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# A proteomic approach envisaged to analyse the bases of oyster tolerance/resistance to bonamiosis

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ABSTRACT

The European flat oyster *Ostrea edulis* is highly susceptible to infection by the protozoan *Bonamia ostreae*, an intracellular parasite able to survive and proliferate within the oyster haemocytes. On the contrary, the Pacific cupped oyster *Crassotrea gigas* is resistant. Two-dimensional electrophoresis was adapted to analyse the proteins of the oyster haemolymph. Comparisons of the haemolymph protein profiles between the two oyster species and between *O. edulis* stocks with different susceptibility to bonamiosis were performed. Differences in the number of proteins detected from each oyster species/stock were recorded; the highest number corresponded to the species resistant to bonamiosis, whereas the lowest number corresponded to the most susceptible *O. edulis* stock. Protein spots exclusive to each species and stock were detected; identification of those proteins could help to understand the key of tolerance/resistance to bonamiosis. Comparison between "healthy" and *B. ostreae* infected oysters was also performed. A substantial reduction of the number of proteins in the oyster haemolymph associated with *B. ostreae* infection was recorded. Protein spots exclusive to healthy and infected groups were detected; identification of haemolymph proteins whose synthesis is induced, repressed, up regulated or down regulated should help to understand the inability of *O. edulis* to neutralise or overcome *B. ostreae* infection. The results support a promising utility of 2-D electrophoresis, applied to the analysis of haemolymph proteins, to understand the oyster–*B. ostreae* interaction and to find the bases of tolerance/resistance to bonamiosis.



## 1. Introduction

Haemocytes, the circulating cells of haemolymph, and humoral factors play an important role in the internal defence of bivalve molluscs (Cheng, 1981). Phagocytosis of foreign particles, pathogens among them, by haemocytes is one of the main immune mechanisms of bivalve molluscs (Cheng, 1981; Fisher, 1986; Carballal et al., 1997; López et al., 1997). Nevertheless, the protozoan *Bonamia ostreae* can survive and multiply inside *Ostrea edulis* haemocytes after being phagocytosed (Comps et al., 1980; Balouet et al., 1983; Chagot et al., 1992; Mourton et al., 1992; Montes et al., 1994). The infection by *B. ostreae* is the most important constraint for the European flat oyster *O. edulis* industry because it causes oyster mass mortalities (Tigé et al., 1982; Hudson and Hill, 1991; van Banning, 1991; Culloty and Mulcahy, 2001a,b; da Silva et al., 2005). As a consequence, oyster growers from many European areas affected by bonamiosis moved to cultivate the exotic oyster species *Crassostrea gigas* that is resistant to this disease (Renault et al., 1995). The company "Atlantic Shellfish Ltd." has produced a European flat oyster strain "Rossmore oysters" through selective breeding, which has displayed increased tolerance to the parasite compared to other European stocks of flat oyster (Culloty et al., 2001; 2004). However, the physiological bases of the differences in susceptibility between oyster species and between flat oyster stocks are unknown. The comparisons of some haemolymph parameters between oysters with different susceptibility to bonamiosis and between infected and non infected oysters have not provided definitive answers thus far (Fisher, 1988; Chagot, 1989; Bachère et al., 1991; Chagot et al., 1992; Mourton et al., 1992; Xue and Renault, 2000; Cronin et al., 2001; Cochennec-Laureau et al., 2003; Comesaña, 2008; da Silva et al., 2008).

The global analysis of protein expression profiles might be invaluable for obtaining a more complete understanding of biological events, such as, development, evolution and pathogenicity (Jungblut et al., 1999, Pennington, 1997). Two-dimensional electrophoresis (2-DE) is a widely used method for analysing complex protein mixtures extracted from cells, tissues, or other biological samples. It was originally described by Klose and O'Farrell (Klose, 1975; O'Farrel, 1975) and involves separation of proteins according to their isoelectric points (pI) and relative molecular masses (Mr). The 2-DE is a unique method for large-scale protein characterisation and, combined with mass spectrometry, allows identification of the protein repertoire of specific tissues (Wilkins et al., 1996). The goal of proteomics is to obtain a comprehensive and

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quantitative description of protein expression and its changes under the influence of biological perturbations (Anderson and Anderson, 1998). Thus, complexity of a biological system can be approached in its entirety as proteomics allows a multiplicity of proteins to be studied simultaneously (Williams, 1999). In the case of molluscs, this technology has been used occasionally to detect differences in protein expression between mussel populations from different environments (López et al., 2001) and between mussels from pure and hybrid crosses from three genetically divergent European populations (Fuentes et al., 2002); to evaluate the genetic variability (Mosquera et al., 2003) and to search for specific larval proteins (López et al., 2005) of the mussel *Mytilus galloprovincialis*; and to analyse the changes in protein expression in molluscs exposed to environmental pollutants (Shepard et al., 2000; Rodríguez-Ortega et al., 2003; Olsson et al., 2004; McDonagh et al., 2005; Dowling et al., 2006; Mi et al., 2007). Vergote et al. (2005) investigated the involvement of haemolymph plasmatic factors in the snail *Biomphalaria glabrata* resistance/susceptibility to the trematode *Echinostoma caproni* larval stages by comparing protein patterns of plasma collected from susceptible and resistant snails.

On the other hand, developments of functional genomics (subtractive suppressive hybridization (SSH) and oligonucleotide microarrays among them) allow identification of up and down regulated genes expressed in specific tissues under particular circumstances. In the last years large amount of work has been conducted in the production of expressed sequence tags in bivalve molluscs (Carlsson and Reece, 2007; Jenny et al., 2007; Quilang et al., 2007, Yu and Li, 2007, 2008; Wang et al., 2008; Shi et al., 2009). These genomic approaches are being used to identify genes related with the immune response of the bivalve molluscs (Gueguen et al., 2003; Tanguy et al., 2004; Gestal et al., 2007; Tanguy et al., 2008; Prado-Alvarez et al., 2009). Candidate genes involved in the tolerance to bonamiosis are being identified using a SSH approach in current studies (Martin-Gómez et al., 2008; Morga et al., 2008). Information derived from genomics and proteomic approaches are complementary, thus the combination of both approaches facilitates comprehensive views.

In the present work, a proteomic approach was applied to evaluate if the differences in susceptibility to bonamiosis are associated with haemolymph protein expression patterns and to explore the utility of this approach to find the bases of the tolerance to bonamiosis. The study includes comparative analysis, through two-dimensional electrophoresis (2-DE), of protein expression maps of the haemolymph of the resistant species *C. gigas* and three *O. edulis* stocks with different susceptibility to the disease. Comparison between "healthy" and *B. ostreae* infected oysters is also provided.

## 2. Materials and methods

### 2.1. Oyster sampling

Three different oyster stocks of the susceptible species *O. edulis* and one of the resistant oyster *C. gigas* were used for comparisons. Cupped oysters *C. gigas* that had been grown from a raft in the Ría de Arousa (Galicia, NW Spain) were collected for the study. The *O. edulis* stocks were: (1) "Rossmore", that has been produced through selective breeding for increased resistance within a programme involving the use of survivors from an area heavily affected by bonamiosis close to Cork harbour (Ireland) as broodstock and repeating the process through several generations (Culloty et al., 2001); (2) "Tralee", naïve oysters from Tralee Bay (Ireland) where the parasite has not previously been observed; and (3) "Ortigueira", oysters from a natural bed in the Ría de Ortigueira (Galicia, NW Spain) that is heavily affected by bonamiosis since early 1980s; bonamiosis has exerted a selective pressure probably favouring increased tolerance in oysters of that population (da Silva et al., 2005). Unfortunately, there was no natural flat oyster population in Galicia that could be reputed as free of *B. ostreae*, therefore no naïve Galician stock could be involved in the experiment. On December 2003, around 1000 "Rossmore" oysters, 1000 "Tralee" oysters, 250 "Ortigueira" oysters and 500 *C. gigas* were set in trays and hung from a raft located in an area of the Ría de Arousa affected by bonamiosis. All the oysters were 2+ years old. On July 2004, oysters from each species/stock were randomly taken from the raft and carried to the laboratory for analysis. The oysters were kept for 24 h in a tank with running seawater before bleeding. A longer stay in the tank could have depressed the functionality of oyster haemocytes. The hinge of every oyster was severed with a knife, then the shell was slightly opened with a wedge (a disposable pipette tip) and haemolymph was withdrawn from the adductor muscle using a 21-Gauge needle attached to a 1 ml syringe; the haemolymph was transferred into a vial (one vial per oyster) and kept cold on crushed ice until used. A drop of haemolymph was observed with light microscopy to assess whether it was clean or contaminated with gametes or debris from pallial cavity; contaminated haemolymph was discarded. Then 150 µl of haemolymph were centrifuged (92×*g*, 5 min, 4 °C) onto a slide using a cytocentrifuge. The resulting haemolymph cell monolayer was fixed and stained with a Hemacolor Kit (Merck). The haemolymph cell monolayers were examined with light microscopy to diagnose *B. ostreae* infection (da Silva and Villalba, 2004). In total, 8 "Ortigueira" oysters, 9 "Tralee" oysters, 56 "Rossmore" oysters and 12 *C. gigas* were examined and just two oysters were found infected by *B. ostreae*; the infection was heavy in both cases. The haemolymph of 6 "healthy" oysters of each stock was pooled, whereas the haemolymph from the 2 *B. ostreae* infected oysters (one from Rossmore and another from Ortigueira) was individually kept. Thus, 6 haemolymph (oyster) groups were available for comparisons: *C. gigas*, healthy Rossmore, healthy Tralee, healthy Ortigueira, *B. ostreae* infected Rossmore and *B. ostreae* infected Ortigueira. No oyster from the Tralee stock was found infected. The haemolymph groups were frozen and lyophilised, and then stored at −80 °C until electrophoresis.

### 2.2. Protein extraction

Proteins were extracted by suspending lyophilised haemolymph immediately in lysis buffer [7.6 M urea, 2 M thiourea, 4%Chaps, 0.2% ampholytes, pH 7 to 10 and 100 mM dithiothreitol (DTT)]. Proteins were solubilised for 4 h at 4 °C with vigorous shaking. The mixture was centrifuged at 12,000×*g* for 30 min. The supernatant was desalted by dialysis and purified by removing albumin (Affi-gel® Blue Gel from Bio-Rad). Samples were either used immediately for electrophoresis or stored at −80 °C. Protein concentration was measured according to the method of Bradfod (1976).

### 2.3. Two-dimensional gel electrophoresis

For iso-electric focusing (IEF), samples of 100 µg of proteins in 250 µl of rehydration buffer [7 M urea. 2 M thiourea, 4% Chaps, bromophenol blue traces, 0.3% DTT and 0.5% immobilized linear pH gradient (IPG)-buffer] were applied in the strip holder channel. The solubilised proteins were loaded onto an IPG strip, pH 5–8, 11 cm (Bio-Rad). Thus the IPG strips were lowered, gel side down, onto the oil. Iso-electric focusing was performed in a horizontal apparatus (Protean®IEF System from Bio-Rad) at 20 °C as follows: 50 V was applied during the rehydration step for improving entry of high molecular weight proteins of the sample into the polyacrylamide gel (Görg et al., 1999).

IEF was then started with 250 V for 15 min, and finally 8000 V until 35 kVh was achieved. After iso-electric focusing the strips were equilibrated for two intervals of 15 min in equilibration buffer [6 M urea, 50 mM Tris, 2% sodium dodecylsulfate (SDS), 30% glycerol]. For the first equilibration step, dithiothreitol 1% (w/v) was added to reduce the proteins. Thereafter the proteins were carbamidometlylated with 2.5% (w/v) iodoacetamide. The equilibrated IPG strips were transferred for the second dimension (SDS-PAGE) onto 13.5% gels. Second dimension electrophoresis was carried out at 15 mA per gel for 15 min followed by 50 mA per gel for 4 h. The SDS-PAGE gel was then silver