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REVIEW

Genomics, immune studies and diseases in bivalve aquaculture

A Romero, B Novoa, A Figueras

Instituto de Investigaciones Marinas (IIM)-Consejo Superior de Investigaciones Científicas (CSIC). Eduardo Cabello, 6. 36208-Vigo, Spain

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Abstract

Diseases are a critical bottleneck for the culture of bivalves causing important yield losses. The study of bivalve diseases has relied on histological techniques and has focused on pathogen morphology, the effect of external factors on the pathogens and infectivity, and on the development of immune and molecular diagnostic techniques. Recently, significant advances in the study of bivalve pathology have been reported; however, increased efforts using “omics tools” are required to explain key physiological/immunological processes. Transcriptomic analysis in parallel with detailed functional studies of gene expression and cell biology in *in vitro* and *in vivo* experimental models are needed. Another important factor is the identification of “resistance traits” and a deeper understanding of the processes that contribute to the welfare of bivalves in culture. Additionally, the definition of "abnormal mortalities" is critical for managing and legislating bivalve aquaculture. The new technologies clearly “opens the door” for the directed manipulation of bivalves to improve the modern intensive aquaculture systems. The future of bivalve research is exciting, and there is an obvious need to develop multidisciplinary international research studies involving research groups and growers organisations to work on shellfish pathology.

Key Words: genomics; immunity; molluscs; diseases

Introduction

Over the past few decades, the global production of aquaculture species has increased, and mollusc aquaculture has doubled its production in that time (FAO, 2006). In 2006, the total production of molluscs was estimated at 14 million tonnes (FAO fishery statistics) (Lee *et al*., 2008), representing approximately one quarter of the total global aquaculture production. Asian countries are the main producers of molluscs; China, Japan and Thailand produced more than 30,000 tons in 2006 (FAO, 2006) and the production levels are increasing. Mollusc production on a global scale is based on more than 42 different species; however, the top five cultivated mollusc species are the Pacific oyster (*Crassostrea gigas*), the Japanese carpet shell (*Ruditapes philippinarum*), the Yesso scallop (*Patinopecten yessoensis*), the blue and Mediterranean mussels (*Mytilus edulis* and *M. galloprovincialis*) and the blood cockle (*Anadara granosa*). In 2008, Europe produced 658,000 tonnes of molluscs, contributing 26 % of the total volume of aquaculture production in Europe (16.6 % value) (FAO, 2010). Spain, France and Italy are the main bivalve-producing countries, although other countries, such as Greece and Norway, are demonstrating an increase in production. Spain is the major producer of mussels, representing 71 % of the total mollusc production. Clam and cockle production represented 10.9 % of the total molluscs produced in 2008, mainly in Italy. The culturing of clams is primarily based on the introduced Japanese carpet shell (*Ruditapes phillipinarum*) rather than local clam species (*Ruditapes decussates*). Oyster culture is based on the introduced Pacific cupped oyster *Crassostrea gigas* and the native oyster (*Ostrea edulis*). Oyster production is largely dominated by the French industry, which contributed 19.9 % of Europe’s total mollusc aquaculture in 2008.

The high mortality of bivalves in the larval stage and in juveniles and adults are the main problem in mollusc cultures. Mortalities decrease the global bivalve population and cause dramatic economic losses. Physical environmental factors, such as prolonged periods of high/low temperatures, extreme salinities and industrial and domestic pollution as well as the increase in bacterial and

Corresponding author:
Antonio Figueras
Instituto de Investigaciones Marinas (IIM)-Consejo Superior de Investigaciones Científicas (CSIC)
Eduardo Cabello, 6, 36208-Vigo, Spain
E-mail: antoniofigueras@iim.csic.es

viral pathogens in seawater leads to mortality at each developmental stage (Pipe and Coles, 1995; Braby and Somero, 2006; Carstensen *et al.*, 2010). The mortalities in bivalves have been associated with different viruses, bacteria and protozoa. Bacterial and viral diseases in molluscs are associated with pathogens from the Vibrio genus, including brown ring disease and summer disease (Borrego *et al.,* 1996; Paillard*,* 2004; Gómez-León *et al.,* 2005; Huchette *et al.,* 2006), and with the Herpesviridae, Iridoviridae and Picornaviridae families, among others (Marteil, 1976; Jones *et al.,* 1996; Hine and Wesney, 1997; Renault and Novoa, 2004; Batista *et al.,* 2007). Examples of disease-causing protozoa are *Haplosporidium nelsoni*, *Bonamia ostreae*, *Marteilia refringens*, *Mycrocitos mackini*, *M. roughley* and *Perkinsus marinus* (Carnegie *et al.,* 2003; Berthe *et al.,* 2004; Burreson and Ford, 2004; Villalba *et al.,* 2004).

How can we fight these diseases?

The effectiveness of any treatment against diseases in molluscs is limited by the physiology of the animals as well as by the characteristics of the culture system (Berthe, 2008). Molluscs lack an adaptive immune response; therefore, they cannot produce antibodies (Bayne, 2003; Ottaviani, 2011) and vaccination strategies are not possible for these animals. Moreover, the culture system is in an open environment, which makes treatments in the field difficult, and the impact on the environment must be considered. Therefore, therapeutic measures have not been applied to mollusc aquaculture in the open environment, although a number of treatments for diseases and parasites have been successful in the laboratory or on a reduced scale in the field (Calvo and Burreson, 1994; Nel *et al.,* 1996; Faisal *et al.,* 1999; Friedman *et al.,* 2007).

Rigorous procedures are applied to minimise the risk of introducing an infectious disease into a disease-free area. Standards, guidelines and recommendations for the introduction of new stocks are provided at international, regional and national levels (ICES, 2004; MAPA, 2008; OIE, 2011). Moreover, important efforts have been made to improve diagnostic methods for diseases that affect molluscs (Bondad-Reantaso *et al.,* 2001; OIE, 2011). Historically, most of the descriptions of mollusc pathogens are based on structural and ultra-structural studies. Traditionally, the diagnosis of mollusc diseases has primarily been achieved using histological methods and transmission electron microscopy (TEM) (Boulo *et al.,* 1989; Azevedo *et al.,* 1990; Anderson *et al.,* 1994; Bushek *et al.,* 1994; Hervio *et al.,* 1996; Longshaw *et al.,* 2001; Howard *et al.,* 2004). The limitations of these methods include the need for highly qualified personnel, the techniques are time-consuming, and they often demonstrate low specificity/sensitivity. To overcome the limitations of histology and TEM for diagnosis, the European and National Reference Laboratories have developed, validated and standardised a series of rapid, specific and reliable molecular diagnostic methods. These entirely new DNA-based assays allow the rapid detection of pathogens (Stokes and Burreson, 1995; Berthe *et al.,* 1999; Le Roux *et al.,* 1999; Carnegie *et al.,* 2000*;* Cochennec *et al.,* 2000; Walker and Subasinghe, 2000; Yarnall *et al.,* 2000; Kleeman *et al.,* 2002; OIE, 2011).

Health management policies in areas where disease outbreaks occur attempt to reduce the incidence or the severity of a disease (Grizel *et al.,* 1986) using the following strategies: changes in culture management procedures (Korringa, 1950, Lauckner, 1983; Andrews and Ray, 1988; Montes *et al.,* 2003), increasing the tolerance of the individuals to disease through stimulating the immune system (Macey and Coyne, 2005; Xue *et al.,* 2008; Van Hai *et al.,* 2009), producing tolerant strains by genetic handling (Ford and Haskin, 1987; Martin et *al.,* 1993; Gaffney and Bushek, 1996; Hand *et al.,* 1998; Nell *et al.,* 2000; Culloty *et al.,* 2004; Nell and Perkins, 2006; Samain *et al.,* 2007; Villalba *et al.,* 2007) or replacing susceptible species with resistant ones (Grizel and Héral, 1991; Mann *et al.,* 1991). However, the introduction of foreign species could introduce pathogens, predators or competitors that may perturb the equilibrium of the ecosystem and affect adjacent areas or neighbouring countries (Carriker, 1992; Shatkin *et al.,* 1997).

Genomics focuses on the study of genes and their function. The use of these tools can compensate for the near absence of clinical manifestations and symptoms in diseased molluscs by identifying key molecules (RNAs or proteins) expressed in response to injures or disease. Moreover, genomics can help determine the relationship between the genes and environmental factors in biological processes, such as diseases. These genetic markers will allow the identification of genes involved in the defence against pathogens, and the generation of specific bioassays to measure their production, activity or expression can be used as markers of host physiology, the activation state of the pathogen or the immune status of the host. The use of genomic tools will facilitate the study of molluscs and increase our understanding of the biological processes that are critical for the success of molluscan aquaculture (Saavedra and Bachère, 2006).

Several problems currently affecting bivalves would benefit from the genomics approach (Saavedra *et al.,* 2009). The presence of toxins of phytoplanktonic origin produces red tides and causes several diseases in humans after consumption (Bricelj and Shumway, 1998). Genomic tools can be used to study the depuration processes for each toxin. Additionally, the study of the larval stages at cellular and molecular levels, diet design, and growth as well as factors related to disease resistance or stress are aspects of bivalve physiology that can be studied using genomic techniques.

The genomic approach: the beginning

When the genomic approach was first initiated in bivalves, the focus was on the effects of environmental pollution. Because of the small number of validated and biologically relevant markers, together with inadequate information on the cellular processes occurring in normal and

stressed mussels, the development of effective biomonitoring programmes was delayed (Viarengo *et al.,* 2000; Moore, 2002). Mussels (*M. edulis* and *M. galloprovincialis*) have been used as model organisms because of their ecological importance (Gosling, 1992) and their economic value in shellfish farming (Beadman *et al.,* 2002). Since the mid-1970s, because of the ubiquitous and sessile characteristics of the adult mussel, its efficient filtering activity, limited xenobiotic metabolism and plasticity to environmental changes, mussels have been used as bio-indicators of chemical pollution (Goldberg and Bertine, 2000; Whitfield, 2001). Different proteins have been identified in mussels after their exposure to toxic contaminants (Lopez *et al.,* 2001; Aardema and MacGregor, 2002). The relationship between changes in the pollution status of the water and changes in the protein profile, the generation of free radical scavengers, and the copper and zinc content have been studied in mussels from dirty and clean areas (Gorinstein *et al.,* 2006). The transcriptome of mussels was used to select markers that trace contaminants (Venier *et al.,* 2003b, 2006; Dondero *et al.,* 2006a).

The first attempts to describe important immune-related genes in bivalves were performed using homology cloning; however, this approach was not successful because of the limited information on bivalve genes in databases. Most of the information on bivalve innate immunity is based on biological activities, and only indirect evidence for the role of particular genes in the bivalve resistance against disease has been described. For example, the protistan *Perkinsus marinus* is a lethal parasite in *Crassostrea virginica*; however, the pathogen is not known to cause lethal infections in *Mytilus edulis* or *Geukensia demissa*. In 2001, Anderson and Beaven suggested that the anti-*P. marinus* properties of plasma proteins in *Mytilus edulis* or *Geukensia demissa* exhibit many hundred-fold greater activity than the plasma proteins in *C. virginica* and may be responsible for the low susceptibility of this species. In another example, Meyers *et al.* (1991) reported that *Crassostrea gigas* is less susceptible to *Perkinsus marinus* than *C. virginica*, suggesting that *C. gigas* is tolerant to *P. marinus*. It has been demonstrated that *P. marinus* proteases compromise the immune defence mechanisms of the eastern oyster (Garreis *et al.,* 1996) and favour the protozoan's propagation (La Peyre *et al.,* 1996). Faisal *et al.* (1998) and Oliver *et al.* (1999) associated the presence of highly specific protease inhibitors in *C. gigas* with their lower susceptibility to *P. marinus*.

The genomic approach: the next step

The next step in the genomic approach was to describe the differentially expressed immune-related genes in bivalves using different methodologies, such as BAC libraries, EST collections from cDNA or subtractive hybridisation libraries (SSH) or microarray technology. These methodologies have been used to select key genes linked to resistance that could be used as markers for selection, and to study in detail the interaction between the host immune system and different pathogens.

BAC libraries

Only two BAC libraries have been reported in molluscs, one for the Eastern and Pacific oyster (Cunningham *et al.,* 2006; Hedgecock *et al.,* 2005) and the other for the Zhikong scallops, *Chlamys farreri* (Zhang *et al.,* 2008). The two libraries produced 73,728 and 7,680 clones, respectively, and the combined libraries contain 81,408 BAC clones in total. The oyster BAC libraries are available to the research community at www.genome.clemson.edu. Because BAC libraries facilitate the handling of long genomic fragments, their use should rapidly expand to include other intensively exploited commercial bivalves.

cDNA and SSH libraries

Several expressed sequence tag (EST) collections from cDNA or subtractive hybridisation libraries (SSH) have been generated in the last few decades. Those libraries are powerful tools for the study of differential gene expression and the identification of genes involved in specific biological functions, especially in organisms where genomic data are not available (Zhang *et al.,* 2001; Satou *et al.,* 2002). The EST approach was used to identify genetic markers in salmon (Davey *et al.,* 2001) and environmental stress indicators in the American oyster (Jenny *et al.,* 2002); it was also used to characterise immune genes in shrimp (Gross *et al.,* 2001). In molluscs, most studies have focused on analysing the immune system with the aim of identifying the molecular basis of the most common pathologies (Saavedra *et al.,* 2009). Although these libraries have provided sequences that are homologous to previously described sequences, a significant number of the generated ESTs did not show similarity to known genes in a BLAST search using the available databases.

In oyster, the first published library was constructed in *C. virginica* with the aim of identifying genes to be used as bioindicators of exposure to environmental pollutants, toxins and infectious agents (Jenny *et al.,* 2002). Approximately 40 % of the 1,000 ESTs generated were novel sequences and demonstrated homology to C-type lectin and scavenger receptors, proteinases, metallothionein, precerebellin, collectins and antimicrobial peptides. Gueguen *et al.* (2003) performed a similar study in *C. gigas* and described genes involved in defence mechanisms after exposure to four pathogenic strains of Vibrio sp. This approach allowed the characterisation of 55 sequences out of 1,142 ESTs that have potential immune function. Interestingly, the authors reported the expression of a low number of antimicrobial peptides and a high number of proteases, inhibitors of proteases and adhesive proteins. Additionally, Roberts *et al.* (2009) reported the construction of a library from *Crassostrea gigas* hemocytes in which 2,198 ESTs were generated. Suppression-subtractive hybridisation (SSH) libraries were also constructed in oyster. Huvet *et al.* (2004) identified differentially regulated genes in *C. gigas* in the F2 progeny with different susceptibility to the summer mortality disease. In this study, several transcripts were detected that exhibited homology to heat shock proteins, and the antimicrobial peptide defensin B was identified. A

study of genes expressed in response to *Perkinsus marinus* challenge was performed in the hemocytes and gills from *C. virginica* and *C. gigas* (Tanguy *et al.,* 2004). The genes involved in the immune system, cell communication, protein regulation and transcription, cell cycle, respiratory chain and cytoskeleton were detected and were overexpressed in challenged oysters. The SSH technology has also been applied to the identification of antimicrobial peptides belonging to the mussel defensin family in oysters (Peatman *et al.,* 2004; Gueguen *et al.,* 2006).

The first clam cDNA library was generated in *Ruditapes philippinarum* infected with *Perkinsus olseni*. Kang *et al.* (2006) sequenced 1,850 clones in total, and 29 ESTs were shown to be related to immune genes, such as C-type lectins, lysozyme and cystatin B. Moreover, lectins, which were the largest group of immune-function ESTs, appeared to be pathogen specific because they appeared after Perkinsus infection but not after a Vibrio infection. SSH libraries have also been constructed in carpet shell clams (*Ruditapes decussatus*), one after bacterial stimulation of the clams (Gestal *et al.,* 2007) and a second one after *Perkinsus olseni* infection (Prado-Alvarez *et al.,* 2009). Gestal *et al.* (2007) first identified clam myticin isoforms 1, 2 and 3, and clam mytilin. Moreover, they reported a low number of ESTs related to AMPs. Prado-Alvarez *et al.* (2009) generated 305 differentially expressed sequences, 42 of which were associated with immunity and stress related functions; the adiponectin-C1q and DAD-1 genes in *R. decussates* were also identified. Finally, Tanguy *et al.* (2008) reported 124 contigs and 1,814 singletons for *R. decussatus*, including immune genes with homologies to lectins and ferritins.

In mussel, the first systematic production and annotation of ESTs was performed in *M. galloprovincialis* by Venier *et al.* (2003a); however, due to the lack of available mussel sequences in the databases, most of the sequences did not exhibit homology. A small number of ESTs were homologous to immune-related genes, such as the β-glucan binding protein, the LPS binding protein, and some proteasome components, lectins and antimicrobial peptides. These ESTs were combined with other cDNA and SSH libraries (Pallavicini *et al.,* 2008) from bacteria-stimulated mussels. Venier *et al.* (2009) constructed, sequenced and annotated 17 cDNA libraries from different Mediterranean mussel tissues (gills, digestive gland, foot, anterior and posterior adductor muscle, mantle and hemocytes) challenged with toxic pollutants, temperature variations and potentially pathogenic bacteria. In total, 24,939 clones were sequenced from these libraries generating 18,788 high-quality ESTs, which were assembled into 2,446 overlapping clusters and 4,666 singletons, resulting in 7,112 non-redundant sequences. This annotated catalogue represents a valuable platform for expression studies, marker validation and genetic linkage analysis, which was the basis for constructing the important mussel database, the MytiBase (http://mussel.cribi.unipd.it) (Venier *et al.,* 2009). The information available in the MytiBase led to the detection of a considerable number of antimicrobial peptides (AMPs). The importance of the AMPs in the bivalve immune response is reflected by the large number of recent publications on this subject, such as the high diversity of MytC (Costa *et al.,* 2009) and its antiviral activity and immunoregulatory properties (Balseiro *et al.,* 2011); the genomic organisation, molecular diversification, and evolution of myticin-C (Vera *et al.,* 2011); studies on the variability of the AMPs (Rosani *et al.,* 2011); the characterisation of big defensins and mytimacins (Gerdol *et al.,* 2012); mytilins (Roch *et al.,* 2008; Parisi *et al.,* 2009); and mytimycins (Sonthi *et al.,* 2012).

Microarrays

The construction of the numerous libraries described has led to a significant increase in the number of ESTs in databases. These collections of ESTs contain genes that are modulated in response to environmental, chemical or biological stimuli and can be used to design probes in microarrays. Microarrays have various applications, including the analysis of expression profiles, the detection of SNPs, genotyping analysis and checking for mutations.

In oyster, Jenny *et al.* (2007) constructed a cDNA microarray in a consortium from Norway, France and the US based on EST libraries from several different tissues of *C. gigas* and *C. virginica* exposed to hypoxia, pesticides, bacterial and protozoan infections, hydrocarbons, hyperthermia, and summer mortality conditions. This cDNA microarray is available to the research community and has already been used (Lang *et al.,* 2009). Moreover, an *in situ* oligonucleotide microarray was developed by Wang *et al.* (2010) using existing ESTs to analyse gene expression profiles during dermo disease caused by *Perkinsus marinus*.

In mussel, the first study using microarrays was performed by Dondero *et al.* (2006b) in the Mediterranean mussel, *M. galloprovincialis*. This microarray was enriched for genes involved in pollutant and xenobiotic responses. The same research group developed the MytArray 1.0, which includes 1,714 probes (Venier *et al.,* 2006) and also an oligo array using RNAs from mussels injected with *Vibrio splendidus* (Venier *et al.,* 2011). Another oligonucleotide-microarray was developed using 4,488 different genes from *M. galloprovincialis* and *M. trossulus* to analyse the effects of heat stress on gene expression (Lockwood *et al.,* 2010) and to study the transcriptional responses of the two species to an acute decrease in salinity (Lockwood and Somero, 2011).

The "big jump": the next-generation sequencing technologies

The "big jump" in genomic research will occur with the development of new-generation sequencing technologies (NGS). These new technologies have been developed because of the high demand for tools that deliver fast, inexpensive and accurate genome information. The primary advantage of NGS over conventional methods is the ability to produce large volumes of data inexpensively (in some cases in excess of one billion short reads can be achieved per run). Multiple platforms for NSG

coexist in the marketplace, some having clear advantages for particular applications over others. Commercially technologies are available from Roche (454 Life Sciences), Illumina (Solexa Sequencing Technology), Applied Biosystems (Life Technologies/APG), Helicos Biosciences, the Polonator, and the Pacific Biosciences (Near-term Technology).

Sequencing technologies combine various stages that can be broadly grouped as template preparation, sequencing and imaging, and data analysis. The unique combination of specific protocols distinguishes one technology from another and determines the type of data produced from each platform (Harismendy *et al.,* 2009; Metzker, 2010).

The two most common methods for template preparation are emulsion PCR (emPCR) (Dressman *et al.,* 2003) and solid-phase amplification (Fedurco *et al.,* 2006). The emPCR prepares sequencing templates in a cell-free system and generates a library of DNA fragments. The DNA is separated into single strands and is captured on beads (one DNA molecule per bead). Millions of beads are immobilised on a microscope slide, on an amino-coated glass surface or are deposited into individual PicoTiterPlate wells, according to the platform used (Polonator, Life/APG and Roche/454, respectively). Solid-phase amplification is used to produce randomly distributed, clonally amplified clusters from the fragments onto a glass slide. This amplification can produce 100 - 200 million spatially separated template clusters (Illumina/Solexa).

The four main sequencing and imaging strategies used include single-nucleotide addition or pyrosequencing (Ronaghi *et al.,* 1996, 1998), cyclic reversible termination (Metzker, 2005), sequencing-by-ligation (Tomkinson *et al.,* 2006) and real-time sequencing (Eid *et al.,* 2009) methods. A hard computational study is used after the generation of millions of reads to perform an alignment to a known reference sequence or an assembled *de novo* (Pop and Salzberg, 2008; Chaisson *et al.,* 2009; Trapnell and Salzberg, 2009).

Recently, the next-generation sequencing technologies have been applied to the study of different processes in molluscs. In non-bivalve molluscs, 454 sequences have been used in the fields of developmental biology, cell biology, larval ecology, ecotoxicology, parasitology, and chemical ecology for the investigation of different species, such as the marine snails *Littorina saxatilis* (Galindo *et al.,* 2010) and *Ilyanassa obsolete* (Lambert *et al.,* 2010), the pulmonated snails *Radix balthica* (Feldmeyer *et al.,* 2011) and *Conus bullatus* (Hu *et al.,* 2011), the mollusc *Crepidula fornicate* (Henry *et al.,* 2010) and the photosynthetic slug *Elysia timida* (Wägele *et al.,* 2011).

In bivalves, this technology has been applied to investigate different physiological processes, such as shell deposition and repair in the Antarctic clam (*Laternula elliptica*) (Clark *et al.,* 2010) and biomineralisation in *Pinctada margaritifera* (Joubert *et al.,* 2010). The technology has also been used to generate genomic tools for environmental monitoring in the Manila clam, *Ruditapes philippinarum* (Milan *et al.,* 2011). *M. galloprovincialis* and the Yesso scallop (*Patinopecten yessoensis*) have been used to study molecular aspects of their reproduction, the skewed sex ratios of their offspring and their adaptation to climatic and pollution factors (Craft *et al.,* 2010; Hou *et al.,* 2011). The developmental process has been analysed at different larval stages in the clam *Meretrix meretrix* (Huan *et al.,* 2012). In an attempt to identify genes potentially involved in bivalve innate immunity, high-throughput sequencing was performed in the deep-sea hydrothermal vent mussel *Bathymodiolus azoricus* (Bettencourt *et al.,* 2010) and in the Manila clam *Ruditapes philippinarum* using in vitro immune-stimulated hemocytes (Moreira *et al.,* 2012), producing more than 700,000 sequencing reads.

In the next few years, the European project ReproSeed has planned to perform 454 sequencing projects in different bivalve species (*P. maximus*, *M. edulis*, *M. galloprovincialis* and *R. decussates*) using larvae, different adult tissues and hemocytes (stimulated with PAMPs *in vivo* and *in vitro*). The main objective is to enrich the database with specific immune-related sequences. Moreover, the ontogeny of the immune system in bivalves and the immune response at the larval stages will be analysed.

Identification and characterization of relevant physiological and immune processes in bivalves

Genomic methods offer useful tools for studying the molecular basis of biological characteristics of great interest in aquaculture, such as disease resistance and stress. Because information concerning the molecular mechanisms of the immune response in bivalves is scarce and fragmentary, the 454-pyrosequencing will lead to the identification of genes involved in the immune defence against infectious diseases. In this context, Moreira *et al.* (2012) described a large number of immune related genes in *Ruditapes philippinarum*, showing the presence of putative members of several immune pathways and processes, such as apoptosis, the Toll-like signalling pathway and the complement cascade. This study also identified sequences from molecules that have not been described in bivalves, such as the complement pathway where almost all components were shown to be present. Information obtained from the MytiBase database was used by Romero *et al.* (2011) to identify for the first time the most important molecules involved in the apoptotic process induced after exposure to UV light in *Mytilus galloprovincialis*.

Apoptosis is a gene-directed mechanism that eliminates unnecessary or dangerous cells without the release of the intracellular components (Kerr *et al.,* 1972; Tittel and Steller, 2000). This mechanism is an essential biological process in all animals and is critical in many aspects of development and for the maintenance of immune system homeostasis (Opferman and Korsmeyer, 2003). Moreover, apoptosis is a host defence mechanism against viral and bacterial pathogens (Koyama *et al.,* 2003; DeLeo, 2004). In mammals, two major pathways of apoptosis activation have been characterised in

detail. The intrinsic pathway, initiated in response to oxidative stress, genotoxic substances or specific signals, leads to the release of mitochondrial cytochrome c into the cytosol (Armstrong, 2006). Cytochrome c activates caspase-9 and the caspase signalling cascade through the formation of the apoptosome (Li *et al.,* 1997). The extrinsic pathway is activated by the death domain-containing transmembrane receptors through interaction with their extracellular ligands, which results in the direct activation of the caspase cascade and/or the induction of the mitochondrial membrane permeabilisation (Bridgham *et al.,* 2003).

Although genes encoding caspase proteins have been described in vertebrates, in almost all invertebrate phyla (Lamkanfi *et al.,* 2002) and a caspase 8-like gene has been reported in the gastropod *Haliotis diversicolor* (Huang *et al.,* 2010), limited information regarding the genes that mediate this mechanism and their regulation under different stimuli is available for bivalves (Sokolova, 2009; Kiss, 2010). Romero *et al.* (2011) characterised 6 different caspase genes in *M. galloprovincialis* and analysed their regulation under the experimental exposure to genotoxic substances and several PAMPs. The initiator caspase group was shown to be composed of 2 sequences with high homology to caspase-2 and caspase-8, whereas the executioner group was composed of 4 members with high homology to caspase-3/7. Evaluation of the tissue expression patterns of the genes revealed extremely high expression levels within the gland and gills where the apoptotic process is highly active due to the clearance of damaged cells. The hemocytes also exhibited high levels of expression of these genes, likely due to the role of apoptosis in the defence against pathogens.

To understand the mechanisms of caspase gene regulation, Romero *et al.* (2011) treated hemocytes with UV light, environmental pollutants and pathogen-associated molecular patterns (PAMPs), and evaluated the apoptotic process using microscopy, flow cytometry and qPCR techniques. The results suggested that the apoptotic process is tightly regulated in bivalve molluscs by the overexpression/suppression of caspase genes; additionally, there was evidence of caspase-specific responses to pathogens and pollutants. The apoptotic process in molluscs has a similar complexity to that of vertebrates; however, apoptosis in molluscs exhibit unique features that may be related to their recurrent exposure to environmental changes, pollutants and pathogens imposed by their sedentary nature. It is important to highlight the considerable capability of molluscs to scope with different typologies of stress, since it represent a fundamental link between environmental changes, immunity and infection lethality.

Advancements in the understanding of the apoptotic pathway in *Mytilus galloporvincilais* were achieved following the description of the mitochondrial pathway. Romero *et al.* (unpublished data) described six novel genes related to the intrinsic pathway (Bcl-2, Bax, BI, P53, PDRG and DFF) and studied the relative contribution of the intrinsic pathway to mitochondrial cell death through the inhibition of different stages of the intrinsic apoptotic pathway.

Concluding remarks

The recent use of new sequencing technologies has drastically increased the number of mollusc genomic sequences in the databases. These sequences are the basis for understanding physiological processes and the immune response against diseases and for solving problems in the bivalve industry.

Genomic information will help find specific answers to a number of questions: Can this information be used to discover specific genes linked to resistance? Can the resistance be enhanced or manipulated by stimulating specific responses? Can the resistant individuals be selected, and will this resistance be maintained and passed to descendants? Will this information open alternatives to selection? Which is the influence of the environmental factors? And finally, of critical importance, is this information a priority to the bivalve industry?

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