression analyses

Mapping of RNA-Seq reads against the reference transcriptome was performed in CLC GENOMIC WORKBENCH version 10.1.1with the following parameters: mismatch cost 2, insertion cost 3, deletion cost 3, length fraction 0.8 and similarity fraction 0.8. For each pool, between 85.97% and 87.54% of the total reads were successfully mapped. Gene counts were then carried out using EdgeR to analyse differential gene expression (Robinson, McCarthy, & Smyth, 2010). Samples were grouped according to sampling area and were normalized using the Trimmed Mean of *M*-values (TMM) method. After normalization, only annotated transcripts were kept for subsequent analysis. Likelihood-ratio test (LRT) was carried out with EDGER to assess differentially expressed genes (DEGs), with significant log fold change (log FC) threshold set to >1 and false discovery rate (FDR) set to <0.05. A functional interpretation of differentially expressed genes was obtained by enrichment analysis using Database for Annotation, Visualization, and Integrated Discovery (DAVID ver. 6.8) software (Dennis et al., 2003; Huang, Sherman, & Lempicki, 2009), considering GO Biological Process (BP), Cellular Component (CC) and Molecular Function (MF) Database and KEGG pathways. DAVID retrieves the functional annotation of differentially expressed genes through enrichment analyses based on an integrated biological knowledge-base containing over 40 annotation categories. Since DAVID databases contain functional annotation data for a limited number of species,

TABLE 1 Summary of results obtained through microbiological analyses

	CG_Ju	CG_Se	T4_Ju	T4_Se	T7_Se
Viable counts data (CFU/g)	7.2×10^4	1.5×10^7	4.8×10^7	2.3×10^7	1.2×10^7
Parasites	*1/30 Nematopsis* sp.	*Rare Nematopsis*	*3/30 Nematopsis* sp.	*Rare Nematopsis*	*Rare Nematopsis*
Virus	Negative	Negative	Negative	Negative	Negative
Bacteria detection through MALDI-TOF					
Bacteria/total colonies investigated	6	7	10	8	8
Vibrio diabolicus	3	1	3		
Vibrio harvey	2	1	2	2	2
Vibrio tubiashii	1	1	2	2	
Vibrio orientalis		1			
Vibrio fortis		1		2	
Vibrio alginolyticus		1		1	3
Vibrio pomeroyi			1		
Vibrio rotiferianus		1	1		1
Vibrio orientalis			1		
Vibrio campbelli				1	
Vibrio pelagius					2
Relative quantification of total bacteria and total *Vibrio* load (qPCR)					
Bacterial 16S relative quantification ($2^{-\Delta\Delta CT}$)	0.998 ± 0.713	9.904 ± 5.961	2.154 ± 2.07	24.339 ± 33.689	10.791 ± 18.774
Total *Vibrio* 16S relative quantification ($2^{-\Delta\Delta Cq}$)	0.735 ± 0.369	0.493 ± 0.432	12.895 ± 9.306	1.009 ± 0.202	9.826 ± 18.791

Note: For each investigated sites, "Viable counts data" (*C. gallina* homogenate), "Parasites," "Virus," "Bacteria/yeast" detections are reported. Identification of colonies in TCBS Agar through MALDI-TOF mass spectrometry is also reported: number of total investigations for each sampling site/time and the corresponding number of each *Vibrio* spp. identified are reported. Relative quantification of total bacteria and total Vibrio load obtained by qPCR are also reported. qPCR has been performed in the same pools of samples considered for gene expression and microbiota analyses. The $2^{-\Delta\Delta Cq}$ here reported represent the mean value of the 5 pools analysed for each sampling time/site. CG, Cupra Grottamare; T4, San Vito; T7, Francavilla. "Ju" and "Se" indicate sample time: Ju, July; Se, September.

C. gallina transcripts were associated with sequence identifiers that could be recognized in DAVID (e.g., Swissprot and Zebrafish Gene IDs). This was carried out using dedicated BLAST searches performed with blastx. The assignment of a putative homologue to a larger number of clam transcripts was obtained using Swissprot (Uniprot). IDs corresponding to differentially expressed striped venus transcripts and to all genes represented in the *C. gallina* transcriptome were obtained and were then used in DAVID to define a "gene list" and a "background," respectively. A functional annotation was obtained for significant genes identified by each pairwise comparison (CG vs. T4; CG vs. T7; T4 vs. T7). DAVID settings: gene count = 2 and ease = 0.1.

3 | RESULTS

3.1 | Microbiological analyses and quantification of bacteria and *Vibrio* spp. by qPCR

The results obtained by microbiological analyses are summarized in Table 1. The viable count data indicate that the number of colony forming units (CFU) per bivalve (*C. gallina* homogenate) was higher than those detected during routine controls carried out by the authorities responsible for guaranteeing food safety (10^3–10^4 CFU/g; personal communication), in both sites subject to mortalities (T7 and T4) in July (10^7 CFU/g) and in the control site CG in September (1.5×10^7 CFU/g). Based on histological examination performed on 30 individuals for each investigated site/time of sampling, the percentage of parasites detected in the clams was generally low and the species detected are usually not pathogenic to *C. gallina*. Bacterial infection and extent of necrosis were detected in only 2 of 30 analysed individuals collected in T7. Virus detection by electronic microscopy was negative. Random colonies of *Vibrio* spp. from TCBS plates were also identified to the species level by MALDI-TOF mass spectrometry. Although the number of identified species was limited, this analysis showed that sites subject to mortality (T4 and T7) were not characterized by any specific *Vibrio* spp. Relative quantification of total bacterial communities obtained by qPCR in *C. gallina* digestive gland confirmed the results obtained by viable count data (Table 1 and Figure S1). A significant increase in total bacterial abundance was observed in CG clams collected in September with a value similar to those detected in mortality sites T4 and T7. However, the relative quantification of total *Vibrio* spp. revealed higher Vibrio load in T4 and T7 compared with CG at both sampling times, suggesting that the increase in total bacteria communities revealed in CG_Se is not associated with an increased presence of potentially pathogenic *Vibrio* species.

3.2 | Microbiome analyses

Similar to what previously found in the Manila clam, mussels and oysters (Cleary, Becking, Polónia, Freitas, & Gomes, 2015; King, Judd, Kuske, & Smith, 2012; Milan et al., 2018; Trabal et al., 2012), the overall composition of clam-associated bacterial communities showed a predominance of the phyla *Proteobacteria* (60%) and *Tenericutes* (33.4%), of the classes *Alphaproteobacteria* (42.8%) and *Mollicutes* (33.2%) and of the genus *Mycoplasma*. The overall distribution of phyla is reported in Figure S2. β-diversity was evaluated through ANOSIM among sampling times (July [Ju] vs. September [Se]), sites (three groups CG vs. T4 vs. T7), sampling time and sites (five groups, CG_Ju, CG_Se, T4_Ju, T4_Se, T7_Se). All comparisons

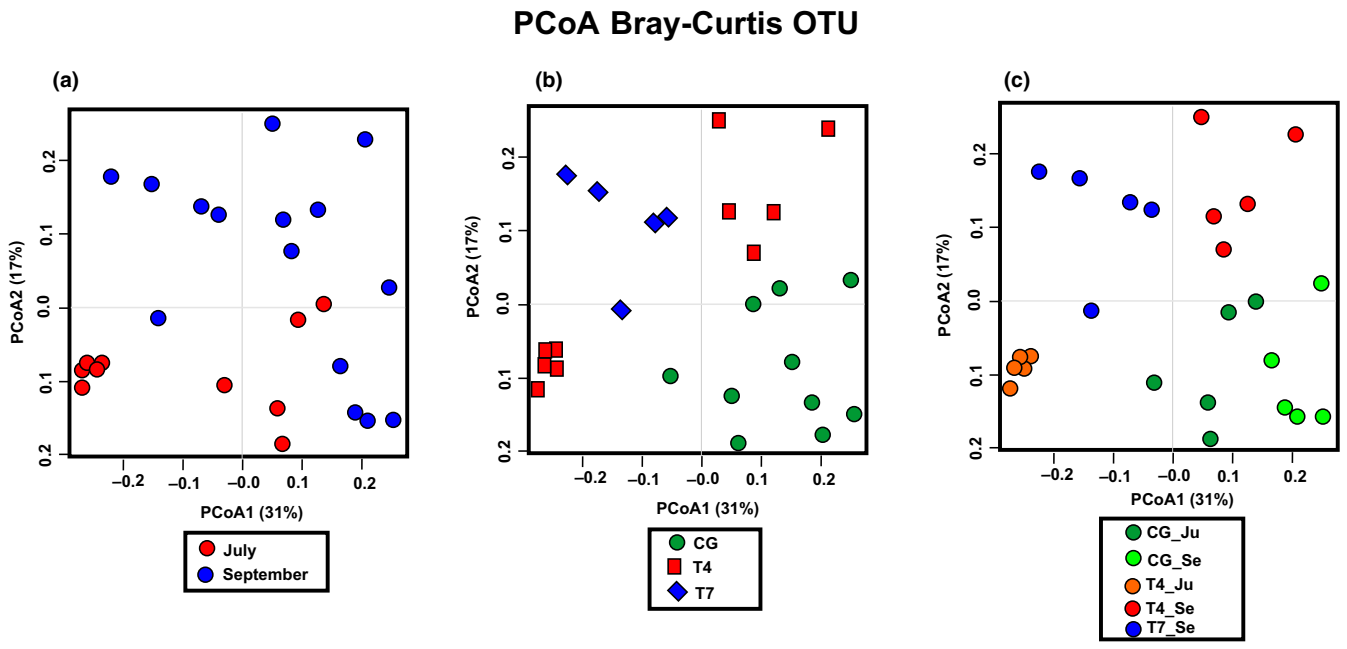

FIGURE 2 Unweighted Principal Coordinates Analysis (PCoA). (a) Different colours indicate the two investigated sampling period (July 2016, September 2016); (b) Different colours indicate sampling sites: samples collected in CG (control site) and in sites subject to mortality (T4, T7) are indicated in green, red and blue, respectively. (c) Each colour indicates different sampling site/time

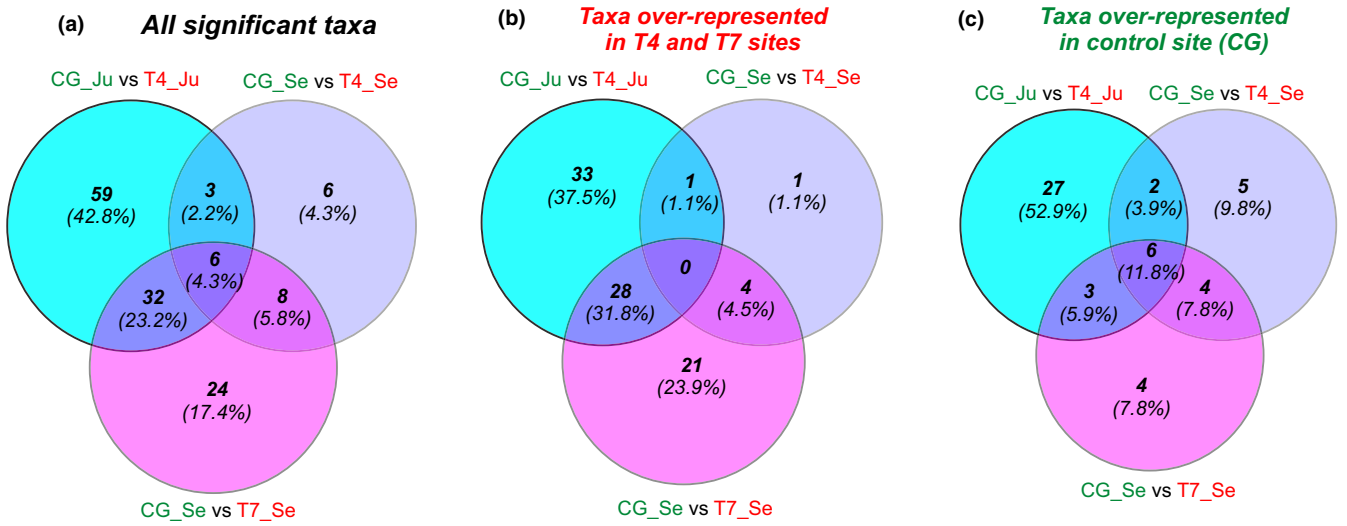

FIGURE 3 Venn diagram showing taxa differentially represented in the comparisons between control site (CG) and sites subject to mortality (T4 and T7), that are CG_Ju vs. T4_Ju, CG_Se vs. T4_Se; CG_Se vs. T7_Se. (a) All significant taxa obtained by each comparison (ANOVA) have been considered. (b) Taxa over-represented in mortality sites compared with CG were considered. (c) Taxa over-represented in control site compared with T4 and T7 were considered

showed significant "time" and "site" heterogeneity among sample groups ($p < .05$) with higher statistical significance in the comparison between "sites" ($p < .001$).

The PCoA analysis confirmed the temporal and geographical heterogeneity among investigated sites and across sampling times and showed a clear separation between July and September in site T4. The control site (CG) appears separated from T4 and T7 independently of the sampling time (Figure 2). Overall, PCoA analyses highlighted temporal and geographical heterogeneity among investigated sites and across sampling times. Considering the temporal proximity of sampling activities (July and September 2016), these findings indicate that bivalve microbiota can be highly diverse and easily influenced by environmental factors (Cleary et al., 2015; Green and Barnes, 2010; Lokmer et al., 2016; Milan et al., 2018; Trabal Fernanndez, Mazon-Suastegui, Vazquez-Juarez, Ascencio-Valle, & Romero, 2014).

To better understand the differences between sampling sites, a one-way ANOVA analysis was performed to identify significant differences in microbial communities between CG, T4 and T7. Four separate pairwise comparisons were carried out within each sampling time to avoid the confounding effect of seasonal variation: (a) CG_Ju vs. T4_Ju; (b) CG_Se vs. T4_Se; (c) CG_Se vs. T7_Se; (d) T4_Se vs. T7_Se. The complete list of significant taxa for each comparison is reported in File S3. The comparison between the microbial profiles of clams collected in July in control site CG and in the mortality site T4 showed the highest number of differentially represented taxa with (100 OTUs, p-value < .05). This result could be related to the higher "total colony counts" detected in T4 (10^7 CFU/g) compared with CG (10^4 CFU/g; Table 1). Among the 62 over-represented taxa in T4, 31 OTUs (50%) belong to *Vibrio* genera or members of the *Vibrionaceae* family, and 12 OTUs represent *Photobacterium* genera (19%). Unfortunately, only few OTUs were able to reliably identify species, such as *Vibrio fortis* (2), *Vibrio shilonii* (2), *Vibrio ichtyoenteri*, *Photobacterium angustum*, *Photobacterium damselae*, *Photobacterium rosenbergii* (3). Similar results were found when comparing the mortality site T7 to the control site CG in September, with the 30% of over-represented OTUs in T7 belonging to the *Vibrio* genus or *Vibrionaceae* family and 7 OTUs (13%) belonging to the *Photobacterium* genera. Finally, the pairwise comparison between the two sites subjected to mortality (in September) identified 34 and 11 over-represented OTUs in T7 and T4, respectively. Among them, T7 showed an over-representation of the *Vibrio* genus or *Vibrionaceae* family (12 OTUs; 35%), the *Pseudoalteromonadaceae* family (8 OTUs; 25%) and the *Photobacterium* genera (4 OTUs; 11%), while site T4 showed an over-representation of the *Chlamydiales* order (4 out of 11 over-represented OTUs).

Venn diagrams were then constructed to identify commonly differentially represented taxa in sites subjected to mortality events (T4 and T7) compared with the control site (CG; Figure 3). Six taxa, represented by the *Mycoplasma* genus (3), the *Ehrlichia* genus, the *Rhodospirillaceae* family and the *Rickettsiales* order were found consistently under-represented in mortality sites, regardless of sampling season (Figure 3c). A total of 38 commonly differentially represented taxa were obtained comparing T4 and CG in July and T7 and CG in September. Among them, a total of 28 OTUs were over-represented in both mortality sites, representing the *Vibrio* (14) and *Photobacterium* (6) genera, the *Pseudoalteromonadaceae* family (6) and the *Aeromonadaceae* family (2).

Functional analysis using PICRUST and STAMP was then carried out for each comparison. The full list of significant terms is reported in File S4. Over-representation of several pathways involved in "Xenobiotic biodegradation and metabolism," such as "Benzoate degradation," "Naphthalene degradation," "Bisphenol degradation," "Atrazine degradation," "Polycyclic aromatic hydrocarbon

degradation" and "Dioxin degradation" in the microbiota of samples collected in T4 and T7 was observed, as well as pathways related to "Aminoacid metabolism" and "Biosynthesis of secondary metabolites." ANOVA results were confirmed by the over-representation of "*Vibrio cholerae* pathogenic cycle" and "*Vibrio cholerae* infection" pathways in T4 and T7 in July and September while the KEGG pathways related to "genetic information processing" were over-represented in CG.

3.3 | Gene expression analyses

To identify persistent transcriptional changes among the investigated sites, pairwise comparisons through EdgeR were performed (log FC > 1; FDR < 0.05). Comparisons between sites affected by mortality events (T4 and T7) and the control site CG showed a high number of DEGs (1,040 and 705, respectively), while fewer transcriptional differences were found between T4 and T7 (157). The complete lists of significant genes for each comparison are reported in File S5. A total of 394 transcripts were found to be differentially regulated in common in T4 and T7 when compared to CG with the same trend (Figure S3). Noteworthy, among the 321 DEGs found commonly up-regulated in mortality sites, a total of 40 genes encoded members of the *cytochrome p450 family* (*CYP450*), with fold changes ranging from 30 to 2.3 (File S5). In addition, 4 putative *Glutathione S-transferase* (*GSTs*), 32 putative *Sulfotransferase* (*SULT*) and *Glutathione peroxidase* (*GPX*) were also found commonly up-regulated in mortality sites. Up-regulated were also three genes coding for *Nose resistant to fluoxetine protein 6 (NRF6)*, *Inhibitor of apoptosis protein* (*IAP*) and *Growth arrest and DNA-damage-inducible protein GADD45 gamma* (GADD45; File S5). Among genes found down-regulated in common at sites T4 and T7, several genes involved in immune response stand out, such as *Interferon-induced protein 44*-like (IFI44L), *Macrophage-expressed gene 1 protein* (MPEG1) and *Toll-like receptor 5* (see File S5).

When up-regulated genes in mortality sites were considered, a significant enrichment of the KEGG pathways *Arachidonic acid metabolism*, *Chemical carcinogenesis*, *PPAR signaling pathway*, *Drug metabolism—cytochrome P450*, *Metabolism of xenobiotics by cytochrome P450* and *Peroxisome* was observed. Conversely, down-regulated genes showed significant enrichment for the BP terms "defense response" (31 and 14 down-regulated genes in T4 and T7, respectively), "activation of immune response" (16 and 6 down-regulated genes in T4 and T7, respectively), "inflammatory response" (17 and 7 down-regulated genes in T4 and T7, respectively), "defense response to bacterium" (9 and 4 down-regulated genes in T4 and T7, respectively), "complement activation" (6 and 3 down-regulated genes in T4 and T7, respectively) and "regulation of response to wounding" (5 and 4 down-regulated genes in T4 and T7, respectively). Enrichment analyses also revealed significant down-regulation in T4 and T7 clams of several pathways typically involved in virus response such as *response to virus* (6 genes down-regulated in T4), *defense response to virus* (5 genes down-regulated in T4), *viral processes* (17 and 8 genes down-regulated in T4 and T7, respectively), *viral life cycle* (12 and 5 genes down-regulated in T4 and T7, respectively) and of genes involved in "response to stress" (43 and 25 down-regulated genes in T4 and T7), "regulation of response to stress" (22 and 11 down-regulated genes in T4 and T7) and "positive regulation of response to stimulus" (32 and 8 down-regulated genes in T4 and and T7). The full lists of enriched Biological Process, Cellular Component, Molecular Function and Molecular Pathways for up- and down- regulated genes within each comparison are reported in File S6.

4 | DISCUSSION

In this study, we seek to decipher the complex host-microbiota interactions underlying the mass mortalities affecting the clam *Chamelea gallina*, providing a more complete representation of the factors potentially involved in the recent population decline. In our previous study, gene expression profiles of the striped venus *C. gallina* in a mortality site (T4) revealed the up-regulation of several genes involved in immune response. The results obtained suggested bacterial and/or viral infection as the major cause of clam mortality (Milan, Matozzo, et al., 2016; Milan, Palazzo, et al., 2016). Here, by using a combination of microbiota characterization (16S) and host-gene expression profile through next-generation sequencing (NGS), we provide a new hypothesis on the mechanism that may have impaired the physiological response of clams and favoured infection.

4.1 | Impacts of chemical pollutants in natural populations affected by mass mortality

Indirect evidence of potential exposure to chemical pollutants is provided by *C. gallina* gene expression profiles, which show a large number of up-regulated genes involved in xenobiotic metabolism and in the antioxidant defense system in sites T4 and T7 when compared to CG, such as CYP450 (Livingstone, Kirchin, & Wiseman, 1989; Stegeman, 1985; Zanette, Goldstone, Bainy, & Stegeman, 2010), SULT (Milan, Ferraresso, et al., 2013; Milan, Matozzo, et al., 2016; Milan, Palazzo, et al., 2016; Pessatti et al., 2016), GSTs (Mezzelani et al., 2016; Milan, Matozzo, et al., 2016; Milan et al., 2015; Park, Ahn, Kim, Lee, & Shin, 2009; Xu, Pan, Liu, Wang, & Miao, 2010) and GPX (Cossu et al., 1997). Noteworthy, GADD45G, that is involved in DNA repair, cell cycle control and apoptosis (Fornace, Jackman, Hollander, Hoffman-Liebermann, & Liebermann, 1992), showed the highest fold changes in mortality sites when compared to the control site. This up-regulation has also been previously described in the Manila clam and in mussels exposed to ibuprofen and pyrene-contaminated plastics (Avio et al., 2015; Milan, Pauletto, et al., 2013), further corroborating its involvement in counteracting genotoxic stress (Takekawa & Saito, 1998). Finally, three transcripts coding for putative NRF6 were also commonly up-regulated in sites T4 and T7. NRF6 plays a role in the uptake of a range of molecules including xenobiotic compounds from the intestine to surrounding tissues in the nematode *Caenorhabditis elegans* (Choy, Kemner, & Thomas, 2006; Choy & Thomas, 1999) and was found positively correlated

with increased concentrations of PCBs, PCDD/Fs, HCB and PBDEs in the Manila clam (Milan, Ferraresso, et al., 2013, 2015).

Although it is a hypothesis that requires further validation, it is possible that the observed microbiome-transcriptome response is triggered by chemical pollutants originating from the freshwater conveyed to the coast by several rivers that flow into the Adriatic Sea (e.g., the Pescara, Foro, Arielli and Feltrino rivers; Figure 1). Waters from these rivers are transported southwards by the Western Adriatic Current, increasing the probability that southern sites along the coast become regularly contaminated by untreated urban sewage and industrial and agricultural pollutants. The Pescara river, the longest river in Abruzzo, collects runoff waters from a densely populated area that includes several urban settlements and industrial facilities. Historically, toxic waste was illegally discharged in this river in the industrial area of Bussi sul Tirino, where water flows down from the Apennine Mountains into the Pescara river. In 2007, Bussi sul Tirino was claimed to be the biggest illegal toxic waste dump in Europe, containing years' worth of dangerous industrial waste responsible for contaminating almost 2 million tons of soil, as well as both the superficial and deep-water tables, with dozens of toxic compounds (Di Francesco, 2010; Piccoli et al., 2010).

In a previous study by Milan, Matozzo, et al. (2016) and Milan, Palazzo, et al. (2016), chemical pollution was ruled out as a primary trigger of clam mortality based on the chemical analyses and gene expression profiles of *C. gallina* in sites T4 and T7. In this study, the combination of transcriptomic and microbiota profiles and the documented high contamination load in river run-off suggest a different scenario, whereby xenobiotic exposure may instead play a crucial role in clam mortality. Three reasons may explain the discrepancy between this study and the one by Milan, Matozzo, et al. (2016) and Milan, Palazzo, et al. (2016). First, lack of transcriptional differences between T4 and T7 related to xenobiotics response in Milan, Matozzo, et al. (2016) and Milan, Palazzo, et al. (2016) might be ascribed to geographical proximity of the two sites, considered to be equally impacted by chemical stress (Figure 1). In this study, we introduced a new control site (Cupra Grottamare; CG) located north to the Pescara river mouth and therefore less affected by the river plume and discharge. In this site, no mass mortality events have ever been recorded in the control site CG. Comparisons among mortality sites and control site suggest that chemical contaminants affect clams in T4 and T7 with equal severity. Secondly, only a few compounds such as heavy metals, PAHs, and pesticides have been tested, to date, in chemical analyses of sediments and clams in the affected area (Milan, Matozzo, et al., 2016; Milan, Palazzo, et al., 2016; Visciano et al., 2015). Considering the complexity of chemical pollutants potentially contaminating the Pescara river waters, we cannot exclude that other, previously unassessed, chemical compounds may be present in marine sediments where clams live. Thirdly, chemical pollution in the areas affected by mass mortality might be episodic rather than constant, with acute peaks of exposures triggered by events such abundant rainfall. Episodic exposure peaks, being transitory, would be more difficult to detect through periodic chemical analysis of the sediment and water column or by bioaccumulation analysis. Unfortunately, for several reasons chemical analyses to determine the concentrations of potential pollutants in the water column, sediments and biota could not be carried out on 2016 samples. That said, we believe that these analyses may not prove conclusive unless future sample collection and chemical analyses are carried out frequently and promptly after abundant rainfall.

In keeping with transcriptomic data, functional analysis of microbial communities also highlighted significant over-representation of several pathways involved in xenobiotic metabolism in clams from mortality sites. A similar pattern was observed in Manila clams collected in highly polluted sites (Milan et al., 2018). Likewise, other significant pathways ("Amino acid metabolism," "Biosynthesis of secondary metabolites," "Genetic information processing") showed a consistent behaviour in the two species. As previously suggested (Milan et al., 2018), exposure to pollutants might open the way to opportunistic infection leading to host-inflammation and enhanced protein turnover. In turn, the reduction in carbohydrate supply in inflamed tissues may select bacterial taxa that mostly rely on amino acid metabolism for energy production (Davenport et al., 2014).

4.2 | Abundance of opportunistic pathogens in sites subjected to mass mortality

Overall, histological, microbiological and electron microscope analyses showed no differences between the investigated sites for the presence of viruses and parasites, while bacterial infection and extent of necrosis were found in only T7 site in 2 of 30 investigated individuals. Considering that at the time this manuscript was written (March 2019) no mortality had been found in CG while mortality persisted in T4 and T7 sites, the higher total number of bacteria detected in CG in September may be linked to seasonal variations such as rainfall and high summer temperatures (Tabanelli et al., 2017) and is likely not associated with increased presence of potentially harmful bacterial species. This is supported by results obtained through quantification of *Vibrio* spp. using quantitative qPCR, which showed a higher total *Vibrio* spp. load in T4 and T7 compared with the control site CG in both the July and September sampling times (Table 1). MALDI-TOF analyses showed different combinations of *Vibrio* spp. in each sampling site/time, while sites subject to mortality were not represented by specific *Vibrio* spp. However, MALDI-TOF analysis was applied to a limited number of colonies (from 6 to 10 per sampling site/time), and we should exercise caution in interpreting this result. Indeed, when 16S microbiota data are considered, pairwise comparisons revealed 38 common taxa differentially represented in T4 and T7 compared with CG. The dominant organisms belonged to *Vibrio* spp. (14 OTUs) and *Photobacterium* spp. (6 OTUs) while the prevailing significant pathways were "Vibrio cholerae pathogenic cycle" and "Vibrio cholerae infection". Vibrios are ubiquitous marine bacteria with high metabolic versatility and genetic variability that confer a high colonization potential (Hazen, Pan, Gu, & Sobecky, 2010; Le Roux, Wegner, & Polz, 2016; Reen, Almagro-Moreno, Ussery, & Boyd, 2006). *Vibrio* spp. have been suggested to be among the major

causes of mass mortality in oyster beds, mostly affecting larvae and juveniles and resulting in losses of up to 80%–100% of oyster production (Eiston, Hasegawa, Humphrey, Polyak, & Hase, 2008; Le Roux et al., 2016; Petton et al., 2015; Sugumar, Nakai, Hirata, Matsubara, & Muroga, 1998). The elevated abundance of *Vibrio* spp. in oyster beds has been recently confirmed by 16S microbiota analyses that demonstrated the implication of the *Vibrio* community either as infective agents or opportunistic colonizers in oyster mass mortality events (King et al., 2019). In our study, 50% and 30% of significantly over-represented taxa in T4 and T7, respectively were assigned to *Vibrio* genera or *Vibrionaceae* family. Species-level identification, however, was possible only for 7 OTUs, which were represented by *Vibrio fortis*, *Vibrio ichthyoenteri* and *Vibrio shilonii*, already proposed as potential pathogens in others marine species (Austin, Austin, Sutherland, Thompson, & Swings, 2005; Ding, Dou, Wang, & Chang, 2014; Ishimaru, Akagawa, & Muroga, 1996; Romalde, Diguez, Lasa, & Balboa, 2014; Rosenberg, Kushmaro, Kramarsky-Winter, Banin, & Yossi, 2009; Wang, Zhang, Qin, Luo, & Lin, 2016).

Potentially pathogenic *Photobacterium* species were found in high abundance in the microbiota of *Vibrio*-infected *C. gigas* (Lokmer & Mathias Wegner, 2015). *Photobacterium* is one of the oldest genera in the family *Vibrionaceae* and is commonly found in the marine environment and in the intestinal contents of marine animals. In the present study, members of the genus *Photobacterium*, such as *Photobacterium damselae*, were over-represented in T4 and T7. *Photobacterium damselae* is a pathogen of a variety of marine animals including cultured fish species (Labella, Manchado, et al., 2010; Labella, Sanchez-Montes, et al., 2010; Labella et al., 2006; Pedersen, Skall, Lassen-Nielsen, Bjerrum, & Olesen, 2009; Romalde et al., 2014). *P. damselae* has also been isolated in *Octopus joubini* (Hanlon et al., 1984) and commercial bivalve species such as *Mytilus galloprovincialis* (Lozano-León, Osorio, Nuñez, Martínez-Urtaza, & Magariños, 2003) and oysters (Richards, Watson, Crane, Burt, & Bushek, 2008).

Although multiple infections involving a virus cannot be excluded in *C. gallina*, the down-regulation in both mortality sites of putative *Interferon-induced protein 44-like* (IFI44), a key element for effective antiviral defense in bivalve species (Olicard, Renault, Torhy, Benmansour, & Bourgougnon, 2005; Pauletto et al., 2017; Renault, Faury, Barbosa-Solomieu, & Moreau, 2011; Segarra et al., 2014) and the significant down-regulation of several pathways typically involved in virus response suggests that viral replications should be excluded in *C. gallina*. Furthermore, the absence of viral detection in the histological analysis carried out in our study supports the premise that viral replication is not at the root of *C. gallina* mortality as recently proposed in *C. gigas* (De Lorgeril et al., 2018).

4.3 | Interactions between pollutants and pathogens could play a key role in *C. gallina* mortality events

The abundance of opportunistic pathogens in clams from sites T4 and T7 might be explained by the disruption of host defense strategies, as suggested by transcriptional profiles. When compared to the control site, both mortality sites showed a significant down-regulation of several pathways and genes typically involved in immune defense, such as *Macrophage-expressed gene 1 protein*, *Toll-Like Receptor 5* and *Hemagglutinin/amebocyte aggregation factor* among others (Benard et al., 2015; Fujii et al., 1992; Moreira et al., 2012; Renault et al., 2011). These results suggest that clams in sites T4 and T7 may have reached a threshold of tolerance that hinders any further response to environmental stress. Several studies suggest a mechanism in which an environmental trigger compromises the host-immune response, thus promoting viral or bacterial infections in bivalves. In a recent study, using 16S rRNA amplicon sequencing, changes in the oyster microbiome were induced by water temperature increase to 25°C and included a significant increment of the abundance of the putative bacterial pathogens *Vibrio harveyi* and *Vibrio fortis* (Green et al., 2018). Therefore, a marine heat wave could potentially trigger a mass mortality event of *C. gigas* by boosting the replication of specific members of the oyster's bacterial community which become able to overwhelm the immunological capacity of physiologically stressed oysters (Green et al., 2018).

Pollution, even at low concentrations, has documented dramatic effects on the physiology, immune response and ecology of bivalves (e.g., Morley, 2009; Rittschof & McClellan-Green, 2005) and in combination with infectious diseases can trigger synergistic responses that are difficult to disentangle (detailed in Morley, 2010). Loss of efficiency in phagocytosis and clearance rate of bacteria owing to pollution toxicity are documented in bivalves (Cheng, Hsiao, & Chen, 2004a, 2004b; Gagnaire et al., 2007; Galloway & Depledge, 2001; Parry & Pipe, 2004; Pipe & Coles, 1995; Pipe & Coles, 1995). With a similar mechanism, we hypothesize that high concentrations of pollutants in T4 and T7 could have disrupted host physiological homeostasis in *C. gallina* and consequently could have facilitated opportunistic pathogens such as *Vibrio* and *Photobacterium* genera in immune-compromised individuals.

This hypothesis needs to be validated through routine chemical analyses and an experimental design implementing clam translocations between control and mortality sites, followed by gene expression analyses, microbiota characterization and microbiological analyses.

5 | CONCLUSIONS

Climate change, chemical contamination and the spread of emerging pathogens combined with fishery pressure are likely to have dramatic effects on marine ecosystems and aquaculture (e.g., Mohanty, Mohanty, Sahoo, & Sharma, 2010; Rizzi et al., 2016). These are major challenges for the shellfish farming industry and researchers for the years to come. Growing evidence on microbial compositions in marine organisms emphasizes the importance of host-microbial interactions in the response to environmental changes. In this study, we provide a revised hypothesis to explain mortality events occurring in *C. gallina*, demonstrating the potential of combining RNA-seq and

16S rRNA high-throughput sequencing to investigate the responses of marine organisms to changes in environmental conditions. This approach can represent a highly effective method for anticipating and interpreting the risks inherent in pollutant-pathogen interactions in rapidly changing environments.

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CONFLICT OF INTERESTS

The data of this study are original, and no part of this manuscript has been published or submitted for publication elsewhere. The authors declare no competing financial interest.

AUTHOR CONTRIBUTION

M.M., G.M., L.B., B.C. and T.P. conceived and designed the project. G.M., A.I., C.M., N.F., M.I. and L.G. performed *C. gallina* sampling. A.I., C.M., N.F., M.I. performed clam dissection and sample preparation. R.B. and G.A. performed histological, microbiological and electron microscope analyses. L.C., G.D.R. and B.C. performed laboratory experiments. L.C., M.S., G.D.R. and B.C. carried out 16S statistical analyses. M.M. performed gene expression analyses. G.D.R., S.I. and A.Z. performed qPCR. M.M., M.S., C.P., C.C., and L.B. wrote the manuscript and prepared the figures. All listed authors edited the final version of the manuscript. All authors read and approved the manuscript.

DATA AVAILABILITY STATEMENT

16S Sequence data are deposited in SRA database (BioProject PRJNA494711). RNAseq data sequencing files are available in NCBI Sequence Read Archive (SRA; https://www.ncbi.nlm.nih.gov/sra; BioProject PRJNA494495).

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SUPPORTING INFORMATION

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Ecotoxicological effects of the herbicide glyphosate in non-target aquatic species: Transcriptional responses in the mussel *Mytilus galloprovincialis*

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Ecotoxicological effects of the herbicide glyphosate in non-target aquatic species: Transcriptional responses in the mussel *Mytilus galloprovincialis*☆

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ABSTRACT

Glyphosate has been the most widely used herbicide worldwide over the last three decades, raising increasing concerns for its potential impacts on environmental and human health. Recent studies revealed that glyphosate occurs in soil, surface water, and groundwater, and residues are found at all levels of the food chain, such as drinking water, plants, animals, and even in humans. While research has demonstrated that glyphosate can induce a broad range of biological effects in exposed organisms, the global molecular mechanisms of action still need to be elucidated, in particular for marine species. In this study, we characterized for the first time the molecular mechanisms of action of glyphosate in a marine bivalve species after exposure to environmentally realistic concentrations. To reach such a goal, Mediterranean mussels *Mytilus galloprovincialis*, an ecologically and economically relevant species, were exposed for 21 days to 10, 100, and 1000 μg/L and digestive gland transcriptional profiles were investigated through RNA-seq. Differential expression analysis identified a total of 111, 124, and 211 differentially regulated transcripts at glyphosate concentrations of 10, 100, and 1000 μg/L, respectively. Five genes were found consistently differentially expressed at all investigated concentrations, including SERP2, which plays a role in the protection of unfolded target proteins against degradation, the anti-apoptotic protein GIMAP5, and MTMR14, which is involved in macroautophagy. Functional analysis of differentially expressed genes reveals the disruption of several key biological processes, such as energy metabolism and Ca^{2+} homeostasis, cell signalling, and endoplasmic reticulum stress response. Together, the results obtained suggest that the presence of glyphosate in the marine ecosystem should raise particular concern because of its significant effects even at the lowest concentration.

1. Introduction

Glyphosate, which represents the primary active constituent of the commercial pesticide Roundup (RD), is a broad-spectrum, nonselective, systemic herbicide, currently among the most widely used agricultural chemicals on a global scale (Annett et al., 2014; Cattani et al., 2014; Myers et al., 2016). This organophosphorus compound is highly effective because of its ability to reduce protein synthesis through specific inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an enzyme of the shikimate pathway that governs the synthesis of the aromatic amino acids phenylalanine, tyrosine, and tryptophan in plants, algae, fungi, and some microorganisms (Barry et al., 1992; Devine et al., 1993; Franz et al., 1997; Shehata et al., 2013).

Due to its widespread use in agriculture, forestry, urban areas,

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and aquaculture as a plant growth regulator, glyphosate can easily spread throughout ecosystems, including surface waters, thus reaching plants, animals, and the food chain. Despite the fact that glyphosate is generally considered to have a low contamination potential in surface waters or groundwater (Vereecken, 2005), recent investigations on glyphosate and amino-methylphosphonic acid (AMPA), the microbial transformation product of glyphosate, have raised increasing concerns about the potential risks to the aquatic environment. Glyphosate concentrations ranging between 10 and 45 μg/L have been measured in rivers (e.g. Struger et al., 2008), while much higher concentrations (>320 μg/L) were detected in wetland environments and ponds after glyphosate application to nearby fields (e.g. Battaglin et al., 2009). Kemp et al. (1983) estimated that up to 2% of the herbicides applied to agriculture fields end up in coastal waters, and persistence of glyphosate in seawater has been demonstrated to be closely related to light exposure and temperature, with a half-life ranging between 47 and 315 days (Mercurio et al., 2014). In Italy, the Italian National Institute for Environmental Protection and Research (ISPRA) highlighted that glyphosate has been responsible for exceeding the environmental quality standards of water in 76 cases (25.2%) out of 302 surface water monitoring stations (ISPRA, 2016). Regarding marine environments, Wang et al. (2016) measured glyphosate concentrations ranging from 13 μg/L to 1377 μg/L in seawater samples collected during a cruise in the Western Pacific in April 2015. In water samples from ten German Baltic estuaries, glyphosate concentrations ranged from 28 to 1690 ng/L and decreased along the salinity gradient, demonstrating the transport of this compound to the Baltic Sea (Skeff et al., 2015). A survey of emerging contaminants in sediment samples from the estuarine receiving environment around Auckland (New Zealand) demonstrated the presence of relatively high levels of glyphosate (up to 950 ng/g) at residential sites (Stewart et al., 2014). In addition, the biodegradation of glyphosate was quantified using standard "simulation" flask tests with native bacterial populations and coastal seawater from the Great Barrier Reef. The half-life for glyphosate at 25 °C in low-light was 47 days, 267 days in the dark at 25 °C and 315 days in the dark at 31 °C (the longest persistence reported for this herbicide) (Mercurio et al., 2014). The authors concluded that the long persistence of glyphosate in flask experiments was indicative of reduced degradation in particular conditions such as flood events and suggested the release of dissolved and sediment-bound herbicide into the lagoon (Mercurio et al., 2014).

While the mode of action of glyphosate is specific to plants, several studies have demonstrated a wide range of toxicological effects in vertebrate and invertebrate species, including aquatic organisms. Glyphosate and its commercial formulation Roundup have been shown to increase oxidative stress in fish species by enhancing ROS generation and inhibiting antioxidant systems, as demonstrated in *Prochilodus lineatus* exposed for 6, 24 and 96 h to 10 mg/L of Roundup (Cavalcante et al., 2010). In addition, exposure to 10 mg/L Roundup was shown to inhibit acetylcholinesterase (AChE) activity in brain and muscle tissues from *Prochilodus lineatus* (Modesto and Martinez, 2010). Genotoxicity of glyphosate formulation was demonstrated in the goldfish *Carassius auratus* (Çavaş and Könen, 2007) while DNA damage was found in *Anguilla anguilla* exposed to Roundup (58 and 116 μg/L) and in the catfish *Corydoras paleatus* after treatment with a Roundup concentration of 6.67 μg/L, corresponding to 3.20 μg/L glyphosate (Guilherme et al., 2010, 2012; De Castilhos Ghisi and Cestari, 2013). Potential effects on reproduction through the disruption of steroid hormone synthesis and increased oxidative stress were also reported in zebrafish (*Danio rerio*) after exposure to 10 mg/L of glyphosate and Roundup (Uren Webster et al., 2014). The same authors also demonstrated the altered expression of several transcripts coding for components of the antioxidant system, stress-response proteins, energy metabolism, and pro-apoptotic signalling molecules in the brown trout *Salmo trutta* exposed to 0.01, 0.5 and 10 mg/L of glyphosate and Roundup (Uren Webster and Santos, 2015). Several studies on the biological effects of glyphosate and other herbicides have also been carried out in aquatic invertebrates. Among the most interesting results, the Pacific oyster *Crassostrea gigas* showed changes in xenobiotic detoxification activity, energy production, and immune system response (Tanguy et al., 2005; Epelboin et al., 2015), while increased glutathione-s-transferase (GST) and alkaline phosphatase (ALP) activities and lipid peroxidation were observed in the mussel *Limnoperna fortunei* treated with nominal glyphosate concentrations of 1, 3 and 6 mg/L (Iummato et al., 2013).

Despite the aforementioned studies, which represent only a fraction of the current literature, there is still little information concerning the molecular mechanisms of action in marine organisms, particularly in bivalves. Transcriptional analyses may represent a powerful tool for the investigation of molecular responses to chemicals, as well as provide valuable data to predict potential adverse health effects on organisms and ecosystems. Recently, several studies performed using DNA microarrays in bivalve species demonstrated that transcriptional data provide extremely useful information regarding the main metabolic pathways involved in the response to environmental pollutants (Milan et al., 2013a, 2013b, 2015, 2016; Avio et al., 2015; Mezzelani et al., 2016). The recent development of Next Generation Sequencing (NGS) technologies has made it possible to conduct transcriptional analyses on any environmentally relevant species through tools such as RNA sequencing (RNA-seq), providing detailed information on the global molecular mechanisms involved in toxicity. To date, these tools are still scarcely used in ecotoxicology, though their potential is increasingly acknowledged by the scientific community.

This study was aimed at evaluating, through a RNA-seq approach, the mode of action of glyphosate in the mussel *M. galloprovincialis*, an edible bivalve species of great commercial interest that is widespread in marine coastal areas and extensively used as a model organism in ecotoxicological studies. To this end, transcriptional profiles were investigated in mussels after a long-term exposure (21 days) to environmentally realistic concentrations of glyphosate (10, 100, 1000 μg/L).

2. Materials and methods

2.1. Mussel exposure to glyphosate and tissue sampling

Specimens of *M. galloprovincialis* (6.5–7 cm shell length) were collected from a reference site located in the southern basin of the Lagoon of Venice (Italy). Before glyphosate exposure, mussels were acclimated in the laboratory for 5 days using large aquaria with aerated natural seawater (salinity of 35 ± 1 psu, temperature of 18 ± 0.5 °C); they were fed daily with microalgae (*Isochrysis galbana*, at an initial concentration of about 500,000 cells/L). Only mussels that showed signs of good health status (secretion of new byssal threads and reattachment to aquarium surface) were subsequently selected for exposure to glyphosate.

A stock solution (0.1 g/L) of glyphosate ($C_3H_8NO_5P$; CAS: 1071-83-6; molecular weight: 169.07; log K_{ow}: −4.59 to −1.70) (Sigma-Aldrich, Milano, Italy) was prepared in distilled water, whereas working solutions were prepared by diluting the stock solution in seawater.

In order to avoid spawning and to reduce possible additional stress, the exposure was performed far from the sexual maturity period of the mussels (June 2016). Animals (in total 70 per condition) were exposed for 21 days to 0 (control), 10 μg/L (LG: low concentration), 100 μg/L (MG: medium concentration) or 1000 μg/L

(HG: high concentration) of glyphosate. The nominal concentrations were chosen based on glyphosate concentration data in aquatic ecosystems (see references in the Introduction section). Mussels (35 per tank) were maintained in 35 L glass aquaria (two aquaria per condition tested) containing aerated seawater under the same thermohaline conditions used during the acclimatisation period. Seawater, glyphosate concentrations and microalgae in exposure tanks were renewed every two days.

After 21 days of exposure, digestive glands were collected from mussels (20 per experimental condition), pooled to obtain five different pools of four mussels each, divided in aliquots, frozen in liquid nitrogen, and stored at −80 °C until processing. Part of sampled tissues (digestive gland, gills, and haemolymph) were used by Matozzo et al. (2018) for cellular and biochemical analyses, in order to assess the effects of glyphosate on biomarkers indicative of cellular and oxidative stress. In this study, four pools of digestive glands for each glyphosate concentration were used for transcriptomic analyses through RNA-seq.

2.2. Chemical analyses

Glyphosate concentrations in seawater samples from the experimental tanks were measured approximately 15 min after the first application (t = 0) and after 48 h (before water renewal and the second application of glyphosate). Chemical analysis was carried out trough LC-MS that was equipped with an Ultimate 3000 UHPLC chromatograph coupled with a Q Exactive™ hybrid quadrupole-Orbitrap™ mass spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) to measure the actual concentrations of glyphosate. The quantitation of glyphosate and AMPA was performed by using a four-point matrix-matched calibration curve in seawater, ranging from 5.0 to 1500 μg/L. Detailed information is reported in Matozzo et al. (2018).

2.3. RNA extraction and library preparation

Total RNA was extracted from 16 samples, 4 pools per concentration using RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA concentration and quality were assessed by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA Integrity Number (RIN) index was calculated for each sample using Agilent 2100 Expert software. RIN provides a numerical assessment of the integrity of RNA that facilitates the standardization of the quality interpretation. In order to reduce experimental biases in RNA-Seq analysis due to poor RNA quality, only RNA samples with RIN number >7 were further processed. The cDNA libraries were constructed using a Sure Select Strand-Specific mRNA Library (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's protocol. Briefly, before fragmentation, oligo d(T) beads were used to purify poly(A) mRNA from total RNA. First-strand cDNA was synthesized from the fragmented mRNA using random hexamer primers, and the cDNA libraries were prepared in accordance with the Illumina protocol. After a purification step, the libraries were quantified with a Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA) and pooled together according to their relative concentrations. The concentration and quality of the pool was assessed by Agilent 2100 Bioanalyzer. The library pool was sequenced on a HighSeq 4000 (Illumina, Davis, CA, USA) with a single 1 × 50 bp setup using 'Version2' chemistry at the UC Davis Genome Center (University of California, California, USA) obtaining a total of 381,004,835 reads. The sequences obtained are available in NCBI Sequence Read Archive (SRA; https://www.ncbi.nlm.nih.gov/sra; BioProject PRJNA407401).

2.4. RNA-seq data analyses

A FastQC report was used to perform an initial quality check of the raw sequencing data (Babraham Bioinformatics, Babraham Hall, Cambridge, UK). Adapter trimming and reads mapping were carried out on CLC Genomics Workbench v.10.1.1 (CLCbio, Aarhus, Denmark). Trimmed RNA-Seq reads of each sample were mapped on the reference *Mytilus galloprovincialis* transcriptome (151,320 contigs) published by Moreira et al. (2015). Transcriptome annotation was updated and improved by running Blastx similarity searches on several protein databases, such as Swissprot (Uniprot), *Homo sapiens* protein Ensembl database, *Danio rerio* protein Ensembl database, and *Crassostrea gigas* protein Ensembl (E-value < 0.0001). Mapping of RNA-Seq reads against the *M. galloprovincialis* transcriptome was performed in CLCbio with the following parameters: mismatch cost 2, insertion cost 3, deletion cost 3, length fraction 0.8, and similarity fraction 0.8. The 92–98% of the total reads were successfully mapped, with the total number of mapped reads per sample ranging between 15 and 28 Mln reads. Detailed information about sequencing and mapping of each analysed sample is reported in Supplementary File S1. Gene expression data was then analysed with Multi Experiment View (MEV) cluster software (https://www.mev.tm4.org; Howe et al., 2011). After TMM Normalization (*trimmed mean of M-values normalization method*), pairwise comparisons (CTRL vs LG; CTRL vs MG; CTRL vs HG) were performed using EdgeR (Robinson et al., 2009) implemented by MEV (p-value < 0.05; false discovery rate; Fold Change>2). A more systematic functional interpretation of differentially transcribed genes was obtained by enrichment analysis using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) software (Dennis et al., 2003; Huang et al., 2008), considering GO Biological Process Database (BP), GO Cellular Component Database (CC), GO Molecular Function Database (MF), and KEGG pathways. To investigate enriched GO and KEGG pathways between our groups, DAVID analysis was performed considering the entire lists of differentially expressed genes, as well as considering up- and down-regulated genes separately, with the following settings, gene count = 2 and ease = 0.05.

3. Results

3.1. Chemical analyses and biological observation

At t = 0, the actual concentrations of glyphosate in the experimental tanks were < LOD (limits of detection) in control tanks, and 7.3, 78.8 and 1057 μg/L in the tanks where glyphosate was added at three nominal levels (10, 100 and 1000 μg/L) (Matozzo et al., 2018). A moderate depletion of glyphosate concentrations in the seawater was recorded, being 20% lower than the nominal values for the LG and MG exposure concentrations. After 48 h (before water renewal), a slight decrease in glyphosate concentrations was noted in all the seawater samples (5.9 μg/L, 59.5 μg/L and 909.8 μg/L for LG, MG and HG exposure concentrations, respectively). No mussel mortality was observed throughout the exposure.

3.2. Transcriptomic analyses

In order to assess transcriptional changes in response to glyphosate exposures, pairwise comparisons between the control and treated mussels were performed, revealing a total of 111, 124 and 211 differentially expressed genes (DEGs) in the LG, MG and HG groups, respectively (FDR-adjusted p-value<0.05; FC > 2). At all glyphosate concentrations, the majority of DEGs were down-regulated in exposed groups (87, 83, 185 DEGs at LG, MG and HG,

respectively), while fewer genes were up-regulated (24, 41, 25 DEGs at LG, MG and HG, respectively). The complete lists of differentially expressed genes with their relative fold change and corresponding annotations are reported in Supplementary File S2 (LG), Supplementary File S3 (MG), and Supplementary File S4 (HG). Venn diagrams were then constructed to represent the DEGs identified at each glyphosate concentration (see Fig. 1). This chart clearly identifies a core of four consistently down-regulated transcripts at all investigated concentrations. These core genes code for SERP2 (*stress-associated endoplasmatic protein 2*), MTMR14 (*myotubularin-related protein 14*), GIMAP5 *(GTPase, IMAP family member 5)*, and ATF7IP *(Activating transcription factor 7-interacting protein 1)*. Unfortunately, no annotation was found for the fifth consistently over-expressed transcript. The complete list of genes commonly found differentially expressed at different glyphosate concentrations is reported in Supplementary file S5.

3.3. Functional analyses

Enrichment analyses through DAVID software were carried out to obtain a functional annotation at each glyphosate concentration, considering the full list of differentially expressed genes as well as up- and down-regulated genes separately. Overall, the functional analysis of differentially expressed genes identified a total of 129, 118 and 133 differentially represented GO terms/KEGG pathways for LG, MG, and HG treatments, respectively. Among the most interesting enriched GO terms were the BP_GO terms “muscle system process” (GO:0003012), “spermatogenesis” (GO:0007283), “cytoskeleton organization” (GO:0007010), “proteolysis” (GO:0006508), and “digestion” (GO:0007586), the CC_GO term “membrane part” (GO:0044425), and the MF_GO term “neurotransmitter transporter activity” (GO:0005326), as well as several terms related to energy metabolism. Concerning KEGG pathways, “ABC transporter” was found enriched when considering down-regulated genes in LG and HG groups, while significant enrichment of “Calcium signalling pathway” stood out in the MG treatment. The complete list of enriched terms from the categories Biological Process (BP), Cellular Component (CC), Molecular Function (MF), and KEGG pathways are reported in Supplementary File S6.

4. Discussion

In previous study, we demonstrated that glyphosate affects haemocyte and biochemical parameters in *M. galloprovincialis* (Matozzo et al., 2018). In particular, exposure to glyphosate negatively influenced total haemocyte count, haemolymph pH, lysozyme and acid phosphatase activities in haemocyte lysate, as well as lactate dehydrogenase activity in cell-free haemolymph and AChE activity in gills. The present study, which is a continuation of that study, investigated for the first time the gene expression profiles of mussels exposed to three environmentally realistic concentrations of glyphosate and provided new information on its putative molecular mechanisms of action in marine organisms. Overall, the three tested glyphosate concentrations induced similar transcriptional changes, as demonstrated by the 134 transcripts (43.7% of the total DEGs) found commonly differentially regulated in at least two investigated concentrations. However, if the five common transcripts differentially expressed in all three treatments are excluded, no DEGs were shared between LG and MG (see Fig. 1). Conversely, similarities in transcriptional profiles were observed between LG and HG (53 DEGs; 17.3%), as well as between MG and HG (76 DEGs; 24.8%). These results suggest that above the glyphosate threshold of 10 μg/L, different molecular mechanisms of defense/response may be activated, while at the highest glyphosate concentration there was a wide response that includes all of the major modifications identified in the LG and MG groups, which share 52% and 65% of the total identified DEGs, respectively.

In order to simplify the discussion of the results obtained from

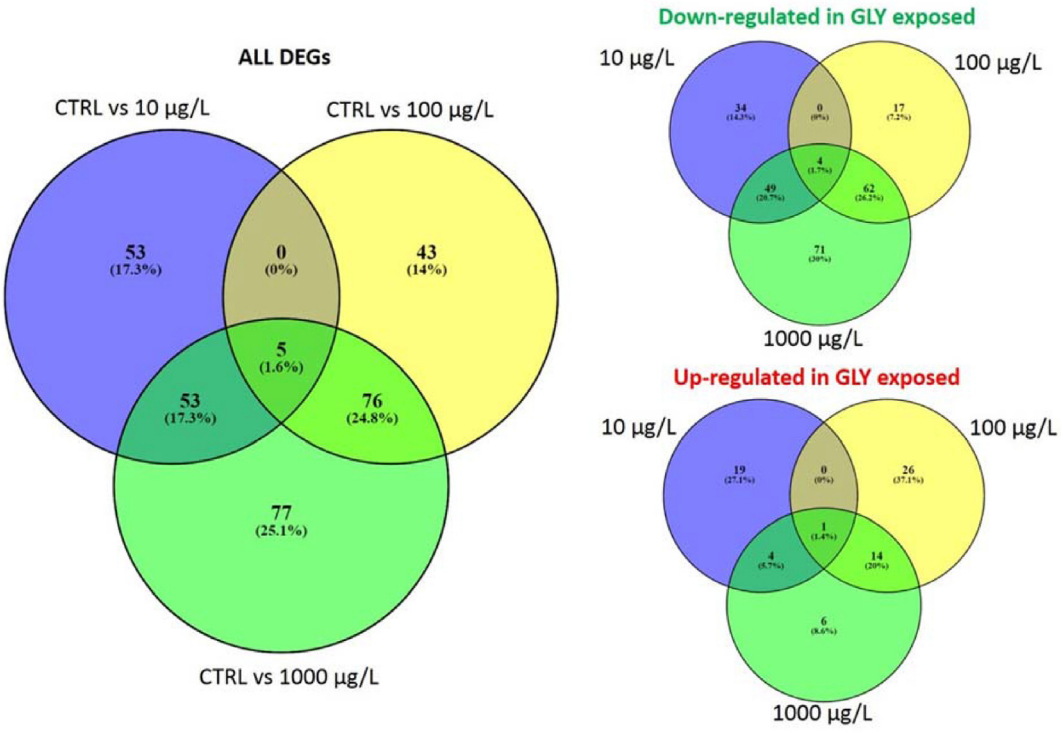

Fig. 1. Venn diagrams reporting the number of differentially expressed genes for each pairwise comparison among control and exposed groups.

the functional analysis (Supplementary File S6), the most pertinent biological processes and molecular pathways involved in the response to glyphosate exposure have been grouped into different chapters discussed below. Although transcriptional activity represents a prerequisite for protein synthesis by providing information about the putative MOA of drugs, the efficiency of translation, protein stability and protein folding could affect protein activities. In this context, it is important to highlight that the discussion on gene transcription data relies on the assumption that the observed transcriptional changes are directly related to protein activities.

4.1. Membrane components and cellular stress response

Among the most represented CC terms, “Membrane part” was enriched for all glyphosate concentrations, represented by a total of 86 down-regulated transcripts. Other enriched terms related to membrane components were “membrane-bounded vesicle” (GO:0031988), “plasma membrane part” (GO:0044459), and “vesicle” (GO:0031982), all represented mainly by down-regulated genes (Table 1; Supplementary File S6). Regarding DEGs belonging to the aforementioned GO terms, mussels exposed to LG and HG showed 15 down-regulated transcripts belonging to the solute carrier group (SCG). The SCG is a group of membrane transport proteins that include over 400 members organized into 52 families able to transport a range of extraordinarily diverse solutes, including both charged and uncharged organic molecules, and inorganic ions (Hediger et al., 2004). The same trend was detected for six transcripts that represent putative *multidrug resistance-associated protein 5* (ABCC5) and *multidrug resistance-associated protein 9* (ABCC9). These putative proteins belong to the enriched BP “transmembrane transport” (GO:0055085) and to the KEGG pathway “ATP-binding cassette (ABC)”, found in both LG and HG treatments. Overall, the main function of solute carrier families and ABC transporters is to handle endogenous metabolites and toxins in order to distribute and maintain effective concentrations of nutrients and antioxidants in the organ. These processes/pathways are centered on transcellular movement of solutes crossing a single layer of epithelial cells by “matching” pairs of Solute carrier families importers and ABC exporters (Wu et al., 2011). This system is extremely sensitive to environmental changes, in particular to xenobiotics that can hitchhike endogenous carrier systems leading to higher toxicity. While the disruption of several Solute carrier families membrane-bound transporters was also revealed at the transcriptional level in *Salmo trutta* exposed to glyphosate and Roundup (Uren Webster and Santos, 2015), the up-regulation of ABC transporter genes has been related to glyphosate resistance in plants, probably due to their participation in the sequestration and excretion of toxic compounds (Yuan et al., 2007. Ge et al., 2010, 2010; Nol et al., 2012; Nol et al., 2012; Doğramacı et al., 2015). These findings suggest that the transcriptional changes observed in these multi-purpose transporters and the modifications of transport capacity to handle endogenous toxins and metabolites may represent one of the main mechanisms of defence/response to glyphosate exposure in mussels.

Among the five transcripts consistently differentially expressed at all the investigated glyphosate concentrations, one is the putative SERP2 homolog, which represents an intrinsic component of membranes. This gene, found down-regulated in all treatments with fold change ranging between 30 (LG) to 8 (MG) (see Supplementary File S5), plays a role in the translocation of target proteins to the endoplasmic reticulum (ER) and in the protection of unfolded target proteins against degradation. Recently, SERP2 was described in the mantle of the Antarctic bivalve *Laternula elliptica* and in the Yesso scallop *Patinopecten yessoensis.* These studies suggested an antioxidant capacity and a role in the stabilisation of membranes in response to stress and in the defence against the cold (Clark et al., 2010; Hou et al., 2011). Overall, *Stress-associated endoplasmic reticulum proteins* participate in pathways controlling membrane protein biogenesis in the ER, and the overexpression of these proteins suppresses the degradation of integral membrane proteins under stress (Yamaguchi et al., 1999). We suggest that the significant down-regulation of SERP2 could be linked to an increased need to degrade damaged membrane proteins and to the disruption of the endoplasmic reticulum, considered among the main targets of glyphosate in plants (Doğramacı et al., 2015).

The normal functioning of the ER is crucial for Ca^{2+} signalling, thus the disruption of Ca^{2+} homeostasis may disrupt protein folding and induce ER stress. Calcium may enter the cell through plasma membrane channels or be released from the ER in response to intracellular messengers. Imbalances in these processes may lead to Ca^{2+} overload, eliciting oxidative stress and apoptosis. In our study, two exposure levels (MG and HG) showed a significant enrichment of the GO terms “response to calcium ion” (GO:0051592) and “calcium ion binding” (GO:0005509), entirely represented by up-regulated genes (Table 1). Conversely, the “Calcium signalling pathway” was represented in MG and HG groups by three down-regulated transcripts coding for three *Calcium- and calmodulin-dependent phosphodiesterase type 1* (PDE1A, PDE1B, PDE1C). PDE1 is a family of phosphodiesterases that exhibit different affinities for cAMP and cGMP. In detail, PDE1A and PDE1B preferentially hydrolyse cGMP, whereas PDE1C degrades both cAMP and cGMP with high affinity, playing pivotal roles in mediating the cross-talk between cAMP and Ca^{2+} signalling (Kakkar et al., 1999). Among the four genes recurrently down-regulated at all glyphosate concentrations, the putative *GTPase IMAP family member 5* (GIMAP5) stands out, being 11-fold down-regulated at all glyphosate concentrations (see Supplementary File S5). GIMAP5 belongs to the immuno-associated nucleotide (IAN) subfamily. Likely, due to its role in Ca^{2+} regulation in the mitochondrial and lysosomal compartments, GIMAP5 is considered an antiapoptotic protein with a pro-survival function (e.g. Chen et al., 2013; Serrano et al., 2017). The GIMAP family represents an expanded gene family in oysters (McDowell et al., 2016) and its potential role as a negative regulator of apoptosis has been also suggested in *Crassostrea virginica* (McDowell et al., 2014). Overall, the disruption in Ca^{2+} homeostasis is among the most cited effects of glyphosate also in vertebrate species (e.g. de Liz Oliveira Cavalli et al., 2013; George and Shukla, 2013; Cattani et al., 2014; Uren Webster and Santos, 2015). Our results confirm these findings, suggesting that an imbalance in Ca^{2+} physiology and ER stress in mussels may also occur at low environmentally realistic concentrations of glyphosate.

A third transcript found consistently differentially expressed at all the tested glyphosate concentrations codes for the protein MTMR14. This transcript, down-regulated 14-fold in LG- and MG-exposed groups, has never been described in a bivalve species, at least to our knowledge. MTMR14 (also known as Jumpy) is a phosphoinositide phosphatase that specifically dephosphorylates phosphatidylinositol 3,5-biphosphate (PtdIns(3,5)P2) and phosphatidylinositol 3-phosphate (PI3P), which are two of the seven phosphoinositides found in eukaryotic cell membranes. PI3P is a cell membrane phospholipid able to recruit a range of proteins involved in protein trafficking to the membranes, while PtdIns(3,5)P2 plays a role in the regulation of endosomal operations (fission and fusion) and in the maintenance of endomembrane homeostasis (Takasuga and Sasaki, 2013). Considering the importance of PI3P in autophagy initiation, a novel role for *Jumpy* in autophagy regulation was recently proposed (Vergne et al., 2009). Autophagy is the primary cellular degradative pathway to recycle cytoplasm, remove damaged organelles, generate energy under stress conditions, and

Table 1
Lists of the main biological processes (BP), cellular components (CC), molecular functions (MF) and KEGG pathways modulated by glyphosate at each investigated concentrations. The arrow ↑ indicates enriched BP and KEGG pathways considering up-regulated genes in response to glyphosate, while ↓ indicates enriched BP and KEGG pathways considering down-regulated genes in response to glyphosate. None arrows indicate that the significant term has been obtained considering the full list of DEGs. N°: number of differentially expressed genes for each enriched BP and KEGG pathways; FE: Fold enrichment.

Glyphosate concentration	Category	Term	N° DEGs	p-value	FE
10 μg/L	BP	↓ membrane part	31	1.61E-04	1.7
	BP	regulation of neurotransmitter levels	4	0.030	5.8
	BP	↓transmembrane transporter activity	18	1.13442038880143E-08	5.2
	BP	↓ transmembrane transport	14	1.04E-05	4.2
	BP	↓ proteolysis	9	0.072	1.9
	BP	↑RNA metabolic process	5	0.071	2.49
	BP	↑RNA processing	4	0.008	7.7
	BP	↑mRNA splicing, via spliceosome	3	0.007	20.3
	BP	response to metal ion	4	0.0671	4.18
	CC	↓ membrane-bounded vesicle	20	3.75E-04	2.27
	CC	↓ plasma membrane part	19	1.38E-05	3.02
	MF	metal ion transmembrane transporter activity	7	0.004	4.43
	KEGG	↓ ABC transporters	2	0.085	21.51
100 μg/L	BP	↓ membrane part	37	0.004	1.47
	BP	↑ response to calcium ion	2	0.038	43.94
	BP	↓ generation of precursor metabolites and energy	5	0.001	9.13
	BP	↓ oxidative phosphorylation	4	2.08E-04	33.06
	BP	↓ carbohydrate derivative metabolic process	9	5.40E-04	4.34
	BP	↓ phosphorus metabolic process	11	0.0121	2.25
	BP	↓ phosphate-containing compound metabolic process	11	0.011	2.25
	BP	↑ muscle contraction	2	0.0865	19.08
	BP	↑ muscle tissue morphogenesis	2	0.0193	87.88
	BP	↑ skeletal muscle myosin thick filament assembly	2	0.004	377.88
	BP	↑ striated muscle contraction	2	0.0414	40.63
	CC	↓respiratory chain	7	2.06E-08	38.07
	CC	↑ muscle myosin complex	2	0.0059	287.12
	CC	↓ mitochondrial membrane	8	3.32E-04	5.71
	MF	↑calcium ion binding	3	0.0227	9.94
	KEGG	↓ calcium signalling pathway	2	0.0252	65.41
1000 μg/L	BP	↓carbohydrate derivative metabolic process	10	0.0333	2.190
	BP	↓digestion	3	0.0401	9.292
	BP	↑muscle contraction	2	0.0865	19.08
	BP	↑muscle tissue morphogenesis	2	0.0193	87.88
	BP	↑skeletal muscle myosin thick filament assembly	2	0.0045	377.88
	BP	↑striated muscle contraction	2	0.0414	40.632
	BP	↓transmembrane transport	16	1.46E-05	3.671
	BP	↑response to calcium ion	2	0.038	43.940
	BP	↓macroautophagy	4	0.085	3.787
	BP	↓spermatogenesis	5	0.0710	3.142
	CC	↓respiratory chain	3	0.054	7.821
	CC	↑muscle myosin complex	2	0.0069	251.232
	CC	↑membrane-bounded vesicle	5	0.0221	3.515
	CC	↓membrane-bounded vesicle	19	0.0612	1.505
	CC	↓ plasma membrane part	22	1.15E-04	2.424
	MF	↑calcium ion binding	4	0.0022	11.369
	MF	↓ neurotransmitter transporter activity	5	5.19E-05	23.798
	KEGG	↓ABC transporters	2	0.0871	21.0548

maintain cellular homeostasis (Feng et al., 2014). Changes in the activity of autophagy pathways in glyphosate exposed mussels is also suggested in the HG group and in the LG group by the enriched BP terms “macroautophagy” (GO:0016236) and “proteolysis” (GO:0006508), represented by 4 and 9 down-regulated transcripts, respectively. Macroautophagy involves the formation of double-membrane-bound autophagosomes, which enclose the cytoplasmic constituent targeted for degradation in a membrane-bound structure, and represents the major inducible pathway for the general turnover of cytoplasmic constituents and long-lived proteins in eukaryotic cells.

Within the LG group, it is important to note an over-representation of up-regulated genes involved in “RNA metabolic process” (GO:0016070), “RNA processing” (GO:0006396) and “mRNA splicing, via spliceosome” (GO:0000398) (Table 1). Transcriptome microarray analyses of the liver and kidneys of rats exposed for two years to ultra-low doses of Roundup via drinking water showed very similar results, with the up-regulation of several splicing factors and other genes involved in RNA splicing (Mesnage et al., 2015). The authors suggested that, as do other small molecule chelators of zinc, glyphosate can disrupt spliceosome assembly as well as exert direct effects on spliceosome function due to its metal chelating properties.

While our study was focused on the transcriptional profiles of the digestive gland of mussels, transcriptional changes of genes involved in “spermatogenesis” (GO:0007283) were also revealed in the HG group. Among the genes involved in this biological process, *LIM and SH3 domain protein* (LASP1) and four transcripts coding for putative *Testis-specific serine/threonine-protein kinase 2* (TSSK2) and *Testis-specific serine/threonine-protein kinase 4* (TSSK4) were found significantly down-regulated with fold changes ranging between 3.7 and 5.1. LASP1 is a cAMP- and cGMP-dependent signalling protein that functions as an actin-binding protein that is thought to play a role in cytoskeletal organization (e.g. Chew et al., 2000). Recently, a possible function of LASP1 in anchoring the stem cell niche and spermatid individualization was suggested in *Drosophila*

(Lee et al., 2008). TSSK2 and TSSK4 belong to a family of serine/threonine kinases required for spermatid development (e.g. Hao et al., 2004; Zhang et al., 2010). Despite the fact that TSKKs are highly expressed in testis, some isoforms were also found expressed in the pancreas, heart, brain and placenta in humans (Hao et al., 2004), as well as in the ovary, immature gonad, mantle, gill and digestive gland of the marine scallop *Argopecten purpuratus* (Boutet et al., 2008). At last, it should also be mentioned the significant down regulation of *Coiled-coil domain-containing protein 63* (CCDC63) in HG group, a protein involved in the elongation of flagella and the formation of sperm heads (Young et al., 2015). Despite several studies suggested possible glyphosate-Roundup effects on male reproduction (Dallegrave et al., 2007; Hokanson et al., 2007; Romano et al., 2010; Uren Webster et al., 2014), the physiological significance of TSSK and CCDC63 in other tissues and the possible repercussions in mussels spermatogenesis due to changes in transcriptional regulation certainly need further investigations.

4.2. Energy metabolism

Disruption of the energy metabolism is suggested by several enriched BP and CC terms such as “generation of precursor metabolites and energy” (GO:0006091), “oxidative phosphorylation” (GO:0006119), “respiratory chain” (GO:0070469), and “carbohydrate derivative metabolic process” (GO:1901135), all entirely represented by down-regulated genes, particularly in the MG and HG treatments. Effects of glyphosate on mitochondrial function are also suggested by enriched “mitochondrial membrane” (GO:0031966) term, represented by 8 down-regulated genes coding for 5 subunits of NADH dehydrogenase (ubiquinone) (MT-ND1, MT-ND2, MT-ND4, MT-ND5, MT-ND6), ATP *synthase subunit a* (ATP6), *Cytochrome c oxidase subunit 1* (CO1), *and Cytochrome b* (CYB). Several studies have demonstrated the disruption of energy metabolism during controlled exposures in rat liver and kidney (e.g. Peixoto, 2005; Mesnage et al., 2015), as well as in aquatic species (e.g. Glusczak et al., 2007; Dutra et al., 2011; Avigliano et al., 2014), particularly to components of the Roundup formula. Current literature suggests at least five non-mutually exclusive hypotheses to explain how glyphosate may damage mitochondria and disrupt energy metabolism. The first is associated to the possible imbalance of intracellular Ca^{2+} homeostasis discussed above. In particular, intracellular Ca^{2+} overload can underlie mitochondrial dysfunction, resulting in disruption of the energy metabolism by the activation of signalling pathways and an increase in reactive oxygen species (Kim et al., 2013; Nazıroğlu et al., 2012; de Liz Oliveira Cavalli et al., 2013). The second hypothesis is related to the potential ability of glyphosate to bind to the substrate binding site of mitochondrial succinate dehydrogenase, thus blocking substrate bioavailability (Ugarte, 2014). The third hypothesis, recently proposed by Samsel and Seneff (2015), is based on the ability of glyphosate to chelate minerals, particularly transition metals such as manganese (Mn), which is a key mechanism for glyphosate's disruption of the shikimate pathway (Cerdeira and Duke, 2006). Glyphosate-mediated Mn sequestration may lead to the deficiency of this essential trace element in mitochondria, where it is a key component of the superoxide dismutase (SOD) enzyme whose function is to partition the superoxide (O_2^-) radical into the less toxic hydrogen peroxide (H_2O_2). Interference with this important process that protects the mitochondria from oxidative stress may in turn lead to mitochondrial dysfunctions and reduced energy metabolism. The ability of glyphosate to form strong complexes with transition metals and metal ions (Motekaitis and Martell, 1985; Undabeytia et al., 2002) is also supported by the enriched terms “response to metal ion” (GO:0010038) and “metal ion transmembrane transporter activity” (GO:0046873) detected in the LG and HG treatments.

The fourth hypothesis is linked to a possible decrease in the feeding rate of the organism as a result of glyphosate exposure. In this regard, a marked reduction in faeces production was observed in exposure tanks during the last days of exposure at the highest glyphosate concentration tested, suggesting a possible decrease in the feeding rate of mussels. It is well known that toxicant exposures or environmental stress may lead to decreased food ingestion rate in organisms, including bivalve molluscs (e.g. Fleeger et al., 2003; Tuffnail et al., 2009).

The last hypothesis is related to the known capability of herbicides to modify the algal and microbial composition (Solomon et al., 2013). Recent studies have raised concerns about the effects of glyphosate on gut microbiota, indirectly affecting farm animals when fed feed containing residues of glyphosate. The antibiotic action of glyphosate has been demonstrated to alter the gastrointestinal microbiome in vertebrates, which could favour the proliferation of pathogenic microbes (e.g. Shehata et al., 2013; Ackermann et al., 2014). Considering that fluctuations in the host microbiota (dysbiosis) may lead to the disruption of the symbiotic relationship and contribute to the onset of pathological conditions, we cannot exclude that the significant up-regulation of several genes involved in immune response, such as the antimicrobial peptides *Myticin-A* (8-fold over-expressed in both LG and HG groups) and *Mytilin-B* (over-expressed 12-fold in both MG and HG groups), is a response to the microbial changes following glyphosate exposures (Bevins and Salzman, 2011). Accordingly, part of the transcriptional changes detected in enzymes involved in energy metabolism and digestion may be related to the indirect effects of changes occurring in host-microbiota composition following glyphosate exposures.

4.3. Neurotransmitters and muscle proteins

Exposures to glyphosate concentrations of 10 (LG) and 1000 (HG) μg/L showed a significant enrichment of the MF term “neurotransmitter transporter activity” (GO:0005326) and of the BP “regulation of neurotransmitter levels” (GO:0001505) (see Table 1). Among the most interesting genes, these terms were represented by two down-regulated transcripts coding for putative *4-aminobutyrate aminotransferase* (ABAT), down-regulation of *Sodium- and chloride-dependent glycine transporter 1* (SLC6A9) and *Sodium- and chloride-dependent glycine transporter 2* (SLC6A5), and over-expression of *protein piccolo* (PCLO). ABAT is responsible for the catabolism of gamma-aminobutyric acid (GABA), an important, mostly inhibitory, neurotransmitter in the central nervous system, while PCLO is involved in the organization of synaptic active zones and in synaptic vesicle trafficking. SLC6A9 is one of two transporters that limit the signalling of glycine, an inhibitory neurotransmitter in the central nervous system, by removing it from the synaptic cleft. Given the structural similarity between glycine, glutamate, and glyphosate, the latter has been suggested to disrupt neurotransmission by replacing the two former (Myers et al., 2016). Currently, acute and chronic exposures to glyphosate are considered to potentially accelerate neurodegeneration and increase risk factors for neurodegenerative disorders, such as Parkinson's and Alzheimer's disease (Barbosa et al., 2001; Wang et al., 2011). A recent study performed by Cattani et al. (2014) suggests that excessive extracellular glutamate levels induced excitotoxicity (nerve cell damage due to over stimulation by neurotransmitters) and oxidative stress in rats exposed to relatively high levels of glyphosate. Bearing in mind that a stronger body of evidence on glyphosate-induced neurotoxicity is crucial, the down-regulation of ABAT and SLC6A9, two proteins that may “block” GABA and the inhibitory action of glycine neurotransmitters, could represent a

mechanism to compensate for neurotransmitter disruption and excitotoxicity, suggesting that there may also be neurotoxic effects of glyphosate in bivalve species.

Most of the enriched BP and MF terms found in the MG and HG groups were related to muscle tissue, including “muscle contraction” (GO:0006936), “muscle tissue morphogenesis” (GO:0060415), “muscle myosin complex” (GO:0005859), “skeletal muscle myosin thick filament assembly” (GO:0030241), and “striated muscle contraction” (GO:0006941). These pathways were mainly represented by up-regulated transcripts, such as three transcripts coding for *titin* (TTN) which were up-regulated 2.5 fold in both the MG and HG treatments. An investigation of glyphosate-based herbicide-induced hepatotoxicity in the guppy *Poecilia reticulata* using a histopathological assessment associated with a proteomic approach revealed decreased levels of titin and titin b, as well as the suppression of proteins related to myosin and to the heavy chain of the skeletal muscle (Dos Santos et al., 2017).

5. Conclusion

Approximately one-third of the total volume of glyphosate-based herbicides applied since the compound's registration in 1974 has been sprayed in the last decade alone, with an annual farm-sector usage having increased to approximately 108.8 million kilograms by 2014 (Myers et al., 2016; Benbrook, 2016). The large-scale use of this compound highlights the importance of obtaining as much information as possible regarding the possible consequences of glyphosate in aquatic organisms, marine in particular. In this study, we shed light on the molecular mechanisms of action of glyphosate in the marine bivalve *M. galloprovincialis*.

Overall, transcriptional profiling obtained through a RNA-seq approach reveals that glyphosate induces alterations of many molecular pathways and biological processes, reflecting both extensive compensatory responses and acute toxicity. Gene expression profiles of mussels exposed to the three environmentally realistic glyphosate concentrations revealed a considerable degree of similarity in mechanisms of action and cellular response, although as expected, the most significant effects were found at the two highest glyphosate concentrations. Significant changes were found in the transcriptional regulation of genes playing roles in key cell functions, such as transmembrane transport, neurotransmission, Ca^{2+} homeostasis, and energy metabolism. Our data raise further concerns about the potential adverse effects of environmental concentrations of glyphosate in marine species, highlighting the need to continue investigations in order to clarify the glyphosate hazard for non-target species and ecosystems.

Author information

Notes

The authors declare no competing financial interest.

Author contributions

MM, VM, MGM, TP, and LB conceived and designed the project. PP and SB performed chemical analyses. GDR and MS performed RNA extractions and libraries preparation. MM and SF executed all statistical analyses. MM performed functional annotation analyses. MM, GDR and MS wrote the manuscript. All listed authors edited the manuscript. All authors read and approved the manuscript.

Data accessibility

All sequencing files are available in NCBI Sequence Read Archive (SRA; https://www.ncbi.nlm.nih.gov/sra; BioProject PRJNA407401).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2018.02.049.

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