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Maternal transference of phenoloxidase and lysozyme activity in eggs from *Penaeus vannamei*

Transferencia materna de la actividad de fenoloxidasa y lisozima en huevos de Penaeus vannamei

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Abstract

Shrimp's immune system has been extensively described and associated with environmental conditions. However, there are few reports of immune enzyme activities during ontogenetic development in shrimp larvae, the most vulnerable stage of the shrimp life cycle. Indeed, lysozyme, a crucial enzyme against bacterial pathogens, has never been described before in shrimp larvae. Hence, there are no reports of the effects of acute ammonia stress on shrimp lysozyme activity. This study aims to evaluate the lysozyme activity through all the early ontogenetic stages of *P. vannamei*, using the evaluation of phenoloxidase activity as a parallel indicator of immune status during the evaluated ontogeny time frame. We determined the enzymatic activity of Phenoloxidase and

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lysozyme during shrimp early ontogeny, from eggs to postlarvae 8. Also, we evaluated the immune response of three shrimp larvae stages after acute ammonia stress with a sublethal dose by determining phenoloxidase and lysozyme enzymatic activity. The phenoloxidase and lysozyme activity was detected in all shrimp stages, including in eggs, reaching their highest values in protozoea I and zoea III, respectively. Also, the activity of phenoloxidase and lysozyme decreased 12 hours after ammonia exposition in Mysis II. Meanwhile, phenoloxidase activity decreased 6 hours after the treatment in postlarvae 2. Our results constitute the first report of lysozyme activity in shrimp larvae stages and the influence of acute ammonia stress on this activity.

Keywords: Lysozyme activity, Phenoloxidase activity, ontogenetic development, immune system, acute ammonia stress**.**

1. Introduction

Shrimps present an immune system that can be classified into two main classes: the passive (exoskeleton and peritrophic membrane) and active elements (hemostatic mechanisms, humoral and cellular response). Moreover, the focus on active elements has led to a new kind of immunity in invertebrates: trained immunity (1). However, the humoral response still stands out as the main parameter to evaluate the shrimp's immune status,, mainly based on the activity of immune enzymes. Among the most used enzymes activities assays to test the immune status of shrimps are phenoloxidase (PO), superoxide dismutase (SOD), lysozyme (Lys), and peroxidases (PX) (2,3,4,5). Thus, lower values of PO activity are related to immunodepression in shrimp; therefore, it can be used to describe the general immune status of shrimps (6). Notwithstanding, other enzymes like lysozyme could describe a better and more specific perspective of shrimp's immune capacity against bacterial pathogens.

Moreover, shrimp, like other crustaceans, have a very long and complex ontogenetic development characterized by short metamorphosis changes (7,8). Hence, the ontogenetic development of the shrimp immune system has to be understood as a continuous process instead of separated larvae stages. Indeed, the study of ontogenetic development of the immune system in shrimp has been conducted as a continuity before, reporting the activity of several immune enzymes like PO, SOD,

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and PX during the early stages of shrimp larvae (9). Also, the expression analysis of several immune genes in shrimps during ontogenetic development has been described (10,11). However, there are no reports about lysozyme activity in penaeid shrimp larvae or even more during the ontogenetic development of early stages of shrimps. Almost all the studies of shrimp immune response are developed in juvenile and adult stages since the small size of the bodyprecludes direct research.

The most vulnerable stages of shrimp biological development are the larvae stages when early shrimp larvae lack structured defenses against pathogens and face harmful environmental conditions (12). In culture tanks, where larvae are farmed in high densities, the concentrations of nitrogen toxic compounds, such as ammonia, reach higher levels than in natural culture systems (13,14). The effect of ammonia on shrimp physiological and pathophysiological processes such as growth, metamorphosis, osmotic regulation, immunity, reproduction, metabolism, survival, and excretion in shrimp has been extensively reported (15,16,17,18,19,20). However, its effect on immune response, specifically on shrimp larvae is still unclear(21) Hence, the present study aims to describe the immune response of early ontogenetic development stages of *P. vannamei* and the effect of acute stress of ammonia on lysozyme and phenoloxidase response.

2. Materials and methods

2.1 Experimental design

A randomized experimental design was employed to evaluate the effect of a sublethal dose of TAN (Total Ammonium Nitrogen) in three larvae stages (Protozoeas II (ZII), Mysis II (MII), and PostLarvae II (PLII)), all from the same broodstock. For each experiment, water from culture tanks was used instead of filtered water, with culture parameters measured using multiparameter equipment (Hanna Instrument). TAN doses were adjusted at 20 mg/L (2,3 mg/L of ammonium, at a temperature of 30 ± 1 ° C), pH (8,3 ± 1), an oxygen concentration of 6.3 mg/L, and a salinity of 32 PSU). The experiments included nine replicates for the TAN treatment and six for the control group (without

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TAN). Samples were taken at three-time points (0h, 6h, and 12h) after TAN exposure, including those from the control groups. All the experiments were initiated at 8 am every three different days. During the experiments, shrimps were fed with an equal amount of Artemia once at 6 hours after TAN dose administration.

2.2 Samples Collection

Samples were collected from the hatchery Yaguacam, Cienfuegos, Cuba. From each larvae stage, three replicates of ten production tanks were taken from eggs after shrimp females oviposition to post larvae eight stage. Also, samples of each stage were stored in 1.5 mL vials, with ARN later solution to determine the expression profile of each stage. The shrimp response to an ammonium challenge was evaluated using larvae from three stages and cultured in tanks. The samples were stored at -20 ^{0}C for posterior analysis.

2.3 Sample processing

Laboratory analyses were performed in Camaguey University Laboratories of Agricultural sciences. Each sample was homogenized in PBS solution and then centrifugated at 8 000 g for 3 minutes in Mini spin plus Eppendorf centrifuge. The supernatant was collected, placed in a new 1.5 mL vial, and stored at -20 ^{0}C. The quantification of its protein concentrations was determined through the Bradford assay (22) in a Biotek plate spectrophotometer (ELx 800, 2014).

2.4 Enzymatic assays

Lysozyme activity

The enzymatic activity of lysozyme in shrimp larvae was detected by the turbidimetric method of De-La-Re-Vega et al. (2006). The substrate solution was prepared with 100 mM buffer phosphate (pH = 8) and *M. lysodeikticus* of 1.2 m g/mL.The essay was performed on a 96-well plate scale, adding 20 µL of processed sample and 180 µL of the substrate solution, resulting in a final volume of 200 µL per well. The absorbance was measured at 630 nm of weave length two times (0 and 10 minutes) in

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a Biotek plate spectrophotometer (ELx 800). A unit of enzymatic activity was defined as a change of 0.001 absorbances per minute. Each replicate was evaluated two times on the plate.

Phenoloxidase activity

PO enzymatic activity was determined according to the protocol of (23) with some changes. The essay was adapted to a 96-well plate scale, adding 20 µL of the samples and 20 µL of trypsin solution (5mg/mL: in PBS solution) and letting it in incubation for 10 minutes, then the substrate solution (L-DOPA, 1.5 mg/mL in PBS solution) was added, measuring the absorbance at 490 nm, two times (0 and 30 minutes) in a Biotek plate spectrophotometer (ELx 800). A unit of enzymatic activity was defined as a change of 0.001 absorbances per minute. Each replicate was evaluated two times on the plate.

2.5 Statistical analysis

Statistical analyses were performed with GraphPad Prism software (version 9.0). Data were initially tested for normality and homoscedasticity of variances, excluding aberrant values. Data are expressed as mean ± standard deviation (SD) and subjected to one-way ANOVA. Significant differences ($P < 0.05$) between experimental groups were detected using the post-hoc Student– Newman–Keuls multiple comparisons test.

3. Results

3.1 Lysozyme activity through shrimp ontogeny.

Lysozyme activity was reported through all the ontogeny stages of *P. vannamei* (See Graph #1). However, Lysozyme activity fluctuated depending on the shrimp stage. After Nauplii I, Lysozyme activity followed an increasing trend until 16 hours after the spawning. Then, enzymatic activity with several minimums and maximums through the shrimp ontogeny, reaching the higher value at 92 h,

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with 1,73 U/mg. In PostLarvae, the enzymatic activity remained steady except for a significant increase at 284 hours, corresponding to the Postlarvae 5 stage.

3.2 Phenoloxidase activity through shrimp ontogeny.

Phenoloxidase activity was reported through all the ontogeny stages of *P. vannamei (See Graph #2, a)*. However, Phenolxidase activity fluctuated depending on the shrimp stage, remaining steady in the eggs and reaching a low in the first stage of nauplii, just 12h after the spawn. After Nauplii I, phenoloxidase activity followed an increasing trend, reaching the maximum value at Protozoea I (44 U/mg). Then, enzymatic activity decreased to a minimum at Mysis I and grew up to Mysis III. Furthermore, the lower values in the study were reached in the Postlarvae stages.

3.3 Ammonium challenge in larvae stages

Ammonium challenges were developed in three larvae stages: Protozoea II, Mysis II, and Postlarvae 2. The immune profile was evaluated by determining the enzymatic activity of lysozyme and phenoloxidase. In Protozoea II, phenoloxidase activity showed no differences after acute ammonium stress in all the study periods compared to control groups. Meanwhile, there was no report of significant lysozyme activity in Protozoea II in both groups,in Mysis II, lysozyme and phenoloxidase activities increased in control groups during the experimental development. However, ammonium-treated groups did not follow the same behavior, decreasing both activities compared to control groups at 12 hours after the ammonium challenge. Furthermore, in Postlarvae 2, phenoloxidase activity increased until 6 hours in control groups. Nevertheless, in treated groups, the phenoloxidase decreased compared to control groups. Notwithstanding, lysozyme activity did not show differences between treated and control groups.

4. Discussion

4.1 Lysozyme activity through shrimp ontogeny.

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Lysozymes are included in the family of antibacterial peptides based on their low molecular weight and their non-specific bacteriostatic effect (24). These enzymes exert their function through the hydrolysis of glycosidic bonds that are linked to the bacterial cell wall (25). Lysozymes are specifically synthesized in hemocytes, mainly the granular ones. In addition, they are well characterized in Penaeid shrimp, where they have lytic activity against several Gram-negative and Gram-positive bacteria, including the pathogenic *Vibrio* spp. (26,27,25,28).

In our experiments, lysozyme activity was detected in all the ontogenetic stages, from the early hours of being eggs to postlarvae 8. Both fertilized and unfertilized eggs showed a steady lysozyme activity that continued invariable until 14 hours after spawn (Nauplii II). During the nauplii stage, lysozyme activity showed a significant increase in eggs, which could be attributed to the proliferation of bacteria associated with non-fertilized eggs and the egg envelope. The egg envelope contains a variety of microorganisms, including pathogenic bacteria that eventually proliferate during incubation, and colonize the nauplius surface (29). The nauplii were disinfected with chlorine to prevent significant losses; however, the recolonization automatically takes place from hatching water, which constitutes a kind of biological reactor for emerging bacteria with several nutrients from non-fertilized eggs and eggs envelopes (30,31). However, the expression of lysozyme has been reported to be extremely low in eggs or not present at all (10,32). Hence, we suggest these levels of lysozyme activity are due exclusively to maternal transference.

After Nauplii II, lysozyme activity fluctuates with its highest value during protozoea III. Quispe and collaborators demonstrated that in protozoea lysozyme expression reached its highest value. Protozoea is the first stage when shrimp larvae are fed with algae and rotifers;the microorganisms ingested together with food may induct excessive immune response or overexpressed for digestion, enhancing the expression of lysozyme and subsequently its activity (11). Also, their activity values fluctuate according to metamorphosis time frames, when shrimp larvae are most susceptible to pathogens. In post-larvae 5, we also reported a significant increasewhen shrimp larvae change from

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planktonic to benthic habits. This change could affect its immune and digestion status, and lysozyme activity could significantly prevent bacterial infections.

Phenoloxidase activity through shrimp ontogeny.

The proPO system is a crucial component of shrimp immune response, based on a complex cascade of enzymatic reactions, where phenoloxidase (PO) plays the leading role (33). PO is a Cu-dependent metallo-type protease that catalyzes multiple reactions: the production of quinones, ortho-hydroxylated phenols, L-DOPA, L-DOPA quinone, and melanin. The result of all reactions is the formation of toxic metabolites with antimicrobial activityand the creation of covalent crosslinking of nearby molecules to form melanin in affected sites and around invading organisms (34). The decrease in PO is closely related to a state of immunosuppression; however, under physiological conditions, this enzyme increases in the face of infections caused by viruses and bacteria (6). PO activity is an indicator that is taken as a reference to analyze the immunological status of crustaceans (35).

In our experiments, we reported PO activity in eggs even just after spawn reached a constant level of phenoloxidase activity in eggs. The peak at protozoea I could be due to the exposure of larvae to the external medium after the metamorphosis, where they could be overwhelmed by microorganisms if not for activation of this enzymatic activity. Glas and collaborators demonstrated the vulnerability of eggs in the early stages of crustacean development and highlighted the crucial role of peroxidase activity in hardening the extra-embryonic coat during these periods (36). Hence, PO activity could also be essential to protect eggs against pathogens. Interestingly, the proPO expression does not start in eggs in *P. vannamei*, suggesting that the PO activity we reported is not due to eggs synthesis de nuovo of PO but due to mother ovum PO activity itself which is kept during the first eight hours after spawn (10,11). However, we demonstrated that this activity does not remain steady, decreasing in Nauplii I, and increasing its level through the following nauplii stages when finally PO expression starts (11).

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The highest value of 298.20 U/µg was detected in the protozoea stage. This could be a crucialrole of PO in protozoea stages, preparing shrimp larvae to metamorphosis following stages where the expression of PO remains steady (11). That could explain the sharp decrease of PO activity from protozoea to the mysis stage and its quick recovery. Then, PO activity decreased during postlarvae stages, where shrimp larvae were less vulnerable. In these final stages of early ontogenetic development, the surface area-volume ratio of larvae decreases, therefore affording lower interactions with pathogens or opportunistic microorganisms on the epidermis (9,37).

3.4 Ammonium challenge in larvae stages

Our experiments constitute the first report of acute stress ammonia's effect on lysozyme and phenoloxidase activity in larvae stages. However, it is known that the negative effect of ammonia stress on several vital processes in shrimps affects immune enzyme expression (38). Also, high ammonia levels suppress immune parameters (39) such as total hemocyte count (THCs) and expression profile (40), phagocytic activity (Chen et al., 2003), phenoloxidase (PO) (Lingxu et al., 2004) that mediates melanin synthesis (1), and antibacterial activity (42).

In our experiments, ammonia stress has been shown to reduce the immune response of shrimp larvae, specifically on mysis IIof aquatic animals by inhibiting PO activity and phagocytosis (35,43), as well as decreasing (up to 60%) proPO gene expresión.

Conclusions

P. vannamei ontogenetic development is characterized by sharp morphological and immunological changes. During the early stages of shrimp larvae, the enzymatic activity of several immune enzymes has been reported, but almost all are not expressed in early stages, suggesting their maternal origin. Instead, they are synthesized in nuovo by eggs and nauplii. That is the case of lysozyme and

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phenoloxidase activity, which have been described in all the larvae stages. In addition, the ammonia effect on the shrimp immune system was shown to be associated with shrimp larvae stages.

Our results provide useful information about shrimp's immune response to pathogens under farming conditions and stressful conditions such as acute ammonia exposition. This information may help in designing efficient strategies for disease control at these stages and thereby help ensure the long-term survival of shrimp aquaculture.

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