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Proteomic profile of *Ostrea edulis* haemolymph in response to bonamiosis and identification of candidate proteins as resistance markers

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ABSTRACT: European flat oyster *Ostrea edulis* populations have suffered extensive mortalities caused by bonamiosis. The protozoan parasite *Bonamia ostreae* is largely responsible for this disease in Europe, while its congener *B. exitiosa* has been detected more recently in various European countries. Both of these intracellular parasites are able to survive and proliferate within haemocytes, the main cellular effectors of the immune system in molluscs. Two-dimensional electrophoresis was used to compare the haemolymph protein profile between *Bonamia* spp.-infected and non-infected oysters within 3 different stocks, a Galician stock of oysters selected for resistance against bonamiosis, a non-selected Galician stock and a selected Irish stock. Thirty-four proteins with a presumably relevant role in the oyster–*Bonamia* spp. interaction were identified; they were involved in major metabolic pathways, such as energy production, respiratory chain, oxidative stress, signal transduction, transcription, translation, protein degradation and cell defence. Furthermore, the haemolymph proteomic profiles of the non-infected oysters of the 2 Galician stocks were compared. As a result, 7 proteins representative of the non-infected Galician oysters selected for resistance against bonamiosis were identified; these 7 proteins could be considered as candidate markers of resistance to bonamiosis, which should be further assessed.

KEY WORDS: *Bonamia ostreae* · *Bonamia exitiosa* · Two-dimensional electrophoresis · Proteomics · Immune response · Selective breeding

INTRODUCTION

Oysters are ecosystem engineers, producing reef habitats for entire ecosystems (Jones et al. 1994). Oyster reefs provide many ecosystem services such as water filtration, food and habitat for other animals, shoreline stabilisation, carbon burial, oyster production, nutrient regeneration and coastal fisheries (Grabowski & Peterson 2007); they should therefore be recognised as a priority for habitat management, conservation and restoration (Beck et al. 2011). Since the late 19th century, overfishing combined with disease outbreaks, habitat transformation and the introduction of non-native competitors has wiped out wild European flat oyster *Ostrea edulis* reefs around much of the European coastline (Lotze et al. 2006, Airoldi & Beck 2007, Beck et al. 2011). Aquaculture contributed to maintain European flat oyster production to some extent in various countries (Korringa 1976), but successive epidemic outbreaks, especially that of bonamiosis starting in the late 1970s (Pichot et al. 1980), which is now endemic in many countries (Engelsma et al. 2014), led to a dramatic reduction of flat oyster landings in Europe, from a peak of 32 995 t in 1961 to 3915 t in 2015 (www.fao.org/fishery/statistics/global-production/es).

Any European flat oyster restoration programme aiming to recover oyster ecosystem services and/or to exploit oysters for human nutrition must involve measures to fight bonamiosis, at least in the areas

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affected by this disease. Using therapeutic products to fight oyster bonamiosis in the open sea is not practical, standard vaccination is not an option for molluscs because it does not induce production of antibodies or other molecules conferring long-term protection to previously susceptible individuals, and eradication of bonamiosis from endemic areas does not seem feasible (Grizel et al. 1987, van Banning 1991, Lynch et al. 2007); therefore, using bonamiosis-resistant flat oyster strains appears to be the most promising approach to overcome this disease in endemic areas (Elston et al. 1987, Naciri-Graven et al. 1999, da Silva et al. 2005, Lynch et al. 2014). Selective breeding programmes have been successful at increasing oyster resistance against various diseases (Beattie et al. 1988, Dove et al. 2013, Frank-Lawale et al. 2014, Lynch et al. 2014).

O. edulis is highly susceptible to infection with the protozoan parasites *Bonamia ostreae* (Pichot et al. 1980) and *B. exitiosa* (Abollo et al. 2008), which are included in the list of notifiable diseases of the World Organisation for Animal Health (www.oie.int/en/animal-health-in-the-world/oie-listed-diseases-2017/) as being responsible for extensive oyster mortality. Both parasites are mainly intracellular, infecting host haemocytes (Comps et al. 1980, Hine 1991), which are the main cell effectors of the mollusc immune system (Cheng 1981, Fisher 1986). *Bonamia* spp. appear to have some counter mechanism that turns haemocyte metabolic destructive capacity off, so that the parasite survives and proliferates within haemocytes without being degraded, causing haemocyte disruption and inducing huge haemocyte infiltration in the digestive gland, gonad, mantle and gills (Balouet et al. 1983, Hine 1991, Cochennec-Laureau et al. 2003).

A better understanding of interactions between the oyster immune system and the pathogen could help to control bonamiosis. Different susceptibility to bonamiosis among *O. edulis* strains could be related to variations in their defence systems (Cochennec et al. 1995, Naciri-Graven et al. 1998, Cochennec-Laureau et al. 2003). Whereas several key genes for the immune response against the parasite have been identified through genomic approaches (Morga et al. 2011a,b, 2012, Martín-Gómez et al. 2012, Pardo et al. 2016), little is known about the proteins involved in such processes. Proteomics is recognised as one of the most powerful tools in the study of biological systems, being especially useful for identifying modified protein expression patterns under altered conditions. Thus, proteomic analyses are needed to completely understand mechanisms underlying biological events (Kaji et al. 2000). The proteomic approach has been applied to study the immune response of bivalve molluscs against pathogens, such as antiviral responses of *Crassostrea gigas* (Corporeau et al. 2014, Green et al. 2016, Masood et al. 2016), *Saccostrea glomerata* (Masood et al. 2016) and *Chlamys farreri* (Chen et al. 2011), and the antibacterial responses of *C. farreri* (Huan et al. 2011, Sun et al. 2014) and *Mytilus galloprovincialis* (Ji et al. 2013, Wu et al. 2013). Furthermore, proteomics has been used to identify molecular markers of disease resistance in *S. glomerata* (Simonian et al. 2009a,b, Vaibhav et al. 2016). If molecular markers of resistance against bonamiosis were identified, their use could shorten and increase the efficiency of and thus shorten selective breeding programmes to produce resistant oyster strains.

Here we describe a proteomic approach envisaged to investigate differences in haemolymph protein expression between oysters infected with either *B. ostreae* or *B. exitiosa* and non-infected oysters, involving 3 oyster stocks with different susceptibility to bonamiosis: Irish and Galician oyster stocks both selected for bonamiosis resistance, and a non-selected Galician stock. The oysters used in the study had been grown in areas affected by bonamiosis in Galicia and Ireland; the oysters of the 2 Galician stocks shared the same environmental conditions throughout their whole life. Furthermore, the haemolymph proteomic profile was compared between non-infected oysters of the 2 Galician stocks, trying to identify proteins associated with resistance to bonamiosis. Haemolymph proteins were separated by 2-dimensional electrophoresis (2-DE); mass spectrometry (MS) was used to identify proteins with differential expression. Thirty-four proteins involved in the host–parasite interaction were identified, from which 7 could be considered as resistance marker candidates, which need further study for confirmation.

MATERIALS AND METHODS

Oyster stocks

Three flat oyster stocks with different susceptibility to bonamiosis were used in this study. Two consisted of hatchery-produced lines derived from 2 different Galician brood-stocks: (1) a Galician selected stock (GSS), including oysters of the third generation of a selective breeding programme for bonamiosis resistance (da Silva et al. 2005, Villalba et al. 2007) that used oysters derived from a natural bed in Coroso

(Ría de Arousa, NW Spain; Fig. 1), and (2) a Galician non-selected stock (GNSS), including oysters that were collected from a natural oyster bed in the Ría de Pontevedra (Fig. 1), which has been affected by bonamiosis since the late 1990s. The spat of both stocks was produced simultaneously in the hatchery facilities of the Centro de Investigacións Mariñas in the summer of 2009 and transferred for growing-out onto a raft located in the raft area named Cambados D (Ría de Arousa; Fig. 1), which has been affected by *B. ostreae* since the early 1980s and where the occurrence of *B. exitiosa* has been documented since 2006 (Abollo et al. 2008, Ramilo et al. 2014); thus, the oysters used in this study from both Galician stocks shared the same environmental conditions throughout their whole life, in hatchery facilities and when grown-out from the raft. Unfortunately, both oyster stocks suffered mass mortality due to infection with a herpes-like virus immediately after transferring them from the hatchery to the raft (Villalba et al. 2010), which interfered with the planned comparison of performance through growing out between oyster stocks and prevented us from drawing conclusions on differential susceptibility to bonamiosis. (3) The third stock consisted of 'Rossmore' (RS) oysters that had been produced within an Irish selective breeding programme for bonamiosis resistance. In this programme, performed by Atlantic Shellfish Ltd. since 1988, bonamiosis-surviving oysters and oysters from a nearby bed in the North Channel at Rossmore, Cork Harbour, Ireland (Fig. 1) were set to collect oyster spat, which was grown-out (Culloty et al. 2001, 2004). Both the Galician and the Irish selective breeding programmes used bonamiosis-surviving oysters as brood-stock for the next generation, which was exposed to bonamiosis in the field; this process was repeated through successive generations by mass selection.

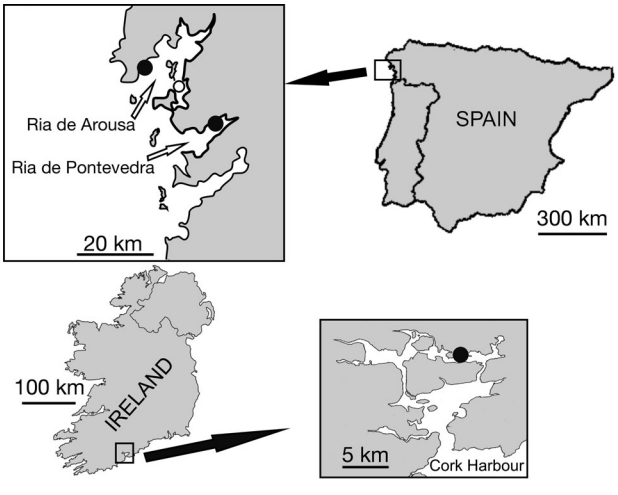

Fig. 1. Locations of the oyster culture raft (in the Ria of Arousa, open circle) and the oyster beds (solid circles) from which the 3 oyster stocks used in the study were derived: the Galician selected stock (GSS) from a bed close to Coroso (Ría de Arousa; Spain), the Galician non-selected stock (GNSS) from a bed close to Illa de Tambo (Ría de Pontevedra, Spain) and the Rossmore stock (RS) from a bed in the North Channel of Cork Harbour (Ireland)

Haemolymph collection

In May and June 2011, 89 GSS and 91 GNSS oysters (2 yr old) were taken from the raft in the Ría de Arousa, and 100 RS oysters (4 yr old) were removed from the Rossmore bed and transported to the laboratory, where they were kept in running seawater tanks and analysed after 1 wk of acclimation. The reason for the age difference between Galician and Irish oysters used in the study is that flat oysters grow faster in Galicia, and high mortality due to bonamiosis occurs usually at younger age than in Ireland (Culloty & Mulcahy 1996, da Silva et al. 2005). As much haemolymph as possible was withdrawn from the adductor muscle of each oyster using a 21 or 23 gauge needle attached to a 1 ml syringe. Right after extraction, haemolymph samples were kept in ice-cold vials to avoid haemocyte aggregation and degradation. A drop from each haemolymph sample was observed under a light microscope to assess quality and to estimate haemocyte viability by using the Trypan blue test. Haemolymph samples contaminated with debris, bacteria, gametes or other tissues were discarded. Clean haemolymph was frozen, lyophilised and stored at −80°C. Furthermore, small pieces of gills and gonad from each oyster were preserved in 96% ethanol to diagnose *Bonamia* spp. infections by PCR.

DNA extraction and diagnosis of *Bonamia* spp. by PCR

DNA extractions from both gills and gonad fragments together (25–50 mg) were performed employing the commercial Wizard Genomic DNA Purification Kit (Promega), according to the manufacturer's protocol. DNA quality and quantity were checked in a Nanodrop® ND-1000 spectrophotometer (Nanodrop Technologies). DNA obtained was analysed by specific conventional PCR assays for *B. ostreae* and for *B. exitiosa*, using the pair of primers BOSTRE-F/

BOSTRE-R and BEXIT-F/BEXIT-R, respectively (Ramilo et al. 2013). PCR assays were performed in a total volume of 25 µl containing 1 µl of genomic DNA (200 ng), PCR buffer at 1× concentration, 1.5 mM $MgCl_2$, 0.2 mM nucleotides (Roche Applied Science), 0.3 µM of each specific primer for either *B. ostreae* (BOSTRE-F/R) or *B. exitiosa* (BEXIT-F/R) and 0.025 U $µl^{-1}$ *Taq* DNA polymerase (Roche Applied Science). A positive control for *B. ostreae* or *B. exitiosa* (DNA from an oyster infected with either *B. ostreae* or *B. exitiosa*) and a negative control (no DNA) were used in every PCR assay. The PCR assays were carried out in a T gradient thermocycler (Biometra), under the following reaction parameters: 94°C for 2 min, 35 cycles at a melting temperature of 94°C for 30 s, an annealing temperature of 55°C for *B. ostreae* and 58°C for *B. exitiosa* for 45 s, and an extension temperature of 72°C for 1 min; followed by a final extension period of 72°C for 1 min. After PCR, 10 µl aliquots of amplified DNA were analysed by electrophoresis on 2% agarose gels, in 1× Tris acetate EDTA buffer, stained with ethidium bromide and scanned in a GelDoc XR documentation system (BioRad).

Haemolymph sample pooling and protein extraction

Individual analysis of oyster haemolymph proteome was not pursued because the protein quantity in some of the individual samples would be below the minimum required for a correct protein separation by 2-dimensional electrophoresis (2-DE); to overcome this problem, samples were pooled. Pooling of samples obscures individual variation, although it is a common approach when performing 2-DE analysis (Monsinjon & Knigge 2007). Accordingly, after bonamiosis diagnosis by PCR, haemolymph samples were grouped into negative and positive pools, within each oyster stock, as shown in Table 1. Individual haemolymph samples with high volume and haemocyte density were selected to make up the pools. In the GSS oysters, the number of cases positive for *B. ostreae* (3 oysters) and *B. exitiosa* (2 oysters) was much lower than in the GNSS (8 oysters positive for *B. ostreae*, 17 for *B. exitiosa* and 3 for both parasite species); for this reason, in the case of GNSS, separate pools were prepared with haemolymph from oysters positive for each *Bonamia* species (the 3 oysters positive for both parasites were not used), whereas a single pool with haemolymph from GSS oysters positive for either *Bonamia* species was produced. In the RS oysters, no positive case for *B. exitiosa* was detected, and 3 different haemolymph pools were made: oysters negative for *Bonamia* spp., oysters with a weak positive signal (a tenuous band in the electrophoresis with the PCR amplified products) for *B. ostreae*, and oysters with a strong positive signal (an intense band) for *B. ostreae* (Table 1). Lyophilised samples were solubilised in lysis buffer (7.6 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulphonate [CHAPS], bromophenol blue traces, 0.2% ampholytes, 100 mM dithiothreitol [DTT] and phenylmethanesulphonyl fluoride [PMSF], a protease inhibitor, pH 3–10) with shaking for 2 h 30 min at 4°C. The homogenate was centrifuged at 16 000 × *g* (30 min at 4°C) to remove debris. Remaining supernatants were collected, and protein content was determined using the commercial kit RC DC Protein Assay (BioRad), based on the Lowry assay (Lowry et al. 1951). For removing salts and albumin, samples were purified with a ReadyPrep™2-DCleanup kit (BioRad). Each sample, containing 500 µg of protein, was diluted in 400 µl of rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, bromophenol blue traces, 0.3% DTT and 0.5% immobilised linear pH gradient [IPG] buffer). Aliquots of protein samples were kept at –80°C until use.

2-DE

2-DE was performed to separate the proteins occurring in the haemolymph pools. Most proteins in *Ostrea edulis* haemolymph have an isoelectric point (pI) between 5.0 and 8.0 (Cao et al. 2009), thus narrow pH strips of 5–8 and 12.5% SDS-PAGE gels were employed for a better separation of haemolymph proteins. Each aliquot was loaded onto 17 cm IPG strips, pH 5–8 (BioRad). After 6 h of passive and 6 h active (50 V) rehydration, iso-

Table 1. Summary of the different haemolymph pools analysed within each oyster stock and number of oysters (n) from which each haemolymph pool was obtained

Stock	Haemolymph pool	n
Galician selected stock (GSS)	Negative for *Bonamia* spp.	7
	Positive for *Bonamia* spp.	5
Galician non-selected stock (GNSS)	Negative for *Bonamia* spp.	7
	Positive for *B. ostreae*	7
	Positive for *B. exitiosa*	7
Rossmore stock (RS)	Negative for *Bonamia* spp.	7
	Weak signal for *B. ostreae*	5
	Strong signal for *B. ostreae*	7

electric focussing was carried out in a horizontal apparatus (Protean®IEF System from BioRad) at 20°C using a 5-step program: 500 V for 1 h and 30 min, followed by 1000 V, 2000 V and 4000 V, each step during 1 h and 30 min; finally 8000 V to reach a total of 52 000 V h^{-1}. Current did not exceed 50 µA per strip. After the first dimension, strips were incubated for 15 min in equilibration buffer (6 M urea, 50 mM Tris, 2% sodium dodecylsulphate [SDS], 30% glycerol) containing 1% DTT and then for 15 min in the same buffer supplemented with 2.5% iodoacetamide. The equilibrated IPG strips were loaded on the top of 12.5% SDS polyacrylamide gels, and the second dimension was performed at 15°C in a vertical apparatus (Protean®Plus Dodeca Cell, BioRad), with capacity to carry out SDS-PAGE in up to 12 gels simultaneously keeping the same conditions: 2.5 W per gel for 30 min followed by 12.5 W until the running front reached the bottom of the gel. To assess the reproducibility of the gels, 6 replicate gels were produced from each health condition (non-infected and infected) within each stock; all replicates were performed in the same conditions. To determine the coordinates of experimental molecular mass (M_r) and pI for each single spot, 2-D gels were calibrated using 2-D SDS-PAGE standards (BioRad), a selected set of reliable identification landmarks distributed throughout the entire gel.

Protein visualisation and image analysis

After 2-DE, protein spots were visualised by silver staining, using a protocol compatible with MS (50% [v/v] ethanol, 1.2% [v/v] acetic acid and 0.05% [v/v] formaldehyde for 1 h or overnight; 50% [v/v] ethanol for 20 min [×3]; 0.02% [w/v] sodium thiosulfate for 1 min; wash step with H_2O milli-Q for 20 s [×3]; 0.01% [w/v] silver nitrate for 30 min; wash with H_2O milli-Q for 1 min [×3]; 0.05% [v/v] formaldehyde, 3% [w/v] sodium carbonate, 0.02% [w/v] sodium thiosulfate until complete appearance of the spots and finally 1.5% [w/v] EDTA for 10 min to stop the staining). For image analysis, silver-stained gels were digitised at 63.5 × 63.5 µm resolution using a Bio-Rad GS-800 calibrated densitometer, and proteomic analyses were performed with PD Quest 8.0.1 software (BioRad). In order to obtain image patterns as similar to the original gels as possible, visual corroboration and manual edition of digitised gels were performed, and streaks, speckles and artefacts were removed. Fig. 2 shows how the comparison between haemolymph pools was carried out within stocks. A synthetic master gel was built by combining the best 4 gels obtained from each pool including only the spots present in all gels of that pool. Then, the creation of a final synthetic gel for each stock, which compared master gels of each pool of that stock, allowed the discrimination of spots unique to each

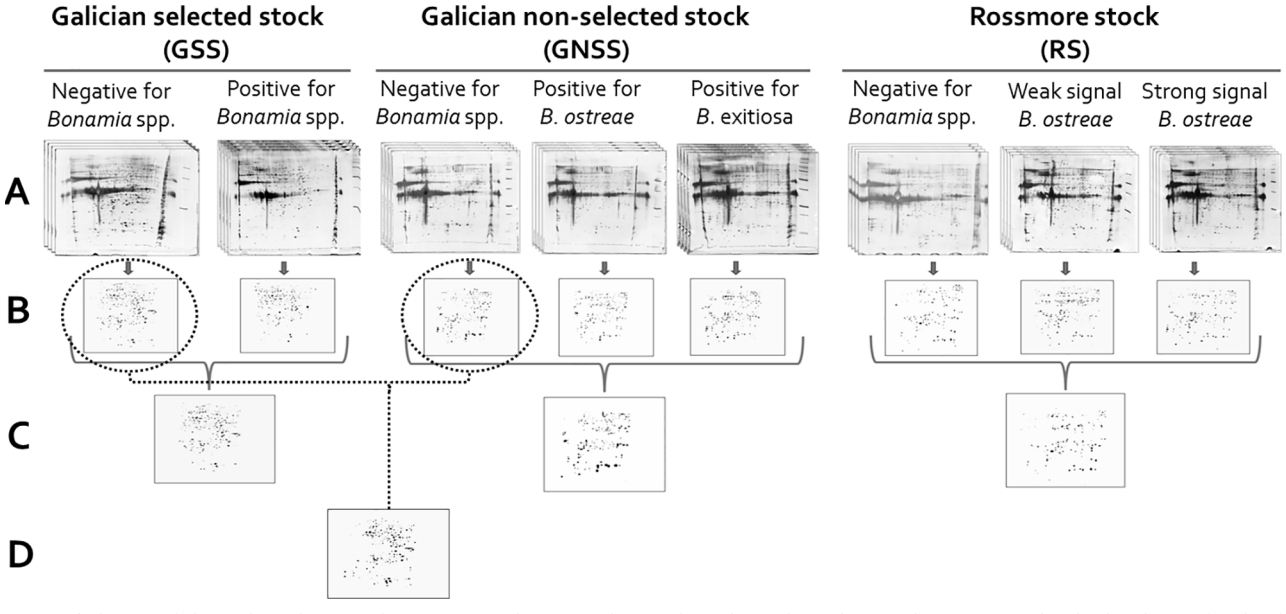

Fig. 2. Scheme of the gel analysis and comparison between haemolymph pools within each oyster stock. (A) Analysis of gels of each haemolymph pool to produce a master gel including only the spots shared by all 4 replicate gels. (B) Comparison between master gels of the haemolymph pools within each stock. (C) Final master gel of each stock discriminating the spots shared by all the haemolymph pools and the spots unique to each of them. (D) Final master gel resulting from the additional comparison between the master gels from oysters negative for *Bonamia* spp. in the 2 Galician stocks

pool from spots shared by all pools within each stock. Those unique spots were considered 'representative' of the haemolymph of that particular pool because they appeared in every gel from that pool but they did not appear in every gel of the other pool/s within the same stock (they might or might not appear in some gels of the other pool/s). A conservative strategy was followed, discarding all results that were ambiguous, badly defined, showed overlapping spots or spots detected at the boundaries of gels that did not resolve properly. Highly reproducible replications were observed within each haemolymph pool. The spots considered representative of each haemolymph pool were excised and processed for protein identification as explained below.

Additionally, particular attention was paid to oysters of the 2 Galician stocks in which *Bonamia* spp. were not detected despite the occurrence of *B. ostreae* and *B. exitiosa* in the farming area. Thus, a comparison between master gels from haemolymph pools of oysters negative for *Bonamia* spp. of GSS and GNSS was performed (samples from oysters negative for *Bonamia* spp. of the RS were not included in the comparison, as their environmental background was different). With this comparison, characterization of spots (1) shared by and (2) unique to or representative of each Galician stock was possible. Unfortunately, such spots could not be further processed for sequencing due to funding limitations. No comparison of the Galician stocks with the Irish stock was made because the environmental conditions of their respective locations was different, and thus discriminating the influence of stock and infection from that of environment in the protein profiles would not have been possible.

Differences in the number of spots occurring in the master gels between stocks were analysed through stock paired comparisons using 2 × 2 contingency tables and Fisher's tests with Bonferroni corrections, in which the PCR diagnosis (positive or negative) were organised into rows and the stocks into columns. IBM SPSS 20 software was used in the statistical analysis.

MS and database search

Selected protein spots were manually excised and sent to the Proteomic Service of the Ramón Dominguez Foundation (Complejo Hospitalario Universitario de Santiago de Compostela, Spain) for sequencing. Spectrometry approaches such as matrix-assisted laser-desorption/ionisation time-of-flight (MALDI-TOF) MS and MALDI-TOF/TOF tandem MS (Gogichaeva et al. 2007) were used in protein identification of trypsin-digested spots (Shevchenko et al. 1996). MS data were obtained in an automated analysis loop using a 4800 MALDI-TOF/TOF analyser (Applied Biosystems). Peptide mass fingerprinting and peptide fragmentation spectra data of each sample were combined through the GPS Explorer Software v3.6 (Applied Biosystems) using Mascot software v2.1. (Matrix Science) to search against the non-redundant protein databases of Mollusca, Alveolata and Rhizaria taxa included in the National Center for Biotechnology Information (NCBI). Protein databases of Alveolata and Rhizaria (protozoan infra-kingdoms, the latter including the genus *Bonamia*) were included in the search to increase the probability of matching in case some excised spots corresponded to *Bonamia*. Due to the poor protein and DNA sequence database coverage for *Ostrea edulis,* most proteins were identified by de novo sequencing and BLAST similarity searching following a procedure outlined by Liska & Shevchenko (2003). All MS/MS spectra for a sample were sequenced de novo using Pro BLAST 1.4 software (Applied Biosystems) (Altschul et al. 1997), and the top 6 candidate sequences for each MS/MS were combined into a single text-format search string, which was then submitted to MS BLAST for sequence similarity searching using a server version of the Paracel BLAST software. Protein identification significance was judged using the MS BLAST scoring algorithm; identifications were considered correct with a score ≥50, although sequences with a lower score but with a percentage of coverage of 100% were also accepted as correct. Additionally, fragmentation patterns calculated from the assigned peptide sequences were matched against raw MS/MS data using links provided by the Pro BLAST software. Only proteins matching with a minimum of 3 peptide sequences with at least 20 identical amino acid residues were included in the result list, providing more stringent criteria for the identification according to the procedure of Jorge et al. (2005).

RESULTS

Comparison of protein patterns between haemolymph pools within each stock

Table 2 summarises the analysis of the gels produced in this study. In the GSS oysters, 2 pools were made: one corresponding to oysters negative for *Bonamia* spp., with an average of 804 spots in the replicate gels, of which 495 were shared by the 4 replicates; and another corresponding to oysters pos-

Table 2. (A) Results of the 2D-gel analysis, showing the average number of spots in the replicate gels from each haemolymph pool, the number of spots in the master gels of each haemolymph pool and the numbers of spots shared by all pools within each stock and spots representative of (unique to) each pool when comparing the pools within each stock (GSS: Galician selected stock; GNSS: Galician non-selected stock; RS: Rossmore stock). The final column shows the number (and percentage) of the representative spots that were further identified. (B) Results corresponding to the comparison between oysters negative for *Bonamia* spp. of the 2 Galician stocks

Stock	Haemolymph pool	No. of spots (4 replicates)	No. of spots (master gel)	Comparison between haemolymph pools within each stock		
				Shared spots	Representative spots	Identified representative spots (%)
(A)						
GSS	Negative for *Bonamia* spp.	804	495	302	43	20 (47)
	Positive for *Bonamia* spp.	707	381		8	3 (38)
GNSS	Negative for *Bonamia* spp.	434	270	143	10	4 (40)
	Positive for *B. ostreae*	505	258		22	2 (9)
	Positive for *B. exitiosa*	533	297		13	0 (0)
RS	Negative for *Bonamia* spp.	321	150	99	2	1 (50)
	Weak signal for *B. ostreae*	622	365		16	4 (25)
	Strong signal for *B. ostreae*	526	203		4	0 (0)
(B)						
GSS	Negative for *Bonamia* spp.	804	495	158	109	–
GNSS	Negative for *Bonamia* spp.	434	270		25	–

itive for *Bonamia* spp., with an average of 707 spots in the replicate gels, of which 381 were shared by the replicates. A total of 302 spots were shared by the 8 gels used for the analysis of this stock. Comparison between haemolymph pools within GSS showed that 43 spots were representative of (unique to) the haemolymph pool from oysters negative for *Bonamia* spp. and 8 spots were representative of the pool from oysters positive for *Bonamia* spp. (Figs. 3A & 4).

In the case of GNSS, 3 haemolymph pools were produced, one from oysters negative for *Bonamia* spp., with an average of 434 spots in the gel replicates, of which 270 spots were shared by the 4 replicates and 10 were representative spots. The second pool comprised oysters positive for *B. ostreae*, with an average of 505 spots in the replicate gels; 258 spots were shared by the 4 replicates and 22 were representative spots. The third pool was from oysters positive for *B. exitiosa*, with an average of 533 spots in the replicate gels; of these, 297 spots were shared by the replicate gels and 13 were representative spots (Figs. 3B & 5). The 12 gels of this stock shared 143 spots.

In the case of RS, 3 haemolymph pools were produced, including one from oysters negative for *Bonamia* spp., with an average of 321 spots in the replicate gels (150 spots were shared by the 4 replicates and 2 were representative spots). The second pool was from oysters with a weak positive signal for *B. ostreae*, with an average of 526 spots in the replicate gels (203 spots shared by the 4 replicates and 4 representative spots). The third pool comprised oysters with a strong positive signal for *B. ostreae*, with

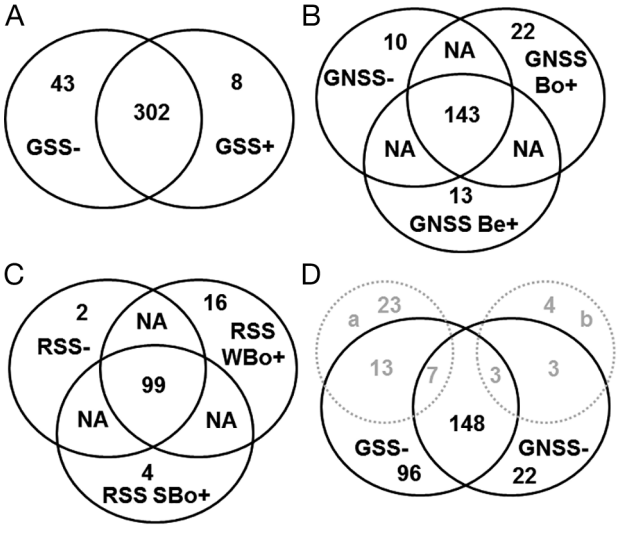

Fig. 3. Comparisons of spot patterns between master gels of different haemolymph pools, showing the number of spots representative of each pool and the numbers of spots shared by all the pools in each comparison. (A) Oysters PCR-negative for *Bonamia* spp. of the Galician selected stock (GSS–) vs. oysters PCR-positive for *B. ostreae* or *B. exitiosa* of the same stock (GSS+). (B) Oysters PCR-negative for *Bonamia* spp. of the Galician non-selected stock (GNSS–), vs. oysters PCR-positive for *B. ostreae* (GNSS Bo+) and PCR-positive for *B. exitiosa* (GNSS Be+) of the same stock. (C) Oysters PCR-negative for *Bonamia* spp. of the Rossmore selected stock (RSS–) vs. oysters with a weak PCR signal for *B. ostreae* (RSS WBo+) and oysters with a strong PCR-signal for *B. ostreae* (RSS SBo+) of the same stock. (D) GSS– vs. GNSS–; in this case, the grey circles correspond to the distribution of the 43 spots found representative of GSS– in comparison 'A' (grey circle a) and that of the 10 spots found representative of GNSS– in comparison 'B' (grey circle b). NA: not analysed

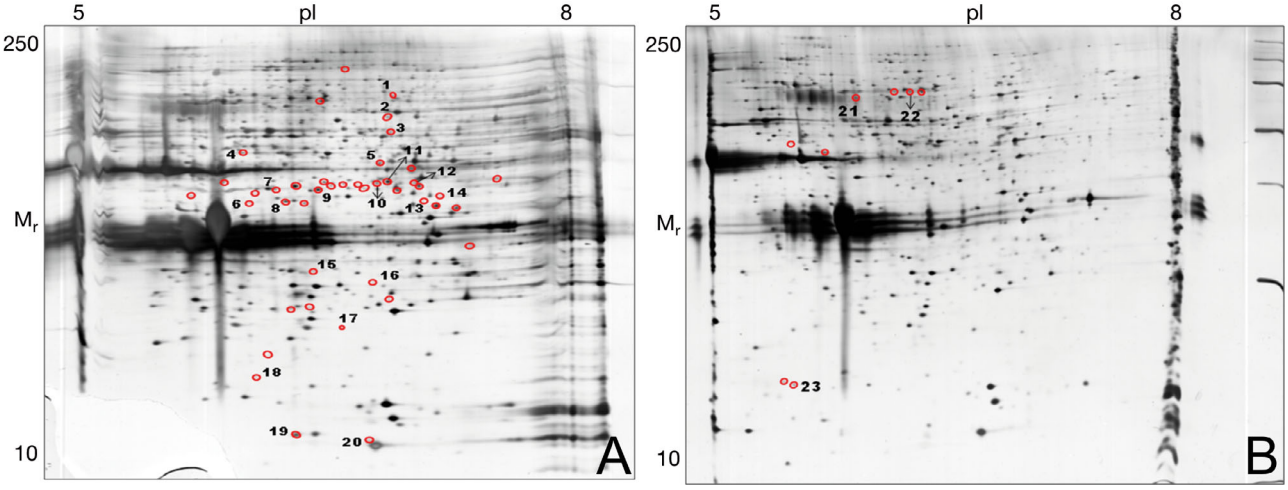

Fig. 4. Digitised images of silver-stained gels produced by 2D-SDS-PAGE showing protein profiles of the haemolymph pools from the oysters *Ostrea edulis* of the Galician selected stock (GSS). Gel corresponding to the haemolymph pool from oysters (A) negative and (B) positive for *Bonamia* spp. Red circles indicate spots representative of each haemolymph pool. Numbers correspond to identified proteins, listed in Table 3. pI: Isolectric point; M_r: apparent molecular mass (kDa). A representative image is shown for each pool; 4 gels were run and analysed for each pool

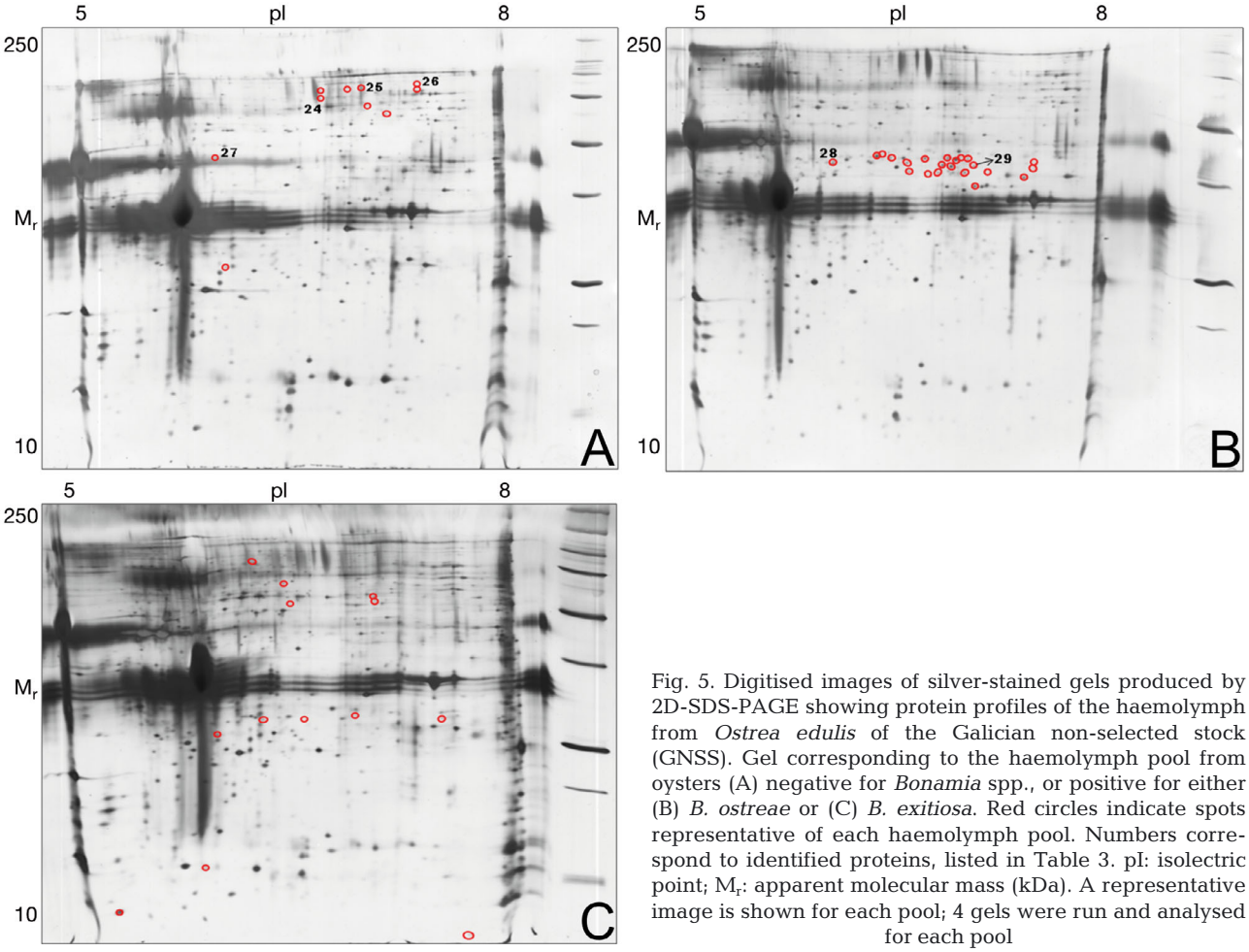

Fig. 5. Digitised images of silver-stained gels produced by 2D-SDS-PAGE showing protein profiles of the haemolymph from *Ostrea edulis* of the Galician non-selected stock (GNSS). Gel corresponding to the haemolymph pool from oysters (A) negative for *Bonamia* spp., or positive for either (B) *B. ostreae* or (C) *B. exitiosa*. Red circles indicate spots representative of each haemolymph pool. Numbers correspond to identified proteins, listed in Table 3. pI: isolectric point; M_r: apparent molecular mass (kDa). A representative image is shown for each pool; 4 gels were run and analysed for each pool

an average of 622 spots in the replicate gels (365 spots shared by the 4 replicates and 16 representative spots) (Figs. 3C & 6). The 12 gels of this stock shared 99 spots.

Differences in the distribution of the number of spots depending on PCR diagnosis (positive or negative) were significant in the comparisons GSS vs. GNSS ($p = 0.002$), GSS vs. RS ($p < 0.001$) and GNSS vs. RS ($p < 0.001$). Remarkably, the PCR-negative pool of the GSS oyster stock had more spots than the PCR-positive pool of the same stock, whereas the PCR-negative pools of the GNSS and RS stocks had fewer spots than the PCR-positive ones of the respective stocks.

Comparison of protein patterns between Galician oysters negative for *Bonamia* spp.

The additional comparison between the master gels from the haemolymph pools of oysters negative for *Bonamia* spp. of the 2 Galician stocks showed that both master gels shared 158 spots, while 109 spots were unique to the master gel of GSS and 25 spots were unique to the master gel of GNSS (Table 2B). The group of 158 spots occurring in both pools of this comparison included 7 spots that were considered representative of the GSS oysters negative for *Bonamia* spp. when compared with GSS-positive ones, and 3 spots that were considered representative of GNSS oysters negative for *Bonamia* spp. when compared with GNSS-positive ones. The group of 109 spots unique to GSS-negative oysters when compared with GNSS-negative ones included 13 spots that were representative of GSS-negative oysters when compared with GSS-positive ones. The group of 25 spots unique to GNSS-negative oysters when compared with GSS-negative ones included 3 spots that were representative of GNSS-negative oysters when compared with GNSS-positive ones (Fig. 3D).

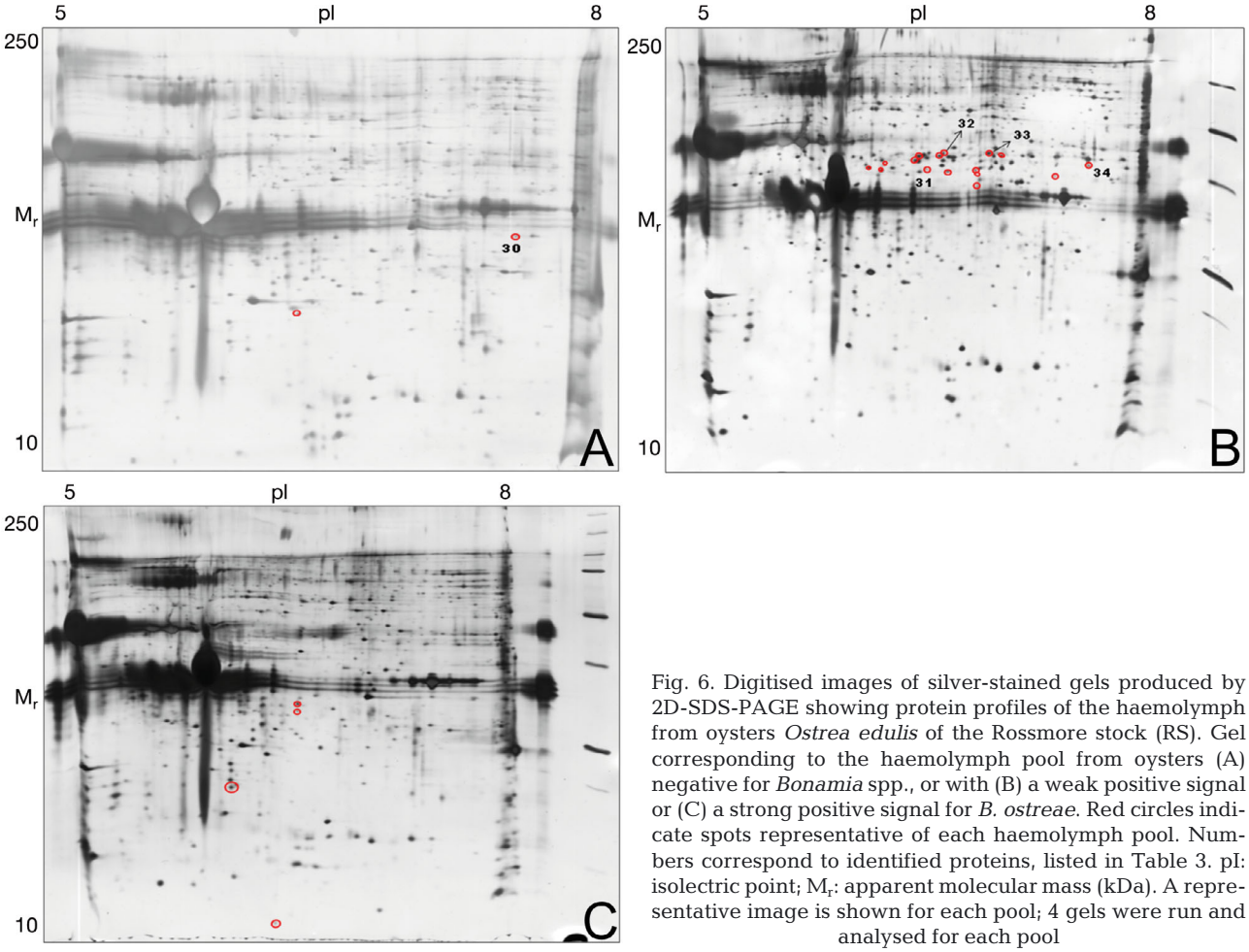

Fig. 6. Digitised images of silver-stained gels produced by 2D-SDS-PAGE showing protein profiles of the haemolymph from oysters *Ostrea edulis* of the Rossmore stock (RS). Gel corresponding to the haemolymph pool from oysters (A) negative for *Bonamia* spp., or with (B) a weak positive signal or (C) a strong positive signal for *B. ostreae*. Red circles indicate spots representative of each haemolymph pool. Numbers correspond to identified proteins, listed in Table 3. pI: isolectric point; M_r: apparent molecular mass (kDa). A representative image is shown for each pool; 4 gels were run and analysed for each pool

Protein identification

A total of 118 spots were found representative of a particular pool when comparing the pools within each stock (Table 2). Those representative spots were excised, digested with trypsin and sequenced by MS. Sequencing and database searching allowed the identification of 34 proteins, of which 2 corresponded to 'hypothetical' matched non-identified proteins of molluscs included in databases. All of the accepted matches were obtained against the Mollusca database and none against the Rhizaria and Alveolata databases. The list of identified proteins representative of each haemolymph pool is included in Table 3. In the GSS stock, 20 proteins from oysters negative for *Bonamia* spp. were identified; they were mainly involved in energy metabolism, respiratory chain, acting as chaperones, RNA processing, protein biosynthesis and degradation, cytoskeleton, oxidation-reduction process, apoptosis, and antioxidant and signal transduction. In the GSS oysters positive for *Bonamia* spp., 3 proteins were identified, which were involved in transcription, protein biosynthesis and antioxidant processes. In the GNSS oysters negative for *Bonamia* spp., 4 proteins were identified, involved in energy metabolism and defence, while 2 proteins involved in glutamate biosynthesis and in defence were identified in GNSS oysters positive for *B. ostreae*. Within the RSS stock, 1 protein involved in cell growth was identified in oysters negative for *Bonamia* spp., while 4 proteins involved in cytoskeleton, protein degradation and detoxification were identified in oysters with a weak signal for *B. ostreae*.

Regarding the comparison between the master gels from the haemolymph pools of oysters negative for *Bonamia* spp. of the 2 Galician stocks, 7 of the 109 spots unique to the master gel of GSS oysters negative for *Bonamia* spp. corresponded to identified spots (codes 1, 3, 5, 10, 12, 14 and 17 in Table 3), while 2 of the 25 spots unique to the master gel of GNSS oysters negative for *Bonamia* spp. corresponded to identified spots (codes 24 and 26 in Table 3).

DISCUSSION

Differences in haemolymph proteomic profile were detected between oysters infected with *Bonamia* spp. and non-infected oysters within each oyster stock. Considering that infected and non-infected oysters of the same stock shared the same environmental conditions throughout their life, the

Table 3. Identified proteins corresponding to 2-DE spots that were considered representative of *Ostrea edulis* haemolymph pools when comparing the pools within each oyster stock (GSS: Galician selected stock; GNSS: Galician non-selected stock; RS: Rossmore stock). pI: Isolectric point; M_r: apparent molecular mass in Daltons; Exp: experimental value; Theo: theoretical value; Score: highest alignment score between the query sequence and the database sequence segment (the higher the value of the score, the better the peptide match); E-value: the number of distinct alignments, with a score equivalent to or better, that are expected to occur in a database search by chance (the lower the E-value, the better the peptide match); Sequence: only the best match sequence is shown in the table; Match/(%Cov): number of amino acids of the protein sequence that corresponded to matched peptides and percentage

Code	Oyster stock	*Bonamia* spp. diagnosis	Protein identification	NCBI acc. no.	M_r (Theo)/(Exp)	pI (Theo)/(Exp)	Biological function	E-value	NCBI score	Sequence	Match (%Cov)
1	GSS[a]	Negative	Cytochrome c oxidase subunit I, partial (mitochondrial) (*Chelidonura berolina*)	AFR24232.1	23.65/80	5.26/6.67	Energy metabolism, respiratory chain	0.088	29.1	GPIAHGSSS	9/9 (100)
2	GSS	Negative	T-complex protein 1 subunit alpha (*Crassostrea gigas*)	EKC42768.1	75.32/67.68	6.82/6.64	Chaperone	9×10^{-21}	89.3	KSVVPGGGAVEAALSIYLENFATSLGSRE	29/29 (100)
3	GSS[a]	Negative	Adenylosuccinate synthetase-like (*Crassostrea virginica*)	XP_022333667.1	48.60/60.98	6.09/6.65	Energy and amino acid metabolism	3×10^{-5}	42.6	KNYDSFPALDEDLCRL	12/12 (100)
4	GSS	Negative	Lupus La homolog protein (*C. virginica*)	XP_022320812.1	48.50/51.22	5.49/5.98	RNA processing	1×10^{-9}	54.9	RNPDMSIPENTEERRN	15/16 (94)
5	GSS[a]	Negative	Eukaryotic initiation factor 4A-III (*C. gigas*)	EKC18878.1	137.9/48.1	6.79/6.58	Protein biosynthesis	5×10^{-23}	96.9	KVILALGDYMSVQCHACIGGTNIGDDIRK	29/29 (100)
6	GSS	Negative	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial-like (*C. gigas*)	XP_022305803.1	80.41/38.70	5.84/5.96	Energy metabolism, respiratory chain	1×10^{-13}	68.1	KNPPQVLFMLGADEGVLTRDDLPKD	21/25 (84)
7	GSS	Negative	RNA helicase p47 (*Spisula solidissima*)	AAK85400.1	50.70/42.70	7.62/6.09	RNA processing	1×10^{-17}	79.1	KITELGYSCFYIHARMNQQHRN	22/22 (100)
8	GSS[b]	Negative	FH1/FH2 domain-containing protein 3 (*Mizuhopecten yessoensis*)	OWF39623.1	294.80/39.48	9.34/6.13	Cytoskeleton organisation	0.50	28.6	PPPPPGAP	8/8 (100)

Table 3. (continued)

9	GSS	Negative	Cellulase (*Haliotis discus hannai*)	BAD01504.1	64.61/41.95	5.95/6.14	Digestion	8×10^{-17}	76.1	KQTDMFFDMIKWPLDYFLKC	20/20 (100)
10	GSS[a]	Negative	26S protease regulatory subunit 8 (*C. virginica*)	XP_022304231.1	45.72/43.19	7.14/6.56	Protein degradation	4×10^{-15}	77.4	RMLREELQLLQEQGSYVGEVVKP	23/23 (100)
11	GSS	Negative	Arginine kinase (*C. gigas*)	EKC24881.1	39.86/43.66	6.40/6.66	Energy metabolism	9×10^{-16}	75.7	RSHASYGFPPVVNAEQCKEMEKI	23/23 (100)
12	GSS[a]	Negative	Kelch-like protein 24 (*C. gigas*)	EKC36218.1	63.16/42.88	5.17/6.66	Cytoskeleton	1×10^{-13}	72.7	RLASGGTVVTEFIGYSFSNKKW	22/22 (100)
13	GSS	Negative	Protein iolS (*C. gigas*)	EKC27394.1	28.96/38.59	6.96/6.78	Oxidation–reduction process	2×10^{-12}	63.8	KAALSWLVNQPNVPVAIVGART	20/22 (91)
14	GSS[a]	Negative	Cathepsin B (*Sinonovacula constricta*)	AGL33709.1	37.80/39.17	6.31/6.87	Apoptosis	7×10^{-12}	67.7	KLLLLLVAVGMATAYKAPPKD	21/21 (100)
15	GSS	Negative	Biliverdin reductase A (*C. gigas*)	EKC34745.1	34.43/31.47	6.39/6.24	Antioxidant	2×10^{-16}	75.3	RDFYNFMEEVTSGKIDQEMKD	21/21 (100)
16	GSS[b]	Negative	NADH dehydrogenase subunit 1 (*Notodoris gardineri*)	YP_004222602.1	34.41/27.84	6.71/6.50	Energy metabolism, respiratory chain	5×10^{-14}	68.5	KGPNKVSIWGIAQPLADAVKL	21/21 (100)
17	GSS[a]	Negative	Von Willebrand factor A domain-containing protein 3B-like isoform X1 (*C. virginica*)	XP_022327901.1	222.85/21.18	9.49/6.18	Cytoskeleton	9×10^{-20}	85.9	KLELFDVLGSVAFKHQDGAVDINMKP	26/26 (100)
18	GSS[b]	Negative	Synaptojanin-2-binding protein-like isoform X1 (*C. virginica*)	XP_022333491.1	17.71/ 20.04	4.51/5.97	Signal transduction	9×10^{-14}	68.1	KRVTHNEAVQHFVNAGEVVTLKV	21/22 (95)
19	GSS[b]	Negative	Farnesoicacid-o-methyltransferase, partial (*Ostrea edulis*)	AFJ91732.1	16.91/16.42	6.21/6.1	Growth and development	3×10^{-18}	80.8	RDVNNYEVLVLPSDKDGYYEWKK	23/23 (100)
20	GSS	Negative	Extracellular superoxide dismutase (Cu-Zn)-like (*C. virginica*)	XP_022307231.1	20.77/16.00	5.17/6.41	Antioxidant	1×10^{-16}	76.6	RSLAILQGDHTSHTSIIACCVVGRS	23/25 (92)
21	GSS	Positive	60S acidic ribosomal protein P2 (*Cryptochiton stelleri*)	O61463.1	11.47/62.83	4.35/5.91	Protein elongation	1×10^{-12}	60.1	KLASVPSGGGVAAAAPAAGGGGADPAEAKEEKK	33/33 (100)
22	GSS	Positive	Zinc finger protein ZFAT (*C. gigas*)	EKC21126.1	91.12/66.45	7.91/6.27	Transcription regulation	0.20	29.5	MKHTCKM	7/7 (100)
23	GSS	Positive	Thioredoxin-like protein 1 (*C. virginica*)	XP_022309459.1	31.95/17.08	4.92/5.48	Antioxidant	0.36	29.1	VKVDEMKG	8/8 (100)
24	GNSS[c]	Negative	Succinate dehydrogenase (ubiquinone) flavoprotein subunit B,mitochondrial (*C. gigas*)	EKC34820.1	83.91/ 78.75	6.28/6.75	Energy metabolism, respiratory chain	1×10^{-19}	85	KYDTSYFIEYFALDLIMEDGECRG	24/24 (100)
25	GNSS	Negative	Complement C1q-like protein 2 (*C. gigas*)	EKC35881.1	42.90/82.50	5.38/7.06	Defence	6×10^{-14}	68.9	RTMSHNTASYMTGTNMAVLQLFKG	20/24 (83)
26	GNSS[c]	Negative	Hypothetical protein CGI_10024713 (*C. gigas*)	EKC32925.1	43.01/76.23	6.03/7.49	Unknown	4×10^{-22}	92.7	KDMDAVLRFLTQPCQQPVNIENHEAKL	27/27 (100)
27	GNSS[b]	Negative	Adenylate kinase isoenzyme 5 (*C. gigas*)	EKC37609.1	99.84/49.33	8.57/5.98	Energy metabolism	4×10^{-20}	86.7	KFEEQITTPKCVLYFDVSDDTMTKR	25/25 (100)
28	GNSS	Positive for *B. ostreae*	Glutamate synthase (NADH), amyloplastic (*C. gigas*)	EKC34018.1	244.77/ 44.03	5.76/6.21	Glutamate biosynthesis	2×10^{-23}	96.5	KAFYRWSAFAMEPWDGPALLTFCDGRY	27/27 (100)
29	GNSS	Positive for *B. ostreae*	Protein fmtA (*C. gigas*)	EKC28751.1	51.38/45.43	7.69/7.06	Defence	4×10^{-23}	96.1	RLGMTSTSFFTSPAFNPFLQDVAQGYVKD	29/29 (100)
30	RS	Negative	Developmentally regulated GTP-binding protein 1 (*C. gigas*)	EKC33015.1	39.12/32.35	8.99/7.30	Cell growth and differentiation	5×10^{-16}	74.0	KWNFDDLLEKMWEYLGLVRI	20/20 (100)
31	RS	Weak positive	Dynein heavy chain, cytoplasmic (*C. gigas*)	EKC21066.1	561.57/40.11	6.19/6.50	Cytoskeleton	0.04	35	LSCGPMVKWA	9/10 (90)
32	RS	Weak positive	Ubiquitin-associated protein 2 (*C. gigas*)	EKC34321.1	149.10/43.78	6.93/6.61	Protein degradation	0.024	32.9	SYGSGSSYNS	10/10 (100)
33	RS	Weak positive	GST omega, partial (*Reishia clavigera*)	AET43965.1	27.47/44.09	6.09/6.92	Detoxification	4×10^{-17}	77.4	KPAFIDFMIWPWHERLPSLKK	21/21 (100)
34	RS	Weak positive	Hypothetical protein LOTGIDRAFT_169702 (*Lottia gigantea*)	XP_009066249.1	59.95/41.52	5.76/7.60	Unknown	6.3	26.1	VSKKIRTEIP	7/7 (100)

[a]Spot representative of the haemolymph pool GSS-negative in the comparison between oysters negative for *Bonamia* spp. of the 2 Galician stocks. [b]Spot found in the master gels of the haemolymph pools GSS-negative and GNSS-negative in the comparison between oysters negative for *Bonamia* spp. of the 2 Galician stocks. [c]Spot representative of the haemolymph pool GNSS-negative in the comparison between oysters negative for *Bonamia* spp. of the 2 Galician stocks

differences in proteomic profile suggest that the pattern of protein expression changed due to infection with *Bonamia* spp. Changes in protein expression might be caused by the parasite, which could block host metabolism in order to escape host defence mechanisms, or reflect a host metabolic dysfunction as a consequence of the disease. Parasites have frequently been associated with alteration of protein variety and concentration in the host haemolymph. A substantial reduction in protein variety in the haemolymph of *Ostrea edulis* associated with infection with *B. ostreae* was reported by Cao et al. (2009). Protein levels lower than in their corresponding healthy counterparts were noted in *Perkinsus marinus*-infected *Crassostrea virginica* (Chu et al. 1993) and in MSX-infected *C. virginica* (Barber et al. 1988). Decreased levels may be due to rapid utilisation of host nutrients by the parasite (Cronin et al. 2001). The oyster stocks used in the study showed different patterns regarding differences between oysters negative for *Bonamia* spp. and positive ones in the number of protein spots; this number was higher in negative than in positive oysters within GSS, whereas GNSS and RSS oysters showed the opposite pattern, i.e. lower number of spots in oysters negative for *Bonamia* spp. Thus, different stocks seem to have different behaviours in response to infection with *Bonamia* spp. Cao et al. (2009) found that bonamiosis-resistant *O. edulis* stocks expressed a wider variety of proteins in the haemolymph than susceptible stocks did; the results obtained from the Galician stocks (GSS and GNSS) were in agreement with that observation.

The qualitative proteomic study allowed the identification of proteins representative of each infection group within each oyster stock up to a total of 34. These proteins whose expression is modified by infection with *Bonamia* spp. are presumably key players in the host–parasite interaction. Remarkable discrepancies were found between the theoretical values of molecular weight and/or isoelectric points of some identified proteins and the observed values according to the position of their spots in the gels. Protein isoforms generated by post-translational modifications, alternative splicing or the occurrence of multigene families could account for such discrepancies (De La Fuente et al. 2011).

Results showed that infection with *Bonamia* spp. leads to modified expression of *O. edulis* proteins involved in major metabolic pathways, such as energy production, respiratory chain, oxidative stress, signal transduction, transcription, translation, protein degradation and cell defence. Changes in protein expression might be caused by the parasite, which could block host metabolism in order to escape host defence mechanisms, or reflect a host metabolic dysfunction as a consequence of the disease. Several proteins identified in Galician stock (GSS and GNSS) oysters negative for *Bonamia* spp. were involved in energetic metabolism. Host energetic reserves are mobilised in order to generate the energy needed to counter the parasite (Engelsma et al. 2014). Also, immune cells require a constant supply of energy for basic housekeeping and specific immune functions such as migration, phagocytosis and cytotoxicity (Buttgereit et al. 2000, Krauss et al. 2001). Immune stimulation results in increased biochemical activity and therefore a greater demand for ATP. Thus, crucial processes for specific immune functions are rapidly impaired when immune cells are deprived of energy (Coyne 2011). Proteins involved in energy metabolism were identified in non-infected oysters of the GSS and GNSS stocks: (1) adenylosuccinate synthetase (AdSS), which catalyses the first reaction in the synthesis of AMP; its regulation is considered a checkpoint in the maintenance of the ATP:GTP ratio in cells (Raman et al. 2004); (2) adenylate kinase, which plays a significant role in providing ATP for cytoskeletal functions crucial in defence mechanisms and is recognised as a sensitive reporter of the cellular energy state (Dzeja & Terzic 2009, van Horssen et al. 2009); and (3) arginine kinase (AK), which catalyses phosphoarginine and ADP to produce more L-arginine and ATP molecules during the immune response. L-arginine is the unique physiologic source of the nitrogen atom in nitric oxide (Kinsey & Lee 2003), which is regarded as a crucial component of the innate immune response (Apel & Hirt 2004). Thus, AK might play an important role in the immune response against invading pathogens by modulating NO concentration (Shi et al. 2012). Modulation of proteins identified in our study has been previously observed in different pathologic processes (Chen et al. 2011, Wu et al. 2013).

Key components of the respiratory chain were also identified in GSS and GNSS oysters negative for *Bonamia* spp.: cytochrome c oxidase, NADH dehydrogenase and succinate dehydrogenase (ubiquinone) flavoprotein. Modified expression of these proteins (or their genes) has been previously identified in other bivalves infected with different parasites (Tanguy et al. 2004, Kang et al. 2006, Perrigault et al. 2009, Prado-Alvarez et al. 2009, Fernández-Boo et al. 2016) and in *O. edulis* when challenged with *B. ostreae* (Martín-Gómez et al. 2012). The mitochondrial respiratory chain is important for the immune

response of molluscs because (1) immune-stimulated cells present increased metabolic and energetic needs (van Rensburg & Coyne 2009, Coyne 2011); (2) a functional electron transport system is necessary for the phagocytosis of invading pathogens (van Rensburg & Coyne 2009); and (3) an intensive activity of the respiratory chain generates an excess of reactive oxygen species (ROS) (Koopman et al. 2010), which is considered a defence mechanism against invading pathogens (De Zoysa et al. 2009). An inhibition of the electron transport could account for a reduced immune response (van Rensburg & Coyne 2009, Donaghy et al. 2012). Altogether, proteins involved in energy metabolism are crucial for the immune response and could be blocked by *Bonamia* spp. to successfully infect bivalves.

Other major metabolic processes in which identified proteins are involved were protein synthesis (transcription and translation) and degradation, which showed that these processes are also affected in *O. edulis*–*B. ostreae* interactions. Zinc finger protein ZFAT involved in transcription was representative of GSS oysters positive for *Bonamia* spp. Lupus La-like protein and RNA helicase p47, involved in translation, were representative of GSS oysters negative for *Bonamia* spp. Eukaryotic initiation factor 4A (eIF4A) and 60S acidic ribosomal P2, involved in protein synthesis, were representative of GSS oysters negative and positive for *Bonamia* spp., respectively. Underexpression of eIF4A and thus blocking protein translation is associated with parasite–host interaction (Prasad et al. 2014). 26S protease regulatory subunit 8 and ubiquitin-associated protein 2, involved in protein degradation, were representative of GSS oysters negative for *Bonamia* spp. and RSS oysters with a weak positive signal for *B. ostreae*, respectively. Variations in the expression of 26S protease regulatory subunit gene have been observed in flat oysters infected with *B. ostreae* (Martín-Gómez et al. 2012). Modified expression of various proteins related to 26S proteasome was also observed in the haemolymph of the octopus *Octopus vulgaris* infected with protozoan parasites (Castellanos-Martínez et al. 2014). Proteasome plays a critical role in ubiquitin-mediated protein degradation through the ubiquitin–proteasome pathway and is involved in cell proliferation, apoptosis and stress responses; therefore, proteasome is regarded as an essential component of the defence system (Wu et al. 2013).

The cytoskeleton plays a central role in many cell functions, regulating cell shape, cell division, adhesion, motility, migration, signal transduction and phagocytosis, which are crucial elements in the defence mechanisms (May & Machesky 2001). Many parasites are able to challenge the cellular machinery to invade and survive within host cells (Rottner et al. 2004). Parasite–host interactions are involved in cytoskeletal changes (Gruenheid & Finlay 2003). Consistently, various proteins identified in this study were involved in cytoskeletal functions: T-complex protein 1 subunit alpha, FH1/FH2 domain-containing protein 3, Kelch-like protein 24 (KLHL) and von Willebrand factor A domain-containing protein 3B (vWFA). Changes in the cytoskeleton of *O. edulis* after being parasitised by *Bonamia* spp. have been previously reported (Chagot et al. 1992, Morga et al. 2011a, Martín-Gómez et al. 2012). Phagocytosis is a cytoskeleton-dependent process (May & Machesky 2001). Phagocytosis of *Bonamia* spp. by host haemocytes is a key process in oyster–parasite interaction, and even *B. ostreae* seems to actively contribute to its own phagocytosis (Chagot et al. 1992). Consistently, variations in the expression of cytoskeleton proteins suggest that such proteins play an important role in response to *Bonamia* spp. AdSS and adenylate kinase have been mentioned above as relevant proteins in energetic metabolism. These proteins are especially interesting, as they participate in the generation of AMP, which plays a key role in phagocytosis (Lacoste et al. 2001, Fabbri & Capuzzo 2010). The involvement of these 2 proteins in 2 independent processes (energetic metabolism and phagocytosis) involved in response to parasites underlies their importance in the response to bonamiosis.

Another relevant cellular process for which specific proteins were identified is cell defence; the identified proteins were involved in (1) activation of the complement system, (2) apoptosis and (3) redox control. C1q is the first subcomponent of the C1 complex of the classical pathway of complement activation, which is crucial for the clearance of pathogens in invertebrates (Zhang et al. 2008). Proteins related to C1q are among the major classes of pattern recognition receptors in most bivalves (Gerdol et al. 2015); these receptors are crucial in the immune response of bivalves (H. Zhang et al. 2008, L. Zhang et al. 2014). Complement C1q-like protein 2 was expressed in GNSS oysters negative for *Bonamia* spp. The implication of proteins related to C1q in the host–parasite interaction has been reported in other bivalves such as *Ruditapes decussatus* infected with *Perkinsus olseni* (Prado-Alvarez et al. 2009) and *Mercenaria mercenaria* parasitised by QPX (Perrigault et al. 2009). In European flat oysters, C1q expression significantly changes due to bonamiosis and neoplasia (Martín-Gómez et al.

2014). C1q has been proposed as one of the genes that confer resistance to parasitic diseases in oysters, as is the case for *Saccostrea glomerata* against *Marteilia sydneyi* (Green et al. 2009) and *O. edulis* against *B. ostreae* (Morga et al. 2012).

Apoptosis plays a key role in defence against pathogens. The mechanisms and signalling pathways underlying apoptosis are crucial in molluscan immune responses (Schaumburg et al. 2006, Sokolova 2009). In bivalves, the role of apoptosis has been highlighted in the response of the oyster *C. virginica* against *P. marinus* (Hughes et al. 2010) and the clam *R. decussatus* against *P. olseni* (Prado-Alvarez et al. 2009). A large number of genes involved in apoptosis has been recently identified in *C. gigas* and found over-expressed in the context of infection with *Vibrio anguillarum* (Zhang et al. 2011). *O. edulis* specifically responds to *B. ostreae* by inducing apoptosis of haemocytes (Gervais et al. 2016, Morga et al. 2017). Two proteins involved in apoptosis were found representative of GSS oysters negative for *Bonamia* spp.: Lupus La-like protein and cathepsin B. Lupus La-like protein was mentioned above as involved in protein synthesis; it can indeed modulate the stability and/or translation of other apoptosis-associated proteins (Valavanis et al. 2007). Cathepsins are involved in the interaction between clam hosts and *Perkinsus* spp. parasites (Kang et al. 2006, Soudant et al. 2013) and are included in the host defence genes of scallops *Chlamys farreri* (Wang et al. 2009). The cathepsin B gene is overexpressed upon *O. edulis–Bonamia* spp. interaction (Morga et al. 2012). Manipulation of host apoptosis by intracellular parasites occurs in vertebrate and invertebrate hosts (Deveraux et al. 1997, Schaumburg et al. 2006). Thus, *B. ostreae* could inhibit apoptosis in order to survive inside the oyster haemocytes (Morga et al. 2012).

Redox balance is an important process for cell defence. Our study has identified several proteins that participate in the oxidative stress response of the cell. Biliverdin reductase A (BVRA) and extracellular superoxide dismutase[Cu-Zn] (EcSOD) were representative of GSS oysters negative for *Bonamia* spp., whereas thioredoxin 1 and glutathione S-transferase omega (GSTO), also implicated in the oxidative stress response (Lillig & Holmgren 2007, Martins et al. 2014), were representative of GSS oysters positive for *Bonamia* spp. and RSS oysters with a weak positive signal, respectively. The product of BVRA enzyme, bilirubin, has been described as a powerful ROS scavenger with a cyto-protective effect (Kapitulnik & Maines 2009). SOD is involved in the oxidative stress response (Gonzalez et al. 2005), being one of the most important antioxidant defence mechanisms in almost all cells, including those of molluscs (Anju et al. 2013). EcSOD expression in oysters *S. glomerata* resistant to *M. sydneyi* is much higher than in non-resistant ones (Green et al. 2009). Expression of SOD in *O. edulis* varied in *B. ostreae*-challenged haemocytes *in vitro* and *in vivo* (Morga et al. 2011b, 2017). Higher levels of activity of Cu/Zn SOD may result in a more potent and lethal respiratory burst (Goodall et al. 2004). Thus, *B. ostreae*-induced interference with the respiratory burst would help the parasite survive and divide within *O. edulis* haemocytes (Hervio et al. 1989, Morga et al. 2009, 2011b, 2017, Comesaña et al. 2012). Thioredoxin gene has been identified as involved in the immune response of *O. edulis* to bonamiosis (Martín-Gómez et al. 2012). It could be expressed by the protozoan to protect itself from bivalve defence; in *P. marinus*, an increase in the thioredoxin expression was observed after challenge with mucus from *C. virginica* (Pales Espinosa et al. 2014). GST plays an important role in bivalve defence against parasites; in *Ruditapes philippinarum*, the GST gene was over-expressed in clams infected by *P. olseni* (Kang et al. 2006) and in *C. virginica* infected with *P. marinus* (Tanguy et al. 2004). Increased GSTO expression has been previously reported during *O. edulis–B. ostreae* interaction (Morga et al. 2011a, Martín-Gómez et al. 2012).

Special attention was paid to differences in the haemolymph proteomic profile between non-infected oysters of the 2 Galician stocks, the selected and the non-selected one. Because oysters used in the study from both Galician stocks shared the same environmental conditions throughout their whole life, differences between both stocks in their proteomic profile should be associated with genotype differences. Some of the genotype-based variations in protein expression could be a basis for the higher resistance to bonamiosis of GSS oysters compared to GNSS ones. Oysters from both Galician stocks had been grown in an area of the Ria of Arousa affected by bonamiosis and, as expected, the number of PCR positive oysters for both *Bonamia* spp. was higher in the non-selected stock (see 'Materials and methods'). Seven proteins (namely, cytochrome c oxidase subunit I, AdSS-like, eIF4A-III, 26S protease regulatory subunit 8, KLHL, cathepsin B and vWFA domain-containing protein 3B-like isoform X1) were found to be representative of GSS-negative oysters when comparing the master gels of GSS and GNSS oysters negative for *Bonamia* spp. Such proteins would presumably play a relevant role in the interaction with *Bonamia* spp. Importantly, some

of them (cathepsin B, KLHL and vWFA) are involved in apoptosis and phagocytosis processes, which have been previously highlighted as key mechanisms supporting resistance to bonamiosis in *O. edulis* (Morga et al. 2017). Remarkably, proteins previously reported to be differentially expressed in molluscan strains selected for disease resistance (when compared with non-selected stocks) were also found among the aforementioned unique proteins: cytochrome c oxidase (Lockyer et al. 2008, 2012), eIF4A (Hong et al. 2011), vWFA (Bouchut et al. 2006, McDowell et al. 2014) and cathepsin B (Lockyer et al. 2008, Fleury & Huvet 2012, Morga et al. 2012, Zahoor et al. 2014). In all, accumulated evidence suggests that those 7 proteins representative of the GSS-negative oysters could constitute candidate markers of resistance against bonamiosis, which should be further assessed.

The identification of 34 proteins whose occurrence in oyster haemolymph is modulated by *Bonamia* spp. contributes to our understanding of host–parasite interactions in the context of oyster bonamiosis. Furthermore, the candidate protein markers of resistance, if confirmed, could be used in marker-assisted selective breeding programmes to produce bonamiosis-resistant oyster strains.

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LITERATURE CITED

Abollo E, Ramilo A, Casas SM, Comesaña P, Cao A, Carballal MJ, Villalba A (2008) First detection of the protozoan parasite *Bonamia exitiosa* (Haplosporidia) infecting flat oyster *Ostrea edulis* grown in European waters. Aquaculture 274:201–207

Airoldi L, Beck MW (2007) Loss, status and trends for coastal marine habitats of Europe. Oceanogr Mar Biol Annu Rev 45:345–405

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Anju A, Jeswin J, Thomas PC, Paulton MP, Vijayan KK (2013) Molecular cloning, characterization and expression analysis of cytoplasmic Cu/Zn-superoxide dismutase (SOD) from pearl oyster *Pinctada fucata.* Fish Shellfish Immunol 34:946–950

Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol 55:373–399

Balouet G, Poder M, Cahour A (1983) Haemocytic parasitosis: morphology and pathology of lesions in the French flat oyster, *Ostrea edulis* L. Aquaculture 34:1–14

Barber BJ, Ford SE, Haskin HH (1988) Effects of the parasite MSX (*Haplosporidium nelsoni*) on oyster (*Crassostrea virginica*) energy metabolism. 2. Tissue biochemical composition. Comp Biochem Physiol A 91:603–608

Beattie JH, Davis JP, Downing SL, Chew KK (1988) Summer mortality of Pacific oysters. Spec Publ Am Fish Soc 18: 265–268

Beck MW, Brumbaugh RD, Airoldi L, Carranza A and others (2011) Oyster reefs at risk and recommendations for conservation, restoration, and management. Bioscience 61: 107–116

Bouchut A, Roger E, Coustau C, Gourbal B, Mitta G (2006) Compatibility in the *Biomphalaria glabrata/Echinostoma caproni* model: potential involvement of adhesion genes. Int J Parasitol 36:175–184

Buttgereit F, Burmester GR, Brand MD (2000) Bioenergetics of immune functions: fundamental and therapeutic aspects. Immunol Today 21:192–199

Cao A, Fuentes J, Comesaña P, Casas SM, Villalba A (2009) A proteomic approach envisaged to analyse the bases of oyster tolerance/resistance to bonamiosis. Aquaculture 295:149–156

Castellanos-Martínez S, Diz AP, Álvarez-Chaver P, Gestal C (2014) Proteomic characterization of the hemolymph of *Octopus vulgaris* infected by the protozoan parasite *Aggregata octopiana.* J Proteomics 105:151–163

Chagot D, Boulo V, Hervio D, Mialhe E, Bachere E, Mourton C, Grizel H (1992) Interactions between *Bonamia ostreae* (Protozoa: Ascetospora) and hemocytes of *Ostrea edulis* and *Crassostrea gigas* (Mollusca: Bivalvia): entry mechanisms. J Invertebr Pathol 59:241–249

Chen G, Zhang C, Li C, Wang C, Xu Z, Yan P (2011) Haemocyte protein expression profiling of scallop *Chlamys farreri* response to acute viral necrosis virus (AVNV) infection. Dev Comp Immunol 35:1135–1145

Cheng TC (1981) Bivalves. In: Ratcliffe NA, Rowley AF (eds) Invertebrate blood cells, Vol 1. Academic Press, London, p 233–300

Chu FLE, La Peyre JF, Burreson ES (1993) *Perkinsus marinus* infection and potential defense-related activities in eastern oysters, *Crassostrea virginica*: salinity effects. J Invertebr Pathol 62:226–232

Cochennec N, Renault T, Chollet B, Maffart P (1995) Comparison of haemograms from resistant and susceptible European flat oysters, *Ostrea edulis*, exposed to the parasite *Bonamia ostreae*. Abstracts Book of the 7th International Conference 'Diseases of Fish and Shellfish'. European Association of Fish Pathologists, Palma de Mallorca, 10–15 September, p 17

Cochennec-Laureau N, Auffret M, Renault T, Langlade A (2003) Changes in circulating and tissue-infiltrating hemocyte parameters of European flat oysters, *Ostrea edulis*, naturally infected with *Bonamia ostreae.* J Invertebr Pathol 83:23–30

Comesaña P, Casas SM, Cao A, Abollo E, Arzul I, Morga B, Villalba A (2012) Comparison of haemocytic parameters among flat oyster *Ostrea edulis* stocks with different sus-

ceptibility to bonamiosis and the Pacific oyster *Crassostrea gigas.* J Invertebr Pathol 109:274–286
Comps M, Tigé G, Grizel H (1980) Etude ultrastructurale d'un protiste parasite de l'huître *Ostrea edulis* L. CR Acad Sci Hebd Seances Acad Sci D 290:383–384
Corporeau C, Tamayo D, Pernet F, Quéré C, Madec S, Marin E (2014) Proteomic signatures of the oyster metabolic response to herpesvirus OsHV-1 µVar infection. J Proteomics 109:176–187
Coyne VE (2011) The importance of ATP in the immune system of molluscs. Invertebrate Surviv J 8:48–55
Cronin MA, Culloty SC, Mulcahy MF (2001) Lysozyme activity and protein concentration in the haemolymph of the flat oyster *Ostrea edulis* (L.). Fish Shellfish Immunol 11:611–622
Culloty SC, Mulcahy MF (1996) Season-, age- and sex-related variation in the prevalence of bonamiosis in flat oysters (*Ostrea edulis* L.) on the south coast of Ireland. Aquaculture 144:53–63
Culloty SC, Cronin MA, Mulcahy MF (2001) An investigation into the relative resistance of Irish flat oysters *Ostrea edulis* L. to the parasite *Bonamia ostreae* (Pichot et al. 1980). Aquaculture 199:229–244
Culloty SC, Cronin MA, Mulcahy MF (2004) Potential resistance of a number of populations of the oyster *Ostrea edulis* to the parasite *Bonamia ostreae.* Aquaculture 237: 41–58
da Silva PM, Fuentes J, Villalba A (2005) Growth, mortality and disease susceptibility of oyster *Ostrea edulis* families obtained from brood stocks of different geographical origins, through on-growing in the Ría de Arousa (Galicia, NW Spain). Mar Biol 147:965–977
De La Fuente M, Borrajo A, Bermúdez J, Lores M and others (2011) 2-DE-based proteomic analysis of common bean (*Phaseolus vulgaris* L.) seeds. J Proteomics 74:262–267
De Zoysa M, Whang I, Lee Y, Lee S, Lee JS, Lee J (2009) Transcriptional analysis of antioxidant and immune defense genes in disk abalone (*Haliotis discus discus*) during thermal, low-salinity and hypoxic stress. Comp Biochem Physiol B Biochem Mol Biol 154:387–395
Deveraux QL, Takahashi R, Salvesen GS, Reed JC (1997) X-linked IAP is a direct inhibitor of cell-death proteases. Nature 388:300–304
Donaghy L, Kraffe E, Le Goïc N, Lambert C, Volety AK, Soudant P (2012) Reactive oxygen species in unstimulated hemocytes of the Pacific oyster *Crassostrea gigas*: a mitochondrial involvement. PLOS ONE 7:e46594
Dove MC, Nell JA, O'Connor WA (2013) Evaluation of the progeny of the fourth-generation Sydney rock oyster *Saccostrea glomerata* (Gould, 1850) breeding lines for resistance to QX disease (*Marteilia sydneyi*) and winter mortality (*Bonamia roughleyi*). Aquacult Res 44: 1791–1800
Dzeja P, Terzic A (2009) Adenylate kinase and AMP signaling networks: metabolic monitoring, signal communication and body energy sensing. Int J Mol Sci 10: 1729–1772
Elston RA, Kent ML, Wilkinson MT (1987) Resistance of *Ostrea edulis* to *Bonamia ostreae* infection. Aquaculture 64:237–242
Engelsma MY, Culloty SC, Lynch SA, Arzul I, Carnegie RB (2014) *Bonamia* parasites: a rapidly changing perspective on a genus of important mollusc pathogens. Dis Aquat Org 110:5–23
Fabbri E, Capuzzo A (2010) Cyclic AMP signaling in bivalve molluscs: an overview. J Exp Zool A Ecol Genet Physiol 313:179–200
Fernández-Boo S, Villalba A, Cao A (2016) Protein expression profiling in haemocytes and plasma of the Manila clam *Ruditapes philippinarum* in response to infection with *Perkinsus olseni.* J Fish Dis 39:1369–1385
Fisher WS (1986) Structure and functions of oyster hemocytes. In: Brehélin M, Arcier JM, Boemare N, Bonami JR, Vivares CP (eds) Immunity in invertebrates. Springer, Berlin, p 25–35
Fleury E, Huvet A (2012) Microarray analysis highlights immune response of Pacific oysters as a determinant of resistance to summer mortality. Mar Biotechnol 14: 203–217
Frank-Lawale A, Allen SK, Dégremont L (2014) Breeding and domestication of eastern oyster (*Crassostrea virginica*) lines for culture in the mid-Atlantic, USA: line development and mass selection for disease resistance. J Shellfish Res 33:153–165
Gerdol M, Venier P, Pallavicini A (2015) The genome of the Pacific oyster *Crassostrea gigas* brings new insights on the massive expansion of the C1q gene family in Bivalvia. Dev Comp Immunol 49:59–71
Gervais O, Chollet B, Renault T, Arzul I (2016) Flat oyster follows the apoptosis pathway to defend against the protozoan parasite *Bonamia ostreae.* Fish Shellfish Immunol 56:322–329
Gogichaeva NV, Williams T, Alterman MA (2007) MALDI TOF/TOF tandem mass spectrometry as a new tool for amino acid analysis. J Am Soc Mass Spectrom 18: 279–284
Gonzalez M, Romestand B, Fievet J, Huvet A, Lebart MC, Gueguen Y, Bachère E (2005) Evidence in oyster of a plasma extracellular superoxide dismutase which binds LPS. Biochem Biophys Res Commun 338:1089–1097
Goodall CP, Bender RC, Broderick EJ, Bayne CJ (2004) Constitutive differences in Cu/Zn superoxide dismutase mRNA levels and activity in hemocytes of *Biomphalaria glabrata* (Mollusca) that are either susceptible or resistant to *Schistosoma mansoni* (Trematoda). Mol Biochem Parasitol 137:321–328
Grabowski JH, Peterson CH (2007) Restoring oyster reefs to recover ecosystem services. In: Cuddington K, Byers JE, Wilson WG, Hastings A (eds) Ecosystem engineers: concepts, theory and applications. Elsevier/Academic Press, Amsterdam, p 281–298
Green TJ, Dixon TJ, Devic E, Adlard RD, Barnes AC (2009) Differential expression of genes encoding anti-oxidant enzymes in Sydney rock oysters, *Saccostrea glomerata* (Gould) selected for disease resistance. Fish Shellfish Immunol 26:799–810
Green TJ, Chataway T, Melwani AR, Raftos DA (2016) Proteomic analysis of hemolymph from poly(I:C)-stimulated *Crassostrea gigas.* Fish Shellfish Immunol 48:39–42
Grizel H, Bachere E, Mialhe E, Tige G (1987) Solving parasite-related problems in cultured molluscs. Int J Parasitol 17:301–308
Gruenheid S, Finlay BB (2003) Microbial pathogenesis and cytoskeletal function. Nature 422:775–781
Hervio D, Bachere E, Mialhe E, Grizel H (1989) Chemiluminescent responses of *Ostrea edulis* and *Crassostrea gigas* hemocytes to *Bonamia ostreae* (Ascetospora). Dev Comp Immunol 13:449

Hine PM (1991) The annual pattern of infection by *Bonamia* sp. in New Zealand flat oysters, *Tiostrea chilensis.* Aquaculture 93:241–251

Hong Y, Peng J, Jiang W, Fu Z and others (2011) Proteomic analysis of *Schistosoma japonicum* schistosomulum proteins that are differentially expressed among hosts differing in their susceptibility to the infection. Mol Cell Proteomics 10:M110.006098

Huan P, Wang H, Liu B (2011) Comparative proteomic analysis of challenged Zhikong scallop (*Chlamys farreri*): a new insight into the anti-*Vibrio* immune response of marine bivalves. Fish Shellfish Immunol 31: 1186–1192

Hughes FM, Foster B, Grewal S, Sokolova IM (2010) Apoptosis as a host defense mechanism in *Crassostrea virginica* and its modulation by *Perkinsus marinus.* Fish Shellfish Immunol 29:247–257

Ji C, Wu H, Wei L, Zhao J, Wang Q, Lu H (2013) Responses of *Mytilus galloprovincialis* to bacterial challenges by metabolomics and proteomics. Fish Shellfish Immunol 35:489–498

Jones CG, Lawton JH, Shachak M (1994) Organisms as ecosystem engineers. Oikos 69:373–386

Jorge I, Navarro RM, Lenz C, Ariza D, Porras C, Jorrín J (2005) The holm oak leaf proteome: analytical and biological variability in the protein expression level assessed by 2-DE and protein identification tandem mass spectrometry *de novo* sequencing and sequence similarity searching. Proteomics 5:222–234

Kaji H, Tsuji T, Mawuenyega KG, Wakamiya A, Taoka M, Isobe T (2000) Profiling of *Caenorhabditis elegans* proteins using two-dimensional gel electrophoresis and matrix assisted laser desorption/ionization-time of flight-mass spectrometry. Electrophoresis 21:1755–1765

Kang YS, Kim YM, Park KI, Kim Cho S, Choi KS, Cho M (2006) Analysis of EST and lectin expressions in hemocytes of Manila clams (*Ruditapes philippinarum*) (Bivalvia: Mollusca) infected with *Perkinsus olseni.* Dev Comp Immunol 30:1119–1131

Kapitulnik J, Maines MD (2009) Pleiotropic functions of biliverdin reductase: cellular signaling and generation of cytoprotective and cytotoxic bilirubin. Trends Pharmacol Sci 30:129–137

Kinsey ST, Lee BC (2003) The effects of rapid salinity change on *in vivo* arginine kinase flux in the juvenile blue crab, *Callinectes sapidus.* Comp Biochem Physiol B Biochem Mol Biol 135:521–531

Koopman WJH, Nijtmans LGJ, Dieteren CEJ, Roestenberg P, Valsecchi F, Smeitink JAM, Willems PHGM (2010) Mammalian mitochondrial complex I: biogenesis, regulation, and reactive oxygen species generation. Antioxid Redox Signal 12:1431–1470

Korringa P (1976) Farming the flat oysters of the genus *Ostrea*: a multidisciplinary treatise. Elsevier Publishing Company, Amsterdam

Krauss S, Brand MD, Buttgereit F (2001) Signaling takes a breath — new quantitative perspectives on bioenergetics and signal transduction. Immunity 15:497–502

Lacoste A, Malham SK, Cueff A, Poulet SA (2001) Noradrenaline modulates oyster hemocyte phagocytosis via a beta-adrenergic receptor-cAMP signaling pathway. Gen Comp Endocrinol 122:252–259

Lillig CH, Holmgren A (2007) Thioredoxin and related molecules — from biology to health and disease. Antioxid Redox Signal 9:25–47

Liska AJ, Shevchenko A (2003) Expanding the organismal scope of proteomics: cross-species protein identification by mass spectrometry and its implications. Proteomics 3: 19–28

Lockyer AE, Spinks J, Kane RA, Hoffmann KF and others (2008) *Biomphalaria glabrata* transcriptome: cDNA microarray profiling identifies resistant- and susceptible-specific gene expression in haemocytes from snail strains exposed to *Schistosoma mansoni.* BMC Genomics 9:634

Lockyer AE, Emery AM, Kane RA, Walker AJ and others (2012) Early differential gene expression in haemocytes from resistant and susceptible *Biomphalaria glabrata* strains in response to *Schistosoma mansoni.* PLOS ONE 7:e51102

Lotze HK, Lenihan HS, Bourque BJ, Bradbury RH and others (2006) Depletion, degradation, and recovery potential of estuaries and coastal seas. Science 312:1806–1809

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

Lynch SA, Armitage DV, Coughlan J, Mulcahy MF, Culloty SC (2007) Investigating the possible role of benthic macroinvertebrates and zooplankton in the life cycle of the haplosporidian *Bonamia ostreae.* Exp Parasitol 115: 359–368

Lynch SA, Flannery G, Hugh-Jones T, Hugh-Jones D, Culloty SC (2014) Thirty-year history of Irish (Rossmore) *Ostrea edulis* selectively bred for disease resistance to *Bonamia ostreae.* Dis Aquat Org 110:113–121

Martín-Gómez L, Villalba A, Abollo E (2012) Identification and expression of immune genes in the flat oyster *Ostrea edulis* in response to bonamiosis. Gene 492:81–93

Martín-Gómez L, Villalba A, Carballal MJ, Abollo E (2014) Cloning and characterization of neoplasia-related genes in flat oyster *Ostrea edulis.* Infect Genet Evol 23:138–149

Martins JC, Campos A, Osório H, da Fonseca R, Vasconcelos V (2014) Proteomic profiling of cytosolic glutathione transferases from three bivalve species: *Corbicula fluminea, Mytilus galloprovincialis* and *Anodonta cygnea.* Int J Mol Sci 15:1887–1900

Masood M, Raftos DA, Nair SV (2016) Two oyster species that show differential susceptibility to virus infection also show differential proteomic responses to generic dsRNA. J Proteome Res 15:1735–1746

May RC, Machesky LM (2001) Phagocytosis and the actin cytoskeleton. J Cell Sci 114:1061–1077

McDowell IC, Nikapitiya C, Aguiar D, Lane CE, Istrail S, Gomez-Chiarri M (2014) Transcriptome of American oysters, *Crassostrea virginica*, in response to bacterial challenge: insights into potential mechanisms of disease resistance. PLOS ONE 9:e105097

Monsinjon T, Knigge T (2007) Proteomic applications in ecotoxicology. Proteomics 7:2997–3009

Morga B, Arzul I, Chollet B, Renault T (2009) Infection with the protozoan parasite *Bonamia ostreae* modifies in vitro haemocyte activities of flat oyster *Ostrea edulis.* Fish Shellfish Immunol 26:836–842

Morga B, Arzul I, Faury N, Segarra A, Chollet B, Renault T (2011a) Molecular responses of *Ostrea edulis* haemocytes to an in vitro infection with *Bonamia ostreae.* Dev Comp Immunol 35:323–333

Morga B, Renault T, Faury N, Chollet B, Arzul I (2011b) Cellular and molecular responses of haemocytes from *Ostrea edulis* during in vitro infection by the parasite *Bonamia ostreae.* Int J Parasitol 41:755–764

Morga B, Renault T, Faury N, Arzul I (2012) New insights in flat oyster *Ostrea edulis* resistance against the parasite *Bonamia ostreae.* Fish Shellfish Immunol 32:958–968

Morga B, Renault T, Faury N, Lerond S and others (2017) Contribution of *in vivo* experimental challenges to understanding flat oyster *Ostrea edulis* resistance to *Bonamia ostreae.* Front Cell Infect Microbiol 7:433

Naciri-Graven Y, Martin AG, Baud JP, Renault T, Gérard A (1998) Selecting the flat oyster *Ostrea edulis* (L.) for survival when infected with the parasite *Bonamia ostreae.* J Exp Mar Biol Ecol 224:91–107

Naciri-Graven Y, Haure J, Gérard A, Baud JP (1999) Comparative growth of *Bonamia ostreae* resistant and wild flat oyster *Ostrea edulis* in an intensive system II. Second year of the experiment. Aquaculture 171:195–208

Pales Espinosa E, Corre E, Allam B (2014) Pallial mucus of the oyster *Crassostrea virginica* regulates the expression of putative virulence genes of its pathogen *Perkinsus marinus.* Int J Parasitol 44:305–317

Pardo BG, Álvarez-Dios JA, Cao A, Ramilo A and others (2016) Construction of an *Ostrea edulis* database from genomic and expressed sequence tags (ESTs) obtained from *Bonamia ostreae* infected haemocytes: development of an immune-enriched oligo-microarray. Fish Shellfish Immunol 59:331–344

Perrigault M, Tanguy A, Allam B (2009) Identification and expression of differentially expressed genes in the hard clam, *Mercenaria mercenaria,* in response to quahog parasite unknown (QPX). BMC Genomics 10:377

Pichot Y, Comps M, Tigé G, Grizel H, Rabouin MA (1980) Recherches sur *Bonamia ostreae* gen. n., sp. n., parasite nouveau d l'huître plate *Ostrea edulis* L. Rev Trav Inst Peches Marit 43:131–140

Prado-Alvarez M, Gestal C, Novoa B, Figueras A (2009) Differentially expressed genes of the carpet shell clam *Ruditapes decussatus* against *Perkinsus olseni.* Fish Shellfish Immunol 26:72–83

Prasad SV, Hepat R, Kim Y (2014) Selectivity of a translation-inhibitory factor, CpBV15β, in host mRNAs and subsequent alterations in host development and immunity. Dev Comp Immunol 44:152–162

Raman J, Mehrotra S, Anand RP, Balaram H (2004) Unique kinetic mechanism of *Plasmodium falciparum.* Mol Biochem Parasitol 138:1–8

Ramilo A, Navas JI, Villalba A, Abollo E (2013) Species-specific diagnostic assays for *Bonamia ostreae* and *B. exitiosa* in European flat oyster *Ostrea edulis*: conventional, real time and multiplex PCR. Dis Aquat Org 104:149–161

Ramilo A, González M, Carballal MJ, Darriba S, Abollo E, Villalba A (2014) Oyster parasites *Bonamia ostreae* and *B. exitiosa* co-occur in Galicia (NW Spain): spatial distribution and infection dynamics. Dis Aquat Org 110: 123–133

Rottner K, Lommel S, Wehland J, Stradal TEB (2004) Pathogen-induced actin filament rearrangement in infectious diseases. J Pathol 204:396–406

Schaumburg F, Hippe D, Vutova P, Lüder CGK (2006) Pro- and anti-apoptotic activities of protozoan parasites. Parasitology 132:S69–S85

Shevchenko A, Wilm M, Vorm O, Mann M (1996) Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. Anal Chem 68:850–858

Shi X, Wang L, Zhou Z, Yang C, Gao Y, Wang L, Song L (2012) The arginine kinase in Zhikong scallop *Chlamys farreri* is involved in immunomodulation. Dev Comp Immunol 37:270–278

Simonian M, Nair SV, O'Connor WA, Raftos DA (2009a) Protein markers of *Marteilia sydneyi* infection in Sydney rock oysters, *Saccostrea glomerata.* J Fish Dis 32: 367–375

Simonian M, Nair SV, Nell JA, Raftos DA (2009b) Proteomic clues to the identification of QX disease-resistance biomarkers in selectively bred Sydney rock oysters, *Saccostrea glomerata.* J Proteomics 73:209–217

Sokolova IM (2009) Apoptosis in molluscan immune defense. Invertebr Surviv J 6:49–58

Soudant P, Chu FE, Volety A (2013) Host–parasite interactions: marine bivalve molluscs and protozoan parasites, *Perkinsus* species. J Invertebr Pathol 114:196–216

Sun Z, Yang C, Wang L, Wang X and others (2014) The protein expression profile in hepatopancreas of scallop *Chlamys farreri* under heat stress and *Vibrio anguillarum* challenge. Fish Shellfish Immunol 36: 252–260

Tanguy A, Guo X, Ford SE (2004) Discovery of genes expressed in response to *Perkinsus marinus* challenge in eastern (*Crassostrea virginica*) and Pacific (*C. gigas*) oysters. Gene 338:121–131

Vaibhav V, Lepretre M, Thompson E, Raftos D, Haynes PA (2016) Biomarkers of winter mortality resistance in selectively bred Sydney rock oysters (*Saccostrea glomerata*). Aquaculture 465:323–329

Valavanis C, Wang Z, Sun D, Vaine M, Schwartz LM (2007) Acheron, a novel member of the Lupus Antigen family, is induced during the programmed cell death of skeletal muscles in the moth *Manduca sexta.* Gene 393:101–109

van Banning P (1991) Observations on bonamiosis in the stock of the European flat oyster, *Ostrea edulis*, in the Netherlands, with special reference to the recent developments in Lake Grevelingen. Aquaculture 93: 205–211

van Horssen R, Janssen E, Peters W, van de Pasch L and others (2009) Modulation of cell motility by spatial repositioning of enzymatic ATP/ADP exchange capacity. J Biol Chem 284:1620–1627

van Rensburg MJ, Coyne VE (2009) The role of electron transport in the defence response of the South African abalone, *Haliotis midae.* Fish Shellfish Immunol 26: 171–176

Villalba A, da Silva PM, Fuentes J (2007) La producción de estirpes de *Ostrea edulis* tolerantes a la bonamiosis como solución para el engorde de la ostra plana en Galicia. In: Cerviño-Eiroa A, Guerra-Díaz A, Pérez-Acosta C (eds) XI Congreso Nacional de Acuicultura. Centro de Investigacións Mariñas, Consellería de Pesca e Asuntos Marítimos, Xunta de Galicia, Vilanova de Arousa, p 1499–1504

Villalba A, Ramilo A, Abollo E, Fuentes J (2010) Mass mortality of European flat oyster *Ostrea edulis* spat associated with a viral infection in Galicia (NW Spain). In: Aquaculture Europe 10, October 2010, Porto. European Aquaculture Society, Oostende, p 1380–1381

Wang L, Song L, Zhao J, Qiu L and others (2009) Expressed sequence tags from the zhikong scallop (*Chlamys farreri*): discovery and annotation of host-defense genes. Fish Shellfish Immunol 26:744–750

Wu H, Ji C, Wei L, Zhao J, Lu H (2013) Proteomic and metabolomic responses in hepatopancreas of *Mytilus*

galloprovincialis challenged by *Micrococcus luteus* and *Vibrio anguillarum.* J Proteomics 94:54–67

Zahoor Z, Lockyer AE, Davies AJ, Kirk RS and others (2014) Differences in the gene expression profiles of haemocytes from schistosome-susceptible and -resistant *Biomphalaria glabrata* exposed to *Schistosoma mansoni* excretory-secretory products. PLOS ONE 9: e93215

Zhang H, Song L, Li C, Zhao J and others (2008) A novel C1q-domain-containing protein from Zhikong scallop *Chlamys farreri* with lipopolysaccharide binding activity. Fish Shellfish Immunol 25:281–289

Zhang L, Li L, Zhang G (2011) Gene discovery, comparative analysis and expression profile reveal the complexity of the *Crassostrea gigas* apoptosis system. Dev Comp Immunol 35:603–610

Zhang L, Li L, Zhu Y, Zhang G, Guo X (2014) Transcriptome analysis reveals a rich gene set related to innate immunity in the eastern oyster (*Crassostrea virginica*). Mar Biotechnol 16:17–33

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