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Genomics and immunity of the Mediterranean mussel *Mytilus galloprovincialis* in a changing environment

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ABSTRACT

The Mediterranean mussel (*Mytilus galloprovincialis*) is a marine invasive species cultured all over the world. Mussels are an appreciated resource in local aquaculture enterprises because of their robust production and resilience that translates into a reliable economic value. So far, no massive mortalities have been reported in natural or cultured populations of this species. In the last years, the knowledge about its immune system has greatly improved but there are still many questions to be answered. One of them is why mussels, with their high filtering activity, are able to be exposed to a high number of potential pathogens without getting infected and without developing an elevated inflammatory response.

The sequencing of the mussel genome has revealed a very complex organization with high heterozygosity, abundance of repetitive sequences and extreme intraspecific sequence diversity among individuals, mainly in immune related genes. Among those genes, antimicrobial peptides are the most expressed gene families in mussels, highly polymorphic and with antimicrobial effect against molluscs pathogens, but also against pathogens of lower vertebrates and humans. The combination of a complex genome with the adaptation of mussel immune system to a changing environment could explain this high variability, not only in immune-related genes, but also in the functional response among individuals sampled in the same location and date.

1. Introduction

Phylum Mollusca is the second largest animal group and a fossil record of over 540 million years [1]. Molluscs of the Class Bivalvia are present in both marine and freshwater environments. There are infaunal bivalves, attached to substratum (by byssus or cemented), wood and rock-borers, and even predators. This diversity is mainly due to an early evolutive radiation in the Late Cambrian era which allowed bivalves to occupy different ecological niches [2].

Bivalves are economically important as aquaculture resources and as producers of pearls and shells. Moreover, they have an important role in the energy flux in the environment and in the maintenance of the water quality [3]. Given their widespread distribution and their filtering mode of life, bivalves, specifically mussels, are also important pollution sentinels [4].

In 2016, the world aquaculture production of molluscs reached 17,139,140 tons (excluding products not intended for human consumption) [5]. Aquaculture is the animal production activity with the highest growth rate and its importance for global human feeding steadily grows from year to year. According to FAO, the European production of bivalves is the most important in the world after China. It

has a relevant social dimension because most of the companies are micro-enterprises (with less than 10 employees) which hire a high number of employees in this sector [6]. European aquaculture is focused on a limited number of species that are produced at industrial level (mainly mussels, oysters and clams) and it is based on the availability of ecosystems of great environmental quality and food abundance. Mussels are the most produced bivalves in Europe reaching 319,928 tons for *M. galloprovincialis* and 155,067 tons for *M. edulis*.

However, bivalve aquaculture is facing a range of risks and limiting factors that include, as other animal production activities, environmental changes, pollution and infectious diseases [7,8]. Since 2008, high mortalities of the Pacific oyster *Crassostrea gigas* have been registered in several European countries [9–14]. Herpes virus OsHV-1 combined with adverse environmental factors could be the cause of these high mortalities [15]. As a result, the annual production of Pacific oyster in France went from 110,800 tons in 2007 to 64,000 tons in 2016 [5]. Recently there have been reports of high mortalities of Pacific oyster associated to bacterial infections by *Vibrio aestuarianus* and also mussel mortalities associated to infections by *Vibrio splendidus* [16]. In fact, it has been reported that the OsHV-1 infection produces an immunosuppression on oysters that evolves towards a fatal bacterial

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infection caused by opportunistic bacteria [17]. In Galicia (NW Spain), the main producing area of mussels in Europe, the parasite *Marteilia cochillia* is causing a critical decline of the exploited populations of the cockle *Cerastoderma edule* [18]. Unfortunately, there are not easy ways to fight against bivalve diseases: as all invertebrates, they do not produce antibodies, therefore it is not possible to vaccinate them and since they are cultured in open sea disease treatments are not possible.

Bivalves use their filtering activity to feed themselves. It has been reported that, on average, mussels can filter about 7,5 L of water in one hour [19]. This means that they are in permanent contact with millions of potentially pathogenic microorganisms [20]. Stabili et al. (2005) reported that the abundance of *Vibrio* spp. is higher inside mussels than in the surrounding water [21]. This would explain why bivalves are susceptible to numerous diseases that compromise their culture causing economic losses all over the world. Interestingly, mussels cohabitate in the same areas where oysters cockles and clams have suffered massive mortalities due to viral, bacterial or protozoan infections, however, high mortalities have never been reported in the field for *M. galloprovincialis* [14,22]. This resistance is thought to be linked to the development of a complex and efficient innate immune system, whose cellular and molecular components are progressively being studied and unveiled [23–25].

Despite the role of the immune system in fighting diseases and the efforts of many researchers throughout the world, our knowledge is quite limited. Bivalve immunologists have studied different processes such as phagocytosis, encapsulation, aggregation, prophenol oxidase system, or the release of effective molecules such as oxygen and nitrogen radicals or lysozyme, among others [26–30]. However, it was not until the late 90's, early 2000s when a new set of small molecules with antimicrobial activity was described in bivalves, coinciding with emergent biochemical, molecular biology and sequencing techniques [31–33]. The presence of these antimicrobial peptides (AMPs) has been reported in different bivalve species such as carpet shell clam [34], oyster [35], scallop [36] and mussels [23,31,33,37–41]. Unlike other bivalve species, in which these molecules have been described, a remarkable abundance of these small peptides has been reported for the Mediterranean mussel, *M. galloprovincialis* [40]. Besides of their abundance, AMPs present high genetic variability [42]. These two characteristics, added to the lack of pathogen associated mortalities in this species, could be indicative of a selective evolutionary advantage for mussels.

1.1. A complex genome and variable individual transcriptomes

Next generation sequencing techniques (NGSs) are versatile and constantly evolving tools that have been used in the last decade to uncover the molecular bases of the whole biology of *Mytilus galloprovincialis*, from development and environmental stress to immune response. In Table 1 we present the transcriptomic and genomic works that have been done since NGSs are available. The cost of these techniques are constantly dropping and, as a consequence, it is expected that in the next years the genomes and transcriptomes of many bivalve species are sequenced.

Although we have already published a draft for the Mediterranean mussel genome [43], its complexity made that we sequenced a new mussel genome with a deeper coverage and sequenced 14 additional individual mussel genomes. This confirmed our suspicions. Mussel genome has a high heterozygosity rate, abundance of repetitive sequences and extreme intraspecific sequence diversity among individuals. A significant number of genes in the reference genome were very variable, but this finding was not explained by the geographical origin or sex of the mussels. Our results suggest that the intraspecific sequence diversity has played a major role in the evolutionary success of mussels, endowing them with a large repertoire of immune receptors and effectors that might have significantly strengthened the social immunity of this bivalve species (Figueras and Gerdol, personal

communication). Many open questions remain about the genetic and evolutionary mechanisms underpinning this unique peculiarity and about how this amazing intraspecific diversity is maintained in contemporary populations (Gerdol et al., submitted for publication).

This high variability can be also observed at transcriptomic level [46]. We have demonstrated the great variability among individual transcriptomes in a study using Illumina sequencing of the transcriptome of individual mussels before and after being infected with *Vibrio splendidus* and how each animal showed an exclusive repertoire of genes not shared with other individuals [67]. In this work, a clear response against the injection of filtered seawater was also found, suggesting a reaction against a tissue injury in which the myticins, the most expressed antimicrobial peptides in mussel, appeared significantly up regulated, in contrast with the down-regulation observed after the infection, suggesting that myticins are involved in the response to a danger signal.

Alterations in gene expression due to a pathogenic stimulus lead to changes not only in the immune response but also in the metabolism, the endocrine system and the neural response to stimulus. Today it is of great interest the global study of all these functions and the terms immunometabolism [68] and neuroendocrine immunomodulation [69] are also new research topics in bivalves. Both of them could play relevant roles in key defense processes that still remain unrevealed.

Other interesting aspect of the mussel transcriptome is the high presence of cancer-related genes [55]. Killing and cancer seem to be two of the most frequent gene ontology terms found in mussel transcriptome. Recently, a transmissible cancer has been described in cockles but only rare and sporadic presence of neoplastic cells has been reported in mussels [70–72]. Cockles are cultured close to mussels, but mussels from the species *Mytilus galloprovincialis* do not seem to be affected by this type of disseminated neoplasia.

1.2. Mussel immune system is in permanent contact with microorganisms

The lack of the classical antibody-based adaptive immunity forced mussels to generate alternative defense strategies to protect themselves. These adaptations allow them to live in a close contact with the big population of microorganisms in the oceans [73] and should avoid an excessive immune reaction against innocuous microorganism but react against potential pathogens. The mucosal immunity is the first line of immune defense, with the external epithelium and mucus acting as a simple physical barrier but also playing a significant immune role [74]. Despite its critical role in immune defense it has not been sufficiently studied. Once this mucosal immune barrier is trespassed, circulating hemocytes in the hemolymph are able to discriminate self from non-self and get rid of potential pathogens. The lack of specific cell markers has prevented, so far, the detailed characterization of the function and the possible differences of specific cell populations.

In mussel hemocytes, almost half of the expressed genes are antimicrobial peptides [40] being myticins, mytilins, and mytimicins, the most expressed of these AMPs. We already have evidences suggesting that AMPs confer resistance to *M. galloprovincialis* against diseases in contrast to other bivalves: the treatment of oyster hemocytes with mussel hemolymph, or with synthetic myticin C peptides, inhibits the replication of OsHV-1 in oyster hemocytes [75]. Moreover, myticins inhibit the replication of bacteria [76], fish viruses [77] and human herpesvirus [75]. Therefore, myticin C and other bioactive peptides of mussels are examples of the biotechnological and therapeutic potential of this species.

The complexity of bivalve immune responses is also evidenced by the fact that their immune system can be primed, leading to short-term memory. For example, an enhanced cellular immune response has been reported in *Crassostrea gigas* after a secondary challenge with *Vibrio splendidus* [78]. Also, recent investigations have shown that experimentally infected juvenile oysters can mount a long-lasting antiviral immune memory that protects them from subsequent viral infections

Table 1
Genomic and transcriptomic works published for *Mytilus galloprovincialis*.

Sequencing	NGS technique	Tissue	Reference
Genome (high quality)	Illumina HiSeq2000 paired end and mate pair Fosmid library PacBio	Mantle	Gerdol et al., submitted
Genome (low quality)	Illumina HiSeq2000	Muscle	[43] Murgarella et al., 2016
Genome (low quality)	Illumina HiSeq 2000	Mantle	[44] Nguyen et al., 2014
Genome (partial)	Targeted 454 pyrosequencing	Foot	[45] Rosani et al., 2014
Transcriptome	2 × 105 K Agilent oligo-DNA microarray	Gill	[46] Lockwood et al., 2010
Transcriptome	Mytarray V1.0	Gill, digestive gland, foot/adductor muscle/ligaments and mantle/gonad	[47] Venier et al., 2006
Transcriptome	454 pyrosequencing	Digestive gland, foot, gill and mantle	[48] Craft et al., 2010
Transcriptome	2 × 105 K Agilent oligo-DNA microarray	Gill	[49] Lockwood and Somero, 2011
Transcriptome	Targeted 454 pyrosequencing	Hemocytes	[50] Rosani et al., 2011
Transcriptome	454 pyrosequencing	Digestive gland	[51] Suárez-Ulloa et al., 2013
Transcriptome	Mytarray V1.1	Females digestive gland	[52] Negri et al., 2013
Transcriptome	Mytarray V1.1	Females digestive gland	[53] Mohamed et al., 2014
Transcriptome	Illumina GAI	Digestive gland	[54] Gerdol et al., 2014
Transcriptome	Illumina HiSeq 2000	Hemocytes, muscle, mantle, gill	[55] Moreira et al., 2015
Transcriptome	Mytarray V1.1	Digestive gland	[56] Banni et al., 2016
Transcriptome	Illumina HiSeq 1500	Mantle edge	[57] Bjärnmark et al., 2016
Transcriptome	Mytarray V1.1	Females digestive gland	[58] Maria et al., 2016
Transcriptome	8 × 60 K Agilent oligo-DNA microarray	Whole animal	[59] Mezzelani et al., 2016
Transcriptome	Low-density Agilent microarray	Digestive gland and gill	[60] Banni et al., 2017
Transcriptome	Illumina HiSeq	Whole animal	[61] Saarman et al., 2017
Transcriptome	Illumina HiSeq 2000	Digestive gland	[62] Pazos et al., 2017
Transcriptome	454 pyrosequencing	Hemocytes	[63] Moreira et al., 2018
Transcriptome	8 × 60 K Agilent oligo-DNA microarray	Digestive gland	[64] Mezzelani et al., 2018
Transcriptome	8 × 60 K Agilent oligo-DNA microarray	Oocytes and larval stages	[65] Moreira et al., 2018
Transcriptome	Illumina HiSeq 2000	Digestive gland and gill	[66] Prego-Faraldo et al., 2018
Transcriptome	Illumina HiSeq 2000	Mantle and digestive gland	[8] Détrée and Gallardo-Escárate, 2018
Transcriptome	Illumina HiSeq 4000	Hemocytes	[67] Rey-Campos et al., 2019

[79]. Furthermore, oysters treated with poly I:C produce offspring with enhanced protection against OsHV-1, suggesting a trans-generational immune priming [80].

In mussels, the study of hemocyte transcriptomes before and after a primary and a secondary sublethal infection with *Vibrio splendidus* revealed that the first infection significantly regulated genes related to inflammation, migration and response to bacteria. However, after the second infection, the analysis of the differentially expressed genes suggests that mussel hemocytes try to control and resolve the inflammatory response avoiding subsequent cellular death and DNA damage. These results suggest the existence of a modified immune response of mussels after the second challenge oriented to tolerate and not to fight the infection minimizing the tissue damage (Rey Campos et al., submitted for publication).

The specific mechanisms leading to and involved in the immune priming process and also in the transgenerational immune priming are completely unknown, a new frontier in bivalve research. It is probable that regulatory mechanisms of gene expression and translation such as epigenetics and non-coding RNAs may play relevant roles. It is already known that epigenetics alters defense response and development in bivalves [81,82], and it has been recently published the mechanism to generate small RNAs in mussels and oysters by Rosani et al. [83]. These non-coding RNAs can alter, among other physiological processes, the immune response [84,85]. Both, epigenetics and non-coding RNAs, are current challenges in bivalve research which will deliver interesting results in the next years.

1.3. Mussel immunity is altered by the anthropogenic influence in the environment

Global climatic change and the increasing level of new pollutants such as micro and nanoparticles are already influencing bivalve homeostasis in a way never experienced before. For example, micro

(smaller than 5 nm) and nanoparticles (smaller than 100 nm) of diverse origin have been shown to disrupt mussel metabolism, microbiota and immune function, increasing the expression of genes related to stress, and resulting in a decrease of energy allocated to growth [8,86,87].

The “mussel watch” project is an international pollution monitoring program that propose mussels as bioindicators of classical and emergent pollutants such as nanomaterials, personal care products and drugs [88,89]. Due to their wide distribution, sessile status and the occupation of vital ecological niches, mussels are a proper tool to monitor contaminants of emerging concern. Bivalve immune system is a target for the effects of nanomaterials due to the efficient immunological system against non-self-particles and the internalization of nano and microparticles by hemocytes [86,89]. *In vitro* assays with mussel hemocytes to test emerging pollutants are recent and powerful tools which allow a fast screening of their immunomodulatory effects and the identification of mechanistic pathways such as reactivity, inflammation and cellular uptake of emergent pollutants [90,91]. Furthermore, *in vitro* ecotoxicological tests with hemocytes are easily reproducible under controlled conditions, and they are the cornerstone of nanotoxicological studies [92–94]. Another advantage of *in vitro* assays with mussel hemocytes is the similar behavior with human cells. In the study by Katsumity et al. (2018), *M. galloprovincialis* hemocytes and lung human cell line TT1 showed similar sensitivity to CuO nanoparticles exposure, reaching up similar levels of ROS production [95]. The limitation of the *in vitro* assays is that they need to be validated for their relevance for *in vivo* hazard [96].

In addition to their toxic properties, the nanoparticles suspended in environmental and biological matrixes acquire secondary properties [97]. The negative zeta potential of nanoparticles is key in the formation of the protein-corona when they are suspended in hemolymph serum [89,98]. The protein-corona, possess long-term stability in biological environments, making the nanoparticles able to interact with membrane receptors, particle wrapping, trafficking, cellular uptake and

Table 2
Molecular and -omic techniques applied to toxicological studies with mussels and pollutants of emerging concern.

Species	Pollutant	Technique	Results	Reference
<i>M. edulis</i>	Au NPs	1-DE, 2-DE	Decreased thiol-containing proteins. Changes in spot patterns consistent with greater protein thiol oxidation in response	[100] Tedesco et al., 2010
<i>M. galloprovincialis</i>	Ag NPs	MALDI-TOF-TOF	Up-regulation of: paranyosin, catchin and major vault protein in gills; Myosin heavy chain in digestive gland	[101] Gomes et al., 2013
<i>M. galloprovincialis</i>	TiO ₂ NPs	qPCR	Induction of metallothionein gene expression in gills	[102] D'Agata et al., 2014
<i>M. edulis</i>	CuO NPs	2-DE and MALDI-TOF-TOF	General increase in protein carbonyls and decrease in protein thiols with increasing dose of CuO NPs	[103] Hu et al., 2014
<i>M. galloprovincialis</i>	Ag NPs	qPCR	No modification in gene expression related to general and oxidative stress, apoptosis, immune system and morphology after 15 days of exposure	[104] Bebbianno et al., 2015
<i>M. edulis</i>	CuO NPs	qPCR	Greater effects of CuO NPs on GST, SOD, MT, Actin, ATP synthase gene expressions	[105] Châtel et al., 2018
<i>M. galloprovincialis</i>	PE microplastics	Illumina HiSeq2000	up-regulation of immune-receptors and stress-related proteins (glutathione peroxidase, hsp70)	[8] Détrée and Gallardo-Escárate, 2018
<i>Mytilus edulis</i>	HDPE and PLA microplastics	HRAMS and Dionex Ultimate 3000	Altered the hemolymph proteome. Proteins involved in immune regulation, detoxification, metabolism and structural development	[106] Green et al., 2019

immune responses [86,99].

Another challenge for the scientific community is the detection of early warning toxicity and to understand complex effects in the specific pathways that could be affected. The molecular and -omic approaches are powerful tools to figure out the mechanisms leading to the response and the adaptation of bivalves to environmental changes [51]. Table 2 shows several toxicological studies with mussels exposed to pollutants of emerging concern applying different molecular and -omic approaches. Of note is the study by Détrée and Gallardo-Escárate [8] that demonstrated stress-memory in mussels after a second exposure of microplastics. It highlights, once again, the complexity of the innate immune system of bivalves.

All these antecedents should lead researchers to be aware that bivalve immune response is strongly influenced by this new pollution source. More attention should be paid to the interaction of this insidious new scenario and its impact on bivalve immune system function and resilience.

1.4. Conclusions and future perspectives

Our intention in this article was not to conduct an exhaustive review of genomics and immunology of bivalves, or specifically mussels, but to offer an overview of some interesting aspects that we found studying the immune response of these animals. As stated in Rey-Campos et al. (2019) «Currently, we undoubtedly can say that mussels are anything but simple and that more revelations will appear in the study of “non-model mussel immunity”».

Competing interests

The authors declare no competing interests.

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