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# Gene expression analysis of *Ruditapes philippinarum* haemocytes after experimental *Perkinsus olseni* zoospore challenge and infection in the wild

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## ABSTRACT

The production of Manila clam (*Ruditapes philippinarum*) is seriously threatened by the protistan parasite *Perkinsus olseni*. We characterized and compared gene expression of Manila clam haemocytes in response to *P. olseni* in a time-course (10 h, 24 h, 8 d) controlled laboratory challenge (LC), representing the first step of infection, and in a more complex infection in the wild (WI), using a validated oligo-microarray containing 11,232 transcripts, mostly annotated. Several immune-genes involved in NIK/NF-kappaB signalling, Toll-like receptor signalling and apoptosis were activated at LC-10 h. However, down-regulation of genes encoding lysozyme, histones, cathepsins and heat shock proteins indicated signals of immunodepression, which persisted at LC-24 h, when only down-regulated genes were detected. A rebound of haemocyte activity occurred at LC-8 d as shown by up-regulation of genes involved in cytoskeleton organization and cell survival. The WI study showed a more complex picture, and several immune-relevant processes including cytoskeleton organization, cell survival, apoptosis, encapsulation, cell redox- and lipid-homeostasis were activated, illustrating the main mechanism of host response. Our results provide useful information, including potential biomarkers, to develop strategies for controlling Manila clam perkinsosis.

*Abbreviations:* *A2ML1*, Alpha-2-macroglobulin; *ABL1*, Tyrosine-protein kinase abl1; *ACTA2*, Alpha-2 actin; *ACTB*, Beta-actin; *ALG1*, Chitobiosyldiphosphodolichol beta-mannosyltransferase; *APOD*, Apolipoprotein D; *ATOX1*, Copper transport protein ATOX1; *ATP5A1*, Mitochondrial ATP synthase alpha subunit; *ATP5B*, Mitochondrial ATP synthase beta subunit; *BIRC2*, Baculoviral IAP repeat-containing protein 2; *C1q*, C1q domain containing protein; *C1QL4*, Complement c1q-like protein 4; *CAPRIN1*, Caprin-1; cDNA, Complementary deoxyribonucleic acid; *CTSK*, Cathepsin K; *CTSL*, Cathepsin L; *CYP450*, Cytochrome P450; *DDOST*, Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 4; DE, Differentially expressed; *EEF1G*, Elongation factor 1-gamma; FC, Fold change; FDR, False discovery rate; *FGR*, Tyrosine-protein kinase Fgr; *FRMPD2*, FERM and PDZ domain-containing protein 2; *FHOD3*, fh1 fh2 domain-containing protein 3; *GHITM*, Growth hormone-inducible transmembrane protein; GO, Gene ontology; HC, Hierarchical cluster; *HIST3H3*, Histone H3.3; HS-transcriptome, Manila clam transcriptome reported by Hasanuzzaman et al.; *HSP*, Heat shock protein; *HSPA8*, Heat shock cognate 71 kda; *IkB*, Inhibitor of NF-kB; *IRF2*, Interferon regulatory factor 2; LC, Laboratory challenge; *LRP2*, Low-density lipoprotein receptor-related protein 2; *MACF*, Microtubule-actin cross-linking factor; ML-transcriptome, Manila clam transcriptome reported by Milan et al.; MR-transcriptome, Manila clam transcriptome reported by Moreira et al.; mRNA, Messenger ribonucleic acid; *MT-CYB*, Cytochrome b; *ND4*, NADH dehydrogenase subunit 4; *NDUFS4*, NADH dehydrogenase [ubiquinone] iron-sulfur protein 4, mitochondrial; PCA, Principal component analysis; *PCNA*, Proliferating cell nuclear antigen; *PSAP*, Saposin; qPCR, Quantitative real-time PCR; *RPL18*, 60S ribosomal protein L18; *SAA*, Serum amyloid A; SD, Standard deviation; *SPTSSB*, Small subunit of serine palmitoyltransferase B; *ST14*, Suppressor of tumorigenicity 14 protein homolog; *TMSB4*, Thymosin beta-4; *TPx*, Thioredoxin peroxidase; *TXNDC17*, Thioredoxin domain-containing protein 17; *TLR*, Toll-like receptor; *TUBA3*, Tubulin alpha-3 chain; *TUBB*, Tubulin beta; *UBA52*, Ubiquitin-60s ribosomal protein l40; *UBB*, Ubiquitin B; *UBE2D3*, Ubiquitin-conjugating enzyme e2; *USPA*, Universal stress protein A; WI, Wild infection

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## 1. Introduction

Manila clam (*Ruditapes philippinarum*; Adams and Reeve, 1850), also known as Japanese carpet shell, is a commercially important species (more than 4.0 Mt world production in 2015 [1]) mainly produced in China (> 97% production). In European clam aquaculture, Manila clam is the top contributor to clam landings, and it has become the most important shellfishery in Galicia (NW Spain) with a production of 1.8 tonnes in 2015 (official records in www.pescadegalicia.com). But, Manila clam production is at risk from diseases, particularly perkinsosis caused by the protistan parasites *Perkinsus* spp. [2,3]. Due to perkinsosis, caused by *Perkinsus olseni*, high mortality of Manila clam has been reported in Europe [4] and Asia [5,6] and, accordingly, this parasite has been included in the list of notifiable diseases of the World Organisation for Animal Health (http://www.oie.int/en/animal-health-in-the-world/oie-listed-diseases-2015/).

The life cycle of *P. olseni* includes parasitic and free stages: the trophozoites proliferate within the host through palintomy; these trophozoites turn into resistant cysts (called hypnospores) because of unfavourable environment in the host and/or host death; finally, these cysts undergo zoosporulation when being in contact with seawater, each producing hundreds of biflagellate zoospores; every life stage is infective [2,7]. There are studies of perkinsosis in Manila clam addressing different biological issues such as infection prevalence and intensity [8]; interference of growth and reproduction [9,10]; and immune-responses including haemocytes infiltration [11,12], polypeptide synthesis, lectin expression, phenoloxidase activity, antimicrobial activity and serine protease inhibition [13–22].

Like other molluscs, Manila clam has an innate immune system composed of cell-mediated defence, principally carried out by freely circulating haemocytes throughout all tissues, and humoral factors. The haemocytes of infected Manila clam secrete humoral factors including lectins and antimicrobial peptides [15–19,23–25]. In response to *P. olseni* infection, Manila clam haemocytes may promote phagocytosis and/or encapsulation of the parasite [26], mediated by secreted polypeptides [13,14] after parasite opsonisation by lectins [16–19,24]. Recently, gene expression in *R. philippinarum* haemocytes challenged with *P. olseni* trophozoites [21] and transcriptomic profile of Manila clam haemocytes exposed to *P. olseni* [27] have reported innate immune responses including pathogen recognition, cell adhesion and apoptosis.

Despite these aforesaid studies of perkinsosis in Manila clam, more research is yet needed to understand the molecular mechanisms underlying *R. philippinarum*-parasite interaction in perkinsosis. Specifically, it is not well known how Manila clam responds to different infectious cells of *P. olseni* in natural beds [3], where different environmental factors and re-infection phenomenon make the infection process complex. This important issue can now be addressed by conducting challenge tests in controlled conditions, given the feasibility of producing millions of zoospores *in vitro* from heavily infected clam tissues [7] and the currently available refined control of the *P. olseni in vitro* culture [28]. Using microarrays, a universal tool for analysing mRNA expression of thousands of genes simultaneously, consistent gene expression profiles can be obtained to understand the molecular response of Manila clam.

In the present work, based on the reported transcriptome databases in Manila clam [23,27,29], we developed and validated an immune-enriched haemocyte oligo-microarray. This oligo-microarray was used to evaluate and compare for the first time the gene expression of Manila clam haemocytes in response to *P. olseni* in two different scenarios: (i) *in vivo*, controlled laboratory challenge (LC) of naïve clams with *P. olseni* zoospores along a time series, and (ii) wild infection (WI). The comparison of gene expression profiles obtained in both scenarios aided us to understand the progress of infection and the molecular response of natural infected clams, enriching our knowledge on perkinsosis for its prevention and control.

## 2. Materials and methods

### 2.1. Sampling and RNA extraction

The study was carried out on Manila clams naturally infected by *P. olseni* in a natural bed (wild infection, WI) and after a laboratory challenge (LC) in controlled conditions accomplished at the facilities of the Centro de Investigacións Mariñas (CIMA, Spain). For the WI study, 200 market-sized (≥40 mm in length, + 2 years old) adult Manila clams were collected from a *P. olseni* affected sea bed (Placeres, Ría de Pontevedra, NW Spain). The level of infection was determined using gill lamellae of each clam processed following the RFTM method [30] and ranked from 0 (null infection) to 5 (very heavy infection) according to the infection intensity scale of Mackin [31] adapted by Villalba et al. [32] for estimating perkinsosis intensity in *Ruditapes decussatus*. The clams with null infection could be considered resistant to *P. olseni* to some extent, because they were exposed to the parasite for more than two years in a *P. olseni*-heavily affected bed. Three haemocyte pools for control and treatment group were obtained by pooling haemocytes from ~10 null-infected and infected clams, respectively. For each infected pool, a mix of haemocytes from three moderately (score 3), five heavily (score 4) and two very heavily (score 5) infected clams was used.

In the case of the LC experiment, clams were collected from a *P. olseni* infection-free natural bed in Camariñas (Galicia, NW Spain) and challenged with *P. olseni* zoospores. The lack of infection was known by previous sampling events and it was further confirmed by PCR [7]. Zoospores were obtained from heavily parasitized grooved carpet-shell clams (*R. decussatus*) following the procedure described [7]. The clams were kept at 14-17 °C in filtered (1 μm) seawater (35‰) with aeration for 1 week to adapt to indoor conditions. Laboratory challenge was performed, mimicking the natural infection path, using one hundred and twenty clams (40-45 mm shell length) which were immersed individually in glasses containing 250 mL of filtered aerated seawater, either without parasite cells (control group, 30 clams) or with a suspension of $1 \times 10^6$ *P. olseni* zoospores (treatment group, 90 clams). After 24 h, the clams were transferred from the individual glasses to two 50 L tanks, one for control and the other for treatment clams, with aerated seawater. Seawater was changed daily and clams were kept for 7 days by feeding daily with a mix of cultured algae (T-Iso and *Skeletonema costatum*). Three haemocyte pools for the control and three for the challenged groups were obtained at each sampling time (10 h, 24 h and 8 d after the onset of challenge). Each pool was made using haemocytes isolated from haemolymph obtained (200 - 1000 μl) from the posterior adductor muscle of each clam. To avoid contamination of haemolymph with gametes or bacteria from unintentional gonad puncture or pallial fluid collection, we eluded touching any other organ during haemolymph collection; besides haemolymph samples contaminated with bacteria or gametes (2.8%) were discarded after light microscope examination. We collected haemolymph samples from several clams, as many as necessary, to reach the required amount of RNA for gene expression analysis. Haemocytes were isolated from plasma by centrifuging haemolymph samples at 800 g for 10 min at 4 °C. The supernatant was then discarded and the pellet resuspended in 750 μL of RNAlater (Qiagen) solution and stored at -80 °C until RNA extraction. Total RNA was extracted from haemocytes pools (~10 clams) using Qiagen RNeasy mini kit with DNase following manufacturer's instructions. RNA quality and quantity were evaluated using a Bioanalyzer (Bonsai Technologies) and a NanoDrop® ND-1000 spectrophotometer (NanoDrop® Technologies Inc), respectively.

### 2.2. Microarray design and analysis

To design a robust *R. philippinarum* oligo-microarray, we considered well-annotated genes of the three previously reported Manila clam transcriptomes: i) the haemocyte transcriptome in response to *P. olseni*