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Immune responses during the larval stages of *Mytilus galloprovincialis*: Metamorphosis alters immunocompetence, body shape and behavior

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ABSTRACT

We investigated the development of the immune system during the larval stages of the mussel *Mytilus galloprovincialis*. The ability of trochophore and veliger larvae to phagocytose foreign particles (*Escherichia coli* and zymosan) was measured. Phagocytosis was detected as early as 24 h post-fertilization (hpf) using flow cytometry and fluorescence microscopy. However, although there was a high basal production of reactive oxygen and nitrogen species (ROS and NRS), the phagocytosis of zymosan did not trigger an associated increase in radical production. In addition, a panel of immune-related mussel genes (Myticin B, Myticin C, Mytilin B, Mytimycin precursor 1, Macrophage migration inhibition factor, lysozyme, C1q, membrane attack complex protein and fibrinogen-related protein) was selected for expression profile analysis throughout the different developmental stages (trochophore, veliger, metamorphosis, post-settlement and spat). The expression of these genes increased during the transition from trochophore to spat, and the level of expression was higher in oocytes than in trochophores, suggesting that gene expression during the first larval stages might be maternal in origin. Metamorphosis was identified as a crucial stage when larvae increased the expression of immune-related genes and responded to environmental signals. Whole-mount *in situ* hybridization studies showed the mantle edge as an important area in the development of immunocompetence in bivalve larvae. Larvae responded to both live and heat-inactivated bacteria by modulating expression of immune-related genes. Altogether, our results support that during the early stages of *M. galloprovincialis* development, immune mechanisms emerge to aid larvae in managing infections.

1. Introduction

Marine invertebrate reproduction generally occurs by the release of a huge number of eggs into the water column and the formation of larvae after egg fertilization. After a period of free living, larvae undergo metamorphosis, transitioning from free-swimming larvae to benthonic postlarvae or juveniles [1]. This mechanism ensures the spread of the species throughout nature and can partially explain the rapid colonization of new habitats by marine animals. Embryonic and larval development are important phylogenetic events because bilateral animals are divided into deuterostomes or protostomes based on the embryonic origin of the mouth and anus. Mollusks are lophotrochozoans, a group characterized by the presence of trochophore larvae [2]. The ontogeny of mollusks is a complex process that is only completed by a small percentage of the original larvae produced after fecundation. The main threats to the completion of the larval life cycle are predation and poor environmental conditions, although larvae also have to manage pathogenic infections [3,4].

Among Protostomia, research into larval immune capacity as well as development, genetics and physiology has focused on model species such as the arthropod *Drosophila* or the nematode *Caenorhabditis*, which belong to the *superphylum* Ecdysozoa [5–7], paying less attention to the *superphylum* Lophotrochozoa, which includes mollusks. Mollusks are an interesting group, not only in terms of aquaculture production [8] but also because they have an important ecological role in the depuration of waters and as environmental sentinels [9]. Additionally, this group includes intermediate hosts for serious parasitic human diseases [10].

Larval settlement and metamorphosis is a vital transition period that is associated with the evolution of metazoans, as well as differentiation and speciation [11]. This stage is also very important because settlement can modify the larval dispersal capacity, leading individuals to settle near their source or to be dispersed with a

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concomitant mortality risk. Therefore, competent and metamorphosing larvae are important for the ecological and evolutionary success of natural populations of marine invertebrates [12]. Even in cultured bivalves, metamorphosis is considered a crucial step for the overall success of the aquaculture facility because animals that have correctly settled usually show increased survival. However, it is unknown if competence is related to the expression of particular genes or if the immune system plays a role in the protection of larval stages. In fact, studies about the gene expression of mollusk larvae during development have been focused mainly on anatomical structural ontogenesis [13,14], and little is known about bivalve larval immune defense or bivalve response to external stimuli [15–17]. Phagocytosis, antimicrobial peptide production and chemical defenses that serve both to control pathogens and to protect larvae from predation have been proposed as immune mechanisms in veliger larvae [18]. It has been reported that larvae initiate metamorphosis when they are developmentally competent to respond to environmental signals [19–21]; however, the factors that regulate this process have been difficult to identify. In this work, we have focused on the immune competence of several developmental stages of *Mytilus galloprovincialis* because immune-related genes are essential for responding to external stimuli. We have identified the immune functions occurring at larval stages and profiled gene expression during each developmental stage to understand this complex process.

2. Materials and methods

2.1. Larval rearing

Mature Mediterranean mussels (*M. galloprovincialis*) were obtained from a commercial shellfish farm (Vigo, Northwestern Spain) during the spawning season. Mussels were subjected to mechanical (brush cleaning) and environmental stress (left at least 1 h without water), and spawning was induced with 1 μm filtered seawater (FSW) at 30 °C containing *Isochrysis galbana*. The mature animals responding to spawning induction were placed in individual containers and oocyte and sperm quality was observed (bright orange color, cell integrity and sperm motility) under a Nikon Optiphot light microscope (Nikon Instruments Inc., NY, USA).

Fertilization was performed by mixing high quality oocytes and sperm at a ratio of 1:10 in a sterile glass container containing 2 L of FSW. After trochophore development for 24 h at 21 °C, larvae were placed into 150 L tanks. Larvae were fed with *I. galbana* during the first week post-fertilization and a combination of *I. galbana* and *Phaeodactylum tricornutum* after the first week. Samples were taken and observed under a Nikon Eclipse E600 light microscope or a Nikon SMZ800 stereomicroscope. Measures were performed on pictures taken with a DXM1200 operated with ACT-1 v2.70 software (Nikon instruments Inc., NY, USA).

2.2. Immune stimulation

Bacterial challenge of larvae was performed with both heat-inactivated and live *Vibrio anguillarum* at all developmental stages. Larvae were stimulated with 10^6 CFU mL^{-1} of either live or inactivated bacterial challenges for 3 h at 15 °C. Three biological replicates of stimulated larvae or controls were used for each stage. Larvae were concentrated, counted and then distributed into 6-well plates at a final volume of 7 mL per well.

2.3. Functional immunology

The capacity of larvae to phagocytose foreign particles and to produce radical oxygen and nitrogen species was studied from 24 to 72 h post-fertilization (hpf). Briefly, *M. galloprovincialis* larvae were mechanically disaggregated in calcium–magnesium free saline (CMFS) buffer (137 mM sodium chloride, 4 mM potassium chloride, 0.4 mM sodium dihydrogen phosphate, 0.2 mM potassium hydrogen phosphate, 12 mM sodium bicarbonate, and 10 mM D-glucose), and the cells were resuspended in FSW prior to flow cytometry experiments.

Phagocytosis assays were performed using the larval cell suspensions and two different fluorescein-labeled (FITC) particles: *Escherichia coli* or zymosan (Molecular Probes, Life Technologies, Carlsbad, CA, USA). Cells and FITC particles were incubated for 90 min at 17 °C in the dark and counterstained with trypan blue to quench non-phagocytosed particles. The level of phagocytosis was determined using a FACSCalibur flow cytometer (Becton and Dickinson, San Jose, CA, USA). In addition, phagocytosis was also studied with fluorescence microscopy using pHrodo-labeled *E. coli* and Texas Red-labeled zymosan (Molecular Probes). Whole larvae were incubated with fluorescent particles as explained above and fixed overnight at 4 °C with 4% paraformaldehyde (Sigma Chem. Co, St. Louis, MO, USA) in FSW. After fixation, larvae were stained with DAPI to visualize nuclei and mounted with ProLong Gold Antifade Reagent (Life Technologies Carlsbad, CA, USA). Larvae were observed with a Leica TCS SPE confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany).

The production of reactive oxygen species (ROS) was measured with flow cytometry using a 5,6-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate probe (CM-H_2DCFDA, Molecular Probes, Life Technologies, Carlsbad, CA, USA). After the probe penetrates the cells, it is oxidized by ROS, producing green fluorescence. Similarly, the production of reactive nitrogen species (RNS) was measured with flow cytometry using the 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM diacetate) probe. In both cases, fluorescence in the FL-1 channel was measured after trypan blue counterstaining in a FACSCalibur flow cytometer (Becton and Dickinson, San Jose, CA, USA). Zymosan at 0.1 μg mL^{-1} was used to stimulate the immune response. The DNA-specific stain 7-amino-actinomycin D (7AAD) was used to determine the integrity of cell membranes.

2.4. Gene expression analysis using quantitative PCR

Larval gene expression was measured both at basal conditions and in challenged larvae. Each experiment was conducted using three different biological replicates, and each replicate belonged to a different family. For ontogeny studies at basal conditions, larvae were collected, concentrated using nylon mesh and centrifuged. The pellet was resuspended in 500 μL of Trizol (Life Technologies Carlsbad, CA, USA), and total RNA extraction and cDNA synthesis were performed using the previously described standard protocols [22]. In the studies of challenged larvae, RNA isolation was performed using the Maxwell 16 LEV Simply RNA Tissue Kit (Promega, Madison, WI, USA) following manufacturer's instructions. cDNA was synthesized as previously described.

We examined the expression of several immune-related genes previously described in mussel [23]. The following genes were included in the analysis: the antimicrobial peptides Mytimycin precursor 1, Mytilin B, Myticin B and Myticin C; the putative genes implicated in pathogen recognition, C1q and Fibrinogen-related protein (FREP); the pore-forming molecule MacP; the inflammatory regulator macrophage inhibition factor and lysozyme. The expression of these immune-related genes was also performed in hemocytes from adult mussels.

Quantitative PCR was performed using the PCR primers summarized in Table 1, which were selected according to qPCR restrictions. Oligo Analyzer 1.0.2 was used to assess dimer and