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Title: Long-term affected flat oyster (*Ostrea edulis*) haemocytes show differential gene expression profiles from naïve oysters in response to *Bonamia ostreae*

Ronza P. [a], Cao A. [b], Robledo D. [c], Gómez-Tato A. [d], Álvarez-Dios J.A. [e], Hasanuzzaman A. F. M. [f], Quiroga M. I. [a], Villalba A. [b,g], Pardo B. G. [h]*, Martínez P. [h]*

[a]Departamento de Anatomía, Producción Animal e Ciencias Clínicas Veterinarias, Facultad de Veterinaria, Universidade de Santiago de Compostela, 27002 Lugo, Spain

[b]Centro de Investigacións Mariñas (CIMA), Consellería do Mar, Xunta de Galicia, 36620 Vilanova de Arousa, Spain

[c]The Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh, Midlothian EH25 9RG, UK

[d]Departamento de Matemáticas, Facultad de Matemáticas, Universidade de Santiago de Compostela, 15782 Santiago de Compostela, Spain

[e]Departamento de Matemática Aplicada, Facultad de Matemáticas, Universidade de Santiago de Compostela, 15781 Santiago de Compostela, Spain

[f]Fisheries and Marine Resource Technology Discipline, Khulna University, Khulna, 9208, Bangladesh

[g]Departamento de Ciencias de la Vida, Universidad de Alcalá, 28871 Alcalá de Henares, Spain

[h]Departamento de Zoología, Genética y Antropología Física, Facultad de Veterinaria, Universidade de Santiago de Compostela, 27002 Lugo, Spain

* Corresponding author

Paulino Martínez

Departamento de Zoología, Genética y Antropología Física, Facultad de Veterinaria. Universidade de Santiago de Compostela, 27002 Lugo, Spain. Tel./fax: +34 982 822428.

Abstract

European flat oyster (*Ostrea edulis*) production has suffered a severe decline due to bonamiosis. The responsible parasite enters in oyster haemocytes, causing an acute inflammatory response frequently leading to death. We used an immune-enriched oligo-microarray to understand the haemocyte response to *Bonamia ostreae* by comparing expression profiles between naïve (NS) and long-term affected (AS) populations along a time series (1 d, 30 d, 90 d). AS showed a much higher response just after challenge, which might be indicative of selection for resistance. No regulated genes were detected at 30d in both populations while a notable reactivation was observed at 90 d, suggesting parasite latency during infection. Genes related to extracellular matrix and protease inhibitors, up-regulated in AS, and those related to histones, down-regulated in NS, might play an important role along the infection. Twenty-four candidate genes related to resistance should be further validated for selection programs aimed to control bonamiosis.

Key words

bonamiosis, parasite, oligo-microarray, immune response, mollusk, bivalve

Introduction

The European flat oyster *Ostrea edulis* has traditionally been a highly appreciated marine resource for human nutrition. Overexploitation has led to the exhaustion of most flat oyster beds through Europe in the last century, and its production has been mostly sustained through aquaculture (Korringa 1976). However, flat oyster farming has been affected at specific areas by disease outbreaks, such as the "shell disease" caused by the fungus *Ostracoblabe implexa* (Alderman and Jones 1971), or at larger areas involving

different countries, such as infections with the protist *Marteilia refringens* (Grizel et al. 1974; Berthe et al. 2004) and *Bonamia ostreae* (Pichot et al. 1980). The latter is a parasite widely distributed throughout the European coast, constraining European flat oyster production (da Silva et al. 2005; Culloty and Mulcahy 2007; Engelsma et al. 2010, 2014; Laing et al. 2014). Thus, controlling bonamiosis is required for a successful recovery of the European flat oyster production, but its eradication from affected areas seems not to be a simple task (Grizel et al. 1986; van Banning 1991; Lynch et al. 2007), and the use of resistant oyster stocks appears to be the most promising strategy (Elston et al. 1987; Naciri-Graven et al. 1999; da Silva et al. 2005, Lynch et al. 2014). Successful breeding programs to produce disease resistant oysters, such as those against summer mortality or infections with *Haplosporidium nelsoni*, *Perkinsus olseni*, *Roseobacter crassostreae*, *Marteilia sydneyi* or *B. ostreae*, have been based on selecting broodstock among survivors after long exposure in heavily affected areas (Ford and Haskin, 1987; Beattie et al. 1988; Ragone Calvo et al. 2003; Dove et al. 2013; Frank-Lawale et al. 2014; Lynch et al. 2014; Dégremont et al. 2015). This procedure relies on the cumulative benefit of successive generations of selection to achieve profitable increased resistance levels. Considering that the maximal oyster mortality due to *B. ostreae* occurs when the oysters have almost reached market-size (Culloty and Mulcahy 1996; Baud et al. 1997; da Silva et al. 2005), the evaluation of each generation in the field to select breeders and obtaining the next generation should take at least 4-5 years. Such procedure would need a long-term program to reach acceptable oyster survival in affected areas (Lynch et al. 2014). Availability of molecular markers associated with resistance would avoid long field evaluation of breeders, thus shortening the process and making it more efficient. Such resistance markers have been successfully found for some diseases in mollusks (Nikapitiya et al. 2014; Normand et al. 2014; Raftos et al.

2014; Nie et al. 2015; Vaibhav et al. 2016), and even a specific mutation conferring resistance to perkinsosis has been identified in the eastern oyster (He et al. 2012).

Mollusc immunity relies on innate cellular and humoral mechanisms, where haemocytes, the circulating cells present in haemolymph and infiltrating through other tissues, play a key role as responsible cells for phagocytosis of foreign particles, among other functions. *B. ostreae* is an intracellular parasite able to proliferate and survive inside the oyster haemocytes, inducing a strong inflammatory response and disturbing the immune capacity of the host (Bucke 1988). Oyster response involves cytotoxic mechanisms as well as non-oxidative and oxidative pathways, including lysosomal enzymes and reactive oxygen/nitrogen intermediates. The haemolymph serum contains humoral defense factors, such as soluble lectins, hydrolytic enzymes and antimicrobial peptides (Allam and Raftos 2015; Bachère et al. 2015). The evaluation of immune-gene expression profiles of oyster and their interactions along the process of infection is crucial to understand the response to bonamiosis and requires the development of large-scale genomic resources and appropriate tools to identify genes and signalling networks related to disease resistance. Recently, a comprehensive transcriptomic haemocyte database has been used to design and validate an oligo-microarray to assess gene expression profiles of flat oyster in response to *B. ostreae* (Pardo et al. 2016).

In this study, we used this oligo-microarray to identify genes and pathways differentially expressed along a time series after challenge with *B. ostreae* in two flat oyster stocks with differential susceptibility to bonamiosis: i) a naïve stock from a free-bonamiosis area; and ii) a stock from a long-term affected area, which had shown some resistance to *B. ostreae*. The comparison of the pattern of response along the infection process and the differentially expressed genes and pathways between both stocks

provided candidate genes and pathways to be validated for their application on breeding programmes for a more efficient selection.

Materials and Methods

Biological material and challenge

Flat oysters were collected from Limfjord (Denmark) and Ortigueira (NW Spain) in 2010. Surveillance since 1996 has shown Limfjord to be a *Bonamia*-free area until 2014, when *B. ostreae* was detected for the first time in November (Madsen and Thomassen 2015). Thus the Limfjord oyster population was still naïve regarding bonamiosis in 2010 (NS: naïve stock onwards). Conversely, bonamiosis affects oysters in Ortigueira since early 1980's (AS: long-term affected stock onwards) and oysters from this bed are more resistant to *B. ostreae* than oysters from other geographic origins, as shown in an experiment where families from Ortigueira showed significantly less prevalence and lower infection intensity (da Silva et al. 2005).

Oysters of both origins were experimentally challenged with *B. ostreae* and compared with their corresponding non-challenged controls. The challenge was performed by individually immersing oysters in beakers containing an aerated suspension of 300,000 *B. ostreae* cells in filtered seawater for 24 h at the Centro de Investigacións Mariñas. The *B. ostreae* cells for the challenge were isolated from heavily infected oysters collected in Lough Foyle (Ireland), following the procedure described by Mialhe et al. (1988). After challenge, oysters were kept in tanks with running, filtered (1 μm) seawater plus continuous (pumped) supply of mixed cultured algae until the end of the experiment. Haemolymph samples were taken at three different times after the challenge (1, 30 and 90 days post-challenge, dpc) considering previous information on the progression of the infection (Culloty and Mulcahy, 2007); as much haemolymph as

possible (from 0.2 ml to 1.5 ml) was withdrawn from the adductor muscle of each oyster with a 21 gauge needle screwed to a 2 ml cold syringe. The haemolymph was immediately poured into a cold vial and kept in crushed ice to avoid haemocyte clumping. Haemolymph samples were centrifuged (800g, 10 min, 4ºC) and the cell fractions (pellets) resuspended in RNA later; then suspensions, after one night at 4ºC, were stored at -20ºC until further processing. Five biological replicates for each condition (control and challenged), stock (NS and AS) and sample times (1, 30 and 90 dpc) were used.

RNA isolation, cDNA synthesis and library construction

RNA was individually extracted using RNeasy Mini Kit (Qiagen, Germany) following manufacturer's instructions with slight modifications, including two additional RPE washes and DNase digestion. Quantity was determined using a Nanodrop spectrophotometer (Nanodrop Technologies, USA) and quality (RNA integrity number, RIN) using a Bioanalyzer (Agilent Technologies, USA). All samples showed high RNA quality (RIN > 8.0) and consequently, they were further processed for microarray analysis. For each experimental condition, the control group was pooled by combining equimolar RNA quantities of the five biological replicates, while the RNA of each challenged oyster was processed individually (five replicates). Next, 50 ng of total RNA were labeled using the Low Input Quick Amp Labeling Kit, One-Color (Cy3; Agilent Technologies).

Microarray hybridisation and scanning

All samples (control pools and challenged oysters) were hybridized individually in a custom 8 x 15 K Agilent oligo-microarray at the Universidade de Santiago de Compostela (USC) Genomics Platform following the protocols for Agilent one-color

Gene Expression Analysis. This custom microarray was mainly based on OedulisDB, an *Ostrea edulis* database obtained from oyster haemocytes challenged with *B. ostreae* (Pardo et al. 2016). A total of 36 microarrays were used for hybridizations of 6 control (RNA pools from 2 stocks x 3 sample times) and 30 challenged oysters (5 biological replicates x 2 stocks x 3 sample times). Copy RNA (cRNA) was hybridized overnight during 17 h at 65ºC and washed with the corresponding buffers on the following day. Hybridized slides were scanned using an Agilent G2565B microarray scanner (Agilent Technologies). The scanner images were segmented with the protocol GE1-v5_95 from the Agilent Feature Extraction Software (v9.5) and to avoid saturation in the highest intensity range extended dynamic range was implemented. Agilent Feature Extraction produced the raw data for further preprocessing. The processed signal (gProcessed-Signal) value was the chosen parameter for statistical analysis as recommended (Millán et al. 2011). Feature quality filtering was performed following Millán et al. (2010).

qPCR validation

Quantitative real-time PCR (qPCR) was performed to validate microarray data. A set of 16 genes was selected following the random stratified procedure proposed by Miron et al. (2006) with some modifications as described by Millán et al. (2011). A total of 39 cases were selected covering the fold change (FC) range of the experiment and including at least one gene per microarray and two cases per gene. The glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) was chosen as reference gene following literature recommendations (Morga et al. 2010).

Primers for qPCR were designed using *Primer Express* Software v2.0 (Applied Biosystems) with default settings (Table S1). The same RNA samples used for microarray hybridizations were used for qPCR. The concordance between microarray

and qPCR data was assessed using the Pearson correlation and the null hypothesis of no differences between both methods tested by *t*-test (P ≤ 0.05).

Data analysis

Normalization using all microarray data was done by the Quantile method implemented in the Limma R package (Ritchie et al. 2015). All further statistical analyses were performed on the software Multiple Experiment Viewer (MeV) version 4.9.0, after a log2 transformation of the fluorescence intensity values. Fold change (FC) values were obtained as the log2 (treatment / control) after averaging technical replicates within each microarray. Principal component analysis (PCA) was computed with default parameters. Hierarchical Clustering (HCL) trees were constructed using Pearson correlation and default parameters (100 iterations). The SOTA program (Self Organizing Tree Algorithm; Dopazo and Carazo, 1997; Yin et al. 2006) was applied to identify groups of genes with similar expression patterns using Pearson correlation and internal cell diversity at P < 0.05. Regulated genes (RGs) at each experimental condition were detected by Significance Analysis of Microarrays test (SAM; Tusher et al. 2001) with a significance threshold of 5% False Discovery Rate (FDR). A two-way ANOVA considering stock and time as factors was performed to detect differentially expressed genes (DEGs) between stocks and across time (P < 0.001).

Results & Discussion

Experimental challenge and microarray evaluation

The gene expression profiles of *O. edulis* haemocytes challenged with *B. ostreae* were evaluated using an *in vivo* experimental infection, by immersing the oyster in beakers containing a suspension of parasitic cells. These conditions mimic the host-parasite interactions occurring in a natural scenario, so constituting an approach aimed to a more

comprehensive evaluation of haemocytes response than other published approaches consisting of injecting the parasite into the oyster adductor muscle (Martín-Gómez et al. 2012), or challenging haemocytes *in vitro* (Morga et al. 2011a; Morga et al. 2011b; Morga et al. 2012; Gervais et al. 2016). This approach enabled to identify a set of 715 regulated genes (RG) in infected oysters regarding controls (FC $\neq$ 0) along the time series evaluated (Table S2). Moreover, the availability of AS and NS stocks allowed to compare expression profiles of oysters showing some degree of resistance to the parasite with those that never had contact with it, and thus, identify 837 differentially expressed genes (DEG) between stocks (Table S3).

Gene profiles were analyzed using an Agilent custom 8 x 15 K oligo-microarray previously validated by Pardo et al. (2016). This microarray contains 6,882 different oligos and two replicates per oligo, which enabled controlling technical noise. The microarray platform Agilent-038418 and the data presented in this publication have been deposited in the NCBI's Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are available under accession number XXX. Comparison of expression data between microarray and qPCR for selected genes was highly consistent except for two values among the 39 comparisons performed, corresponding to the genes predicted HD phosphohydrolase family protein (ID: 4160e) and tripartite motif-containing protein 2 (4677e). The Pearson correlation coefficient increased from 0.769 to 0.929 after excluding these two cases (P < 0.001 for both correlations), and no significant differences were detected between both data sets including all cases (Wilcoxon test P = 0.137). Similar results have been reported with custom oligo-microarrays in other aquaculture species (Ferrareso et al. 2008; Millán et al. 2010; Ribas et al. 2016).

Gene expression profiles

Hierarchical clustering (HCL) was applied to check the grouping of biological replicates using RGs and considering the two main experimental factors: time after challenging (T1, T2 and T3) and stock evaluated (NS and AS). The codes NT1, NT2, NT3, AT1, AT2 and AT3 combining time and stock will be used onwards to refer to each experimental condition. Most biological replicates clustered in the expected group according to the experimental condition (Figure 1), especially for T1 and T3 in both stocks, which supports the fact that experimental factors (time and stock) override biological variation and that technical noise was kept under control in our hybridization protocol. T2 showed a slightly more erratic pattern because, as shown below, no response to the parasite was detected at this time.

A certain proportion (6.6%) of RGs or DEGs showed the same annotation (Table S4), reflecting the redundancy of the oyster database (Pardo et al. 2016). Probes with the same annotation usually showed correlated profiles along the experimental conditions tested (88.2%; Table S4). For example, the three genes annotated as peptidylglycine alpha-hydroxylating monooxygenase ($r > 0.990$) or the two annotated as collectin-12 ($r = 0.999$) showed highly significant correlation between them, suggesting that they are part of the same gene resulting from limitations of the transcriptome assembly process (Pardo et al. 2016) or they represent true duplicated genes with the same functional role. In the remaining 11.8% cases, no significant correlation or even a significant negative correlation was observed for the same annotation, suggesting that they are duplicated genes belonging to different paralogs with refined functions (neofunctionalization) or they represent alternative splice variants of the same gene. The most remarkable cases were those related to calcium and integrin-binding protein ($r = -0.647$) and complement C1q-like protein ($r = -0.493$), which showed a significant negative correlation value ($P < 0.001$). It has been suggested that stress- and immune-related genes suffered a notable

genomic expansion in molluscs due to their feeding by filtering in an enriched microbial environment, as reported for *Crassostrea gigas* (Zhang et al. 2012). This might explain our observations, especially considering that these genes were modulated after a parasite challenge.

A total of 715 RGs were detected after *Bonamia* challenge in at least one of the 6 experimental conditions (2 stocks x 3 times) tested (Table S2). While an important amount of RGs were detected at 1 dpc and 90 dpc, none was observed at 30 dpc either in NS or in AS stocks, which suggests some kind of latency during the infection progress. It is well known that the infection by *B. ostreae*, an intracellular parasite, presents a long pre-patent period, from one to several months, which is a major issue still unclear regarding the course of this parasitosis (Tigé and Grizel 1982; Elston et al. 1987; Montes et al. 1991; Culloty and Mulcahy 2007). In general, the AS stock showed a more intense response than the NS (536 *vs* 206 RGs, respectively) and a sharply different temporal pattern was observed between both stocks (Table 1). The response was much more intense at 1 dpc in AS (AT1: 507 RGs *vs* AT3: 31 RGs), while in NS more RGs were detected at 90 dpc (NT1: 86 *vs* NT3: 127). The huge difference observed at 1 dpc (six times more RGs in AS than NS) suggests that natural selection in the bonamiosis-endemic area might have favored a more intense and quicker response to the parasite. Interestingly, very few RGs were shared between times (1 dpc and 90 dpc) or stocks (NS *vs* AS), the highest common set being detected between NT1 and AT1 (12 RGs), which suggests a specific response to the challenge at each time and stock (Figure 2).

In general, significant FC values were moderate: mean FC > 0 = 1.204; mean FC < 0 = -1.722; 15.8% RGs showing 2 < FC < -2 (Table S5). FC ranged between 3.943 and -7.486 for the von Willebrand factor type A domain containing protein (NT3) and

leucine-rich repeat-containing G-protein coupled receptor 6 (NT1), respectively; down-regulated genes were more frequent than up-regulated among those showing the more extreme FCs (41 *vs* 18); and the AS stock showed a higher proportion of genes in this list than NS, especially among the up-regulated ones (14 out of 18) (Table S5).

The two-way ANOVA identified a total of 837 significant DEGs between stocks, 250 DEGs across time and 637 DEGs due to interaction of stock x time ($P < 0.001$) (Table S3). The large number of genes regarding interaction between both factors (stock x time) agrees with the particular behavior of each stock across time highlighted in the analysis of RGs. Accordingly, no significantly enriched pathways were detected in the 250 DEGs across time, and thus we focused our attention on the 837 DEGs between stocks as the most interesting to extract relevant information for future selection programs. Among them, 196 were in common with the RGs list (Table S2). The clustering of expression profiles of the 43 annotated genes showing the highest differences between both stocks (SD > 0.8) is represented in Figure 3. Among them, several genes related to histones and extracellular matrix (ECM) proteins, up-regulated in the AS stock and down-regulated in NS, were identified.

The application of SOTA analysis to identify clusters of genes showing correlated profiles rendered four RG groups in each of the two stocks (Figure S1, Table S6) and five DEG clusters (Figure S2, Table S7). Significantly enriched GO terms were found in two RG SOTA clusters from the AS stock (AS.RG1 and AS.RG2; Figure 4; Table S8) and in three DEGs SOTA clusters (GS2, GS3 and GS4; Figure 5; Table S9).

Functional analysis of RGs and DEGs: searching for resistance to bonamiosis

Our results support the important role of ECM in the resistance to *B. ostreae*, possibly related to parasite adhesion and host cell invasion. Enriched GO terms related to ECM

were found both in AS.RG2 and GS3 clusters, representing one of the most remarkable observations when comparing the response of NS and AS challenged oysters. This would be in accordance with the lower infection intensity observed in more resistant oysters (da Silva et al. 2005). The importance of ECM restructuring in response to infection has been recently related to resistance in eastern oyster (*Crassostrea virginica*) against the bacteria *Roseovarius crassotreae* (McDowell et al. 2014). In our study, a set of RGs that participate in ECM remodelling, including those encoding for metalloproteases tolloid-like protein 1 and matrix metalloproteinase 17 and 19, as well as those encoding for structural ECM proteins, such as several collagen isoforms, hemicentin-1, tenascin-R and tenascin-N, were identified in AS oysters (Table S2).

Tenascins, additionally, are members of the fibrinogen-related protein family (FREP), also including ficolin 1, up-regulated in AT1 (Table S2), and fibrinogen C domain-containing protein 1, one of the genes with the highest differential response between NS and AS stocks (Table S3, Figure 3). Many FREPs are known to act as pathogens recognition receptors (Dong and Dimopoulos 2009; Thomsen et al. 2011; Piccinini and Midwood 2012), and these molecules are increasingly being considered as essential factors in invertebrates' immune response (Hanington and Zhang et al. 2010; Gordy et al. 2015; Huang et al. 2015). Interestingly, FREPs and complement C1q domain containing proteins (C1qDCs) have been postulated as sound candidate markers for resistance to Quahog Parasite Unknown (QPX) in clams (*Mercenaria mercenaria*) (Wang et al. 2016a), showing up-regulation in resistant clams from an endemic infected area. In the present work, we found three RGs in AT1 related to C1qDCs, two up-regulated (C1QL4 and C1QTNF3) and one down-regulated (C1QBP) (Table S2). Furthermore, another gene related to host defence through pathogen recognition, collectin 12 (Ma et al. 2015), was also up-regulated in AT1.

Several evidences were found on the early activation of AS haemocytes in response to the challenge. In the cluster AS.RG1, significantly enriched GO terms were related to membrane, receptor activity, G-protein coupled signaling pathway and cell response to stimulus, reflecting the expression of several G-protein coupled receptors (GPCRs) and toll-like receptor (TLR) 1 in response to the parasite. GPCRs are essential in several signaling pathways regulating a wide range of cell responses, including the development of the immune response through activation, migration and adhesion of the cells of the immune system (Sun and Ye 2012; Hanlon and Andrew 2015). On the other hand, TLRs are well known as key components of the innate immune system that recognizes pathogen-associated molecular patterns (PAMPs), and a prominent role of TLR signaling was demonstrated in infection-induced haemocyte activation in *C. gigas* (Zhang et al. 2013). We found several GPCRs up-regulated in AT1 (Table S2), mostly classified as rhodopsin-like receptors, including orexin receptor type 2, cholecystokinin A receptor, cephalotocin receptor 1, neuropeptide FF receptor 2, growth hormone secretagogue receptor type 1 and prostaglandin E2 receptor 4. The involvement of G-protein signaling genes in the pathogenesis of bonamiosis in flat oyster was previously hypothesized (Martín-Gómez et al. 2012, 2014). Furthermore, in other invertebrates like the mosquito *Anopheles gambiae*, rhodopsin-like GPCRs have demonstrated an important role in response to the infection with the African malaria parasite *Plasmodium falciparum* (Mendes et al. 2011), and specifically, prostaglandin E2 receptor 4 has been involved in the innate immune response of *Crassostrea hongkongensis* to different pathogens (Qu et al. 2015). Two genes encoding alpha-adrenergic receptors were, by contrast, down-regulated in AT1 (Table S2). Interestingly, those GPCRs have been associated with immunomodulatory functions in the scallop *Chlamys farreri*, and their activation was shown to repress haemocyte phagocytic and

antibacterial capacities (Zhou et al. 2013). In two recent transcriptomic studies on mollusks, GPCRs and TLRs have been associated with defence response of *C. gigas* to the ostreid herpes virus 1 microvariant and with that of *Mercenaria mercenaria* against Quahog Parasite Unknown (QPX) (He et al. 2015; Wang et al. 2016b). In our study, NS oysters barely presented some RGs related to GPCRs, and one was leucine-rich repeat-containing G-protein coupled receptor 6, which was the most down-regulated gene detected in any condition (FC = -7,486 at T1; Table S5).

We found other up-regulated genes related to cell membrane in AS oysters (cluster AS.RG2; Figure S1, Table S6), such as integrin alpha-PS3, calcium- and integrin-binding protein (*CIB1*) and innexin, which might be involved in the early immune response. Integrins are transmembrane proteins that show a wide functional range, including spreading, adhesion and migration, and, particularly, integrin-α PS3 was reported to be activated in haemocytes of Manila clam (*Ruditapes philippinarum*) exposed to zoospores of *Perkinsus olseni* (Fernández-Boo et al. 2016), and involved in phagocytosis of apoptotic cells and bacteria in *Drosophila* haemocytes (Nonaka et al. 2013). Moreover, up-regulation of *CIB1*, encoding for an integrin binding partner that regulates integrin function (Freeman et al. 2013), was observed in *C. gigas* oysters infected by ostreid herpes virus and related to the mechanisms of virus entry (Jouaux et al. 2013). Innexins are transmembrane proteins, better characterized in insects than in bivalves, which are known to be structural components of the gap junctions in invertebrates. In arthropods, the formation of gap junction between haemocytes has been described during defense response, particularly for pathogen encapsulation, which is also a main defense mechanism of bivalves, for example against the previously cited parasites *Perkinsus* spp. and QPX (Soudant et al. 2008; Allam and Raftos 2015).

All in all, results suggest that up-regulation of cell-surface molecules and pathogens recognition receptors occur in AS haemocytes early after challenge, possibly making them "battle ready" to efficiently fight against the antigenic stimuli.

We also found significantly enriched GO terms associated to up-regulated genes encoding protease inhibitors (cluster AS.RG2; Figures 4 and S1, Table S8), which were especially present in AT1 (inter-alpha-trypsin inhibitor heavy chain H3, inter-alpha-trypsin inhibitor heavy chain H4, serine protease inhibitor dipetalogastin, pregnancy zone protein, protein AMBP, murinoglobulin-2). These molecules, often acting as acute phase proteins, represent an important defense mechanism against infectious diseases in bivalves (Allam and Raftos 2015; Hasanuzzaman et al. 2017; Xue et al., 2017), and a polymorphism in a serine protease inhibitor gene has been associated to disease resistance in *C. virginica* (Yu et al. 2011).

Genes related to histones are known to participate in mollusk immune response being up-regulated in different oyster species in response to *Perkinsus* spp. (Nikapitiya et al. 2013; Poirier et al. 2014). These genes were broadly down-regulated in NS as compared to AS oysters (Table S2 and S3). Histones H2A, H2B, H3 and H4 were among those showing the highest differences between stocks (Figure 3) and were clustered in the SOTA group GS2 (Table S7), where significantly enriched GO terms related to these nuclear proteins were found (Table S9, Figure 5).

Conversely, cluster GS4 contained genes mostly up-regulated in the NS stock at 1 dpc and 90 dpc, but down-regulated in the AS stock (Figure S2, Table S7), and included six ubiquitin- and ten proteasome-annotated genes. Accordingly, several enriched related GO terms were related to the ubiquitin-proteasome pathway (Figure 5, Table S9). This pathway is a critical regulator of innate immunity; nonetheless, intracellular pathogens are capable of manipulating the ubiquitin-proteasome system of the host to their benefit

for growth, replication or immune evasion (Olivier et al. 2005; Laliberte and Carruthers 2008; Collins and Brown 2010). Some indications of proteasome activation in wild flat oysters infected by *B. ostreae* were previously reported (Martín-Gómez et al. 2012), and, interestingly, transcripts related to this pathway were found up-regulated in Atlantic salmon susceptible to infectious pancreatic necrosis virus (IPNV) in a study comparing susceptible and resistant specimens (Robledo et al. 2016).

B. ostreae was shown to interfere with essential processes in the defense response of *O. edulis* haemocytes, such as phagocytosis and lysosomal destruction, and reactive oxygen species (ROS) production (Morga et al. 2009, 2012). Nevertheless, NADPH oxidase (NOX5) and cytochrome b-245 light chain, both involved in ROS production (Segal et al. 1992; Bedard and Krause 2007), were found among RGs in AT1 (Table S2). Furthermore, two genes related to antioxidant defense by avoiding the collateral effects of ROS, such as catalase and glutathione S-transferase (Hermes-Lima, 2005), showed a significant activation in the AS stock as compared to NS (cluster GS3, Table S7), which suggests that resistant oysters might be able to circumvent parasite interference showing more active ROS production. In the same way, several cytoskeleton-related RGs were found in AS and among DEGs, mostly up-regulated in AT1. Cytoskeleton plays a key role in cell proliferation, migration and phagocytosis, but also during cell death by apoptosis, and the interaction with host cytoskeleton is essential for a successful invasion by intracellular pathogens (Epting et al. 2010; Desouza et al. 2012; Freeman and Grinstein 2014). Among those genes, we found filamin, previously detected in oyster haemocytes infected with *B. ostreae*, and hypothesized to be involved in parasite internalization (Morga et al. 2011a). Also, several genes related to the actin cytoskeleton structure, such as alpha-actinin and vinculin, and cytoskeleton dynamics, including three members of the Rho family of

GTPase, known to be involved in phagocytosis, cell spreading and motility (Freeman and Grinstein 2014), were identified. Conversely, genes encoding tubulin alpha and beta, the major proteins of microtubules, and kinesin, a motor protein participating in microtubules dynamics, were down-regulated in AT1 (Table S2). The dynamic rearrangement of cytoskeletal components is key for apoptosis, and microtubules appear to have a main role in Fas-mediated apoptosis (Doma et al. 2010). This mechanism was previously hypothesized as important for defense in *Bonamia*-resistant oysters, where a Fas-ligand was up-regulated in haemocytes (Morga et al. 2012).

As highlighted in a recent work (Gervais et al. 2016), the activation of the apoptotic machinery appears as a main defence response of *O. edulis* against *B. ostreae* infection; the controlled death of infected haemocytes would avoid the intracellular development and multiplication of the parasite decreasing the number of parasitic cells. Several genes involved in apoptosis were detected among RGs in AS oysters, such as the proapoptotic genes tumor protein 53-induced nuclear protein 1, caspase 7, caspase 10, growth arrest and DNA-damage-inducible protein alpha, endonuclease G and salvador-like protein 1, as well as three genes related to the Inhibitor of Apoptosis (IAP) family (Table S2).

A refined interpretation of the processes underlying the modulation of the cytoskeleton- and apoptosis-related genes is beyond the scope of this study, where the expression profiles evaluated might include heterogeneous populations of haemocytes, including different cell types. However, our results show consistently that the haemocytes of AS oysters present a wide modulation of these sets of genes at 1 dpc, reflecting cytoskeletal dynamics and remodeling, as well as activation of the apoptosis machinery in response to the parasite challenge, which were unnoticed in NS.

In general, the expression profile of the NS haemocytes at 1 dpc reflected a limited defense response, but an increased activation, reflected in the number of RGs, was

found at 90 dpc. Among these RGs, we detected several immune-related genes, such as universal stress protein (USP), macrophage migration inhibitory factor and interferon-induced protein 44, or galectin 1, shown to be involved in *P. marinus* recognition in *Crassostrea virginica* (Feng et al. 2013). Also some genes activated in AT1, such as beta catenin and kinesin, involved in cytoskeletal organizations, and a caspase family member (caspase 2) acting in the execution-phase of apoptosis (Table S2), were detected in NT3. This might be indicative of a delayed, and probably less effective, response by NS oysters, given the lack of previous contact with the parasite.

Despite mechanisms such as apoptosis of infected haemocytes appears to be a strategy to avoid the spreading of the infection, consistent with the lower infection intensity showed by AS oysters (da Silva et al. 2005), the results of our work suggest that the resistance of AS has a multifactorial nature, depending also on a more efficient defense response by preventing the haemocytes invasion by *B. ostreae*.

A list of 24 candidate genes for resistance/susceptibility of *O. edulis* to *B. ostrae* is presented in Table 2. These genes were chosen among those in common between the lists of DEGs and RGs at the most significant time point for AS response (1 dpc), but also considering their involvement in the functions here hypothesized as relevant after data analysis and interpretation. G-protein, integrin-mediated and ubiquitin signaling pathways are represented, as well as cell-matrix interactions, apoptosis and immune-related genes, such as histones, protease inhibitors and complement. This set of genes represents one of the main outcomes of this work and the basis for further investigation and validation of potential markers for resistance to bonamiosis. This could be tackled either by testing SNP associated allelic variants in resistant/susceptible individuals or by testing the use of target genes (mRNA, proteins) on hemolymph samples from infected oysters as intermediate phenotypes.

Conclusions

The application of a validated haemocyte flat oyster oligo-microarray has contributed to a better understanding of the response against the threatening parasite *B. ostreae* and to the identification of candidate genes related to resistance/susceptibility. The expression profiles observed in the present study revealed that the haemocytes of AS presents a more intense and quicker response as compared to the NS, with activation of several signal transduction pathways, early after challenge with *B. ostrae*. This aspect might represent a key factor for the resistance characteristics observed for this stock. Also, a main role was found for cell-ECM interactions and proteases inhibitors in the defense response of AS, while the broad down-regulation of histones-related genes in challenged haemocytes from NS suggests that these proteins might have an important part during the infection process. Further work is needed to deepen into these aspects and to investigate the validity and potential of the candidate genes for genetic selection.

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Legends to Figures

Figure 1.- Hierarchical clustering of samples from two flat oyster (*O. edulis*) stocks (N: naïve, A: long-term affected) challenged with *B. ostreae* along a time series (T1: 1 day post-challenge (dpc), T2: 30 dpc, T3: 90 dpc) based on regulated genes (fold change $\neq$ 0).

Figure 2.- Venn diagram showing the number and percentage of regulated genes shared between flat oyster (*O. edulis*) stocks (N: naïve, A: long-term affected) challenged with *B. ostreae* along a time series (T1: 1 day post-challenge (dpc), T3: 90 dpc).

Figure 3.- Hierarchical clustering of the differentially expressed genes (ANOVA, P < 0.001) showing the highest difference (standard deviation > 0.8) between the naïve (N) and long-term affected (A) flat oyster *(O. edulis)* stocks after challenge with *B. ostreae* along a time series (T1: 1 day post-challenge (dpc), T2: 30 dpc, T3: 90 dpc).

Figure 4.- Relevant enriched GO terms of regulated genes groups showing correlated expression profiles (SOTA groups) along the time course in the long-term affected (AS) flat oyster (*O. edulis*) stock after challenge with *B. ostreae* along a time series (T1: 1 day post-challenge (dpc), T2: 30 dpc, T3: 90 dpc); A: AS.G1; B: AS.G2. In the upper right the profile pattern of the group including T1, T2 and T3 and the five replicates per time (see Figure S1).

Figure 5.- Relevant enriched GO terms of differentially expressed genes between naïve (NS) and long-term affected (AS) flat oyster (*O. edulis*) stocks showing correlated expression profiles (SOTA groups) after challenge with *B. ostreae* along a time series (T1: 1 day post-challenge (dpc), T2: 30 dpc, T3: 90 dpc). A: GS2; B: GS3; C: GS4.

Supplementary Figure 1:SOTA groups of regulated genes (RG) showing significant correlation patterns within stock (NS: naïve; AS: long-term affected) after challenging flat oyster (*O. edulis*) with *B. ostreae* along a time series (NT1, NT2, NT3: naïve stock at 1, 30 and 90 days post-challenge (dpc), respectively; AT1, AT2, AT3: long-term affected stock at 1, 30 and 90 dpc, respectively).

Supplementary Figure 2: SOTA groups of differentially expressed genes (DEG) between stocks showing significant correlation patterns after challenging flat oyster (*O.*

edulis) with *B. ostreae* along a time series (NT1, NT2, NT3: naïve stock at 1, 30 and 90 days post-challenge (dpc), respectively; AT1, AT2, AT3: long-term affected stock at 1, 30 and 90 dpc, respectively).

Legends to Tables

Table 1: Number of regulated genes (RG) in the naïve (NS) and long-term affected (AS) stocks of flat oyster (*O. edulis*) in response to *B. ostreae* along the time series (T1: 1dpc; T2: 30dpc; T3: 90 dpc).

Table 2. List of relevant immune-genes regulated (RG) at the first time post challenge (1 dpc; SAM test, FDR < 0.05) and differentially expressed (DEG) between long-term affected (AS) or naïve (NS) flat oyster (*O. edulis*) (ANOVA; P < 0.001). In parentheses the number of different genes with the same annotation detected either among RG or DEG. Asterisks indicate genes with the same annotation but showing a opposite expression pattern.

Table S1: Primers and qPCR conditions used to validate the oligo-microarray data of flat oyster (*O. edulis*) challenged with *B. ostreae* following the procedure of Miron et al. (2006).

Table S2: Regulated genes (FC: fold change ≠ 0) of the naïve (NS) and long-term affected affected oysters (*O. edulis*) challenged with *B. ostreae* along a temporal series.

Table S3: Fold change (FC) of differentially expressed genes (DEG) between naïve (NS) and long-term affected (AS) flat oyster (*O. edulis*) stocks after challenging with *B. ostreae* along a time series (T1: 1dpc; T2: 30dpc; T3: 90 dpc).

Table S4: Fold change (FC) of duplicated regulated genes (RG) across time and stock or differentially expressed (DEG) between naïve (NS) and long-term affected (AS) flat oyster (*O. edulis*) stocks after challenging with *B. ostreae* along a time series (T1: 1dpc; T2: 30dpc; T3: 90 dpc).

Table S5: Regulated genes (RG) of flat oyster (*O. edulis*) showing the highest fold change (FC > 2 or < -2) after challenging naïve (NS) and long-term affected (AS) individuals with *B. ostreae* along a time series (T1: 1dpc; T2: 30dpc; T3: 90 dpc).

Table S6: Fold change (FC) of regulated genes (RG) showing a significant correlation patterns (SOTA groups) across the time course within stock (NS: naïve, AS: long-term affected) after challenging flat oyster (*O. edulis*) with *B. ostreae* along a time series (T1: 1dpc; T2: 30dpc; T3: 90 dpc).

Table S7: Fold change (FC) of differentially expressed genes (DEG) between stocks showing a significant correlation pattern (SOTA groups) after challenging naïve (NS) and long-term affected (AS) flat oyster (*O. edulis*) stocks with *B. ostreae* along the time series (T1: 1dpc; T2: 30dpc; T3: 90 dpc).

Table S8: Enriched GO terms in SOTA groups of regulated genes (RG) showing a significant correlation patterns (SOTA groups) across the time course within stock (NS: naïve, AS: long-term affected) after challenging flat oyster (*O. edulis*) with *B. ostreae* along a time series (T1: 1dpc; T2: 30dpc; T3: 90 dpc).

Table S9: Enriched GO terms in SOTA groups of differentially expressed genes (DEG) between stocks after challenging naïve (NS) and long-term affected (AS) flat oyster (*O. edulis*) stocks with *B. ostreae* along the time series (T1: 1dpc; T2: 30dpc; T3: 90 dpc).